# Immune6

Potential Statements:

- Contains essential nutrients like vitamins C and D, and selenium, which are thought to provide additional support to your health.*
- Provides antioxidant support.*
- Vegetarian product
- Contains other ingredients like IP-6 (inositol hexaphosphate), graviola and black raspberry.
- IP6 may boost the immune system and offers antioxidant properties* (Matejuk & Abulkalam, 2010 –See research—partial statement).
- Inositol was originally named inos (muscle, in Greek)(Matejuk & Shamsuddin, 2010).
- Excellent source of the antioxidant Vitamin C (Retrieved from **FDA Dietary Supplements Labeling Guide/;** FDA, Regulatory, Antioxidants).
- Vitamin C is needed for the growth and repair of tissues in the body (Medline Plus, NIH, NLM, Vitamin C, Retrieved from NIH Vitamin C).
- Vitamin C promotes healing and helps the body absorb iron, in addition to being important for skins, bones and connective tissue (Medline Plus. NLM, NIH.LM, NIH Medline Plus. Vitamin C. Retrieved from http://www.nlm.nih.gov/medlineplus/druginfo/meds/a682583.html).
- People who eat a limited variety of food may not be getting enough vitamin C." (Retrieved from ODS, NIH Fact Sheet, Vit C, Consumer).
- Selenium is a mineral found in the soil that acts as an antioxidant. Selenium helps the body to make antioxidant enzymes (special proteins) (Medline Plus, NLM, NIH. Retrieved on 6/29/2015. Retrieved from Medline Plus, NLM, NIH Selenium).
- "Adequate calcium and **vitamin D**, as part of a well-balanced diet, along with physical activity, may reduce the risk of osteoporosis" (retrieved from http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/LabelingNutrition/ucm064919.htm).

***These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.**

Formulated with six powerful agents, Immune6 contains the antioxidants vitamin C and selenium, as well as vitamin D as cholecalciferol which have been shown to provide immune support. Our proprietary formula of inositol hyexaphosphate (IP-6), Black Raspberry and Graviola provides a broad spectrum of health benefits.

Dietary Supplements

According to NIH News in Health, Aug 2013 article on supplements "the most popular nutrient supplements are multivitamins, calcium and vitamins B, C, and D." The article further states molecules that help to maintain health and prevent cell damage, antioxidants, are from

Vitamins C and E, while calcium supports bone health and vitamin D helps the body absorb calcium (NIH, News in Health, Aug 2013, feature 1, Should you take Dietary Supplements? Retrieved from http://newsinhealth.nih.gov/issue/aug2013/feature1).

Certain vitamins and minerals work as Antioxidants. Antioxidants (i.e. Selenium, Vitamin A, Beta-Carotene Vitamin C, Vitamin E, Lycopene and Lutein) are man-made or natural substances that may delay or prevent various types of cell damage (Medline Plus, NLM, NIH, antioxidants. Accessed 5/5/15, retrieved from http://www.nlm.nih.gov/medlineplus/antioxidants.html). Each vitamin and mineral, including those working as antioxidants, play a specific role in the body.

Selenium

Selenium is a mineral found in the soil that acts as an antioxidant. Selenium helps the body to make antioxidant enzymes (special proteins) which have a role in preventing cell damage. Some studies suggest that selenium may help with preventing cardiovascular disease and certain cancers, as well as assisting in protecting the body from poisonous effects of heavy metals and other harmful substances (Medline Plus, NLM, NIH. Retrieved on 6/29/2015. Retrieved from Medline Plus, NLM, NIH Selenium). In the U.S. and Canada, a selenium deficiency is quite rare. Selenium deficiency may cause male infertility and a type of heart disease known as Keshan disease, as well as a type of arthritis (Kashin-Beck) that produces swelling, pain, and loss of motion in your joints (ODS, NIH, retrieved 9/15/2015. Retrieved from https://ods.od.nih.gov/factsheets/Selenium-Consumer/).

Selenium is found in many foods naturally with the amount of selenium in plant foods being dependent upon the amount of selenium in the soil used to grow the plant foods. In animal products, the amount of selenium depends on the foods that the animals ate and the related selenium content of those foods. Dietary selenium is available in many foods, such as eggs, poultry, meat, dairy, seafood, and grain products such as cereals and bread (ODS, NIH, retrieved 9/15/2015. Retrieved from https://ods.od.nih.gov/factsheets/Selenium-Consumer/).

Vitamin C (as ascorbic acid)

Vitamin C (ascorbic acid) is a water soluble (not stored in the body) nutrient, which acts in the body as an antioxidant, helping to protect cells from damage caused by free radicals (UMM, University of Maryland Medical Center, Vitamin C, retrieved from UMM Vitamin C; Retrieved from ODS, NIH Fact Sheet, Vit C, Consumer). Vitamin C is needed for the growth and repair of tissues in the body (Medline Plus, NIH, NLM, Vitamin C, Retrieved from NIH Vitamin C). Additionally, vitamin C is used to heal wounds and form scar tissue; repair and maintain bones, cartilage and teeth; and to form an important protein (collagen) used to make ligaments, tendons, and blood vessels (Medline Plus, NIH, NLM, Vitamin C, Retrieved from NIH Vitamin C; University of Maryland Medical Center, UMM, Vitamin C (Ascorbic Acid), retrieved from UMM Vitamin C; ODS, NIH, Vitamin C, retrieved 6/17/2015 from ODS, NIH Fact Sheet, Vit C, Consumer).

Additionally, vitamin C is important to in helping the immune system function properly to protect the body from disease (Retrieved 6/17/2015. Last reviewed 6/24/2011. Retrieved from ODS, NIH Fact Sheet, Vit C, Consumer). Vitamin C from ascorbic acid is used to prevent and treat scurvy (Medline Plus, NIH, NLM, Vitamin C, Retrieved from NIH Vitamin C or http://www.nlm.nih.gov/medlineplus/druginfo/meds/a682583.html ). Vitamin C helps the body absorb iron (Medline Plus, NIH, NLM, Vitamin C, Retrieved from NIH Vitamin C; Medline Plus, NIH, NLM, iron, retrieved from NIH Iron).

Vitamin C promotes healing and helps the body absorb iron, in addition to being important for skins, bones and connective tissue (Medline Plus. NLM, NIH.LM, NIH Medline Plus. Vitamin C. Retrieved from http://www.nlm.nih.gov/medlineplus/druginfo/meds/a682583.html).

Although the role of vitamin C supplement in CVD remains unproven (Benzie & Wachtel-Galor, editors, 2011), the antioxidant properties of vitamin C are thought to act synergistically with those of vitamin E, by blocking lipid peroxidation and decreasing the formation of peroxyl radicals. Vitamin C, by increasing available nitric oxide (NO) has an action on endothelial vasodilator function in heart failure (Benzie & Wachtel-Galor, editors, 2011; Frishman, 1999). According to Mikirova, Ichim & Riordan (2008), high doses of vitamin C have been associated with decreased levels by endothelial cells of NO (Nitric Oxide) production (Benzie & Watchtel-Galor, editors, 2011). Kritchevsky, et al. (1995) reports that a clinical trial showed that vitamin C slowed progression of atherosclerosis in women and men over 55 years of age. However, not all studies show an effect on ascorbic acid and cardiovascular events, such as the Physician's Health Study II, which showed no benefit from taking 500 mg of vitamin C daily to prevent events of CVD (Sesso, Buring, Christen et al., editors, 2008; Benzie & Watchtel-Galor, editors, 2011). In the Nurse's Health Study, vitamin C supplements were associated with a lower risk of CHD in women, according to Osganian, Stampfer & Rimm, et al., editors (2003).

Food Sources: Vitamin C: Vitamin C comes from fruits and vegetables (citrus fruit, red and green peppers, greens, tomatoes, and broccoli) and may be added to other foods, such as juices or cereals (Medline Plus, NIH, NLM, Vitamin C, Retrieved from NIH Vitamin C or http://www.nlm.nih.gov/medlineplus/vitaminc.html).

Vitamin C, Dosage

The best way to take vitamin C supplements is 2-3 times/day with meals; some studies suggest adults may need to take 250-500 mg, twice daily for any benefit; it is recommended to speak with a physician prior to taking more than 1,000 mg of vitamin C on a daily basis and before giving vitamin C to children. In doses as high as 2,000 mg/daily may cause problems with gas, diarrhea, or stomach upset. Smokers or people who use nicotine patches need more vitamin C (nicotine causes vitamin C in the body to be less effective). Infants born to mothers taking 6,000 mg or greater of vitamin C may develop rebound Scurvy (due to intake of vitamin C dropping after birth). For pregnancy, talking to a physician prior to taking more than 1,000 mg of vitamin C is recommended. It is possible that vitamin C may raise blood sugar levels in persons with diabetes. It may interfere with healing to take vitamin C prior to or following angioplasty. Certain medications are thought to interact with vitamin C and it is recommended to speak with a health care provider (UMM, University of Maryland Medical Center, Vitamin C, retrieved from UMM Vitamin C).

A vitamin C deficiency is rare in Canada and the U.S. Scurvy may arise from people who get 10 mg or less per day of vitamin C. Scurvy causes inflammation of the gums, fatigue, small purple or red spots on the skin, poor wound healing, joint pain and corkscrew hairs, in addition to swollen, bleeding gums, loosening or loss of teeth, and depression. Scurvy is fatal if not treated (Retrieved 6/17/2015. Last reviewed 6/24/2011. Retrieved from ODS, NIH Fact Sheet, Vit C, Consumer).

Vitamin D3 (as cholecalciferol)

Vitamin D is found in many dietary sources, such as egg yolk, fish, fortified milk, and cod liver oil. Foods may be fortified with vitamin D2 or vitamin D3. The sun contributes significantly as well to the daily production of vitamin D with as little as 10 minutes of exposure possibly being

enough to prevent deficiencies (Natural Medicines Database, Vitamin D, Professional Handout, retrieved from Natural Medicines Database: Vitamin D).

According to Somjen, Weisman, Kohen, et al., editors (2005), vitamin D receptors are found in vascular smooth muscle, and per Merke, Milde, Lewicka, et al., editors (1989), the endothelium (tissue that forms a single layer of cells lining various cavities and organs of the body); and per Holick (2006), cardiomyocytes (cardiac muscle cells). The literature supports evidence to suggest an association between the accelerated development of CVD and low levels of 25-hydoxyvitamin-D (Dobnig, Pilz, Scharnagl et al., editors, 2008; Moats and Rimm, 2007; Giovannucci, Liu, Hollis, & Rimm, 2008; Wallis, Penckofer, and Sizemore, 2008). Results from the Framingham Offspring Study suggest a direct association between the subsequent incidence of CVD and vitamin D deficiency (Wang, Pencina, Booth, et al., editors, 2008). According to Zittermann and Koerfer (2008), it is not clear what dosage of vitamin D is necessary for the prevention of CVD.

Vitamin D, Dosage

When used long-term orally, doses of vitamin D should not exceed the tolerable upper intake level (UL) of 4000 IU per day for adults (When used orally long-term, doses should not exceed the tolerable upper intake level (UL) of 4000 IU per day for adults (Dietary reference intakes for calcium and vitamin D. Institute of Medicine, November 30, 2010. Available at: IOM, DRI, Calcium and Vitamin D); however, for the short-term treatment of vitamin D deficiency, much higher doses are taken orally for 6-12 weeks, such as 50,000 IU/week (Natural Medicines Database, Vitamin D, Professional Handout, retrieved from Natural Medicines Database: Vitamin D (Natural Medicines Database, Vitamin D, Professional Handout, retrieved from Natural Medicines Database: Vitamin D; Cava & Javier, 2007; Vitamin D dosing: an update. Pharmacist's Letter/Prescriber's Letter 2010;26(7):260707).

Black Raspberry Fruit Extract

Black raspberry is also known as rubus occidentalis, from the family Rosaceae (Natural Medicines Database, Black Raspberry, Professional Handout, retrieved from Natural Medicines. Professional monograph. Black Raspberry). Orally, black raspberry is used for bleeding, stomach pain, and cancer prevention; the applicable parts of black raspberry are the leaf and fruit, with studies being looked at for potential anticancer properties, which may be due to gallic acid (Natural Medicines Database, Black Raspberry, Professional Handout, retrieved from Natural Medicines. Professional monograph. Black Raspberry).

Black raspberry is likely saved when orally used in amounts that are found in foods; insufficient, reliable information is available about medicinal amounts and safety of black raspberry and black raspberry is not recommended during pregnant or breast-feeding due to a lack of information for medicinal amounts (Natural Medicines Database, Black Raspberry, Professional Handout, retrieved from Natural Medicines. Professional monograph. Black Raspberry; Liu et al., 2005). Black raspberries contain high amounts of phenols and anthocyanins, which give it antioxidant properties (Natural Medicines Database, Black Raspberry, Professional Handout, retrieved from Natural Medicines. Professional monograph. Black Raspberry; Liu et al, 2005; Wada & Ou, 2002; Wang & Jiao, 2000; Wang & Lin, 2000 ). Black raspberry also contains vitamins C and E, beta-carotene, glutathione, chlorogenic acid, and flavonoids (Natural Medicines Database, Black Raspberry, Professional Handout, retrieved from Natural Medicines. Professional monograph. Black Raspberry; Wang & Jiao, 2000; Wang & Lin, 2000).

Graviola (Soursop Fruit Extract)

Graviola (soursop), is indigenous to tropic South and North America and is an upright, small evergreen tree that produces an edible, large, heart-shaped commercially sold fruit (Natural Medicines Database. Graviola. Professional Monograph. Retrieved 9/14/2015. Retrieved from Natural Medicines Database. Professional Monograph. Graviola). The uses of graviola are as follows: as a sedative, antibiotic, cathartic, antiparasitic, for coughs, herpes, catarrh, leshmaniasis (a disease transmitted through the bite of sand fleas and caused by parasites) and cancer; topically, graviola is used for arthritis, and in foods graviola is used in beverages and cooking (Natural Medicines Database. Graviola. Professional Monograph. Retrieved 9/14/2015. Retrieved from Natural Medicines Database. Professional Monograph. Graviola).

Mechanism of Action, Graviola

Graviola is rich in isoquinoline alkaloids (organic compounds, plant origin) and lactones (an organic (carbon-containing) compound, that contains as part of a ring, an ester group —OCO—). The seeds, bark, stem, and leaf of graviola contain numerous amounts of a novel groups of potentially biologically active chemicals, or annonaceous acetogenins, of which 220 types have been isolated from graviola (Natural Medicines Database. Graviola. Professional Monograph. Retrieved 9/14/2015. Retrieved from Natural Medicines Database. Professional Monograph. Graviola; Oberlies, Chang, & McLaughlin, 1997). There are no known interactions with food or with lab tests (Natural Medicines Database. Graviola. Professional Monograph. Retrieved 9/14/2015. Retrieved from Natural Medicines Database. Professional Monograph. Graviola). Graviola may exacerbate Parkinson's symptoms due to acetogenin constituents being neurotoxins (Caparros-Lefebvre & Elbaz, 1999; Roman 1998; Natural Medicines Database. Graviola. Professional Monograph. Retrieved 9/14/2015. Retrieved from Natural Medicines Database. Professional Monograph. Graviola).

Effectiveness, Safety, Dosage: Graviola

There is not sufficient or reliable information available about graviola's effectiveness, including for children. In formulation & standardization for graviola, there is no well-known standardization (Natural Medicines Database. Graviola. Professional Monograph. Retrieved 9/14/2015. Retrieved from Natural Medicines Database. Professional Monograph. Graviola). For oral use in adults, based on historical use, 150 ml of boiling water poured over 2 g of dried graviola leaf and stem was used as an infusion and steeped for 5-10 minutes, with product being taken between meals three times daily. As an alternative, 2-4 ml of a 1:2 tincture may have been taken three times daily (Natural Medicines Database. Graviola. Professional Monograph. Retrieved 9/14/2015. Retrieved from Natural Medicines Database. Professional Monograph. Graviola). Regarding adverse effects, graviola is well tolerated when taken in amounts consumed normally in the diet. High doses of graviola could cause hypotension, gastrointestinal upset, myeloneuropathy of the optic nerve, and neuronal dysfunction leading to neurological disorders (Natural Medicines Database. Graviola. Professional Monograph. Retrieved 9/14/2015. Retrieved from Natural Medicines Database. Professional Monograph. Graviola). **Graviola is likely unsafe when used orally by women who are pregnant or lactating** (Caparros-Lefebvre & Elbaz, 1999; Roman, 1998; Natural Medicines Database. Graviola. Professional Monograph. Retrieved 9/14/2015. Retrieved from Natural Medicines Database. Professional Monograph. Graviola).

**Graviola is likely unsafe for adults when used orally.** Movement disorders, similar to Parkinson's disease, may be caused by consumption of graviola fruit (Caparros-Lefebvre & Elbaz, 1999), while tea made from the stems and leaves of graviola is associated with neurotoxicity (Roman, 1998) (Natural Medicines Database. Graviola. Professional Monograph. Retrieved 9/14/2015. Retrieved from Natural Medicines Database. Professional Monograph. Graviola). "Graviola is **UNSAFE**. It can kill nerve cells in the brain and other parts of the body. It may cause movement disorders similar to Parkinson's disease." (Natural Medicines Database. Consumer Education and Info. Retrieved 9/15/2015. Retrieved from Natural Medicines Database. Consumer Education & Info. Graviola). "Special precautions and warnings: **Pregnancy or breast-feeding**: Do not use graviola. It is **UNSAFE**" ((Natural Medicines Database. Consumer Education and Info. Retrieved 9/15/2015. Retrieved from Natural Medicines Database. Consumer Education & Info. Graviola).

IP-6 (Inositol hexaphospate) - Introduction

More than 150 years ago, Josef Scherer, a German chemist, isolated a new molecule from muscle tissue, or inos (muscle in Greek), the original name for inositol (Matejuk & Shamsuddin, 2010; Scherer, 1850). One of nine inositol isomers and the most abundant in nature is Myo-inositol (Matejuk & Shamsuddin, 2010). As an essential nutrient and a member of the B vitamins, inositol is a simple hexa-carbon carbohydrate, derivative of glucose (Matejuk & Shamsuddin, 2010). All 6 carbons when attached to phosphate groups, are known as inositol hexaphosphoric acid, or $IP_6$, Ins$P_6$, phytic acid (Matejuk & Shamsuddin, 2010). IP6 or phytic acid has been studied extensively in animals and been promoted in health food stores as an anti-cancer agent (Fox, 2002).

Phytic acid (IP6) is found naturally in wheat brand, soy foods, and legumes and is believed to be the active ingredient that provides these substances their cancer fighting capabilities (Fox, 2002). IP6 has been recognized in the following ways: as an agent for preventing calcification and kidney stone formation; as a natural antioxidant; for the reduction of pathological platelet activity; and for the normalization of cholesterol concentrations (Matejuk & Shamsuddin, 2010). For cancer prevention, IP6 has been documented to control tumor growth, progression and metastasis (Matejuk & Shamsuddin, 2010). The anti-cancer actions of IP6 involves reducing cell proliferation, boosting immunity, antioxidant properties, and inducing differentiation of malignant cells through lower phosphate inositol phosphates ($IP_{1-5}$) and potentially via highly phosphorylated derivatives (PP-$IP_4$and PP-$IP_5$)(Matejuk & Abulkalam, 2010).

IP-6 (Inositol hexaphosphate) – Animal Studies and Cancer

A review via a Medline search of studies from 1966 to May 2002, using the keywords cancer and phytic acid, with a limit of research to subheadings of 'prevent,' 'therapeutic uses,' and 'adverse effects,' revealed 28 studies, according to Fox (2002). A large majority of the reviewed 28 studies were conducted in animals and indicated that phytic acid had antineoplastic (acting to halt, inhibit, or prevent the development of a neoplasm or tumor) properties in liver, colon, breast, prostate, sarcomas, and skin cancer, but not in human studies (Fox, 2002). Side effects included an increase in renal and bladder papillomas and chelation of multivalent cations, but the increase in papilloma formation occurred only with the sodium salt of phytic acid (not with magnesium or potassium salts)(Fox, 2002). Therefore, a large animal evidence indicates that phytic acid may play a role in the treatment and prevention of various forms of cancer, with enough justified evidence to initiate Phase I and Phase II human clinical trials (Fox, 2002).

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# Inositol phosphates: health implications, methods of analysis, and occurrence in plant foods

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## Abstract

Inositol phosphates (InsPs), especially *myo*-inositol hexakisphosphate ($InsP_6$), are important binders of phosphorus and minerals in plant seeds. However, they have long been considered as anti-nutritional components of plant foods due to their possible negative effects on the absorption of minerals and proteins in mammals. On the other hand, recent findings have found InsPs to be ubiquitous in eukaryote cells and actively participating in multiple cell functions. *In vivo* and *in vitro* studies have also documented the preventive potential of these compounds against the development of a wide range of diseases. In light of these findings, interest in the relationship between these compounds and human health has been renewed. It is suggested that the interactions of InsPs with other nutrients in the gut are complex, that the absorption of dietary InsPs might be implied but is not certain, and that the disease fighting capabilities of InsPs hold both promises and limitations. At the same time, the analysis of these compounds in foods and biological samples still faces many challenges, calling for more advanced modification and developments in the future.

**Keywords:** Inositol phosphates (InsPs); Phytic acid; Phytate; Antioxidant; Chemoprevention.

## 1. Introduction

Phytic acid (*myo*-inositol hexakisphosphate, $InsP_6$), also frequently referred to by its salt form phytate and phytin, is an important component of plant seeds and a controversial constituent in the human diet. The compound is commonly consumed in high amounts from staple cereals and legumes, in which it serves as the main phosphorus reservoir and one of the major binders of minerals for the seeds (Kornegay, 2000). While plant seeds possess endogenous phytases to hydrolyze $InsP_6$ and its salts upon germination, humans produce very minimal amount of this enzyme group endogenously and must rely on microbial phytases, which are often insufficient for the complete digestion of the $InsP_6$ content in foods (Lopez et al., 2002). At the pH levels of the human digestive tract, $InsP_6$ can form insoluble salts and complexes with minerals and macronutrients, rendering these nutrients unavailable for absorption or other activities (Konietzny et al., 2006; Kumar et al., 2010). As a result, this compound has been implied to be an antinutrient even though its negative effects have not been well described *in vivo*. On the other hand, emerging research is showing that $InsP_6$ as well as lower forms of inositol phosphates (InsPs), including *myo*-inositol mono-, bis-, tris-, tetrakis-, and pentakisphosphate ($InsP_{1-5}$), are widely present in eukaryote cells and may have important physiological functions (Irvine and Schell 2001; Sauer and Cooke 2010; Shears 1998). $InsP_6$ has also been found to have anti-inflammatory and anticancer effects in various types of cell lines (Vucenik et al., 2004). At the same time, it is still unclear whether dietary InsPs can be absorbed in the human digestive tract and mobilized for metabolic activities, as no carrier for these compounds has been

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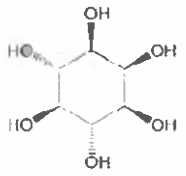


Figure 1. Chemical structure of myo-inositol.

detected, but some studies have reported intracellular InsPs to be dependent on extracellular InsPs (Grases et al., 2002). Overall, there remain many questions on the role of dietary InsPs in human health and disease prevention. This contribution aims to provide an overview on the development of the understanding pertaining to the relationship between dietary InsPs and human health, the efforts to quantify InsPs in major food products, as well as the on-going challenges that are important to the study of these compounds.

## 2. Chemical properties of inositol phosphates

InsPs refer to the phosphorylated compounds of inositol (cyclohexane-1,2,3,4,5,6-hexol), in which one or more hydroxyl moieties on the six-carbon ring of inositol is replaced with a phosphate group. Inositol exists in nine possible stereoisomers, of which *myo*-inositol (Figure 1) is the most abundant species found in nature. As a result, "inositol phosphate" commonly refers to a phosphorylated *myo*-inositol. Depending on the number of phosphate groups on the inositol ring, a compound in this family can be referred to as *myo*-inositol mono-, bis-, tris-, tetrakis-, pentakis-, or hexakisphosphate ($InsP_{1-6}$). All lower inositol phosphates ($InsP_{1-5}$) display isomerism.

To date, *myo*-inositol hexakisphosphate ($InsP_6$; Figure 2), also commonly referred to as phytic acid, has garnered the most attention due to its capability to chelate mineral cations in the digestive system and its high apparent concentration in plant seeds. The compound is sterically stable when one phosphate is in the axial position and five phosphates are in the equatorial position (1ax 5eq) (Bohn et al., 2008). Depending on the pH of the medium and the presence of other cations, $InsP_6$ can invert into the sterically hindered form, in which one phosphate is in the equatorial position and five are in the axial position (5ax 1eq). Under the effect of heat, acid treatment, or enzymatic activity by the enzyme group phytase, $InsP_6$ can be dephosphorylated into $InsP_{1-5}$ as well as *myo*- inositol.

As indicated by their prefixes, the phosphate groups of $InsP_{2-6}$ are not internally connected and these compounds are polydentate ligands (Bohn et al., 2008). InsPs are capable of binding mineral cations such as calcium, zinc, copper, cobalt, manganese, iron, and magnesium, forming the salt phytate (phytin, if it is a mixed salt). The binding capability varies among InsPs and is dependent on the type of mineral. For example, *in vitro* studies at pH 3 to 8 have found that InsPs typically have a greater affinity with copper, zinc, and nickel than with other mineral cations (Persson et al., 1998; Tsao et al.,1997; Vasca et al., 2002). $InsP_6$ and $InsP_5$ have higher binding strength than $InsP_3$ and $InsP_4$, and the distribution of the phosphate groups on the inositol ring also differentiates binding affinity among stereoisomers. InsPs and their salts with monovalent cations, such as sodium and potassium, are typically water soluble, while salts with multivalent cations have varying degrees of solubility depending on the pH of the environment and the presence of competing cations.

At low pH levels, $InsP_6$ can bind to the cationic groups of proteins and forms insoluble complexes (Ellis and Morris, 1983). This binding might include interactions with the $\alpha$-$NH_3$ terminal group, the $\varepsilon$-$NH_3$ group of lysine, the imidazole group of histidine, and the guanidyl group of arginine. At pH levels higher than these proteins' isoelectric points, soluble protein-mineral-phytate complexes may form (Gemede, 2014).

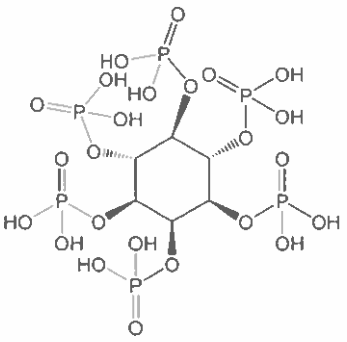


Figure 2. Chemical structures of *myo*-inositol hexakisphosphate ($InsP_6$) or phytic acid.

## 3. The physiological roles of extraneous inositol phosphates

InsPs are ubiquitous in seeds, which include cereals, legumes, and nuts that are frequent components in human and animal diets. $InsP_6$ is said to typically comprise ≥85% of InsP composition and serves as a mineral binder, phosphorus source, and energy source for the seed (Kornegay, 2000). During germination, phosphorus and minerals will be released from phytate by phytases. Many animals, especially ruminant types, have been found to depend on microbial phytases in the gut for InsP catabolism. In humans, phytase secretion appears to be limited, and microbial phytases in the large intestine are likely to be important to the digestion of InsPs (Iqbal et al.,1994; Lopez et al., 2002, Schlemmer et al.,2009).

### 3.1. Inositol phosphates as an antinutrient

Dietary InsPs are often viewed as undesirable due to their capability to chelate mineral cations and to form complexes with protein, carbohydrate, and lipid, which reduce the availability of these nutrients in the body (Kumar et al., 2010). $InsP_6$ and $InsP_5$ are the most abundant InsPs in foods and are also the InsPs with the strongest binding capabilities. In many countries where whole grain cereals and legumes are staples, InsPs, along with other components, such as inorganic phosphates, polyphenols, and nondigestible fiber, may exacerbate on-going nutrient deficiency problems (Schlemmer et al., 2009). Of most concern are the potential adverse effects on the absorption of zinc and iron, two minerals with prevalent deficiencies around the world, as well as calcium and magnesium. Furthermore, the formation of insoluble phytates - phytins and complexes also render phosphorus unavailable for the body (Kornetzny et al., 2006).

Zinc is strongly sequestered by $InsP_6$ and zinc phytate forms readily within the pH range of 3 to 7, which includes the pH range of the duodenum, where the absorption of this mineral occurs (Persson et al., 1998). Among $InsP_{3-6}$, only $InsP_6$ and $InsP_5$ have been found to exert a significant inhibitory effect on zinc absorption (Lönnerdal et al.,1989). The World Health Organization (WHO)

considers zinc absorption to be low when it is <15% (compared to ~30% in most diets), which can be caused by a phytate:zinc mole ratio of more than 15 (World Health Organization, 2014). On the other hand, the International Zinc Nutrition Consultative Group (IZiNCG) set the low level of zinc absorption to be 18% in adult men and 25% in adult women, which may occur when the phytate:zinc mole ratio is more than 18 (IZiNCG et al., 2004). The difference between these prediction models highlights the need for more human studies on this topic. It is notable that other than the phytate:zinc mole ratio, the level of inhibition can also be affected by the presence of secondary cations, such as calcium and magnesium ions, as secondary cations can either displace $Zn^{2+}$ from InsPs' binding sites or form insoluble phytins in conjunction with $Zn^{2+}$(Forbes et al., 1984; Gemede, 2014; Walter et al., 2000).The impact of these interactions in humans is not yet fully understood, and while IZiNCG currently considers calcium and protein to be insignificant factors in predicting zinc absorption, the organization also emphasizes that more studies on the interactions of these nutrients with zinc and phytate during the digestion process are necessary. For example, in a study with forty human subjects, Fredlund et al. (2006) experimented by adding various levels of $InsP_6$ ranging from 0 to 1344 µmol to a low-phytate bread roll containing 47µmol zinc and 66.6 mmol calcium. The author found that zinc absorption decreased from 21.5% to 14.45% when the $InsP_6$ content in the roll reached a phytate:zinc mole ratio of 5.7:1 (260 µmol $InsP_6$/roll). By the time the phytate:zinc mole ratio surpassed 15:1, the overall zinc absorption was only 7.4%, which was much lower than what was predicted by WHO and IZiNCG.

Of the two types of dietary iron, heme and non-heme iron, dietary $InsP_6$ and $InsP_5$ have been implicated with decreased non-heme iron absorption (Reddy et al., 1996; Sandberg et al., 1999). As heme iron only comes from animal foods, there is concern for populations whose diet contains little to no meat, as a high proportion of phytate-containing grains may experience exacerbated iron deficiency. With regard to non-heme iron, $InsP_1$ and $InsP_3$ did not exhibit an inhibitory effect on absorption when they were the only InsPs in foods, but when present in combination with $InsP_5$ and $InsP_6$, the inhibitory efficacy was stronger than when only $InsP_5$ and $InsP_6$ were in the samples (Sandberg et al., 1999). This finding suggested that there could be a synergistic effect between $InsP_{5-6}$ and lower InsPs, where an iron ion at a high degree of covalency could bind with two or three different InsP molecules simultaneously, some of which could be $InsP_{3-1}$. The authors also remarked that different isomeric forms of $InsP_{3-1}$ could have exerted varying levels of effect in this case, as *myo*-1,2,6-inositol trisphosphate and InsPs with the 1,2,3-trisphophate grouping can form more stable complexes with $Fe^{3+}$. On the other hand, the negative effect of InsPs on iron absorption was not found in all studies, showing that this effect can be influenced by factors other than the levels of InsPs and iron that are yet to be identified (House and Welch, 1987; Simpson et al., 1981). Hallberg et al. (1987) have observed that iron phytate in foods can be present in notable quantities as monoferric phytate, which is well absorbed. Yet, when sodium phytate or a mixture of magnesium phytate and potassium phytate were added to the samples, iron absorption decreased, likely due to the formation of insoluble phytins.

Similarly, the inhibitory effect of phytate on calcium and magnesium ion absorption may be suggested, but experimental values have been inconclusive (Crea et al.,2006). Miyazawa et al. (1996) reported a negative effect on the apparent absorption of magnesium but not that of calcium in rats. Lönnerdal et al. (1989) observed inhibition of calcium ion uptake by $InsP_6$ and $InsP_5$, but not by $InsP_1$ and $InsP_3$. It is also unclear whether lowered absorption of these minerals actually translates into health concerns. A survey study by López-González et al. (2008) assessed that dietary phytate is not a risk factor of osteoporosis, and increasing phytate consumption can even be linked with higher bone mineral density.

In pig and poultry nutrition, phytase supplementation to the fodder has been demonstrated to improve amino acid digestibility, which implied that InsPs impedes amino acid absorption in the gut (Selle et al., 2000). The form of InsP can influence this effect, as the magnesium-potassium salt of $InsP_6$ showed a higher inhibitory strength than free $InsP_6$ (Onyango et al., 2008). $InsP_6$ has also been shown to inhibit several digestive enzymes such as α-amylase, lipase, and various proteinases (e.g., pepsin, trypsin, chymotrypsin) in fish and poultry (Denstadli et al., 2006; Khan and Ghosh, 2013; Liu et al., 2010). These effects may be explained by the non-specific bindings of InsPs with cationic moieties of amino acids and proteins, followed by the precipitation of these complexes at low pH levels. In additional, ternary protein–mineral–phytate complexes may form, with a divalent mineral cation serving as the cationic bridge between phytate and an anionic moiety of a protein. Examples of these anionic moieties include the unprotonated imidazole group of histidine and the ionized carboxyl groups of other proteins (Selle et al., 2000). The solubility of these complexes is also dependent on pH. Among soluble mineral–phytate complexes and ternary protein–mineral–phytate complexes, an equilibrium may exist. Preference for either type of complex is influenced by ion concentrations as well as the degree of strain placed on the inositol ring by each type of complex (Champagne et al., 1990). For instance, the equilibrium involving calcium ion can be illustrated as follows:Protein + Ca + Phytate ⇌ [Protein–Ca–Phytate] ⇌ [Ca-Phytate] + Protein

From these perspectives, InsPs appear to be generally antinutritional. Breeding, genetic modification, germination, soaking, heat treatment, and fermentation are methods that have been investigated and recommended to reduce InsP levels in plant foods. On the other hand, it must be noted that InsPs are only some among many dynamic factors that can interact with each other in the digestive tract to affect mineral absorption. For instance, the inhibitory effect of InsPs on zinc ion absorption can be reduced with dietary protein, while administration of ascorbic acid with a phytate-containing meal has been demonstrated to improve iron absorption (Davidsson et al., 1994; Sandström et al., 1989; Siegenberg et al., 1991). Regarding magnesium, many unrefined plant foods rich in phytate are also rich in this mineral, and it has been argued that the eventual amount of magnesium absorbed from these foods may be equal to or higher than from low-phytate containing foods (Levrat-Verny et al., 1999). In rats, resistant starch has been shown to be able to negate the effects of phytic acid on the absorption of iron, zinc, and copper (Lopez et al., 1998). The authors suggested that a potential mechanism behind this effect is the production of short-chain fatty acids (SCFA) during the fermentation process, which adjusts the pH of the environment. Additionally, these SCFA can compete with InsPs to form complexes with mineral cations, which may be low in charge and can enter the enterocytes.

The dynamic of $InsP_6$ protonation and complex formation is difficult to study and summarize because it is strongly affected by several factors at the same time. The molecule can donate up to twelve protons and exhibits multiple acid dissociation constants across a wide range of pH values, depending on the ionic strength of the medium and the presence of other ions (Crea et al., 2008; Heighton et al., 2008). The stability and solubility of the complexes formed between $InsP_6$ and any type of ion are also affected by these aspects. Meanwhile, the condition of the digestive system can vary strongly. The average pH level in the human gastrointestinal tract varies between 1 and 7.5, with possible modifications caused by foods consumed in the meal (Evans et al., 1988; Kong

and Singh 2008). Taking these effects into account, improving mineral absorption by maintaining a diverse diet might be a more positive approach to avoid mineral deficiency than reducing InsP contents in foods. Some amount of dietary InsPs may be important, because InsPs' general capability to chelate mineral ions may help reduce the absorption of toxic trace elements such as cadmium and lead (Schlemmer et al., 2009). Moreover, new findings on the roles of InsPs in normal body functions and disease states propose that InsPs may be essential and beneficial to human health.

### 3.2. Inositol phosphates as participants in cell functions and the role of dietary inositol phosphates

In recent decades, insights on the importance of InsPs to eukaryote cells have emerged. Ins(1,4,5)$P_3$, Ins(1,3,4)$P_3$, Ins(1,3,4,5)$P_4$, and Ins(1,3,4,5,6)$P_5$ have been found to work dynamically in different $Ca^{2+}$ signaling pathways (Irvine and Schell, 2001; Luckhoff and Clapham, 1992; Streb et al., 1983; Tsubokawa et al., 1996; Vajanaphanich et al., 1994). Both *myo*-inositol-1,3,4,5,6-pentakisphosphate and $InsP_6$ have important roles in mRNA export, genomic stability, and apoptosis (Loss et al., 2015; Majerus et al., 2008; Monserrate and York, 2010). More than half of the 63 possible InsP isomers have been identified in various types of cell (Irvine and Schell, 2001; Shears, 1998). Different reactions by InsP pools to specific stimulations imply that these compounds may actually have many more functions to be investigated (Irvine and Schell, 2001; Otto et al., 2007).

Many different pathways for the synthesis of InsPs by cells have been discovered in plants, molds, and animals. Phosphatidylinositol 4,5-bisphosphate can be hydrolyzed into Ins(1,4,5)$P_3$, which, together with inositol, can be rapidly converted by plant and slime mold cells into other InsPs through a series of positionally selective and sequential kinase reactions. In mammalian cells, glucose-6-phosphate is believed to serve as the precursor for Ins(1,4,5)$P_3$, providing a second pathway that is independent of inositol lipid metabolism (Irvine and Schell, 2001; Sasakawa et al., 1995). Yet, both pathways have been found to proceed at extremely slow rates, despite some evidences indicating that the conversion of Ins(1,4,5)$P_3$ into $InsP_6$ in cells can be very fast. The synthesis of InsPs in animals remains an ongoing topic for examination.

It is also worthwhile to mention a class of molecules closely related to InsPs, namely inositol pyrophosphates (PPx-InsPs). Using $InsP_6$ as precursor, these compounds are synthesized by linking additional phosphate(s) to an existing phosphate group on the inositol ring via pyrophosphate bond(s) (Williams et al., 2015). So far, a few members of this family have been described, including several stereoisomers of triphosphoinositol pentakisphosphate (PPP-Ins$P_5$), diphosphoinositol pentakisphosphate (PP-Ins$P_5$), bis-diphosphoinositol trisphosphate ($(PP)_2$-Ins$P_3$) and bisdiphosphoinositol tetrakisphosphate ($(PP)_2$-Ins$P_4$). These compounds are sometimes abbreviated as InsP$_7$ and InsP$_8$, although these names fail to signify the important presence of the high-energy diphosphate or triphosphate chain(s). Similar to InsPs, PPx-InsPs are ubiquitous in eukaryote cells and have been inferred to participate in many cell functions, including energy sensing, inorganic phosphate sensing, and immune response (Williams et al., 2015; Wilson et al., 2013). Because PPx-InsPs are present in very small amounts, dietary PPx-InsPs are unlikely to be absorbed at significant levels by the human body. Consequently, the body may have to rely on the synthesis of these compounds from $InsP_6$.

At the moment, the contribution of exogenous sources to the maintenance of InsP pools in the human body is also not fully understood. Depending on the pH, InsPs can be negatively charged, which hinder their absorption in the gut. The small size of the inositol ring also leads to a high-charge density. So far, no carrier has been detected for InsPs in the human digestive track. Nevertheless, existing studies suggest that cellular intake of these compounds is possible and that InsP levels in the body are influenced by dietary intakes.

Grases et al. (2002) have demonstrated that in rats fed with different levels of $InsP_6$, the concentration of extracellular $InsP_6$ (in plasma and interstitial fluid) increases with dietary intake, while the concentration of extracellular $InsP_5$ is not significantly affected. In the same study, human epithelial and mesenchymal cells grown in culture media with inositol reached an $InsP_6$ concentration of ~46 μmol kg, whether or not $InsP_6$ was supplied in the media, indicating the cells' capability to synthesize $InsP_6$ from inositol. When these cells were treated with 1.8 mM $InsP_5$ for 1 hour, the intracellular $InsP_6$ content did not change, whereas the intracellular $InsP_5$ content doubled. This effect was attributed to dephosphorylation activity by the cell, which varies in rate depending on cell type (Vucenik and Shamsuddin, 1994). The concentrations of $InsP_5$ and $InsP_6$ in plasma fluid and in cells were thus concluded to have partial links to exogenous $InsP_6$.

By monitoring $InsP_6$ intake through diet and administration of oral doses, Grases et al. (2001) showed that the plasma level and urinary excretion of $InsP_6$ in humans are affected by oral ingestion in both the short and long term. A positive correlation was detected between these two measurements. Under the test conditions, the urinary excretion levels were always much lower than the intake level and would plateau at a certain $InsP_6$ dose, indicating that humans have a limited ability to absorb and or excrete $InsP_6$. Another study also found that $InsP_6$ absorption is independent of the condition of the stomach (whether empty, empty with an alkalinizer, or full), suggesting that absorption may occur during intestinal transit (Grases et al., 2006).

Using the ileostomy model, Agte et al. (2005) were able to describe the apparent absorption of all six InsPs from traditional Indian vegetarian meals. The level of apparent phosphorus absorption was found to increase from $InsP_3$ to $InsP_6$, although it must be noted that higher InsPs contributed more phosphorus than lower forms due to their higher relative abundance. $InsP_1$, which was consumed in minimal amount, was completely degraded, while absorption for phosphorus from $InsP_2$ only reached 64%. For $InsP_{3-6}$, which were responsible for ~96% of the phosphorus amount bound by InsPs in these meals, the % phosphorus absorption varied between 29 and 39. The net absorption of InsP phosphorus from these meals was 35%. As only three subjects were able to complete the trial (two men and one woman), this study was severely limited by sample size. For $InsP_{3-6}$ output results, it was not possible to distinguish between the amount of InsPs retained from the original meals and the quantity of InsPs generated by the degradation of higher forms. Nevertheless, these results still suggest that InsPs might be degraded and absorbed at a significant degree in the human digestive system.

In a controlled trial with Korean women, Joung et al. (2007) found that the degradation of dietary $InsP_5$ and $InsP_6$ is greater in elderly women compared to that in young women. A rise in dietary phytate levels also induced a significant rise in phytate excretion in young women, but not in elderly women. In both subject groups, phosphorus absorption was not affected when phytate became the main dietary phosphorus source. These results imply that the human body may have a mechanism to alter phytate degradation activity in the digestive tract according to its needs.

It is important to remember that biological samples are particularly complex to analyze, and some of the studies mentioned above used indirect assays for the measurement of InsPs, which might

result in inaccurate detection. By using a specific assay, Letcher et al. (2008) have found $InsP_6$ to be undetected in the serum and platelet-free plasma (<1 nM $InsP_6$) as well as in the urine (<5 nM $InsP_6$), suggesting that $InsP_6$ was unlikely to be absorbed in the gut. Wilson et al. (2015) also reached this conclusion when they applied a different specific assay on the same types of samples. As a result, the absorption of dietary InsPs still requires further investigation, especially with direct analysis methods.

### 3.3. Inositol phosphates' disease fighting abilities

Recently, many findings have highlighted InsPs' disease fighting capabilities. These include the prevention of pathological calcification, antioxidative activities, and anticancer effects. $InsP_6$ was first considered as an agent against pathological calcification in hypercalcuria patients due to its possible interference with calcium absorption (Nassim and Higgins, 1965). Later, $InsP_6$ was found to have strong crystallization inhibiting property against these unwanted calcium salts, which allows it to be effective even at a relatively low concentration. Experimentation with synthetic urine has demonstrated that phytic acid can completely stop the growth of calcium oxalate stones, which is the predominant form of kidney stones (Costa-Bauzá et al., 2005). In animal models, $InsP_6$ has been shown to reduce calcium deposition in kidneys and related organs, as well as slow down the growth of dystrophic calcification plaques in the cardiovascular system (Grases et al., 1996; Grases et al., 2004b). In humans, lower urinary concentration of $InsP_6$ has been associated with active calcium oxalate stone-formers, and high intake of dietary $InsP_6$ has been suggested to decrease the risk of kidney stones in these subjects (Conte et al., 1999; Curhan et al.,2004; Grases et al., 2000). As a crystallization inhibitor, $InsP_6$ may contribute to the prevention of sialolithiasis and dental calculus formation (Grases et al., 2009; Grases et al.,2003).

InsPs may exert their antioxidative property via generic as well as specific chelating activities. Other than binding iron and copper ions in general, some InsPs has the 1,2,3-trisphosphate arrangement which, upon interacting with $Fe^{3+}$, would occupy all six coordination sites of this ion with hydroxyl groups (Phillippy and Graf, 1997). As a result, these compounds inhibit iron's catalytic action on lipid peroxidation and hydroxyl radical formation in the Fenton reaction. A similar effect has been observed with ferrous ion. Phytate can be used to prevent myoglobin oxidation in meat processing, which changes the color of homogenized meat from red to brown (Park et al., 2004; Stodolak et al.,2007). From *in vitro* studies on rodent liver, Rimbach and Pallauf (1998) did not find phytate to have the capability to reduce oxidation markers in rats, while Bhowmik et al. (2017) found that $InsP_6$ significantly reduced the oxidative stress caused by iron overload in mice. Intravenously-injected phytate appears to provide some degree of myocardial protection to rats subjected to cardiac excision, but not to rat hearts that were removed from hypothermic storage (modeling a heart transplant) (Kazimoglu et al., 2004; Rao et al.,1991). These results suggest that InsPs' performance as antioxidants *in vivo* may be limited to specific working conditions and routes of administration (Iemma et al., 2007).

$InsP_6$ has demonstrated a remarkable preventive effect on tumor development in different cancer cell lines, namely blood, colon, liver, lung, mammary, melanoma, pancreas, prostate, skin, soft tissue, and uterine cervix (Vucenik and Shamsuddin, 2006). Multiple effects beyond antioxidant activity have been recognized, such as the inhibition of cell proliferation, metastasis, angiogenesis, and inflammation (Gu et al., 2010;Kumar et al., 2004;Norazalina et al., 2010; Rana et al., 2013). $InsP_6$ has been shown to support apoptosis as well as the differentiation and maturation of malignant cells, which can recover the normal phenotype (Delthiers et al., 2002;Shamsuddin and Yang, 1995; Singh et al., 2003; Vucenik et al.,2004). Some studies observed that $InsP_6$ enhances the activity of natural killer cells and reduces the depression of these cells by carcinogens (Baten et al., 1989; Zhang et al., 2005). These activities vary depending on dose, time, and the origin of the cells. A synergistic effect upon combination with inositol has also been suggested in a number of animal studies on different cancer types (Shamsuddin et al., 1989; Vucenik et al., 1995).

Dietary phytate exhibits an antineoplastic effect in non-ruminant animal models equally well whether consumed in a fluid or non-fluid system, highlighting the possibility of effective absorption mechanisms for extraneous InsPs that do not depend on microbial phytases. However, a human's ability to absorb InsPs cannot be directly extrapolated from rodent models because phytase activity in our tissue is ~30 times lower than that of rat tissues (Iqbal et al., 1994). On the other hand, it has been hypothesized that the anticancer activities of $InsP_6$depend on the compound's dephosphorylation to lower InsPs, even though the exact metabolism pathways and mechanisms of action still need to be elucidated (Vucenik and Shamsuddin, 2006). From this point of view, extraneous $InsP_{1-5}$ may also play important roles against cancer.

In mouse, $InsP_6$ has demonstrated a protective effect against Alzheimer's disease by decreasing lipid peroxidation and increasing cytochrome oxidase levels in the brain (Anekonda et al., 2011). In the same study, the compound was found to prevent the apoptosis of neurons by protecting MC65 cells against cytotoxicity by amyloid-β peptide. This action is opposite to the effect $InsP_6$ exerts on malignant cells, which has been described above, implying that $InsP_6$ is likely to serve as an apoptosis modulator. Similar protective activities have also been observed in other cell conditions. In studying the rat mesencephalic dopaminergic cell line (N27), Xu et al. (2008) reported that $InsP_6$ decreased pathological apoptosis induced by 1-methyl-4-phenylpyridinium and increase cell viability, suggesting that $InsP_6$ may offer protective therapy for Parkinson's disease. In pig small intestines, $InsP_6$ was found to reduced cell proliferation, apoptosis, and cyclooxygenase-2 expression, which are associated with inflammation (Silva et al., 2014a). The compound also protected cells against hypoxia-induced morphological changes. These effects were attributed to $InsP_6$'s antioxidant property, which may have inhibited the production of cytosolic reactive oxygen species. Notably, $InsP_6$ from rice was found to be more efficient than $InsP_6$ from corn, which was hypothesized by the authors to be due to the different numbers of sodium and water moieties attached to each $InsP_6$ molecule between the two sources (i.e., $InsP_6 \cdot 12Na \cdot 8H_2O$ from rice versus $InsP_6 \cdot 11Na \cdot 7H_2O$ from corn).

Some positive effects of phytate on type-2 diabetes mellitus have been suggested, as phytate intake has been found to negatively correlate with blood glucose response. In mice fed a high-fat diet, $InsP_6$ exerted an antihyperglycemic effect by regulating the activities of glucose-6-phosphatase, glucokinase, and phosphoenolpyruvate carboxykinase (Kim et al., 2010). Larsson et al. (1997) also demonstrated that $InsP_6$ may play a role in regulating insulin secretion, possibly by inhibiting the activity of serine–threonine protein phosphatases, thus opening intracellular calcium channels and leading to insulin release. $InsP_5$ could exert similar effects on calcium ion channels, but required a higher amount than $InsP_6$, while $InsP_4$ was not able to provide any action of the type.

Jariwalla et al. (1990) reported phytate to significantly lower serum cholesterol and triacylglycerol levels. A lower serum zinc level and zinc–copper ratio were observed in conjunction with these effects. Zinc and copper ions share the same mucosal carrier systems, and excess zinc in the diet can lead to reduced copper

absorption, which has been hypothesized to cause coronary heart disease (Klevay, 1975). Because $InsP_6$ has a greater binding affinity for zinc than copper ions, its presence may have helped in lowering the zinc copper ratio (Persson et al., 1998). However, in experimenting with rats fed a high-sucrose diet, Onomi et al. (2004) noted that the hepatic concentrations of total lipids and triacylglycerols, as well as the hepatic activity of glucose-6-phosphate dehydrogenase, were reduced when the sodium phytate level in the feed was as low as 0.02%. Meanwhile, the serum zinc level was not affected until the sodium phytate content reached 2.5%. These authors also detected reductions in cholesterol level and fatty acid synthetase activity, but the effects did not show a clear dose-dependent relationship. As a result, the mechanisms behind the reductive effects of dietary phytate on serum cholesterol still need to be investigated.

Several *in vitro* studies have been conducted on InsP 's antiviral potential on the human immunodeficiency virus (HIV). The compound was found to inhibit the cytopathic effect of this virus and the expression of the HIV-specific antigen (Kumar et al., 2010). In a T-cell line, $InsP_6$ was also shown to be able to suppress the replication of HIV-1 (Otake et al., 1999). Recently, Tateishi et al. (2014) found $InsP_6$ to be able to tightly bind the matrix domain of the $Pr55^{Gag}$ protein, which is a critical component for the assembly of the HIV-1 virus. Subsequently, these authors coupled $InsP_6$ with diacylglycerol moieties to synthesize *myo*-phosphatidylinositol 2,3,4,5,6-pentakisphosphate derivatives. These derivatives were found to have an even stronger binding capacity with the $Pr55^{Gag}$ protein's matrix domain, suggesting a potential molecular design for anti-HIV agents.

The effects of $InsP_6$ against mycotoxins have been investigated, with some results. $InsP_6$ has been shown to impede the histopathological alterations caused by aflatoxin B1 in albino rats, which include heightened oxidative stress and decreased reproductive function (El-Saad and Mahmoud, 2009). In swine, $InsP_6$ exhibited protective effects on the cytoplasmic membrane of intestinal cells when these cells were exposed to deoxynivalenol and fumonisin B1 (Silva et al., 2014b).

Because there is no unambiguous evidence that dietary InsPs can be absorbed, it is still unclear as to whether InsPs in foods can provide significant disease prevention. Yet, the growing number of findings pertaining to the physiological functions and potential benefits of InsPs promise that much remains to be learned about these compounds, and there is a need to separate and quantify InsPs in foods. While accurate and sensitive methods are becoming more available, the diversity of food and biomedical systems also require that modifications be investigated for each sample type.

## 4. Methods of separation and quantification for InsPs

Although $InsP_6$ was identified as far back as 1903, the detection and quantification of InsPs have been of continuous interest for researchers to this day. These compounds do not possess a characteristic spectrophotometric absorbance maximum and are available only at relatively low concentrations. Currently, an optimal method for the complete separation and measurement of all InsPs with their isomers has yet to be developed.

### 4.1. Non-specific methods

Heubner and Stadler (1914) introduced a non-specific method for the determination of phytic acid in ground cereal powder (Rather, 1917). In this method, phytic acid is extracted with hydrochloric acid (HCl) and then titrated with a ferric chloride solution in the presence of ammonium thiocyanate to form a pink-colored chromogen. The quantity of $Fe^{3+}$ that reacts is used to calculate the phytic acid content. Because ferric phytate forms as a white precipitate, the exact end point of this titration is difficult to detect. Furthermore, the mole ratio between $Fe^{3+}$ and $InsP_6$ in the complex is inconsistent. Using the assumption that the total phosphorous content of the sample would originate from phytate, McCance and Widdowson (1935) later modified this method by quantifying the phosphorus content of the ferric phytate precipitate instead of $Fe^{3+}$. A major disadvantage of assays that depend on ferric phytate is the overestimation of phytic acid in samples with high contents of inorganic phosphorus and/or non-InsPs organic phosphates, as these compounds can also precipitate with the ferric ion. Even today, similar research continues. Burgos-Hurtado and Tong (2015) recently described a method based on the back-titration of ferric phytate with EDTA for the measurement of phytic acid in fluid food samples. McKie and McCleary (2016) also reported a modified colorimetric molybdenumblue assay to measure the amount of inorganic phosphate released from extracted phytic acid of foods and animal feeds as a result of dephosphorylation with phytase and alkaline phosphatasetreatment.

To eliminate inorganic phosphorus from samples, researchers have attempted to develop quantitative methods based on ion-exchange interactions. Most notable is a method by Harland and Oberleas (1977), in which the supernatant from an HCl extraction of the sample is placed on an AG 1-X8 chloride form anion-exchange column. The column is rinsed with water and 0.05 M NaCl to elute inorganic phosphates. Organic phosphates trapped on the resin are then eluted with 0.7 M NaCl. Assuming that phytates accounts for all forms of organic phosphates in the sample, an acid digestion of the final fraction followed by the measurement of inorganic phosphates via the Fiske-Subbarow colorimetric method can be used to infer the phytate content. In the Fiske-Subbarow assay, the sample is treated with ammonium molybdate to form phosphomolybdate, which can be reduced to molybdenum blue in the presence of a reductant, such as aminonaphthosulfonic acid (Goldenberg and Fernández, 1966). The intensity of the blue-colored chromogen, with maximum absorbance at $\lambda_{max}$ 640 nm, is directly correlated to the concentration of phosphate in the sample. In an improvement of this method, Ellis and Morris (1983) added EDTA and NaOH to prevent the binding of $InsP_6$ to proteins and metal ions in the extraction. This version was later adopted as the official AOAC method (986.11) for determining phytate in foods (AOAC International 2005).

Due to the non-specificity of this chromatographic method, the $InsP_6$ concentration can still be heavily overestimated in samples where $InsP_{1-5}$ make up more than 20% of the InsP content (Ehrfeld and Morris 1992). Additionally, sugar phosphates, phospholipids, nucleotides, and phosphate derivatives of thiamine also contribute to the organic phosphate levels in foods. Overestimation problems are of particular importance, because foods and diet patterns reported to have high phytic acid contents would often be associated with mineral deficiency concerns.

### 4.2. Specific methods

While working with soil, Smith and Clark (1952) were able to extract $InsP_{3-6}$ from hydrolyzed sodium phytate via anion-exchange chromatography, utilizing a weak-base exchange resin and stepwise elution with increasing HCl concentration (Cosgrove, 1963a). The content of each InsP was deduced via the phosphorus–inositol ratio in each collected fraction. Later, Cosgrove (1963b) demon-

strated that utilizing the strong anion exchange resin AG 1 for this method allowed for separation of different $InsP_5$ isomers. The author, however, did not perform a quantification of InsPs in food samples.

To achieve a shorter elution time, Tangendjaja et al. (1980) then applied high-performance liquid chromatography (HPLC), particularly reversed-phase chromatography, using a μBondapak C18 column and sodium acetate as mobile phase. Graf and Dintzis (1982) later improved this method by adding a purification step with the AG 1 resin after extraction. With both methods, however, the retention time of $InsP_6$ was less than 2 min and other InsPs could not be identified. In a modification, Sandberg and Ahderinne (1986) used formic acid/methanol as the mobile phase and added tetrabutylammonium hydroxide as the ion-pair reagent, thus affording an increase in the retention time and differentiation between $InsP_{3-6}$, although their stereoisomers were still indiscriminable.

Around this time, the application of gas chromatography (GC) in InsP analysis was also explored. Heathers et al. (1989) derivatized InsP fractions collected from HPLC separation into hexatrimethylsilyl derivatives for quantification by GC, and successfully applied this method to isomers of $InsP_{1-3}$. The need for a derivatization process and the inability to measure $InsP_{1-6}$ are considered drawbacks for this method.

Overall, HPLC is still the most common separation technique for InsPs due to its versatility. While ion-pair reversed-phase HPLC has been succesfully used to separate InsPs, anion-exchange HPLC has become more popular due to its ease of use. In recent decades, many different mobile phase gradients and anion-exchangers for HPLC have been applied to separate InsPs down to their isomers. Nevertheless, it remains that $InsP_{3-6}$ are better eluted with an acidic mobile phase while $InsP_{1-2}$ prefer an alkaline mobile phase (Schlemmer et al., 2009). Good separation of all InsPs and their isomers is unlikely to be achieved in the same run, while time-effective separation of $InsP_{1-6}$ in one run will sacrifice information on isomers. It should be noted that a high pH level may reduce the life time of a silica-based stationary phase; when an alkaline mobile phase is utilized, a polymer-based column is preferable. Therefore, there might be some limitations to the choice of column for the sensitive detection of $InsP_{1-2}$ by HPLC (Sjöberg et al., 2016).

Taking advantage of the exchange of $Fe^{3+}$ from the ferric sulfosalicylic acid complex to phytate, Latta and Eskin (1980) developed a colorimetric detection method using a modified Wade reagent. Reaction with phytate in a sample extract would result in a loss in the absorbance at $\lambda$ = 500 nm for this purple reagent. Rounds and Nielsen (1993) then modified this assay by replacing the AG 1 resin in the preceding method with another polystyrene-based strong anion-exchanger equipped on HPLC. Effluent from the analytical column was combined with a post-columnreagent of 0.015% $FeCl_3 \cdot 6H_2O$ (w/v) plus 0.15% (w/v) sulfosalicylic acid; this allowed for decrease absorbance readings at $\lambda$ = 500 nm to be monitored as the eluting phosphates complexed with iron in the reagent. Using this approach, $InsP_{2-6}$ could be separated within 30min. Recently, Agostinho et al. (2016) introduceda similar post-column detection method based on the inhibitory effect by $InsP_6$ on the formation of the red complex between glyoxal bis(2-hydroxyaniline) and calcium ions in alkaline.

In 1988, another post-column dye-detection method was developed based on the exchange of yttrium ionfrom the $Y^{3+}$-4-(2-pyridylazo)resorcinol complex to InsPs, which reduces the absorbance at $\lambda$ = 546 nm of this colored complex (Mayr, 1988). In a modified method utilizing the same approach, Guse et al. (1993) were able to quantify $InsP_{3-6}$ in cell extracts with high sensitivity: the detection limits were approximately 15 pmol for $InsP_3$, 10 pmol for $InsP_4$, 5 pmol for $InsP_5$, and 1 to 3 pmol for $InsP_6$. Yet, this method requires great care to avoid contamination of the eluent with ions such as $Fe^{3+}$ and $Zn^{2+}$, as these ions can complex with both InsPs and 4-(2-pyridylazo)resorcinol.

Ligand exchange reactions between InsPs and fluorescent complexes have also been introduced, including the use of a ferric methylcalcein blue complex, copper(II) gelatin complex, and copper(II) 2,2′-bipyridine complex (Cao et al., 2011; Chen et al., 2007; Irth et al., 1990). Interestingly, $InsP_6$ has been found to activate the oxidation of 1,1′-dipyridyl ketone hydrazone (DPKH) in the presence of $Cu^{2+}$, resulting in an intense fluorescent product (March et al., 1999). The authors suggest that the copper(II) phytate complex may have heightened catalytic activity compared with $Cu^{2+}$ alone on the reaction between DPKH and oxygen. This method is useful for the detection of phytic acid in foods and urine.

A method for on-line detection of InsPs has always been in demand. In qualitative studies on cells and tissue, a number of authors have chosen to radiolabel their samples with *myo*-[$^3$H]inositol or [$^{31}$P]$PO_4^{3-}$. In working on several isomers of $InsP_{1-4}$, Taylor et al.(1990) coupled this technique with separation via HPLC for the quantitation of InsPs. They successfully recorded changes in the distribution of the analytes overtime, proposing a potential approach for the study of InsPs in biological processes, such as cellular signaling. Due to the lack of absolute concentration data, this method is considered unsuitable for many research purposes. Furthermore, it is laborious and less sensitive than some off-line analyses. Instead, several studies have investigated the application of chemically-suppressed conductivity and the researchers were able to separate different phosphorylated organic compounds, including InsPs, in both physiological and food samples (Smith and MacQuarrie, 1988; Talamondet al.,2000). Notable is the coupling of isotachophoresis and zone electrophoresis with contact conductivity, as demonstrated by Kvasnička et al. (2011).

When multiple InsPs are being identified or when matrix effects can cause the elution times of InsPs to deviate from those of standard compounds, reliance on retention time for detection in HPLC methods can also risk inaccurate interpretation. Nuclear magnetic resonance (NMR) spectroscopy, atomic emission spectroscopy (AES), and mass spectrometry (MS) are among detection techniques that can reduce this risk in susceptible samples. $^{31}$P-NMR spectroscopy is prized as a noninvasive and highly specific method for InsP determination. Because it can be applied on intact tissues and cell suspensions, it is especially suitable for the study of InsP metabolism, binding activities, and degradation (Heighton et al., 2008; Zhuang et al., 2010). Alternatively, some authors have used $^1$H-NMR to study the interactions between $InsP_6$ and paramagnetic metal ions such as ferric and ferrous ions (Heighton et al., 2008). NMR can be used to identify and confirm stereoisomers that often coelute during chromatographic separation (Phillippy, 1989). The disadvantages of NMR, however, are that the technique is relatively insensitive, cost-prohibitive, and spectra can be very complex (Kemme et al., 1999; Schlemmer et al., 2009).

In 1996, a method using inductively coupled plasma (ICP) combined with AES for $InsP_6$ determination in urine was reported, but the sample treatment process is long, complicated, and requires a large amount of sample (Grases and Llobera, 1996). Later, a more efficient and sensitive method was developed, attaining a limit of detection of 64 μg/L and limit of quantification of 213 μg/L (Grases et al.,2004a). Although still less sensitive than ICP coupled with MS, this method is considered suitable for routine determination. A preliminary study by Amaro et al. (2004) suggested that coupling HPLC with ICP-AES can improve sensitivity.

Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are powerful approaches to attain high sensitivity and can be

coupled with HPLC, GC, and ICP for different purposes. ICP-MS is suitable for biomedical samples such as urine (Muñoz and Valiente, 2003). For food samples, the use of sector field ICP-MS has been investigated with initial success (Helfrich and Bettmer, 2004). GC-MS has also been applied for food and biological samples, such as rat organs, human plasma, urine and kidney stones (de Koning, 1994; March et al., 2004), but this method still requires a derivatization step of InsPs to trimethysilyl derivatives. HPLC-MS can achieve comparable results without calling for derivatization of the sample (Perelló et al., 2004). Further modification may even eliminate all together the need for a separate chromatographic extraction step preceding detection. Using negative mode electrospray ionization (ESI) with anion-exchange HPLC-MS/MS, Liu et al. (2009) were able to simultaneously separate and quantify $InsP_{1-6}$ from various foods and cell samples, with sensitivity reaching as low as the pmol range and elution time of less than 30 min. This can be considered one of the most efficient methods for measuring InsPs in samples without regard to stereoisomers. A similar method was later successfully applied for the qualitative detection of InsPs from environmental matrices, including soil, manure, and aquatic sediments (Sjöberg et al., 2016). Recently, Zhang et al. (2017) introduced a method coupling ion-pair chromatography with ESI-MS/MS that could detect $InsP_{1-6}$ within 15 min of sample injection and achieved very low limits of detection for $InsP_{1-5}$ (less than 1 pmol). Another notable method which utilized MS/MS was described by Shelor et al. (2015), in which hydroxide eluent ion chromatography was coupled with MS/MS. In addition to simultaneously detecting $InsP_{1-6}$, this method was able to fully separate several stereoisomers of $InsP_{2-6}$ in a 50-min run. Unfortunately, the authors did not attempt to identify the structure of these isomers. An advantage of using MS/MS compared to ICP-MS is the potential of discriminating structural isomers based solely on mass spectrometry information. Utilizing positive-ion mode ESI with low-energy collisional-activated dissociation MS/MS, Hsu et al. (2003) were able to differentiate the stereoisomers of $InsP_3$, $InsP_4$, and $InsP_5$ in bovine brain extract. This method was unable to identify the stereoisomers of $InsP_1$ and $InsP_2$, however, and how it can be applied for the quantification of $InsP_{1-5}$ isomers still needs to be examined.

## 5. Inositol phosphates in plant foods

The distribution of InsPs in plant seeds varies greatly, with the major concentration sites being the bran, germ, and the cotyledon. For cereals, $InsP_6$ has been found to occur mainly in the germ of corn, the aleurone of wheat, the bran of rice, and the germ and bran of pearl millets (O'Dell et al., 1972; Reddy et al., 1989). On the other hand, legumes deposit $InsP_6$ throughout the cotyledon. Further structural characteristic of InsP depositions can be attained via toluidine blue staining, where these compounds would appear red-violet under an optical microscope. In almond meal and hazelnuts, for example, these depositions have been found to be small pockets densely surrounded by protein, suggesting the storage location of InsPs in these foods (Andriotis et al., 2005; Dourado et al., 2004).

Existing data on the $InsP_6$ content of plant seeds have mainly reported only $InsP_6$ and the values have shown strong variations, especially with tree nuts, where the highest $InsP_6$ value is can be 6- to 10-fold higher than the lowest value in the range (see Table 1 and 2). Significant variations in cereal $InsP_6$ content due to cultivar, location, crop year, and fertilization have been observed in some but not all studies (Barrier-Guillot et al., 1996; Hídvégi and Lásztity, 2002; McCall et al., 1953; Nahapetian and Bassiri, 1976; Singh and Sedeh, 1979; Steiner et al., 2007). The contribution of InsPs phosphorus to total phosphorus content in plant seeds has been found to vary widely, ranging from 30 to 95% in cereals and legumes, and 25 to 75% in tree nuts (Deshpande, 2002; Lolas et al., 1976; O'Dell et al., 1972; Reddy et al., 1989; Rodehutscord et al., 2016). Steiner et al. (2007) have identified a positive correlation between $InsP_6$ phosphorus and total phosphorus content in some cereals and legumes, with the relationship stronger in legumes than in cereals.

It is important to note that drawing a general consensus on the occurrence of $InsP_6$ in food products is difficult because not all studies utilized specific methods and some studies have estimated the $InsP_6$ content by assuming that a certain percentage of the total phosphorus content comprises phytic acid, which disregards possible deviations due to cultivation and processing factors. Furthermore, it is unclear whether indirect assays measure $InsP_6$ as phytic acid or its salt forms (phytate phytin), which can vary in number and the type of mineral cation bound to the molecule. In light of the recent findings on the potential roles of different InsPs in human health, it has become important to accurately assess the InsPs levels in foods with specific methods.

Although information on the abundance of $InsP_{1-5}$ in plant seeds is still limited, some differences among different plants have been identified. Stepwise dephosphorylation of $InsP_6$ by phytases extracted from the bran of spelt, rye, barley, and oat have identified the products to be Ins(1,2,3,5,6)$P_5$, Ins(1,2,5,6)$P_4$, Ins(1,2,6)$P_3$, Ins(1,2)$P_2$, and Ins(2)P (Greiner and Alminger, 2001). With phytase from rice bran, dephosphorylation can result in the same products, as well as Ins(1,2,3,4,5)$P_5$, Ins(1,2,3,4)$P_4$, and Ins(1,2,3)$P_3$ (Hayakawa et al., 1990). Similarly, by using phytase extracted from wheat, the major hydrolysis pathway of $InsP_5$ would result the same products listed by Greiner and Alminger (2001), while a minor pathway may instead produce Ins(1,2,3,6)$P_4$ and Ins(1,2,3)$P_3$ as $InsP_4$ and $InsP_3$ products (Nakano et al., 2000). Lim and Jate (1973) have shown that Ins(1,3,4,5,6)$P_5$, Ins(1,2,3,4,5)$P_5$, Ins(1,2,3,4,6)$P_5$, Ins(1,2,3,4)$P_4$, and Ins(1)P may also be produced in wheat, suggesting that multiple types of phytase may be present. This effect has been observed with phytases from lupine seeds, in which the phytase LP2 produces Ins(1,2,3,4,5)$P_5$ and Ins(1,2,3,4)$P_4$ while the two phytases LP11 and LP12 produces Ins(1,2,4,5,6)$P_5$ and Ins(1,2,5,6)$P_4$. After generating these products, all three phytases would produce Ins(1,2,6)$P_3$, Ins(1,2)$P_2$, and Ins(2)P (Greiner et al., 2002).

Interestingly, in surveying multiple genotypes of barley, oats, rye, maize, triticale, and wheat, Rodehutscord et al. (2016) found Ins(1,2,4,5,6)$P_5$ to be present in all samples, whereas Ins(1,2,3,4,5)$P_5$ was only in barley, rye, triticale and wheat samples, and Ins(1,2,3,4,6)$P_5$ was only in rye, triticale, and wheat samples. $InsP_4$ was not identified in any sample. The only $InsP_3$ isomer detected was Ins(1,5,6)$P_3$, which was determined in selected genotypes of barley, maize, and rye. These findings are in agreement with previous results by Kasim et al. (1998), where $InsP_4$ was not detected in the grains of barley, corn, oats, sorghum, and wheat. The only published value of $InsP_3$ in cereal we have found was reported by Helfrich and Bettmer (2004) for corn (maize). We are also unaware of values pertaining to the levels of $InsP_4$ and $InsP_3$ in grains. Overall, cereal grains appear to be quite different from legumes and tree nuts, in which lower InsPs, including $InsP_{1-2}$ were usually detected (Table 3).

Soaking and/or boiling have been found to significantly decreased $InsP_{3-6}$ in both legumes and grains (El Tinay et al., 1989; Karle and Pelela, 2001; Lestienne et al., 2005a; Morris and Hill, 1996). In these processes, InsPs are hypothesized to be hydrolyzed

**Table 1. Content of myo-inositol hexakisphosphate in the grain/seed of major cereals and legumes**

| Sample | Taxonomic names | $InsP_6$ content (μmol/g)* |
|---|---|---|
| Cereal | | |
| Barley | *Hordeum vulgare* L. | 5.7–18.9[2,13,15,16,18,19,26,30] |
| Maize | *Zea mays* L. | 3.3–19.5[2,12,13,15–17,19,20,23,25,26,31] |
| Millet | *Pennisteum* spp. | 3.6–16.5[17,25,28,31] |
| Oats | *Avena sativa* L. | 6.3–21.5[6,11,13,15,18,19,26,30] |
| Rice | *Oryza sativa* L. | 4.7–16.4[6,17,19,23,25] |
| Rye | *Secale cereale* L. | 6.6–14.7[16,19,26,30] |
| Sorghum | *Sorghum* spp. | 5.5–19.8[15–17,19,20,24,25,31,33] |
| Triticale | x *Triticosecale* Wittm. | 3.5–15.2[16,19,26,29,29] |
| Wheat | *Triticum* spp. | 4.9–20.5[1,13,15,6,18,19,22,23,26,30] |
| Legume | | |
| Chickpeas | *Cicer arietinum* L. | 4.2–19.1[3,8,19,21,27] |
| Common beans | *Phaseolus vulgaris* L. | |
| Black beans | | 6.7–14.9[5,21] |
| Kidney beans | | 7.4–16.1[5,4,9,21,31] |
| Pinto beans | | 7.8–12.1[5,21,27] |
| Great Northern beans | | 12.7–13.1[5,21] |
| Navy beans | | 12.4–12.6[5,21] |
| White beans | | 7.4–25.8[8,27] |
| Cowpeas | *Vigna unguiculata* L. | 4.4–13.9[13,17,19–21,31] |
| Lentils | *Lens culinaris* Medikus | 3.7–15.9[3,12,19,21,27] |
| Mung beans | *Vigna radiata* (L.) R. Wilczek | 3.6–5.9[5,17] |
| Peas | *Pisum sativum* L. | 6.5–20.2[13,10,19,34] |
| Peanuts | *Arachis hypogaea* L. | 2.6–10.3[7,19,31,32] |
| Soybeans | *Glycine max* (L.) Merr. | 13.3–28.8[8,13,14,17–20,31] |

*$InsP_6$: *myo*-inositol hexakisphosphate. [1]Barrier-Guillot et al. 1996 [2]Blatny et al. 1995; [3]Burbanoet et al. 1995; [4]Carnovale et al. 1988; [5]Chen 2004; [6]de Koning 1994, [7]Dost and Karaca 2016; [8]El Tinay et al. 1989; [9]Ferguson et al. 1988; [10]Fredrikson et al. 2001; [11]Harland and Oberleas 1986; [12]Helfrich and Bettmer 2004; [13]Hídvégi and Lásztity 2002; [14]Karkle and Beleia 2010; [15]Kasim and Edwards 1998; [16]Lehrfeld 1989; [17]Lestienne et al. 2005a; [18]Lolas et al. 1976; [19]Lott et al. 2000; [20]Marfo et al. 1990; [21]Morris and Hill 1996; [22]Nahapetian and Bassiri 1976; [23]O'Dell et al. 1972; [24]Radhakrishnan & Sivaprasad 1980; [25]Ravindran et al. 1994; [26]Rodehutscord et al. 2016; [27]Simonet et al. 2003; [28]Simwemba et al. 1984; [29]Singh and Sedeh 1979; [30]Steiner et al. 2007; [31]Talamond et al. 2000; [32]Venkatachalam and Sathe 2006; [33]Wang et al. 1959; [34]Zdunczyk et al. 1997.

**Table 2. Content of *myo*-inositol hexakisphosphate in major tree nuts**

| Sample | Taxonomic names | $InsP_6$ content (μmol/g)* |
|---|---|---|
| Almonds | *Prunus dulcis* (Mill.) D.A. Webb | 5.3–32.0[1,2,4–10] |
| English walnuts | *Juglans regia* L. | 2.7–21.0[1,2,3,5,8,10] |
| Cashews | *Anacardium occidentale* L. | 2.3–29.8[1,3,5,8,10] |
| Brazil nuts | *Bertholletia excelsa* Humb. & Bonpl.: Lecythidaceae | 2.9–27.3[3,8,10] |
| Macadamias | *Macadamia integrifolia* Maiden & Betche | 2.3–14.3[1,3,5,10] |
| Pistachios | *Pistachia vera* L. | 3.0–43.0[3,5,8,10] |
| Pecans | *Carya illinoinensis* (Wangenh.) K. Koch. | 1.8–28.9[3,5,10] |
| Hazelnuts | *Corylus avellana* L. | 2.2–35.5[2,3,5-10] |
| Pine nuts | *Pinus* L. | 3.0–11.9[8,10] |

*$InsP_6$: *myo*-inositol hexakisphosphate. [1]Chen 2004; [2]Dost and Karaca 2016; [3]Duong et al. 2017a; [4]Duong et al. 2017b; [5]Harland et al. 2004; [6]Helfrich and Bettmer 2004; [7]Liu et al. 2009; [8]Lott et al. 2000; [9]Simonet et al. 2003; [10]Venkatachalam and Sathe 2006.

Table 3. Lower inositol phosphate contents in selected plant seeds[a]

| Sample | Lower inositol phosphate content (μmol/g) | | | | |
|---|---|---|---|---|---|
| | $InsP_5$ | $InsP_4$ | $InsP_3$ | $InsP_2$ | $InsP_1$ |
| Cereals | | | | | |
| Barley | N.d.–0.8[6,9] | N.d.[6,9] | N.d.–2.9[6,9] | – | – |
| Maize | 0.3–2.5[5,6,9] | N.d.–0.7[5,6,9] | 0.1–0.5[9] | – | – |
| Oat | 0.1–0.2[9] | N.d.[6,9] | N.d.[6,9] | – | – |
| Rye | 0.2–0.4[9] | N.d.[9] | N.d.[9] | – | – |
| Sorghum | N.d.–0.9[6] | N.d.[6] | N.d.[6] | – | – |
| Triticale | 0.2–0.4[9] | N.d.[9] | N.d.[9] | – | – |
| Wheat | N.d.–0.5[6,9] | N.d.[6,9] | N.d.[6,9] | – | – |
| Legumes | | | | | |
| Chickpeas | – 2.1[1,5,10] | 0.04–0.6[1,5,10] | <0.005–0.6[1,6,3] | 0.3[10] | 0.1[10] |
| Common beans | | | | | |
| Black beans | 0.5–3.2[4,5] | 0.1[3] | <0.005[5] | – | – |
| Great Northern beans | 0.6–2.2[4,5] | 0.2[3] | <0.005[5] | – | – |
| Navy beans | 0.6–1.8[4,5] | 0.1[3] | <0.005[5] | – | – |
| Pink beans | 0.6[4] | – | – | – | – |
| Pinto beans | 0.3–3.1[2,3,10] | 0.2–0.7[8,10] | <0.005–0.2[8,10] | 0.2[10] | 0.1[10] |
| Red kidney bean | 0.3–1.8[2,9,10] | 0.2[3] | <0.005[3] | – | – |
| White beans | 3.0[10] | 0.6[10] | 0.2[10] | 0.2[10] | 0.2[10] |
| Cow peas | 2.5[2] | 0.3[3] | 0.01[3] | – | – |
| Lentils | 0.2–3.2[1,5,8,10] | 0.1–0.8[1,5,8,10] | 0.3–0.4[1,8,10] | 0.3[10] | 0.2[10] |
| Mung beans | 1.2[2] | – | – | – | – |
| Tree nuts | | | | | |
| Almonds | 0.4–10.0[2,3,5,7,10] | 0.4–8.5[1,5,7,10] | 1.0–1.8[4,7,10] | 0.7–3.8[1,7,10] | <0.002–1.5[3,7,10] |
| Cashews | 1.3–6.6[2,3,5] | 0.3–1.2[3,5] | 0.2–0.5[3,5] | 0.07–0.2[3,5] | <0.002–0.2[3,5] |
| Hazelnuts | 0.4–1.2[2,5,7,10] | 0.2–1.1[3,7,10] | 0.01–0.5[3,7,10] | 0.04–0.5[3,7,10] | 0.003–0.3[3,7,10] |
| Macadamias | 0.6–1.2[2,3] | 0.2[3] | 0.02[3] | <0.002[3] | 0.06[3] |
| Pecans | 0.3–0.5[2,3] | 0.2[3] | 0.08[3] | 0.07[3] | 0.04[3] |
| Pine nuts | 1.5[10] | 1.1[10] | 0.7[10] | 0.9[10] | 1.1[10] |
| English Walnuts | 0.8–1.6[2,3] | 0.2[3] | 0.02[3] | <0.002[3] | 0.07[3] |

[a]$InsP_{1-5}$ are *myo*-inositol monophosphate, *myo*-inositol bisphosphate, *myo*-inositol trisphosphate, *myo*-inositol tetrakisphosphate, *myo*-inositol pentakisphosphate, respectively; N.d.: not detected and the author(s) did not specify the limit of detection; [1]Barre et al.1995; [2]Chen et al. 2007; [3]Duong et al. 2017a; [4]Duong et al. 2017b; [5]Hefnawi and Beltmer 2004; [6]Kasim and Edwards 1998; [7]Liu et al. 2003; [8]Morris and Hill 1996; [9]Rodehutscord et al. 2016; [10]Simonet et al. 2003

into inorganic phosphorus inside the grain. Phytases are expected to be quickly degraded upon heat application. Festienne et al. (2005b) found the inorganic phosphorus level to increase in soaked soybeans, whereas the soaking fluid showed no similar change or any significant phytase activity. Karle and Belela (2001) also demonstrated that heat application after soaking did not further decrease the $InsP_6$ content of soybeans compared to when only soaking was employed. Marfo et al. (1990) observed a significant decrease in the $InsP_6$ level in several cereals and legumes after fermentation. This effect declined after 48 hours, which might be due to the inhibition of phytase activity by low the pH or increased abundance of inorganic phosphate. In processed foods containing cereals and legumes, the InsP levels can be affected by the processes mentioned above as well as the loss removal of parts of the grain seed and the inclusion of other ingredients, leading to great variation even within the same product type. For example, $InsP_6$ has been found to range from <0.008 to 50 μmol g in ready-to-eat wheat cereals and <0.008 to 16.1 μmol g in wheat breads (Harland et al., 2004; Reddy et al., 1989).

## 6. Conclusions

InsP's and their abundances in plant foods have long been a con-

cern for nutrient absorption, especially for populations that may be more susceptible for nutrient deficiencies, including children, vegetarians, and residents in developing countries. This review has summarized some of the nutrients most affected by InsPs, as well as several factors that may influence InsPs capabilities to inhibit the absorption of this nutrition in the gut. Additionally, InsP roles in normal mammal biological functions, the potential for disease prevention, and the possibility for absorption from food sources were discussed. In each of these areas of interest, it is evident that many questions are still unanswered and further research is needed to improve the state of knowledge. Thus, more research on rapid and sensitive analytical methods to determine InsPs in food and biological systems is warranted.

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# $IP_6$ in Cancer Therapy: Past, Present and Future

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**Abstract:** Inositol hexaphosphate ($IP_6$) and its derivatives are naturally occurring, most abundant polyphosphorylated carbohydrates in organisms as diverse as yeast, endo-parasites, mammals and plants. $IP_6$ represents a major metabolic pool in cellular phosphate pathways involved in vital functions such as signal transduction, regulation of cell proliferation and differentiation, RNA export, DNA repair, energy transduction and ATP regeneration. $IP_6$ has been recognized as a natural antioxidant and agent preventing calcification and kidney stone formation, normalizing cholesterol concentrations, and reducing pathological platelet activity. The most striking effect of $IP_6$ has been documented in cancer prevention by controlling tumor growth, progression and metastasis. Its anti-cancer actions involve boosting immunity, antioxidant properties, reducing cell proliferation and inducing differentiation of malignant cells. This is brought about via lower-phosphate inositol phosphates ($IP_{1-5}$) and probably by the highly phosphorylated derivatives (PP-$IP_4$ and PP-$IP_5$); however the selectivity of its metabolite(s) in anti-cancer activity has not been adequately investigated. Preliminary *in vitro* studies show that $IP_6$ synergistically acts with conventional anti-cancer drugs like tamoxifen overcoming its resistance thus enhancing the anticancer effect of conventional chemotherapy. In this review we present anti-cancer abilities of $IP_6$ and its metabolites and discuss their future use as potential drug(s) for cancer prevention and therapy.

**Key Words:** IP6, inositol, phytate, cancer chemotherapy, nutritional supplement, nutraceuticals.

## $IP_6$ and its Metabolites-Structure and Function

Inositol was originally named inos (muscle in *Greek*) by Josef Scherer a German chemist, who more than 150 years ago isolated new molecule from muscle tissue [1]. The most abundant in nature is *Myo*-inositol, one of the nine inositol isomers. Inositol a simple hexa-carbon carbohydrate, derivative of glucose is an essential nutrient and a member of the B vitamins. When all of its six carbons are attached to phosphate groups, it is known as inositol hexaphosphoric acid ($IP_6$, Ins$P_6$, phytic acid). Almost all plant and mammalian cells contain $IP_6$ and its lower phosphorylated forms with fewer phosphate groups ($IP_{1-5}$). $IP_6$ is the most abundant inositol phosphate with intracellular concentrations of about 100 μM [2]. In mammals the highest levels of $IP_6$ has been found in brain tissue where it serves as a neurotransmitter. Recent reports demonstrate that inositol 1,3,4-trisphosphate 5/6-kinase (ITPK1) a crucial enzyme in the synthesis of $IP_6$ is also essential for neural tube and axial mesoderm development [3]. $IP_6$ is contained in high amounts in most cereals, grains, seeds, legumes; the richest sources being wheat bran and flaxseed (0.4–6.4%) [4]. In cereal grains $IP_6$ accumulates in the germ and aleurone layers [5]. In mature seeds it is preferentially localized as phytate salts of minerals such as K, Mg, Ca, Fe in inclusions called globoids [6]. Only *myo*-inositol hexaphosphate has been found in plants, while *neo*-, *chiro*-, and *scyllo*-inositol hexaphosphates have been isolated from soil [7]. The phosphate grouping in positions 1, 2, and 3 (axial-equatorial-axial) is unique for $IP_6$, providing a specific interaction with iron to completely inhibit its ability to catalyze hydroxyl radical formation, making $IP_6$ a strong physiological antioxidant. Contrary to water soluble inositol phosphates, phosphoinositides, which in addition to inositol and phosphates contain hydrophobic fatty acids, are water insoluble. Inositol and phosphates in conjugation with lipids are mainly present in cell membranes, as phosphatidylinositol. Cells respond to diverse extracellular stimuli from the environment and other cells by activating phospholipase C (PLC) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to generate inositol 1,4,5-$P_3$ ($IP_3$) and 1,2-diacylglycerol. $IP_3$ is the most extensively characterized inositol phosphate. It is known that $IP_3$ acts through allosteric activation of the $IP_3$ receptor that releases calcium from intracellular stores [8]. Furthermore, it has been shown that $IP_3$ serves as a precursor for other inositol phosphates, including $IP_4$, $IP_5$, $IP_6$ and inositol pyrophosphate, containing high energetic pyrophosphate (P-P) bonds, such as recently identified diphosphoinositol pentaphosphate ($IP_7$) and bi-diphosphoinositol tetraphosphate ($IP_8$) [2,8-11]. These inositol polyphosphates are continuously inter-converted in a rapidly turning over inositol phosphate pool [2,8,9,11] (Fig. 1).

Inositol phosphates have important roles in the regulation of diverse cellular activities [12]. $IP_6$ is a natural antioxidant [13] and a neurotransmitter [2]. The existence of receptors and binding proteins for inositol polyphosphates [14] indicate their importance in controlling various cellular functions, such as ion channels and protein trafficking [15,16], endocytosis [17], exocytosis [18], oocyte maturation [19], cell division [8,9], cellular differentiation [8,9], efficient export of mRNA from the nucleus to the cell [20], DNA repair [21,22] and protein folding [23]. In addition to these physiological functions, multiple pharmacological activities

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# IP6 in Cancer Therapy: Past, Present and Future

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of $IP_6$ beneficial for human health have been reported, such as striking anticancer function, strengthening the body's immune system by enhancing natural killer cell activity, inhibition of pathological calcification, reduction of serum lipid levels, etc [24-27].

**$IP_6$ as a Crucial Dietary Factor in Preventing Cancer, Evidence from Epidemiological Studies**

Cancer prevention strongly acknowledges the importance of a diet and lifestyle approaches to reduce cancer risk. Epidemiological studies show that the vast majority of cancers are caused by bad dietary habits and can be prevented by incorporating healthy agents. There seems to be a general agreement that certain dietary factors such as fiber, certain vegetables, and total dietary fat are important in the etiology of several cancers [28]. Nearly four decades ago, Burkitt [29] hypothesized that refining of grains and the lack of fiber in diet may be implicated in colorectal carcinogenesis. Comparative studies in Scandinavia pointed out that, despite similar background of living standards between Finland and Denmark, the incidence of colon cancer in Finland is half to one third of that in Denmark [30]. In the 1980's it was proposed that fiber may also have beneficial effects on breast cancer; while most studies of diet and breast cancer of that time were focused on the role of fat, very few have addressed the effect of fiber [31]. Major supporting evidence came from the observation that in Finland breast cancer incidence is considerably lower than in United States, although the fat intake is relatively high, but the fiber intake is much higher than that in the United States [32]. Although there has been the lack of consistency in current epidemiological findings, recent studies have shown that, indeed, adequate dietary intake of fiber can decrease the risk of colonic adenomas in the Prostate, Lung, Colorectal, and Ovarian (PLCO) a study [33] and colorectal cancer as much as 40% in the European Prospective Investigation into Cancer and Nutrition (EPIC) study [34], and that high consumption of whole grains reduced the risk of colon cancer in women [35]. However, the potential association of dietary fiber with breast cancer is still inconclusive, showing a modest decrease in breast cancer risk [28,36]

Thus growing evidence in epidemiological studies confirmed by clinical interventions, and animal model studies have pointed that minor components of fiber including $IP_6$ present in wheat bran, grains and legumes, may protect against cancer [37]. Furthermore, it was noticed that only fiber with high $IP_6$ content, such as cereals and legumes, show negative correlation with colon cancer, indicating that it could be $IP_6$ and not fiber that suppressed colon cancer [38]. And, indeed, it has been shown that $IP_6$ is one of the biologically active components of fiber, responsible for its anticancer effect.

***In vivo* and *in vitro* Evidence of $IP_6$ Anti-Cancer Properties**

*In vivo:* The effectiveness of $IP_6$ as a cancer preventive agent was first shown in colon cancer models in different species (rats and mice) with different carcinogens (1,2-dimethylhydrazine and azoxymethane) [39-48]. $IP_6$ was effective in a dose-dependent manner given either before or after carcinogen administration. The finding that $IP_6$ was able to reduce the development of colon cancer 5 months after carcinogen administration, when virtually all animals had already developed tumors, suggested its potential use as a therapeutic agent [41]. $IP_6$ decreased the incidence of aberrant crypts, often used as an intermediate biomarker for colon cancer [45,46,49]. It was also tested in a sarcoma (non-epithelial cancer) model - the FSA-1 mouse model of transplantable and metastatic fibrosarcoma [50]. After subcutaneous inoculation of mouse fibrosarcoma FSA-1 cells, mice were treated with intraperitoneal injections of $IP_6$ and a significant inhibition of tumor size and survival over untreated controls was observed. In this model, experimental lung metastases are developed after intravenous injections of FSA-1 cells; intraperitoneal injections of $IP_6$ resulted in a significant reduction of metastatic lung colonies [50]. Adding much higher amounts of $IP_6$ to the diet, Jariwalla *et al.* [51] have reported similar results in a rat fibrosarcoma tumor model. Studies using other experimental models showed that the antineoplastic properties of $IP_6$ were rather broad-spectrum in scope. $IP_6$ significantly reduced experimental mammary carcinoma in Sprague-Dawley rats induced either by 7,12-dimethylbenz[*a*]anthracene (51-54) or *N*-methylnitrosourea [44]. Using a 2-stage mouse skin carcinogenesis model, Ishikawa *et al.* investigated the effect of $IP_6$ on skin cancer, and found a reduction in skin papillomas when $IP_6$ was given during the initiation stage but not when given during the promotion stage [52]. Gupta *et al.* also showed prevention of skin carcinogenesis in a mouse carcinogenesis model where $IP_6$ caused a reduction in the number of skin tumor formation [53]. Lee *et al.* have shown that dietary administration of $IP_6$ and inositol significantly inhibit chemically induced rat hepatocarcinogenesis [54]. A strong anticancer activity of $IP_6$ was also demonstrated against human rhabdomyosarcoma RD cells transplanted in nude mice [55], where the efficacy of $IP_6$ was tested on the tumor-forming capacity of RD cells. Peritumoral treatment with $IP_6$ initiated 2 days after subcutaneous injection of rhabdomyosarcoma cells suppressed the tumor growth by 25 – 49-fold [51]. $IP_6$ was also potent in inhibiting experimental hepatoma [56,57]. A single treatment of HepG2 cells *in vitro* by $IP_6$ resulted in complete loss of the ability of these cells to form tumors when inoculated subcutaneously in nude mice. Additionally, the pre-existing liver cancers regressed when they were treated directly with $IP_6$ [57]. Studies in Agarwala's laboratory demonstrated the efficacy of orally administered IP6 (2%) in drinking water against *in vivo* growth of human prostate cancer xenografts in nude mice [58], in marked contrast to only a modest effect in rather large dosage mixed in diet by Jariwalla *et al.* [51]. Clearly, there is an advantage of giving $IP_6$ in drinking water, for when mixed in diet it may be less available for absorption. Raina *et al.* [59] have shown that orally administered $IP_6$ inhibits not only the cancer, but also the precancerous prostatic intraepithelial neoplasia (PIN). Most recently $IP_6$ has been demonstrated to prevent UVB-induced tumor formation in mice [60].

*Myo*-inositol itself was also shown to have modest anticancer activity. It inhibited colon, mammary, soft tissue and lung tumor formation [40,50,61-64]. Additionally, it was shown that inositol potentiates both the antiproliferative and

antineoplastic effects of $IP_6$ *in vivo* [40,50,61,62] with synergism of $IP_6$ + inositol in colon [40], mammary [61,62] and metastatic lung cancer models [50]. Not only the combination of $IP_6$ and inositol was significantly better in different cancers than was either one alone, but it also consistently reduced *all* tumor growth parameters. Thus, for clinical trials, the combination of $IP_6$ and inositol should be considered for optimal efficacy.

*In vitro*: $IP_6$ inhibited the growth of cancer cell lines in a dose- and time-dependent manner, irrespective of whether they were epithelial or mesenchymal in origin. $IP_6$ inhibited the growth of human leukemia cells [65,66], human colon cancer cells [67,68], both estrogen receptor-positive and estrogen receptor-negative human breast cancer cells [69], laryngeal carcinoma [70], cervical cancer [71], prostate cancer [17,72-74], hepatoma [75], pancreatic [76,77] and melanoma cell line [78]. The growth of mesenchymal tumors such as murine fibrosarcoma [50] and human rhabdomyosarcoma [55] was also inhibited in the presence of $IP_6$. However, cells from different origins have different sensitivities to $IP_6$. Hepatoma and leukemic cell lines seem to be highly susceptible to $IP_6$, suggesting that $IP_6$ affects different cell types through different mechanisms of action. Inhibitory effects of $IP_6$ was also shown in a benzo[*a*]pyrene-induced transformation of rat tracheal epithelial cells [79] and BALB/c mouse 3T3 fibroblasts [80]. Further, $IP_6$ impaired the transformation induced by epidermal growth factor or phorbol ester in JB6 (mouse epidermal) [81], and reduced 12-*O*-tetradecanoyl phorbol-13-acetate–induced ornithine decarboxylase activity, an essential event in tumor promotion in HEL-30 cells, a murine keratinocyte cell line [82].

Along with this reduction in cell proliferation (rather normalization), $IP_6$ induces differentiation and maturation of malignant cells, often resulting in reversion to the normal phenotype, as demonstrated in K-562 hematopoietic cells [65], human colon carcinoma HT-29 cells [67,83], prostate cancer cells [72], breast cancer cells [69], rhabdomyosarcoma [55], pancreatic cancer and hepatoma cell lines [75].

A good anticancer agent needs to be selective: it should only affect malignant cells and spare the normal cells and tissues. This property was shown for $IP_6$. When fresh CD34+ cells from bone marrow was treated with different doses of $IP_6$, a toxic effect (inhibition of the clonogenic growth or as cytotoxicity on liquid cultures) was observed that was specific to leukemic progenitors from chronic myelogenous leukemia patients, but no cytotoxic or cytostatic effect was observed on normal bone marrow progenitor cells under the same conditions [66]. $IP_6$ inhibited the colony formation of Kaposi Sarcoma cell lines, KS Y-1 (AIDS-related KS cell line) and KS SLK (Iatrogenic KS) and CCRF-CEM (human adult T lymphoma) cells in a dose-dependent manner; however, in striking contrast to taxol, $IP_6$ did not affect the ability of normal cells (peripheral blood mononuclear cells and T cell colony-forming cells) to form colonies in a semisolid methylcellulose medium [84]. Malignant and normal cells are known to have different metabolism, growth rate, expression of receptors, etc.; but the mechanism for this intriguing differential selectivity of $IP_6$ for normal and malignant cells deserve further investigations.

**Anticancer Activity of $IP_6$ depends on its Rapid Intake and Dephosphorylation to Metabolites**

The anticancer action of $IP_6$ may be mediated either directly, or *via* lower phosphorylated forms of inositol [39-41]. Though we know that the cellular mechanisms governing the anticancer activity of inositol and $IP_6$ involve cell proliferation and differentiation, we are now only beginning to understand the biochemical pathways and molecular mechanisms, leading to cell growth and cell death. It is known that virtually all animal cells contain inositol phosphates and that the inositol phosphates with fewer phosphate groups, especially $IP_3$ and $IP_4$, have an important role in cellular signal transduction, regulation of cell function, growth and differentiation [8,9]. Shamsuddin originally hypothesized that one of the several ways by which $IP_6$ exerts its action is *via* lower-phosphate inositol phosphates. Exogenously given $IP_6$ is quickly absorbed from the gastrointestinal tract [85] and rapidly taken up by malignant cells [86], showing that orally administered $IP_6$ can reach target tumor tissue distant from the gastrointestinal tract. Studies of absorption, intracellular distribution and metabolism of $IP_6$ in HT-29 human colon carcinoma and cells of hematopoietic lineage (K-562 human erythroleukemia and YAC-1 mouse lymphoma cells), show that exogenous $IP_6$ is rapidly taken up by these cells, transported intracellularly by pinocytosis and/or receptor-mediated endocytosis and dephosphorylated into inositol phosphates with fewer phosphate groups [86]. Similarly, that the anticancer activity of $IP_6$ is a result of its rapid intake by tumor cells was shown when MCF-7 human breast cancer cells were incubated with [$^3$H]-$IP_6$. As early as 1 min after incubation, 3.1% of $IP_6$-associated radioactivity was taken up by MCF-7 cells and 9.5% after 1 h. By differential centrifugation 86% radioactivity was recovered from the cell cytosol and 7.4% from the nuclear pellet. Anion-exchange chromatography showed that 58% of the absorbed radioactivity was in $IP_6$ form, indicating that externally applied $IP_6$ enters the cells followed by dephosphorylation. However, $IP_5$ appeared to be a predominant metabolite of $IP_6$, which possibly might have important role in its anticancer activity. Recently it has been demonstrated that $IP_7$ but not $IP_6$ induced apoptosis in SCC22A squamous carcinoma cells when administrated to mice with targeted deletion of inositol hexakisphosphate kinase 2 (IP6K2) a key enzyme in $IP_7$ synthesis [87]. When [$^3$H]-$IP_6$ was administered intragastrically to rats, it was quickly absorbed from the stomach and upper intestine and distributed to various organs as early as 1 h following administration [85]. While the radioactivity isolated from gastric epithelium at this time was associated with inositol and $IP_{1-6}$, the radioactivity in the plasma and urine was associated with inositol and $IP_1$. These data indicate that the intact molecule was transported inside the gastric epithelial cells, wherein it was rapidly dephosphorylated, and that the metabolism of $IP_6$ was very rapid. When [$^3$H]-$IP_6$ was given *via* oral gavages to rats bearing mammary tumors, a substantial amount of radioactivity (19.7% of total) was found in tumor tissue as early as 1 h after administration. This may explain at least in part the antineoplastic activity of $IP_6$ at sites distant from the gastrointestinal tract. In this study only 50% of the radioactivity was excreted in urine within 72 h following administration; in addition, feces accounted for another 10% of radioactivity, suggesting that at least 40% of the $IP_6$-

associated radioactivity was distributed within the animal tissues. These data indicate that $IP_6$ can reach and concentrate at cellular targets throughout the body. Chromatographic analysis of tumor tissue revealed the presence of inositol and $IP_1$, similar to that in plasma, once again indicating rapid dephosphorylation.

Several obstacles hindered the full scale effort in this emerging field of $IP_6$ research. First, is the misconception that $IP_6$, a highly negatively charged and extremely hydrophilic molecule, can not pass the plasma membrane to enter the cell. Ferry *et al.* [71] demonstrated that $IP_6$ is internalized into the cells by the process of pinocytosis, since colchicines, a pinocytosis inhibitor, completely blocked the uptake of $IP_6$. Measurement of intracellular inositol phosphates after $IP_6$ treatment indeed showed an increased level of lower-phosphate inositol phosphates [65,71,86,88]. Second, Grases *et al.* [88,89] were able to identify $IP_6$ in human urine and plasma and detect $IP_6$ and its less-phosphorylated forms ($IP_{3-5}$) in mammalian cells and in body fluids, as they occur naturally. Therefore, not only that the key misconceptions about this molecule are nullified, but it has also been demonstrated that $IP_6$ is an essential nutrient whose level in plasma and urine fluctuates following deficiency or replenishment [89]. Reversible phosphorylation of specific intracellular proteins is known to be an important and versatile mechanism for regulating their biological activity. After rapid intake and dephosphorylation, $IP_6$ enters inositol phosphate pool and controls a variety of cellular functions, such as growth, differentiation and cell cycle regulation.

## Potential Mechanisms and Molecular Targets for $IP_6$

### a. Potent Antioxidant

The antioxidant role of $IP_6$ is widely recognized; this function of $IP_6$ occurs by chelation of $Fe^{3+}$ and suppression of •OH formation [13] and by inhibiting xanthine oxidase [90]. Therefore, $IP_6$ can reduce carcinogen mediated active oxygen species and cell injury as well as inhibit free radical production in inflammation, radiation, etc. Although $IP_6$ is known as a strong natural antioxidant, *in vivo* data of its antioxidant effect are very limited. Its *in vivo* antioxidant action was recognized in different experimental models of myocardial reperfusion injury [91], pulmonary inflammation [92], and inflammation and ulcer induction [93]. A protective role of $IP_6$ against lipid peroxidation in colon associated with high level of iron was shown in rats [43], mice [94] and pigs [95] affecting glutathione peroxidase and catalase activity. Interestingly, not only $IP_6$, but also inositol has antioxidative properties by inhibiting xanthine oxidase and scavenging superoxide *in vitro* and *in vivo*, and preventing formation of ADP-iron-oxygen complexes that initiate lipid peroxidation [90,96]. The anticancer action of $IP_6$ may be further related to mineral binding ability; $IP_6$ by binding with $Zn^{2+}$ can affect thymidine kinase activity, an enzyme essential for DNA synthesis. Similarly, excess iron, which may augment colorectal cancer formation, can be removed by $IP_6$ [43,48].

There is however a paradox: inasmuch as $IP_6$ is a strong antioxidant, under certain conditions it may actually work to increase even more free radical production. For instance, actual killing of bacteria by the polymorphonuclear neutrophils is brought about by the free radicals. Eggleton *et al.* [97] have demonstrated that priming of neutrophils by $IP_6$ results in increased production of free radicals and therefore more efficient bacterial killing! Coupled with normalization of abnormally increased cell proliferation, differentiation of malignant cells to normal phenotype and elevation of depressed natural killer (NK) cell activity (*vide infra*), this is another example how $IP_6$ helps reverse abnormal parameters back to normal.

### b. Immune Enhancer

$IP_6$ and inositol have been shown to augment NK cell activity *in vitro* and normalize the carcinogen-induced depression of NK cell activity *in vivo* [98,99]. An inverse relationship between NK activity and tumor incidence has been shown in these models of colon carcinogenesis; an increased tumor incidence is correlated with a decreased NK cell activity. Conversely, the animals on $IP_6$ and inositol enjoyed a lower incidence of cancer and had a concomitantly enhanced NK cell activity. But, those animals that received the combination of $IP_6$ + inositol had the highest NK activity and lowest tumor incidence as inositol acts synergistically with $IP_6$ [98]. The mechanism how $IP_6$ and its metabolites including inositol bring about this function needs investigation. Most recently $IP_6$ has been shown to influence expression of pro-inflammatory cytokines like TNFα. In certain tumors TNFα and its receptor activity (TNFRI and TNFRII) are dysregulated. Cholewa *et al.* [100] demonstrated that $IP_6$ influences transcription of genes and coding for TNFα and its receptors in human colon cancer Caco-2 cells. IP6 up-regulates TNF receptor I (TNFRI) and decreases TNFα and TNFRII. Neutrophils, which as a part of the body's innate immune system form a first line of defense, are also affected by $IP_6$. $IP_6$ functions as a neutrophil priming agent and appears to up-regulate a number of diverse neutrophil functions [97].

### c. Molecular Targets Involved in Cell Cycle, Proliferation, Differentiation, Invasion, Angiogenesis and Metastasis

#### *Cell Signaling*

While normal cells divide at a controlled and limited rate, malignant cells escape from the control mechanisms that regulate the frequency of cell multiplication and usually have lost the checkpoint controls that prevent replication of defective cells. $IP_6$ can regulate the cell cycle to block uncontrolled cell division and force malignant cells either to differentiate or to go into apoptosis. $IP_6$ can modulate cellular response at the level of receptor binding; $IP_6$, after sterically blocking the heparin-binding domain of basic fibroblast growth factor (bFGF), disrupted further receptor interactions [101]. $IP_6$ blocks phosphatidylinositol-3 kinase (PI3K) and activating protein-1 (AP-1) [81], protein kinase C (PKC) [18,82,102,103] and mitogen-activated protein kinases (MAPK) [17,81,102,103]. Moreover, it has been shown that $IP_6$ operates *via* a direct control of protein phosphorylation [104]. Interestingly, although inositol phosphate-regulated phosphorylation was shown for $IP_6$, no activated phosphorylation was observed using the lower inositol phosphate in particular $IP_3$ or $IP_4$, both active signal transducers [104]. Despite the fact that $IP_6$ is the most abundant inositol metabolite in cells, all of its cellular functions are yet to be discovered. Even more fascinating is the role of inositol pyrophosphates, which occur physiologically and are implicated in diverse cellular functions. Adding to this complexity is the

recent demonstration that inositol hexaphosphate kinase-2, by generating diphosphoinositol pentaphosphate ($IP_7$) from $IP_6$, provides physiological regulation of apoptosis [10]. Thus, the role of $IP_6$ among all these multiple signaling pathways and their interaction in regulation of cell function needs to be addressed in the future.

### *Cell Cycle Regulators*

Uncontrolled proliferation is a hallmark of malignant cells. $IP_6$ reduces the cell proliferation rate of many different cell lines of different lineage and of both human and rodent origin [65-67,69-76,78,105]. $IP_6$ induces G1 phase arrest and a significant decrease of the S phase of human cancer cell lines by modulations of Cyclins and cdks, up-regulation of p27Kip1 and p21WAF1/CIP1 and decrease in retinoblastoma (Rb) protein phosphorylation [73,74,106,107]. $IP_6$ has been shown to induce up-regulation of p27Kip1 and decrease in expression levels of hyperphosphorylated Rb (ppRb) in both estrogen receptor-positive (MCF-7) and negative (MDA-MB 231) cells. As a consequence, a markedly increased level of hypophosphorylated pRb form (pRb) was observed [73,106] and the effects of $IP_6$ on PKCδ are responsible for the up-regulation of p27Kip1[106]. In leukemia cells, $IP_6$ appears to cause the accumulation of cells in the G2M phase of the cell cycle; a cDNA microarray analysis showed up-regulation of p57 mRNA and a down-modulation of multiple genes involved in transcription and cell cycle regulation by IP6 [66]. Roy *et al.* [108] have recently shown that cyclin dependent kinase inhibitors p21Cip1 and p27Kip1 act synergistically in bringing about $IP_6$-mediated inhibition of cell proliferation and apoptosis.

### *Cell Survival/Apoptosis*

Apoptosis is a hallmark of action of many anticancer drugs. It has been reported that $IP_6$ induces apoptosis *in vivo* [47] and *in vitro* in prostate [73,74], breast [106], cervical cancer [71], pancreas [76], melanoma [78], Barrett's adenocarcinoma [109] and KS (Kaposi's sarcoma) cell lines [84], involving cleavage of caspase 3, caspase 9 and poly ADP-ribose polymerase (PARP), an apoptotic substrate, in a time- and dose-dependent manner. McFadden *et al.* [109] have demonstrated that while $IP_6$ inhibited late apoptosis and necrosis in BIC Barrett adenocarcinoma cell line, in SEG-1 cells it caused inhibition of both early and late apoptosis and necrosis. That the induction of apoptosis by $IP_6$ is not just a finding in the *in vitro* system has been demonstrated by Singh *et al.* [58] in DU 145 prostate cancer cells xenografted *in vivo*! An important role of $IP_6$ in apoptosis is further suggested by findings that inositol hexaphosphate kinase-2 is a physiologic mediator of cell death [10]. In promoting apoptosis $IP_6$ down-regulates cell survival factors BIRC-2 (baculovirus inhibitor-of-apoptosis repeat containing-2) and telomerase and up-regulates calpain and caspase-3 activities in malignant glioblastoma cells [110]. Additionally $IP_6$ has been shown to regulate pro-apoptotic BCL-2 family of genes [111].

In sharp contrast to inducing apoptosis in cancer cells (a desirable effect), in conditions where apoptosis is harmful, $IP_6$ was shown to prevent it. For example, $IP_6$ protects against iron-induced apoptosis in immortalized rat mesencephalic dopaminergic cell model (N27 cells) and subsequent neuronal degeneration [112] to protect the cells and prevent disease. Once again, taken along with its ability to either be an antioxidant or induce free radical formation on demand points to $IP_6$'s ability to protect the organism as a whole. This is further supported by studies in a TRAMP mouse model and in DU-145 human prostate cancers cells whereby $IP_6$ inhibited telomerase activity, crucial for cells to gain immortality and cell survival [113].

Nuclear factor kappa B (NFκB) is an early-response gene involved in cell growth and apoptosis, and has been implicated in various cancers. $IP_6$ inhibits NFκB in different models of cancer such as prostate [114]. Certain chemotherapeutic agents such as paclitaxel can activate NFκB. $IP_6$ has been shown not only to act synergistically in inhibiting squamous cell cancers of the oral cavity, but it also inhibits paclitaxel-mediated increase in NFκB [115]. Recent studies by Kapral *et al.* [116] show that $IP_6$ primarily influences p65 subunit of NFκB and its inhibitor IκBα in human colorectal cancer cell line Caco-2.

### *Angiogenesis and Metastasis*

Tumors depend on the formation of new blood vessels to support their growth and metastasis. Many tumors produce large amounts of vascular endothelial growth factor (VEGF), a cytokine that signals normal blood vessels to grow. $IP_6$ inhibited the growth of endothelial cells [84,117] and inhibited the secretion of VEGF from malignant cells [58,66,84,117]. $IP_6$ can also adversely affect angiogenesis as antagonist of FGF [101].

One important characteristic of malignancy is the ability of tumor cells to metastasize and infiltrate normal tissue. A significant reduction in the number of lung metastatic colonies by $IP_6$ was observed in a mouse metastatic tumor model using FSA-1 cells [50]. Using highly invasive MDA-MB 231 human breast cancer cells, work in our laboratory demonstrated that $IP_6$ inhibits metastasis *in vitro* by affecting cancer cell adhesion, migration and invasion [118,119]. Tumor cells emit substances known as matrix metalloproteinases (MMPs) that allow metastatic cells to breakdown the barriers in vessel wall and enter into blood circulation; $IP_6$ significantly inhibits secretion of matrix metalloproteinase-9 (MMP-9) from MDA-MB 231 cells as well as significantly reducing the invasion properties of cancer cell *in vitro* [119].

### *DNA Repair*

There are other ways by which $IP_6$ could influence the various activities within the cells. For instance, repair of double-strand breaks in DNA is essential for maintaining the stability of the genome, failure to repair may result in loss of genetic information, chromosomal translocation, and even cell death. $IP_6$ has been demonstrated to stimulate non-homologous end-joining; it has been proposed to be brought about by the binding of $IP_6$ to the DNA-dependent protein kinase DNA-PKcs [21,120]. Others report that it is not DNA-PKcs (a large protein of ~3500 amino acids, Mw ~465 kDa), but the DNA end binding protein Ku (consists of Ku70 - 70 kDa, and Ku86 - 83 kDa) that binds to $IP_6$ [22]. Be that as it may, these studies, in spite of their differences in their specific findings clearly show a very important role of $IP_6$ in DNA repair mechanism.

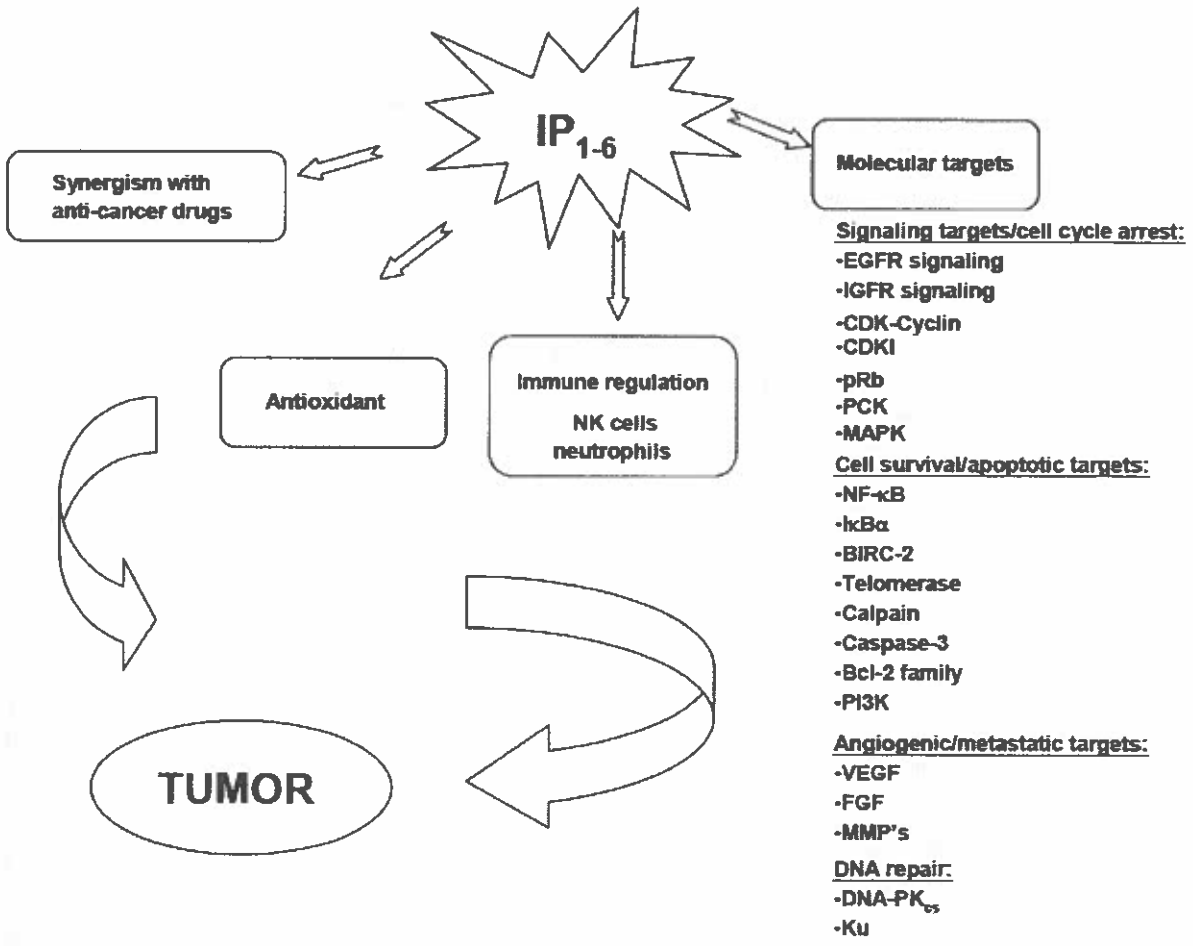



**Fig. (1).** Illustrates the points where $IP_6$ can interact with cellular processes involved with cancer. Multiple steps in the cancer process can be modified and it is possible that $IP_6$ can influence more than one of these processes simultaneously [122]. Thus, its anticancer potential can be expressed by the combination of the antioxidant, anti-inflammatory and immune-enhancing activities, influence on the cells cycle and cell differentiation, induction of apoptosis and suppression of proliferation and angiogenesis, and synergism with conventional drugs. Additionally, $IP_6$ can modulate biochemical events presumed to be mechanistically linked to carcinogenesis and carcinogen blocking activities, modifying enzymes involved in metabolic activation of chemical carcinogens, such as phase I and phase II detoxification enzymes, inhibition of several liver enzymes and reduction of 12-*O*-tetradecanoylphorbol-13-acetate–induced ornithine decarboxylase activity.

Once the assault on the cell has gone past the scope of DNA repair, the otherwise heretofore normal cell is likely to transform to a malignant (cancer) cell. Insofar as the transformation of cells from normal to malignancy is concerned, there are various models and pathways, one of these pathways is the activation of transcription factors activating protein-1 (AP-1) and nuclear factor NFκB via phosphatidylinositol 3-kinase (PI-3 kinase). Using tumor promoter-induced cell transformation of human skin JB6 cells, Huang *et al.* [81] have demonstrated that $IP_6$ blocks epidermal growth factor-induced PI-3 kinase and AP-1 activity. That $IP_6$ also acts as an anti-mutagenic agent has been recently demonstrated by Ra Yoon *et al.* [121].

### *Synergistic Effects of $IP_6$ and Conventional Drugs*

Most of the currently used chemotherapeutic agents do not discriminate between normal and tumor cells, and induce cell death in all actively proliferating cells. A logical approach is to develop drugs that specifically attack tumor cells, while sparing normal cells. Another important aspect of cancer treatment is overcoming acquired drug resistance. Tamoxifen, a cell type-specific anti-estrogen, has been widely used for the prevention and therapy of breast cancer for over two decades [123] and almost all initial responders eventually develop resistance. The mechanisms by which this resistance occurs remain unclear, but it includes changes in cellular signal transduction pathways [124]. Conversion of breast tumors to a tamoxifen-resistant phenotype is associated with oxidative stress, activating protein-1 (AP-1) activity [125] and activation of nuclear factor kappa B (NFκB) [126]. It has been shown that agents which up-regulate cyclin-dependent kinase inhibitor p27Kip1 and inhibit kinases involved in pro-proliferative and pro-survival pathways ERKs [127-129] and PI3K/Akt [130,131] are capable of preventing tamoxifen resistance in breast cancer cells. Interestingly, recent data demonstrate that $IP_6$ acts synergistically with doxorubicin and tamoxifen, being particularly effective against estrogen receptor–negative and doxorubicin-resistant tumor cell lines, both conditions that are challenging to treat [132]. Specifically $IP_6$ blocks AP-1 and NFκB transcriptional activi-

ties [24,71,81], up-regulates p21Cip1 [133] and p27Kip1 [24,106] and inhibits ERK and PI3K/Akt [24,71,81,106] attacks tumor cells without affecting normal cells. These data are particularly important because tamoxifen is usually given as a chemopreventive agent in the post-treatment period and doxorubicin has enormous cardiotoxicity and its use is associated with doxorubicin resistance. These findings make $IP_6$ an excellent candidate as a drug to be used in combination with tamoxifen and other drugs in anti-cancer therapeutic approaches.

A major complication of tamoxifen therapy is thromboembolism. Platelet hyperactivity and platelet aggregation are known to contribute to the initiation and progression of thromboembolism. $IP_6$ has been shown to significantly reduce elevated serum lipids [134] and to inhibit platelet aggregation [135,136]. Rats given 2% IP6 in drinking water for 40 weeks showed a 45% inhibition of platelet aggregation [136]. Aggregation of human platelets was significantly reduced by $IP_6$ in a dose-dependent manner [135]. This strong antiplatelet activity would certainly prevent or ameliorate tamoxifen-associated thromboembolism, besides reducing the risk of not only coronary heart diseases, but also other related conditions, such as ischemic stroke. $IP_6$ probably exerts this effect by interfering with receptor binding and intracellular signaling molecules that control platelet activation and aggregation.

Another complication of long-term tamoxifen use is uterine cancer. As a broad-spectrum anti-cancer agent, $IP_6$ could also prevent this unwanted complication of tamoxifen. Thus, it is imperative to further investigate the combination of $IP_6$ + tamoxifen. Most other anti-cancer agents exert their toxicity *via* free radical generation [137]. The antioxidative function of $IP_6$ would clearly help in reducing the toxicity of those drugs as well. Recently successful combined treatment in form of liposome containing $IP_6$ and irinotecan (CPT-11) was observed in colon tumors [138].

### Clinical trials

An enhanced antitumor activity with improved quality of life by $IP_6$ was demonstrated in a pilot clinical trial involving 22 patients with advanced colorectal cancer (Dukes C and D) with multiple liver and lung metastasis [139]. $IP_6$ + Inositol (*$IP_6$ Gold* by IP-6 International, Melbourne, FL www.ip6gold.com) was given as an adjuvant to chemotherapy according to Mayo protocol. One patient with liver metastasis refused chemotherapy after the first treatment, and she was given only $IP_6$ + Inositol; her control ultrasound and abdominal computed tomography scan 14 months after surgery showed a significantly reduced growth rate. A reduced tumor growth rate was noticed overall and in some case a regression of lesions was noted. Additionally, when $IP_6$ + Inositol was given in combination with chemotherapy, side effects of chemotherapy, such as drop in leukocyte and platelet counts, nausea, vomiting, alopecia, were diminished and patients were able to perform their daily activities allowing them to continue the chemotherapy regimen uninterruptedly [139,140]. Additionally, long-term survival and improved quality of life was noticed in patients with breast cancer [141,142] and advanced lung cancer [143]. Prophylactic dosage of $IP_6$ + Inositol is low (1.0 – 2.0 g/day) and the cancer therapeutic dosage may be 10 times higher.

Clearly, these encouraging pilot data beg for further controlled randomized clinical trials. Phase I trial is needed to determine human dose range and toxicity levels, and Phase II and III to determine the efficacy.

### FUTURE- Haute Couture Therapies for Cancer Patients

$IP_6$ has been shown to be rapidly internalized and metabolized to lower inositol phosphates and inhibit principal pathways of malignancy such as proliferation [55, 65, 67, 69, 75], cell cycle progression [17,18,24,27,69,144], metastasis and invasion [55,118,119], angiogenesis [58,66,117], apoptosis [71,73,106] and differentiation [24,27,69]. In studies of breast and prostate cancers, the inhibition of growth and induction of differentiation was independent of the hormone dependence [69]. Moreover, $IP_6$ selectively inhibits cancer cells without affecting the normal and acts synergistically with standard therapeutics [106,132]. $IP_6$ is well absorbed from the gastrointestinal tract of both human and rodents, without any toxic effects [144]. Thus, $IP_6$ offers itself as an excellent candidate for both cancer prevention and cancer therapy. However, the high dosage of $IP_6$ necessary to achieve its full potency, precludes its use as a drug of choice.

Like many other naturally occurring substances the pharmacological doses of $IP_6$ has been high, since the normal physiological concentration ranges from 0.01-1 mM, and levels found in human plasma are also rather high, 0.4 ± 0.1 μM [88]. The pharmacological doses of $IP_6$ used *in vitro* have been in mM range. On the other hand, it is extremely likely that the metabolites could be effective at much lower concentrations. Indeed Maffucci *et al* has shown that as little as 50 μM $IP_5$ was an effective inhibitor of angiogenesis while the same concentration of $IP_6$ was totally ineffective [145]. Identification of the most potent inositol polyphosphate(s), which can be used in low doses as opposed to high dosage of $IP_6$, is crucial for future drug development and clinical trials. Moreover work in our lab has previously shown that different cancer cell lines yield different profiles of polyphosphates; for example no $IP_5$ was identified in MCF-7 breast cancer cells whereas $IP_{1\text{-}6}$ were observed in HT-29 colon cancer cells [86]. Correlating this is the differential potency of $IP_6$ for different cancer types. For instance the $IC_{50}$ of $IP_6$ for hepatoma is 338 μM as opposed to 2-5 mM for HT-29 colon cancer cells [75]. Thus, it is crucial to study $IP_6$ metabolites for treatment of different types of tumors; individual metabolite(s) or isoform(s) could be used for tailor-made therapy of different cancers. It is to be noted that while inositol has modest anticancer action *in vivo*, it is a growth promoter and essential element for cell culture *in vitro*! This may also suggest that the anticancer action of inositol could very well be *via* phosphorylation to $IP_{1\text{-}6}$ other pathways notwithstanding. Additional advantage for administering $IP_6$ metabolites in conjunction with other drugs like tamoxifen is the ability of $IP_6$ to inhibit platelet aggregation. While $IP_6$ inhibits abnormally elevated rate of platelet aggregation as in diseases, it does not do so for normal platelets [135]. Along with its ability to lower serum cholesterol [146], $IP_6$ could decrease the risk of thromboembolic disease and endometrial cancer, two grave and frequent complications of tamoxifen therapy.

Another avenue of future research should be use of $IP_6$ in combination with citrate. Addition of citrate to $IP_6$ molecule gives rise to a new molecule called inositol hexaphosphate citrate ($IP_{6c}$) [147,148]. While the parent $IP_6$ molecule has 12 valencies, the new one ($IP_{6c}$) has 24, making it a better chelator and hence an even better antioxidant, perhaps the strongest available to date. Preliminary data [unpublished results] indicate that, indeed, $IP_{6c}$ may be more potent in cancer inhibition than $IP_6$.

### Broader Impact of $IP_6$ Metabolites in Global Health

Cancer control is not just finding newer and better therapeutics, but even more importantly, better strategies in cancer prevention. Cancer prevention can be approached through prophylactic supplements and vitamins. However, fortification of food items as in control of various deficiency diseases may be a better approach for protecting the public at large since the socioeconomic and cultural factors in different regions of the planet may preclude effective supplementation. $IP_6$ and its metabolites have great potential to serve in this capacity as well.

In the seeds, $IP_6$ is stored in electron dense spherical particles named globoids which are localized predominantly in the aleurone layer in wheat and barley, or in the embryo in maize. The size of these globoids depends on the amount of $IP_6$ in the grain. In wild type (WT) wheat, the globoids may be as large as up to 4 μm in diameter, whereas a low [$IP_6$] phytic acid (lpa) wheat mutant (Js-12-LPA) with the same amount of phosphate in the grains but lowered $IP_6$ concentration has smaller globoids. Along with smaller size of the beans, low $IP_6$ content of is also associated with them becoming hard-to-cook [149]. Citing the chelating property of $IP_6$ albeit controversial, there has been a recent trend in certain agricultural sectors to reduce $IP_6$ content through lpa mutants *via* knock-out of genes involved in $IP_6$ biosynthesis [150,151]. Greiner *et al* show that the phytase ($IP_6$-specific phosphatase) from Malaysian waste-water bacterium preferably dephosphorylates $IP_6$ in a stereospecific way by sequential removal of phosphate groups via D-I(1,2,3,4,5)$P_5$, D-I(2,3,4,5)$P_4$, D-I(2,3,4)$P_3$, D-I(2,3)$P_2$ to finally I(2)$P_1$ [152]. It was estimated that more than 90% of $IP_6$ hydrolysis occurs via D-I(1,2,3,4,5)$P_5$. Thus, if indeed any of the $IP_6$ metabolite(s) or their combinations is determined to be the most potent, genetic modification of cereal grains could [theoretically] help disease prevention on a global scale. However, since the lower inositol phosphates are crucial factors in plant cell metabolism, production of lpa mutant is quite challenging. The fact that germination ability is affected when $IP_6$ content is reduced also creates serious issues. One cannot be too careful about the use of such approach as Doria *et al.* [153] has most recently shown that reduction of $IP_6$ content through lpa mutation could actually be harmful to the plant itself! Further investigation is clearly warranted before we hastily start modifying the cereal grains, staple food source for much of this planet.

## SUMMARY

Cancer is a major public health burden in not just the United States but throughout the world. While we have made remarkable progress in cancer treatment, unfortunately we are still far from combating this menace effectively. Furthermore, conventional anti-cancer agents so far have side-effects that are not accepted readily by the patients. Thus it is a matter of great urgency to identify drugs that are not only effective, but also safe. $IP_6$ and its metabolites including inositol offer themselves as one such family.

$IP_6$ and its metabolite(s) offer the following advantages as potential drug of choice: 1) $IP_6$ differentially inhibits the proliferation of cancer cells without affecting the normal cells 2) $IP_6$ inhibits cell proliferation of cancer cells irrespective of their hormonal receptor status, 3) individual $IP_6$ metabolite(s) or combinations could be specifically effective against specific cancers, thereby increasing the chances of successful therapy 4) while a high dose of $IP_6$ is needed to achieve therapeutic levels restricting its use in cancer treatment, its metabolite(s) could be effective alone, or in combination with other drugs at a much lower concentration, akin to the conventional anti-cancer drugs and 5) the anti-thrombogenic and broad-spectrum anti-cancer action of $IP_6$ would prevent two of the serious and common complications of conventional anticancer drugs. Additionally, 6) genetically modified cereal grains to produce the most potent of the $IP_6$ metabolite(s) could be the future of public health strategy in global prevention of cancer and other diseases.

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# Cancer Inhibition by Inositol Hexaphosphate ($IP_6$) and Inositol: From Laboratory to Clinic[1,2]

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ABSTRACT Inositol hexaphosphate ($IP_6$) is a naturally occurring polyphosphorylated carbohydrate that is present in substantial amounts in almost all plant and mammalian cells. It was recently recognized to possess multiple biological functions. A striking anticancer effect of $IP_6$ was demonstrated in different experimental models. Inositol is also a natural constituent possessing moderate anticancer activity. The most consistent and best anticancer results were obtained from the combination of $IP_6$ plus inositol. In addition to reducing cell proliferation, $IP_6$ increases differentiation of malignant cells, often resulting in a reversion to normal phenotype. Exogenously administered $IP_6$ is rapidly taken into the cells and dephosphorylated to lower-phosphate inositol phosphates, which further interfere with signal transduction pathways and cell cycle arrest. Enhanced immunity and antioxidant properties can also contribute to tumor cell destruction. However, the molecular mechanisms underlying this anticancer action are not fully understood. Because it is abundantly present in regular diet, efficiently absorbed from the gastrointestinal tract, and safe, $IP_6$ holds great promise in our strategies for the prevention and treatment of cancer. $IP_6$ plus inositol enhances the anticancer effect of conventional chemotherapy, controls cancer metastases, and improves the quality of life, as shown in a pilot clinical trial. The data strongly argue for the use of $IP_6$ plus inositol in our strategies for cancer prevention and treatment. However, the effectiveness and safety of $IP_6$ plus inositol at therapeutic doses needs to be determined in phase I and phase II clinical trials in humans. J. Nutr. 133: 3778S–3784S, 2003.

KEY WORDS: • *prevention* • *treatment* • *differentiation* • *phytic acid*

Cancer remains a major health problem in the United States and in other developed countries (1). In our continuing effort to reduce the public health burden of cancer, there is a constant search for more effective cancer treatment, and increased interest in the concept of prevention, as a promising approach to the control of cancer (2).

A novel anticancer function of inositol hexaphosphate ($IP_6$;[4] also Ins$P_6$ and phytic acid) has been shown both in vivo and in vitro (3–5). $IP_6$ is a polyphosphorylated carbohydrate, contained in high concentrations (0.4–6.4%) in cereals and legumes (6). *Myo*-inositol is a parent compound of $IP_6$. Only *myo*-inositol hexaphosphate has been found in plants; *neo*-, *chiro*-, and *scyllo*-inositol hexaphosphates have been isolated from soil (7). The phosphate grouping in positions 1, 2, and 3 (axial-equatorial-axial) is unique for $IP_6$, providing a specific interaction with iron to completely inhibit its ability to catalyze hydroxyl radical formation, making $IP_6$ a strong antioxidant, probably still the only role of $IP_6$ that is widely recognized and accepted.

Almost all mammalian cells contain $IP_6$ and much smaller amounts of its forms with fewer phosphate groups ($IP_{1-5}$), which are important for regulating vital cellular functions. Inositol occurs ubiquitously in cell membranes in conjugation with lipids, as phosphatidylinositol. Recently, inositol phospholipids in the plasma membrane have received much attention because of their biological significance for signal transduction systems. Phosphatidylinositol 4,5-bisphosphate ($PIP_2$), a phosphoinositide, is a precursor for several informational molecules in signal transduction—inositol 1,4,5-$P_3$ ($IP_3$), 1,2-diacylglycerol, and phosphatidylinositol 3,4,5-trisphosphate—linking receptor stimulation to $Ca^{2+}$ mobilization (8). A second messenger role in intracellular $Ca^{2+}$ homeostasis for $IP_4$ was also shown. It is now recognized that subsequent to $PIP_2$ hydrolysis a cascade of inositol phosphate metabolites are formed and that these multiple isomers show a complex pattern of interconversion (8–10). Inositol phosphates are versatile molecules with important roles in controlling diverse cellular

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[4] Abbreviations used: Ins, inositol; $IP_6$, inositol hexaphosphate; $IP_3$, inositol 1,4,5-$P_3$; KS, Kaposi's sarcoma; $PIP_2$, phosphatidylinositol 4,5-bisphosphate.

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activities (9,10). $IP_6$ may serve as a natural antioxidant (11) and possibly as a neurotransmitter (10). Different binding proteins for inositol polyphosphates have been isolated, indicating their importance for the cellular functions (12) such as effects on ion channels and protein trafficking (13,14), endocytosis (15), exocytosis (16), and efficient export of mRNA from the nucleus to the cell (17).

How can exogenously administered $IP_6$ affect tumor growth? Pioneering experiments showing this novel anticancer feature of $IP_6$ were performed by Shamsuddin et al. (18–20), who were intrigued by the epidemiologic data indicating that only diets containing a high $IP_6$ content (cereals and legumes) showed a negative correlation with colon cancer. Almost 15 y ago, Shamsuddin et al. hypothesized that $IP_6$ can be internalized by the cells and dephosphorylated to $IP_{1-5}$ and then can enter into the intracellular inositol phosphate pool and inhibit tumor growth. It was also hypothesized that the addition of inositol, a precursor of inositol phosphates and also a natural carbohydrate, to $IP_6$ may enhance the anticancer function of $IP_6$ (18–20). Because inositol phosphates are common molecules involved in signal transduction in most mammalian cell systems, it was further hypothesized that the anticancer action of inositol phosphates would be observed in different cells and tissue systems (18–20). All these proposed hypotheses have been confirmed.

Contrary to the dogma and skepticism at that time, we showed that $IP_6$ is taken up by malignant cells (21) and that orally administered $IP_6$ can reach target tumor tissue distant from the gastrointestinal tract (22). Because of the highly charged nature of $IP_6$, it was a common misconception that it could not be transported into the cells. Analyzing absorption, intracellular distribution, and metabolism of $IP_6$ in HT-29 human colon carcinoma and cells of hematopoietic lineage (K-562, human erythroleukemia and YAC-1, mouse lymphoma cells), we found that $IP_6$ is rapidly taken up by mechanisms probably involving pinocytosis or receptor-mediated endocytosis, transported intracellularly, and dephosphorylated into inositol phosphates with fewer phosphate groups (21). Similar data were obtained when MCF-7 human breast cancer cells were incubated with [$^3$H]-$IP_6$ (SA 444 GBq/mmol, 370 Bq/$10^6$ cells): as early as 1 min after incubation, 3.1% of $IP_6$-associated radioactivity was taken up by MCF-7 cells, and 9.5% after 1 h. By differential centrifugation 86% radioactivity was recovered from the cell cytosol. Anion-exchange chromatography showed that 58% of the absorbed radioactivity was in $IP_6$ form. When [$^3$H]-$IP_6$ was administered intragastrically to rats, it was quickly absorbed from the stomach and upper intestine and distributed to various organs as early as 1 h after administration (22). Although the radioactivity isolated from gastric epithelium at this time was associated with inositol and $IP_{1-6}$, the radioactivity in the plasma and urine was associated with inositol and $IP_1$. These data indicate that the intact molecule was transported inside the gastric epithelial cells, wherein it was rapidly dephosphorylated, and that the metabolism of $IP_6$ was very rapid. In our preliminary studies, [$^3$H]-$IP_6$ was given via oral gavage to rats bearing 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors. A substantial amount of radioactivity (19.7% of all radioactivity recovered in collected tissues) was found in tumor tissue as early as 1 h after administration, providing at least partial explanation for the antineoplastic activity of $IP_6$ at sites distant from the gastrointestinal tract. In this study only 50% of the radioactivity was excreted in urine within 72 h after administration; in addition feces accounted for another 10% of radioactivity, suggesting that at least 40% of the $IP_6$-associated radioactivity was distributed within the animal tissues. These data indicate that $IP_6$ can reach and concentrate at cellular targets. Chromatographic analysis of tumor tissue revealed the presence of inositol and $IP_1$, similar to plasma.

Using a novel and highly sensitive method combining gas chromatography–mass spectrometry analysis and HPLC, Grases et al. (23,24) were able to identify $IP_6$ in human urine and plasma and detect $IP_6$ and its less-phosphorylated forms ($IP_{3-5}$) in mammalian cells and in body fluids as they occur naturally. They also showed that the levels of $IP_6$ and its less phosphorylated forms fluctuate depending on the intake of $IP_6$.

That the extracellularly applied $IP_6$ enters the cell and that this intracellular delivery is followed by a dephosphorylation of $IP_6$ was recently confirmed by Ferry et al. (25).

### *Anticancer action of $IP_6$*

As hypothesized, it was demonstrated that $IP_6$ is a broad-spectrum antineoplastic agent, affecting different cells and tissue systems. In vitro studies with $IP_6$ are summarized in Table 1.

$IP_6$ inhibited the growth of all tested cell lines in a dose- and time-dependent manner. The growth of cells of hematopoietic lineage was inhibited: human leukemic hematopoietic cell lines, such as K-562 (26,27) and human normal and leukemic hematopoietic cells (27). The antiproliferative activity of $IP_6$ was further reported in human colon cancer HT-29 cells (28), estrogen receptor–positive and estrogen receptor–negative human breast cancer cells (32), cervical cancer (25), prostate cancer (15,33,34), and HepG2 hepatoma cell lines (31). $IP_6$ also inhibited the growth of mesenchymal tumors, murine fibrosarcoma (39), and human rhabdomyosarcoma (38). However, cells from different origin have different sensitivity to $IP_6$ (the leukemic cell lines seem to be highly susceptible to $IP_6$), suggesting that $IP_6$ may affect different cell types through different mechanisms of action.

**TABLE 1**

*Antitumor effect of inositol hexaphosphate ($IP_6$) in vitro*

| Organ or tissue | Species | Cell line | Investigator |
|---|---|---|---|
| Blood | Human | Erythroleukemia | Shamsuddin et al. (26) |
| | | K562 cell line | |
| | | K562 + human bone marrow | Deliliers et al. (27) |
| Colon | Human | Adenocarcinoma | Sakamoto et al. (28) |
| | | HT-29 cell line | Yang & Shamsuddin (29) |
| Lung | Rat | Tracheal epithelium + B[a]P | Arnold et al. (30) |
| Liver | Human | HepG2 cells | Vucenik et al. (31) |
| Mammary | Human | Adenocarcinoma | Shamsuddin et al. (32) |
| | | MCF-7, MDA-MB 231 cells | |
| Uterine cervix | Human | HeLa cells | Ferry et al. (25) |
| Prostate | Human | Adenocarcinoma | Shamsuddin & Yang (33) |
| | | PC-3 cell line | |
| | Human | DU145 cells | Zi et al. (15) Singh et al. (34) |
| Skin | Mouse | JB6 cells | Huang et al. (35) |
| | Mouse | HEL-30 cells | Nickel & Belury (36) |
| Soft tissue | Mouse | 3T3 fibroblast | Babich et al. (37) |
| | Human | Rhabdomyosarcoma, RD cells | Vucenik et al. (38) |

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The potential of $IP_6$ to induce differentiation and maturation of malignant cells, often resulting in reversion to the normal phenotype, was first demonstrated in K-562 hematopoietic cells (26). $IP_6$ was further shown to increase differentiation of human colon carcinoma HT-29 cells (28,29), prostate cancer cells (33), breast cancer cells (32), and rhabdomyosarcoma cells (38).

The cancer preventive activity of $IP_6$ in vitro was first tested in a benzo[*a*]pyrene-induced transformation in the rat tracheal cell culture transformation assay (30) and then was tested in a model using BALB/c mouse 3T3 fibroblasts (37) with modest efficacy. The observation that $IP_6$ impaired the transformation induced by epidermal growth factor or phorbol ester in JB6 (mouse epidermal) cells (35) strongly suggested the potential role of $IP_6$ as a cancer preventive agent, because this model has been a well-characterized cell system for studying the tumor promotion and molecular mechanisms of antitumor agents. Furthermore, $IP_6$ reduced 12-*O*-tetradecanoylphorbol-13-acetate–induced ornithine decarboxylase activity, an essential event in tumor promotion in HEL-30 cells, a murine keratinocyte cell line (36).

A summary of in vivo studies using $IP_6$ and inositol is shown in **Table 2**. Although experts in the field of nutrition and cancer have been performing in vivo experiments by adding $IP_6$ to the diet, in all our cancer prevention studies, $IP_6$ was given via drinking water in concentrations ranging from 0.4% to 2.0%. We were able to obtain comparable or even stronger tumor inhibition with much lower concentrations of $IP_6$ when it was given in drinking water. For example, much stronger tumor inhibition was achieved with 0.4% $IP_6$ in drinking water compared with the same amount given in a 20% high fiber diet (52).

The effectiveness of $IP_6$ as a cancer preventive agent was shown in colon cancer induced in different species (rats and mice) with different carcinogens (1,2-dimethylhydrazine and azoxymethane) (18–20,40–46). $IP_6$ was effective in a dose-dependent manner given either before or after carcinogen administration. The finding that $IP_6$ was able to reduce the development of large intestinal cancer 5 mo after carcinogen administration, when $IP_6$-treated animals demonstrated a significantly lower tumor number and size, has suggested its potential use as a therapeutic agent (20). $IP_6$ decreased the incidence of aberrant crypts when they were used as an intermediate biomarker for colon cancer (43,44). Studies using other experimental models showed that antineoplastic properties of $IP_6$ were not restricted to the colon. $IP_6$ significantly reduced experimental mammary carcinoma in Sprague-Dawley rats induced either by 7,12-dimethylbenz[*a*]anthracene (51–54) or *N*-methylnitrosourea (42). Using a two-stage mouse skin carcinogenesis model, Ishikawa et al. (55) investigated the effect of $IP_6$ on skin cancer and found a reduction in skin papillomas when $IP_6$ was given during the initiation stage but not when given during the promotion stage (55).

The therapeutic properties of $IP_6$ were demonstrated in the FSA-1 mouse model of transplantable and metastatic fibrosarcoma (39). After subcutaneous inoculation of mouse fibrosarcoma FSA-1 cells, mice were treated with intraperitoneal injections of $IP_6$ and a significant inhibition of tumor size and survival over untreated controls was observed. In this model experimental lung metastases are developed after intravenous injections of FSA-1 cells; intraperitoneal injections of $IP_6$ resulted in a significant reduction of metastatic lung colonies (39). A strong anticancer activity of $IP_6$ was also demonstrated against human rhabdomyosarcoma RD cells transplanted in nude mice (38), where the efficacy of $IP_6$ was tested on the tumor-forming capacity of RD cells. Peritumoral treatment with $IP_6$ (40 mg/kg) initiated 2 d after subcutaneous injection of rhabdomyosarcoma cells suppressed the tumor growth by 25–49-fold (38). $IP_6$ was also potent in inhibiting experimental hepatoma (31,48). We tested the effect of $IP_6$ on tumorigenicity and tumor regression in this model. A single treatment of HepG2 cells in vitro by $IP_6$ resulted in the complete loss of the ability of these cells to form tumors when inoculated subcutaneously in nude mice (48). Additionally, the preexisting

**TABLE 2**

*Antitumor effect of $IP_6$ and inositol in vivo*

| Organ/Tissue | Species | Disease parameter | Mode | Investigator |
|---|---|---|---|---|
| Colon | Mouse | Carcinoma | in drink | Shamsuddin et al. (19) |
| | Rat | Carcinoma | in drink | Shamsuddin et al. (18,20) |
| | Rat | Carcinoma | in drink | Ullah & Shamsuddin (40) |
| | Rat | Carcinoma | in diet | Nelson et al. (41) |
| | Rat | Carcinoma | in diet | Shivapurkar et al. (42) |
| | Rat | Carcinoma | in diet | Pretlow et al. (43) |
| | Rat | Carcinoma | in diet | Challa et al. (44) |
| | Rat | Carcinoma | in diet | Jenab & Thompson (45) |
| | Mouse | Cell proliferation | in diet | Thompson & Zhang (46) |
| Liver | Rat | Hepatocellular Ca | in diet | Hirose et al. (47) |
| | | HepG2 cell line | intratumoral | Vucenik et al. (48) |
| Lung | Mouse | Pulmonary adenoma | in diet | Estensen & Wattenberg (49) Wattenberg (50) |
| Mammary | Rat | Carcinoma | in drink | Vucenik et al. (51–53) |
| | Rat | Carcinoma | in diet | Shivapurkar et al. (42) Hirose et al. 1994 (54) |
| | Mouse | Cell proliferation | in diet | Thompson & Zhang (46) |
| Skin | Mouse | Papilloma two-step | in drink | Ishikawa et al. (55) |
| | | initiat→promotion | in drink | |
| Soft Tissue | Rat | Fibrosarcoma | in diet | Jariwalla et al. (56) |
| | | Transplanted | 12% Mg | |
| | Mouse | Fibrosarcoma | i.p. | Vucenik et al. (39) |
| | | Trans + Metast | | |
| | Human | Rhabdomyosarcoma | peritumoral | Vucenik et al. (38) |
| | | RD cell line | | |

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liver cancers regressed when they were treated directly with $IP_6$ (48).

Myo-inositol itself was also demonstrated to have anticancer function, albeit modest. It inhibited pulmonary adenoma formation in mice (49,50). We found that inositol alone or in combination with $IP_6$ can prevent the formation and incidence of several cancers in experimental animals: in soft tissue, colon, metastatic lung, and mammary cancers. Additionally, we showed that inositol potentiates both the antiproliferative and antineoplastic effects of $IP_6$ in vivo (3–5,19,39,51,52). Synergistic cancer inhibition by $IP_6$ when combined with inositol was observed in colon cancer (Table 3) (19) and mammary cancer studies (Table 4) (51,52). Similar results were seen in the metastatic lung cancer model (39). Thus, the combination of $IP_6$ and inositol was significantly better in different cancers than was either one alone.

### *Mechanisms of action of $IP_6$*

The mechanisms involved in the anticancer activity of inositol compounds are not fully understood. It is known that virtually all animal cells contain inositol phosphates and that the inositol phosphates with fewer phosphate groups, especially $IP_3$ and $IP_4$, have an important role in cellular signal transduction, regulation of cell function, growth, and differentiation (8,9). We hypothesized that one of the several ways by which $IP_6$ plus inositol exerts its action is via lower-phosphate inositol phosphates. Measurement of intracellular inositol phosphates after $IP_6$ treatment showed an increased level of lower-phosphate inositol phosphates ($IP_{1-3}$) (21,24–26); their involvement in signal transduction pathways can affect cell cycle regulation, growth, and differentiation of malignant cells (3–5). Derivatives of phosphatidylinositol transmit cellular signals in response to extracellular stimuli, and enzymes responsible for the phosphorylation and hydrolysis of these signaling lipids play an important role in a broad range of biological effects. A central molecule is a phosphatidylinositol-3 kinase, which primarily phosphorylates the lipid phosphatidylinositol on the 3 position of the D-*myo*-inositol ring, yielding phosphatidylinositol-3-phosphate, but also can use phosphorylated forms of phosphatidylinositol as substrates. $IP_6$ inhibits phosphatidylinositol-3 kinase (35). This action is related to the $IP_6$ structure that is similar to D-3-deoxy-3-fluoro-PtdIns, an inhibitor of phosphatidylinositol-3 kinase (35). In addition to the blocking of phosphatidylinositol-3 kinase and activating protein-1 by $IP_6$ (35), protein kinase C (16,57) and mitogen-activated protein kinases (15,35) are involved in $IP_6$-mediated anticancer activity. The role of $IP_6$ among these multiple signaling pathways and their cross-talk in regulation of cell functions needs to be addressed in the future.

**TABLE 3**

*Synergistic cancer inhibition by $IP_6$ when combined with inositol (Ins) 1,2-dimethylhydrazine (DMH)- induced colon carcinoma in mice*

| Experimental group | Tumor incidence (%) | Total number of tumors | No. of tumors/tumor bearing mice | Mitotic rate (%) |
|---|---|---|---|---|
| DMH | 63[1] | 22 | 12 | 1.92 ± 0.17 |
| DMH + $IP_6$ | 47[2] | 13 | 10 | 1.48 ± 0.15 |
| DMH + Ins | 30 | 9 | 6 | 1.01 ± 0.14 |
| DMH + $IP_6$ + Ins | 25 | 4 | 4 | 1.06 ± 0.13 |

[1] The difference in tumor incidence between DMH-only (carcinogen control group) and DMH + $IP_6$ + Ins is significant at $P < 0.001$.
[2] Between DMH + $IP_6$ and DMH + $IP_6$ + Ins at $p < 0.005$.
Adapted from Shamsuddin et al. (19).

**TABLE 4**

*7,12-Dimethylbenz[a]nthracene (DMBA)-induced mammary carcinoma in rats*

| Experimental group | Tumor incidence (%) | Total number of tumors | No. of tumors/tumor-bearing rat | Rats with ≥5 tumors (%) |
|---|---|---|---|---|
| DMBA | 92.5 | 113 | 3.1 ± 0.4[1] | 17.5 |
| DMBA + $IP_6$ | 71.5 | 69 | 2.5 ± 0.2[2] | 5.3 |
| DMBA + Ins | 75.0 | 64 | 2.1 ± 0.2 | 2.5 |
| DMBA + $IP_6$ + Ins | 76.3 | 51 | 1.8 ± 0.1 | 0.0 |

[1] The difference in total number of tumors, tumor burden (No. of tumors/tumor-bearing rats) and tumor multiplicity (Rats with ≥5 tumors) between DMBA-only and DMBA + $IP_6$ is significant at $P < 0.05$.
[2] Between DMBA and DMBA + $IP_6$ + Ins for tumor burden and multiplicity at $P < 0.05$.
Adapted from Vucenik et al. (52).

$IP_6$ can also modulate cellular response at the level of receptor binding. $IP_6$, after sterically blocking the heparin-binding domain of basic fibroblast growth factor, disrupted further receptor interactions (58). This modulation in binding and the activity of basic fibroblast growth factor is thought to be due to the chair conformation of $IP_6$ mimicking that of the pyranose ring structure in heparin (58).

The observed anticancer effect of inositol compounds could be mediated through several other mechanisms. The antioxidant role of $IP_6$ is known and widely accepted; this function of $IP_6$ occurs by chelation of $Fe^{3+}$ and suppression of $^{\cdot}OH$ formation (11). Therefore, $IP_6$ can reduce carcinogenesis mediated by active oxygen species and cell injury via its antioxidative function. This activity seems to be closely related to its unique structure. The phosphate grouping in positions 1,2,3 (axial-equatorial-axial) is unique to $IP_6$, specifically interacting with iron to completely inhibit its ability to catalyze hydroxyl radical formation, making $IP_6$ a strong antioxidant. This anticancer action of $IP_6$ may be further related to mineral binding ability; $IP_6$ by binding with $Zn^{2+}$ can affect thymidine kinase activity, an enzyme essential for DNA synthesis, or remove iron, which may augment colorectal cancer (3–5,41,46).

Besides affecting tumor cells, $IP_6$ can act on a host by restoring its immune system. $IP_6$ augments natural killer cell activity in vitro and normalizes the carcinogen-induced depression of natural killer cell activity in vivo (59).

### *Value of $IP_6$ as a therapeutic and preventive agent for cancer*

*Safety.* $IP_6$ is a natural compound and an important dietary component. Some concerns have been expressed regarding the mineral deficiency that results from an intake of foods high in $IP_6$ that might reduce the bioavailability of dietary minerals. However, recent studies demonstrate that this antinutrient effect of $IP_6$ can be manifested only when large quantities of $IP_6$ are consumed in combination with a diet poor in oligoelements (60–63). A long-term intake of $IP_6$ in food (60,61) or in a pure form (64) did not cause such a deficiency in humans. Studies in experimental animals showed no significant toxic effects on body weight, serum, or bone minerals (Table 5) or any

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**TABLE 5**

*Effect of inositol compounds on bone minerals*

| Treatment (*n*) | $Ca^{2+}$ (*mg/g*) | $Mg^{2+}$ (*mg/g*) | $Zn^{2+}$ (*μg/g*) |
|---|---|---|---|
| Tap water (*n* = 6) | 116.9 ± 13.9[1] | 1.13 ± 0.14 | 109.2 ± 14.9 |
| 15 mM $IP_6$ (*n* = 3) | 124.8 ± 11.3[2] | 1.19 ± 0.14 | 127.4 ± 11.5 |
| 15 mM Ins (*n* = 4) | 117.4 ± 14.2 | 1.10 ± 0.16 | 116.7 ± 14.5 |
| 15 mM $IP_6$ + 15 mM Ins (*n* = 5) | 125.9 ± 9.0 | 1.14 ± 0.06 | 115.1 ± 9.9 |

[1] Values are mean ± SD.
[2] There was no statistical difference among groups in the levels of bone minerals.
Reprinted with permission from Vucenik et al. (52).

pathological changes in either male F344 or female Sprague-Dawley rats for 40 wk (40,51,52). Grases et al. (65) confirmed our findings and also reported that abnormal calcification was prevented in rats given $IP_6$.

***$IP_6$ does not affect normal cells.*** The most important expectation of a good anticancer agent is for it to only affect malignant cells and not affect normal cells and tissues. That property was recently shown for $IP_6$. When the fresh $CD34^+$ cells from bone marrow was treated with different doses of $IP_6$, a toxic effect (inhibition of the clonogenic growth or as cytotoxicity on liquid cultures) was observed that was specific to leukemic progenitors from chronic myelogenous leukemia patients but no cytotoxic or cytostatic effect was observed on normal bone marrow progenitor cells under the same conditions (27). Recently, we (66) showed that $IP_6$ inhibited the colony formation of Kaposi's sarcoma (KS) cell lines, KS Y-1 (AIDS-related KS) and KS SLK (Iatrogenic KS), and CCRF-CEM (human adult T lymphoma) cells in a dose-dependent manner (66). However, in striking contrast to taxol, used as a control, $IP_6$ did not affect the ability of normal cells (peripheral blood mononuclear cells and T-cell colony-forming cells) to form colonies in a semisolid methylcellulose medium. Malignant and normal cells are known to have a different metabolism, growth rate, expression of receptors, etc., but the mechanism for this different selectivity of $IP_6$ for normal and malignant cells needs to be further investigated.

***$IP_6$ acts synergistically with standard chemotherapeutics.*** Current cancer treatment recognizes the importance of using combination therapy to increase efficacy and decrease side effects of conventional chemotherapy. Another important aspect of cancer treatment is overcoming acquired drug resistance. Our recent data demonstrate that $IP_6$ acts synergistically with doxorubicin and tamoxifen, being particularly effective against estrogen receptor–negative and doxorubicin-resistant cell lines, both conditions that are challenging to treat (67). These data are particularly important because tamoxifen is usually given as a chemopreventive agent in the post-treatment period and doxorubicin has enormous cardiotoxicity and its use is associated with doxorubicin resistance.

***$IP_6$ affects principal pathways of malignancy.*** Our goal is to identify agents that can target tumors at vulnerable sites and interrupt specific pathways of carcinogenesis. From the behavior and characteristics of malignant cells, several principal pathways of malignancy have been established, such as proliferation, cell cycle progression, metastases and invasion, angiogenesis, and apoptosis; interestingly, $IP_6$ targets and acts on all of them.

Uncontrolled proliferation is a hallmark of malignant cells, and $IP_6$ can reduce the cell proliferation rate of many different cell lines of different lineage and of both human and rodent origin (3–5,26,28,31–33,38). Although normal cells divide at a controlled and limited rate, malignant cells escape from the control mechanisms that regulate the frequency of cell multiplication and usually have lost the checkpoint controls that prevent replication of defective cells. $IP_6$ can regulate the cell cycle to block uncontrolled cell division and force malignant cells either to differentiate or go into apoptosis. $IP_6$ induces $G_1$ phase arrest and a significant decrease of the S phase of human breast (68,69), colon (69), and prostate (34) cancer cell lines. However, $IP_6$ causes the accumulation of human leukemia cells in the $G_2M$ phase of the cell cycle; a cDNA microarray analysis showed a down-modulation of multiple genes involved in transcription and cell-cycle regulation by $IP_6$ (27).

One important characteristic of malignancy is the ability of tumor cells to metastasize and infiltrate normal tissue. A significant reduction in the number of lung metastatic colonies by $IP_6$ was observed in a mouse metastatic tumor model using FSA-1 cells (39). Using highly invasive MDA-MB 231 human breast cancer cells, we demonstrated that $IP_6$ inhibits metastasis in vitro through effects on cancer cell adhesion, migration, and invasion (70,71). Tumor cells emit substances known as matrix metalloproteinases that allow metastatic cells to pass into the blood vessels; $IP_6$ significantly inhibited secretion of MMP-9 from MDA-MB 231 cells (70).

Tumors depend on the formation of new blood vessels to support their growth and metastasis. Many tumors produce large amounts of vascular endothelial growth factor, a cytokine that signals normal blood vessels to grow. $IP_6$ inhibited the growth and differentiation of endothelial cells (66,72) and inhibited the secretion of vascular endothelial growth factor from malignant cells (27,66,72). $IP_6$ can also adversely affect angiogenesis as antagonist of fibroblast growth factor (58).

Apoptosis is a hallmark of action of many anticancer drugs. It has been reported that $IP_6$ induces apoptosis in vivo (45) and in vitro in prostate (34) and cervical cancer (25) cell lines, involving cleavage of caspase 3, caspase 9, and poly ADP-ribose polymerase, an apoptotic substrate, in a time- and dose-dependent manner.

***Effectiveness of $IP_6$ as a cancer preventive agent.*** Possible mechanisms of the cancer preventive action of $IP_6$ include carcinogen blocking activities, antioxidant activities, and antiproliferation and antiprogression activities (73). Therefore, the strategy of chemoprevention is to use agents that will inhibit mutagenesis, induce apoptosis, induce maturation and differentiation, and inhibit proliferation (74). The antioxidant activity of $IP_6$ is widely accepted and indisputable (11), and $IP_6$ possesses antiproliferative and antiprogression activities. Its induction of terminal differentiation (26,28,29,32,33,38), restoration of immune response (59), modulation of growth factors (58), modulation of signal transduction pathways (15,16, 35,57), induction of apoptosis (25,34,45), and possibly inhibition of oncogene activity and restoration of tumor suppressor function are well documented. $IP_6$ not only inhibits the activities of some liver enzymes (75,76) but also significantly increases the hepatic levels of glutathione *S*-transferase (44,77), both of which indicate its possible role in carcinogen-blocking activities and cancer protection.

Although $IP_6$ may belong to almost all previously mentioned categories of cancer preventive drugs, affecting almost all phases of cancer prevention, it still appears that $IP_6$ is not a direct antagonist to the carcinogen because of its moderate efficacy in vitro when tested and compared with other chemopreventive agents (30) and a lack of dramatic decrease in cancer incidence when tested in vivo. However, because cancer prevention is a long process, a long administration of

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cancer preventive agent is generally needed, requiring usually 10–40 y of continuous treatment (2,73), and, therefore, it is very important that cancer preventive agents have low or almost no toxicity. $IP_6$, a natural compound with virtually no toxicity, can satisfy this special and very important requirement for cancer prevention.

### *$IP_6$ plus inositol and patients*

An enhanced antitumor activity without compromising the patient's quality of life was demonstrated in a pilot clinical trial involving six patients with advanced colorectal cancer (Dukes C and D) with multiple liver and lung metastasis (78). $IP_6$ plus inositol was given as an adjuvant to chemotherapy according to Mayo protocol. One patient with liver metastasis refused chemotherapy after the first treatment, and she was treated only with $IP_6$ plus inositol; her control ultrasound and abdominal computed tomography scan 14 mo after surgery showed a significantly reduced growth rate. A reduced tumor growth rate was noticed overall and in some cases a regression of lesions was noted. Additionally, when $IP_6$ plus inositol was given in combination with chemotherapy, side effects of chemotherapy (drop in leukocyte and platelet counts, nausea, vomiting, alopecia) were diminished and patients were able to perform their daily activities (78). Further controlled randomized clinical trials are necessary to confirm these observations.

### *Other biological effects of $IP_6$*

In humans, $IP_6$ not only has almost no toxic effects, but it has many other beneficial health effects such as inhibition of kidney stone formation and reduction in risk of developing cardiovascular disease. $IP_6$ was administered orally either as the pure sodium salt or in a diet to reduce hypercalciuria and to prevent formation of kidney stones, and no evidence of toxicity was reported (64,65,79,80). A potential hypocholesterolemic effect of $IP_6$ may be very significant in the clinical management of hyperlipidemia and diabetes (75,76,81). $IP_6$ inhibits agonist-induced platelet aggregation (82) and efficiently protects myocardium from ischemic damage and reperfusion injury (83), both of which are important for the management of cardiovascular diseases.

Many potential beneficial actions of $IP_6$ have been described. The inclusion of $IP_6$ plus inositol in our strategies for prevention and treatment of cancer as well as other chronic diseases is warranted. However, the effectiveness and safety of $IP_6$ plus inositol need to be determined in Phase I and Phase II clinical trials in humans.

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# RASPBERRIES AND RELATED FRUIT



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(For more information visit www.fruit.cornell.edu)

Raspberries are a high-value crop due to their unique flavor, exacting climatic requirements, high costs of production, and perishability. This article describes the relationships among various raspberry species and relatives, discusses the commercial raspberry industry, describes the morphology, anatomy and chemical composition of fruit, reviews harvesting and handling techniques for fresh fruit, and presents various food uses for the raspberry.

## Taxonomy and Commercial Importance

Raspberries are a diverse group of flowering plants that are closely related to blackberries. Both raspberries and blackberries belong to the genus *Rubus*. Taxonomists recognize 12 subgenera within *Rubus*, but only the raspberries *(Idaeobatus)* and blackberries *(Eubatus)* have obtained commercial significance. Species of a third group, the arctic raspberries (*Cylactis*), are harvested from the wild, and are popular in Scandinavia for making liqueurs, but these are not cultivated. The fruit of raspberries detaches from the receptacle when picked, leaving a white torus attached to the plant and a hollow fruit. This characteristic distinguishes them from blackberries in which fruit abscission occurs behind the receptacle.

The genus *Rubus* is a member of the rose family (Rosaceae), which also includes important fruit crops such as apples, pears, cherries, peaches, plums, and strawberries. Wild raspberries occur on five continents, but are most abundant in the Northern Hemisphere. The temperate and subtropical region of eastern Asia is recognized as the centre of origin where the most diversity exists. More than 200 species have been identified, but only a few are important commercially. These include the European red raspberry *(R. idaeus* subsp. *vulgatus* Arrhen.), the North American red raspberry *(R. idaeus* subsp. *strigosus* Michx.), and the black raspberry *(R. occidentalis L.)* of the eastern USA. Hybrids between the red and black raspberry are commonly called purple raspberries because of the fruit and cane color, and these were once given the specific rank of *R. neglectus* Peck. However, most taxonomists do not recognize hybrids as distinct species. Interspecific hybrids with blackberries have also been made; some of these are commercially important, such as Tayberry, Loganberry, Boysenberry and Youngberry.

Several other species within the genus *Rubus* have edible fruit, or have been used by plant breeders to improve cold hardiness and resistance to diseases and insects in raspberries. Examples are *R. glaucus* Benth., a South American tetraploid black raspberry that is probably a raspberry x blackberry hybrid, *R. leucodermis* Torr. and Gr. (the western North American black raspberry), and *R. spectabilis* Pursh. (salmonberry); the Asiatic species *R. coreanus* Miq., *R. phoenicolasius* Maxim. (Japanese wineberry), *R. parvifolius* Nutt. (trailing raspberry), *R. ellipticus* Sm. (golden evergreen), *R. illecebrosus* Focke (strawberry raspberry), *R. kuntzeanus* Hemsl. (Chinese raspberry), and *R. nivens* Thumb.; the Hawaiian species *R. macraei* Gray and *R. hawaiiensis* Gray (Akala berries); and the arctic raspberries of Europe *(R. arcticus L.)* and North America *(R. stellatus* Sm.).

## Commercial Industry

Raspberries were first introduced into cultivation in Europe nearly 450 years ago. By the early nineteenth century, more than 20 cultivars of red raspberry were grown in both England and the USA. English cultivars were then exported to the USA, where crosses between them and North American seedlings gave improved cultivars. Red raspberries are the most widely grown, while black raspberries are popular only in certain regions of the eastern USA. The progeny of black and red raspberries have purple fruits and canes; these types are popular in eastern North America. Yellow-fruited *R. idaeus*, caused by a recessive mutation, is also grown on a limited scale for specialty markets. *R. occidentalis* genotypes with yellow fruit are not grown commercially.

The three major raspberry production regions are (1) Russia, (2) Europe (mostly in Poland, Hungary, Serbia, Germany, and the UK), and (3) the Pacific Coast of North America (British Columbia, Washington, and Oregon). Much of the fruit produced in these regions is harvested mechanically and processed. In other production regions, such as eastern North America, nearly all the production is for the fresh market. Many other countries, such as Chile, New Zealand, and Australia, have significant production as they supply the fresh market during winter in the northern hemisphere. World production is estimated at more than 400 000 t.

Patterns of production in North America shifted dramatically in the early 1900s. In 1920, New York State growers (East Coast) harvested more than 4000 ha. The systemic 'mosaic virus disease' infected most of the planting stock, and the processing raspberry industry collapsed in this region. With the development of tissue culture propagation techniques, virus indexing of nursery stock, and breeding for resistance to the virus vector, the raspberry processing industry redeveloped on the West Coast. In Britain during World War II, little attention was paid to maintaining the health of raspberry stock. It was not until the 1970s that the raspberry industry reorganized with certification programs and heat treatment therapy for the elimination of viruses. Currently, there is interest in both Europe and North America in greenhouse raspberry production to supply local markets during winter and spring.

## Varieties

Two types of bearing habits are found in commercial red raspberries. The first type is called a 'summer-bearing' habit. Canes originate from either crown buds or adventitious root buds in early spring. Canes elongate during the growing season, forming fruit buds in the axils of leaves in the autumn when temperatures decrease and day lengths shorten. The plants become dormant for winter, then the buds on the cane grow the following spring once the chilling requirement has been fulfilled. The chilling requirement varies considerably among summer-bearing varieties, ranging from a few hundred hrs to more than 1800 hrs. The lateral axillary buds on dormant canes contain both leaf and flower primordia. At the onset of warm weather, buds break and flowering occurs about 6 - 10 weeks later. Fruiting occurs in early to late summer, depending on variety, then the entire cane senesces. While these second year canes (floricanes) are flowering, first year canes (primocanes) are growing from the crown or roots. These primocanes will fruit the following year. More than 40 summer-bearing red raspberries are grown commercially, and these change with the release of new, improved varieties. Among the major varieties originating in North America are 'Boyne', 'Canby', 'Killarney', 'Meeker', 'Reveille', 'Taylor', 'Titan', 'Tulameen', and 'Willamette'. The Glen and Malling series are important varieties from the UK. The Scottish Crops Research Institute is the leading institution in the world for raspberry variety development.

The second type of growth habit is called 'fall bearing'. In some varieties, fruiting laterals will develop from the top of first year primocanes after they reach a certain height, without any chilling. If the growing season is sufficiently long, fruit can be harvested from the upper portion of these canes through autumn. The lower portion of the cane will fruit the following summer, if it is allowed to remain in the field. The major variety worldwide is 'Heritage'; other important varieties are 'Amity', 'Autumn Bliss', 'Polana', and 'Autumn Britten.'
At least 13 varieties of black raspberries are grown in North America. The most successful are 'Allen', 'Bristol', 'Haut', and 'Jewel'. 'Royalty' is the most popular purple raspberry. Several yellow or golden raspberry varieties are grown on a small scale for specialty markets.

## Morphology and Anatomy of Fruit

Each raspberry flower contains from 60 to 160 ovaries. Each ovary contains two ovules, but one usually aborts after differentiation. About one month after pollination, the ovaries ripen simultaneously to form the fruit.
Fruits ripen in three phases. Pollination is followed by a period of rapid cell division. In the second phase, cell division slows while the embryo develops and the seed coat hardens. Finally, very rapid growth occurs due to cell enlargement. Each phase lasts from 10 to 12 days. Considerable variation in fruit size exists, with a range from 1 to 5 g. Under certain conditions, fruit can exceed 10 g.
As with most climacteric fruit, ethylene production in the raspberry begins in the receptacle when the fruit starts to color, and peaks when fully ripe. Respiration, however, decreases as ripening proceeds. Growth stresses help to fracture the

and other hydrocarbons (naphthalene and related compounds). A particular ketone, 1-(*p*-hydroxyphenyl)-3-butanone, has an odor very characteristic of raspberry.

Considerable changes occur in phenolic compounds as fruits mature. Raspberry juice from ripe fruit contains 0.10 - 0.14% polyphenols, mainly as catechin and chlorogenic, ferulic and neochlorogenic acids.

Raspberry fruits contain small amounts of vitamins; only vitamin C is present at a significant level. Amino acids include alanine, serine, asparagine, glutamic acid, glutamine, (gamma)aminobutyric acid, valine, leucine, and aspartic acid. The high levels of antioxidants and ellagic acid in raspberries have been highlighted in the popular press as public interest in cancer prevention increases. Table 1 presents data on the vitamin and nutrient content of red raspberries.

*Fruit Color*

The color of raspberry fruit is imparted by anthocyanins. The anthocyanin molecule in raspberry consists of cyanidin and pelargonidin with glucose attached at the 3-position. Additional glucose, rhamnose or xylose sugars may be present in various combinations to give diglycosides or triglycosides. Fruits with a preponderance of pelargonidin glycosides have an orange-red color, as opposed to a deep red color with cyanidin glycosides.

A few major genes control the type and concentration of anthocyanins in the fruit. Fruits with a yellow color are produced when one or more of these genes suppress anthocyanin production. Molecules that are complexed with the anthocyanin also affect color development, and the pH of the fruit has a small effect. During storage, raspberry fruits increase in anthocyanin, darken, and become bluer, partially due to an increase in pH of the cytoplasm.

## Handling and Storage of Fresh Produce

*Harvest Considerations*

Raspberries have one of the highest respiration rates of any fruit. This, coupled with their thin skin and sugary interior, makes them among the most perishable of all fruits. With any given variety, fruit on a bush will ripen over a period of several weeks. Harvesting the same planting frequently (once every two days) is critical. Fruit harvested before it is fully ripe will have a much longer shelf life than fully ripe or overripe fruit, but will be lower in sugar and anthocyanins. The optimum stage of maturity for the raspberry occurs when the berry first becomes completely red, but before any darker hues develop.

Fruit quality for fresh market raspberries usually declines as the season progresses. Marketing channels must be open before the first berries ripen, as these are likely to be the highest quality and have largest size for the season. Berries should not be touched before harvest, and only undamaged berries with good appearance should be placed in the pack. The magnitude of injury caused by human pickers can be so great as to mask any other causes of deterioration.

Overripe or damaged berries should be harvested and discarded because they are susceptible to moulds. *Botrytis* is the most common pathogen of raspberry fruit. Once the mould growing on overripe berries sporulates, large amounts of inoculum will be present to infect other ripening fruit. Overripe berries also attract ants, wasps, and other pests.

Containers holding approximately 150 g of raspberries are typically used. Wide, shallow containers are preferable to deep containers; each should have no more than four layers of raspberries to prevent crushing. Many different types of containers are available, but among the most popular today are plastic clamshells.

*Post-harvest Considerations*
The objective of post-harvest handling of raspberries is to slow the respiration and transpiration rate of fruit. Respiration and transpiration result in shrinkage and reduced sweetness. Conditions that slow the respiration process are low temperatures, high carbon dioxide levels, and low oxygen in the storage chamber. Transpiration is slowed by high humidity.

Temperature is the easiest environmental variable to modify for extended storage of raspberries. A 5C reduction in temperature reduces the respiration rate by approximately 50%: at 0C the respiration rate is 24 mg of carbon dioxide per kg of raspberry per hour; at 5C the rate is 55; at 10C the rate is 92; at 15C the rate is 135; and at 20C the rate is 200. Rapid movement of cold, humid air through the berries is essential during the first few hours after harvest to slow respiration and prevent deterioration. Large growers may have a precooling facility specifically designed for removing field heat, and an additional storage cooler. For every hour delay in cooling, shelf life can be reduced by one day. Growers take advantage of natural night cooling by harvesting fruit as early in the morning as possible. Mechanical harvesting is often done at night.

Once the berries are cool, containers are wrapped in plastic to prevent water loss from the fruit and condensation on the berries when they are removed from the cooler. The plastic is not removed until the temperature of the berries warms to near the ambient temperature.

The storage room can be maintained as low as -1C. Berries will not freeze at or above this temperature because the sugars in the fruit depress the freezing point. When temperature is lowered, the amount of moisture in the air is reduced. For raspberries, it is critical to maintain a humid atmosphere (90-95%) simultaneously with low temperature to prevent water loss from the fruit. Special cooling units designed to maintain a high humidity are required for raspberries. At 25C and 30% relative humidity, fruits lose water 35 times faster than at 0C and 90% relative humidity.

A high carbon dioxide (15-20%) and/or low oxygen (5-10%) atmosphere will reduce respiration and mould growth. *Botrytis, Rhizopus, Alternaria,*

*Penicillium* and *Cladosporium* can cause post-harvest fruit rots, depending on storage temperature and carbon dioxide level. Modified atmospheres are used frequently when raspberries are transported long distances. Special semi-permeable wraps are sometimes used by shippers to create a modified atmosphere (low oxygen, high carbon dioxide) within individual containers. Low-oxygen atmospheres will extend the shelf life of raspberries, but bad-tasting aldehydes and alcohols can accumulate in the raspberry fruit when oxygen is limited for an extended period. Off-flavors and browning also develop when raspberries are held under elevated carbon dioxide levels for an extended period. Raspberries are considered to be at moderate risk of physiological injury from high carbon dioxide or low oxygen atmospheres.

The loss of raspberries from harvest to the consumer's table has been estimated at more than 40%. A 14% loss occurs from farmer to wholesaler, a 6% loss occurs from wholesaler to retailer, and 22% is lost between the retailer and consumer. Most of these losses are due to poor handling of berries after harvest.

## Fruit Uses

Raspberries have been eaten fresh for thousands for years. Medicinal uses of raspberries are frequently found in the literature, with references to raspberry leaf tea dating to the 16$^{th}$ century. In the early 1900s, black raspberry juice was extracted, concentrated, and used as an edible dye for foodstuffs, such as meat. Raspberries were also dehydrated for long-distance transport. Today, raspberry fruits are either harvested by hand and eaten fresh, or machine-harvested and processed. Approximately 27,000 tons are processed each year. The major products of processing are IQF, block frozen, puree frozen (8 – 15° Brix), juice (about 9° Brix), concentrate, canned, aseptic packs, and preserves. These products are then packaged and sold directly to the consumer, or reprocessed into jam, jelly, dessert topping, pie filling, ice cream, yoghurt and other desserts. Raspberry juice is usually blended with apple, pear, or grape juice because the flavor is too intense for direct consumption. Recently, the demand for fruit wines has increased, and raspberries make one of the better wines. Some wineries add raspberry juice to grape wine to obtain a less expensive raspberry-flavored wine. Raspberry beer is also made at breweries and meaderies. The popularity of raspberries continues to grow as many raspberry-containing products are now on supermarket shelves.

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**Table 1.** Reported values or ranges of nutrient content in 100 g of fresh raspberries

| **Nutrient** | **Amount** |
|---|---|
| Water (g) | 84-87 |
| Food energy (kcal) | 31-49 |
| Protein (g) | 0.42-1.40 |
| Fat (g) | 0.20-0.55 |
| Carbohydrate (g) | 5.8-11.6 |
| Fiber (g) | 3.0-7.4 |
| Ash (g) | 0.40-0.51 |

| **Minerals** | **Amount (mg)** |
|---|---|
| Calcium | 22-50 |
| Iron | 0.57-1.20 |
| Magnesium | 18-30 |
| Phosphorus | 12-50 |
| Potassium | 130-221 |
| Sodium | 0-2.5 |
| Zinc | 0.46 |
| Copper | 0.07-0.21 |
| Manganese | 1.01 |
| Sulphur | 17.3 |
| Chlorine | 22.3-22.8 |
| Boron | 71-125 |

| **Vitamins (mg)** | **Amount (mg)** |
|---|---|
| Carotene | 0.05-0.08 |
| Thiamin | 0.01-0.03 |
| Riboflavin | 0.03-0.10 |
| Pantothenic acid | 0.24-0.30 |
| Nicotinamide | 0.20-1.00 |
| Vitamin B6 | 0.06-0.90 |
| Vitamin C | 13-38 |
| Tocopherols | 0.3-4.5 |

CHAPTER

9

# Influence of Dietary Polyphenols on Carbohydrate Metabolism

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## 1. INTRODUCTION

The global prevalence of diabetes mellitus has been increasing dramatically due to the fact that globalization has led to social impacts such as urbanization and lifestyle changes.[1] Diabetes, especially type 2 diabetes mellitus (T2DM), previously thought to be prevalent in developed countries,[2] has now emerged as a potential epidemic in developing countries like China and India.[3] T2DM was once believed to be a metabolic syndrome exclusive to adults, but has now risen as a plague in adolescents and children.[4] In 2010, an estimated 285 million cases of diabetes were reported; this figure is expected to increase by 54% to 439 million by 2030.[3] Despite the availability of a variety of pharmacological agents to control blood glucose level (from insulins, sulfonylureas, biguanides, thiazoledinediones to the most recent dipeptidyl peptidase IV (DPP IV) inhibitors), lifestyle changes involving exercise and dietary intervention are pivotal to achieving the goal of cost-effective management with minimal complications but maximal quality of life. As pharmacological intervention is invariably associated with adverse effects such as hypoglycemia, gastrointestinal discomfort, weight gain and fluid retention, exercise and dietary intervention are vital in the management of diabetic patients and those in the high-risk group, in whom multiple risk factors are present (Table 9.1). Over the years, a wealth of evidence has accumulated, correlating lower consumption of carbohydrate, saturated fat, processed food and higher consumption of fruits, vegetables, legumes, coffee, and tea with lower risk of diabetes and improved glucose and lipid metabolism. Plant-based foods are always rich in a group of phytochemicals, known as polyphenols.

Polyphenols can be found abundantly in fruits (berries, grape), plant-based beverages (coffee, tea, wine), vegetables (broccoli, cabbage), cereals and legumes. There are at least 15 classes of polyphenols, classified according to the number of phenol ring(s) each contains and the structural elements that bind these rings together.[5] Flavonoids represent the major and the most-studied polyphenols, and can be further sub-divided into flavonols, flavones, flavanones, catechins, anthocyanidins, and isoflavones.[6] In addition, phenol rings may be associated with carbohydrates, organic acids and with one another, forming classes of compounds like phenolic acid, lignans and stilbenes. Table 9.2 shows the classes of polyphenols with their respective examples. Polyphenols are considered to be secondary metabolites and do not possess any specific metabolic function in plant cells.[7] They are of interest to researchers and food manufacturers due to their strong antioxidant capacity, which can combat oxidative stress that leads to various complications in diabetics. Earlier studies have shown that these polyphenols regulate various enzymes,[8,9] protein kinases,[10–12] and receptors.[13]

Maintaining glucose homeostasis is one of the most important physiological processes in humans. Plasma glucose is maintained at approximately 90 mg/dL (5 mmol/L), with a maximal postprandial increase not exceeding 165 mg/dL (9.2 mmol/L)[14] or a decrease to not lower than 55 mg/dL (3.1 mmol/L) after exercise[15] or a 60-hour fast.[16] Prolonged hyperglycemia will result in complications that include retinopathy, nephropathy, neuropathy, dyslipidemia and cardiovascular disease. Several organ systems coordinate and regulate glucose homeostasis; these include: (1) the gastrointestinal tract, which regulates carbohydrate digestion and glucose

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TABLE 9.1 Risk Factors for T2DM

| Non-Modifiable Risk Factors | Modifiable Risk Factors |
|---|---|
| Age | Obesity |
| Sex | Sedentary lifestyle |
| Ethnicity | Dietary factors |
| Family history | Smoking |
| Polycystic ovary syndrome | History of impaired glucose tolerance |
| | Abnormal lipid profile |
| | Inflammation |
| | Intrauterine environment |

absorption before release into the systemic circulation, (2) the endocrine pancreatic system, which secretes key regulatory hormones, insulin and glucagon, in response to abnormal glucose levels, (3) the liver, where glucose synthesis, glycogen storage and breakdown are initiated, and (4) insulin-sensitive peripheral tissues like skeletal muscle and adipose tissue, where glucose is metabolized for energy or stored for future use. This review aims to summarize available data on how dietary polyphenols regulate glucose homeostasis through their effects on these organ systems.

## 2. POLYPHENOLS AND GLYCEMIC RESPONSES

Regular consumption of coffee has been associated with a lower risk of T2DM. This finding has been replicated across sexes, geographical locations and obesity levels. [1] Battram *et al.* showed that decaffeinated coffee resulted in a 50% lower glucose response in an oral glucose tolerance test but others found no acute effect on glucose metabolism by coffee. [2] Nevertheless, a very recent cross-sectional study of a population of 954 multi-ethnic non-diabetic adults, showed that acute consumption of coffee was positively related to insulin sensitivity and beta cell function. [3] Notably, these beneficial effects of coffee were not attributable to caffeine. [1,3] Catechin-rich tea was shown to lower blood glucose, improve glucose tolerance and enhance glucose-stimulated insulin secretion in spontaneous *db/db* diabetic mice, [4] streptozotocin (STZ)-, alloxan- and high-cholesterol diet-induced [5] diabetic rats. In Taiwan, 30-day consumption of oolong tea was shown to reduce plasma glucose levels of T2DM patients markedly. [6] However, these effects were not observed in other human studies. [7] Animal [8] and human [9] studies were also conducted on soybean or isoflavones-enriched diet to show their effects in moderating hyperglycemia and insulin levels. However, Hall

TABLE 9.2 Classes and Examples of Polyphenols

| Classes/Subclasses | Examples | Sources |
|---|---|---|
| *FLAVONOIDS* | | |
| 1 Flavonols | Fisetin | Strawberry |
| | Galangin | Propolis |
| | Kaempferide | Aromatic ginger |
| | Kaempferol | Tea, broccoli |
| | Morin | Guava |
| | Myrocetin | Grape, walnut |
| | Quercetin | Tea, apple |
| | Rutin | Buckwheat, asparagus |
| 2 Lavones | Apigenin | Parsley, thyme |
| | Luteolin | Celery, green pepper |
| | Diosmetin | Oregano |
| | Chrysin | Propolis, honey |
| 3 Flavanones | Naringenin | Grapefruit |
| | Naringin | Citrus fruit |
| | Hesperidin | Citris fruit |
| 4 Catechins | (+)-Catechin | Cocoa, tea |
| | (−)-Epicatechin | Cocoa, tea |
| | (+)-Gallocatechin | Green tea, banana |
| | (−)-Epigallocatechin galate | Green tea |
| | Theaflavin | Black tea |
| | Theaflavin gallate | Black tea |
| | Theaflavin digallate | Black tea |
| 5 Anthocyanidins | Cyanidin | Grape, berry |
| | Delphinidin | Cranberry, pomegranate |
| | Pelargonidin | Berry, plum |
| | Peonidin | Berry, plum |
| 6 Isoflavones | Genstein | Soybean, fava bean |
| | Genistin | Soybean |
| | Daidzen | Soybean |
| | Daidzin | Soybean |
| *PHENOLIC ACIDS* | | |
| 1 Benzoic acid derivatives | Gallic acid | Tea |
| | Protocatechuic acid | Mushroom |
| | Paraben | Blueberry |
| | Salicylic acid | Berry, date |

(*Continued*)


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TABLE 9.2 (*Continued*)

| Classes/Subclasses | Examples | Sources |
|---|---|---|
| 2 Cinnamic acid derivatives | *p*-Coumaric acid | Peanut, tomato |
| | Caffeic acid | Coffee |
| | Ferulic acid | Coffee, cereal |
| | Chlorogenic acid | Coffee |
| *LIGNANS* | | |
| | Pinoresinol | Sesame |
| | Matairesinol | Whole grain |
| | Secoisolariciresinol | Linseed |
| *STILBENES* | | |
| | Resveratrol | Grape |
| | Piceatannol | Red wine |
| | Pterostilbene | Grape, blueberry |
| *OTHER* | | |
| 1 Curcuminoids | Curcumin | Turmeric |

*et al.* (2006) reported that no significant effects were observed in plasma glucose, insulin or lipids in postmenopausal women taking an isoflavones-enriched diet.[34] Berry purée (consisting of strawberries, cranberries, bilberries and blackcurrants) was found to decrease the postprandial glucose response in healthy subjects by delaying digestion and/or absorption of sucrose.[35] Grapes, especially procyanidin-rich grape seed extract, were shown to ameliorate diet-induced insulin resistance in animals.[36,37] Also, by using the euglycemic-hyperinsulinemic clamp, red wine consumption was found to enhance whole-body, insulin-mediated glucose disposal in T2DM patients.[38] Spices like cinnamon[39,40] and turmeric[41,42] were shown to possess modulatory effect on blood glucose in humans and animal models. Beneficial effects of polyphenol-rich herbs (such as fenugreek,[43] *Coptis chinensis*[44] and *Vernonia amygdalina*[45]) in diabetic animal models have also been reported.

The potential of individual polyphenols to regulate glucose metabolism has yet to be adequately investigated in human epidemiological studies. However, in animal models, mechanistic studies have shown promising results. Despite the vast amount of epidemiological studies on the protective effects of coffee on the development of T2DM, can the paradoxical effect of caffeine be counteracted by phenolic compounds that are also present in coffee? In a randomized crossover trial in healthy men, ingestion of chlorogenic acid (CGA), a major phenolic compound in coffee,[46] was found to reduce early glucose and insulin responses in oral glucose tolerance tests.[22] Recently, we showed that in the spontaneously diabetic *db/db* mice, CGA caused a significant decrease in fasting blood glucose before and after glucose challenge.[47] Another study also showed that the total area under the curve (AUC) for blood glucose was significantly attenuated in rats fed CGA.[48] Caffeic acid, another polyphenolic compound in coffee, was also shown to reduce blood glucose and glycated hemoglobin levels in *db/db* mice.[49] Last but not least, ferulic acid[50] and isoferulic acid[51] from coffee also exerted hypoglycemic responses in spontaneously or chemically induced diabetic animal models.

Quercetin, which is abundant in green and black tea, has been shown to decrease fasting and postprandial blood glucose and glycated hemoglobin levels, without significant influence on blood insulin levels.[52] Quercitrin, a quercetin glycoside, was found to decrease plasma glucose and increase insulin levels in the STZ-induced diabetic rat.[53] Another flavonol, rutin, decreased blood glucose and improved lipid profile in the STZ-diabetic rat [54] and diabetic human subjects.[55] Ten-day treatment with apigenin increased serum level of insulin with a parallel decrease of serum glucose in alloxan-diabetic animals.[56] Combinatory treatment of naringin with vitamin C[57] and hesperidin[58] decreased blood glucose levels in diabetic animals. Also, naringenin has been demonstrated to attenuate hyperglycemia in fructose-fed[59] and high-fat-LDL-receptor negative[60] insulin-resistant animals. Epigallocatechin gallate (EGCG) was shown to improve glucose tolerance in *db/db* mice and ZDF rats.[61] In addition to improving glucose tolerance, Ortsäter *et al.*[26] found that EGCG increased glucose-stimulated insulin secretion. Studies have reported that several derivatives of cyanidine are also involved in the modulation of glucose homeostasis. In a study in *db/db* mice and high fat diet (HFD)-induced diabetic mice, cyanidin-3-glucoside was found to improve insulin resistance and also glucose levels, compared to their respective controls.[62] Another study using normal rodents suggested that cyanidin-3-rutinoside was effective in suppressing and delaying postprandial hyperglycemia.[63] Well-known for its estrogenic effect, genistein has been shown to ameliorate hyperglycemia, increase insulin secretion and improve glucose tolerance, without altering insulin tolerance in STZ-diabetic rats.[64,65] Oral treatment with gallic acid daily for 21 days alleviated high blood glucose and stimulated insulin secretion in STZ-diabetic rats.[66]

Resveratrol, the most extensively studied stilbene, was consistently demonstrated to lower blood glucose and insulin levels in diet-induced diabetic mice[67,68] and swine fed on a high-cholesterol diet.[69] However, such a decrease was not observed in insulin levels of an STZ-nicotinamide-induced model.[70] Recently, its effect was tested in T2DM subjects. Oral treatment of resveratrol for a month significantly decreased blood

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glucose, delayed the postprandial glucose peak and improved the homeostatic model assessment of insulin resistance ($HOMA_{IR}$).[7] In rats rendered diabetic by a combination of HFD and STZ, supplementation with curcumin reduced the glucose level and improved insulin tolerance.[8] Another study proposed that the anti-hyperglycemic and insulin-sensitizing properties of curcumin may be attributed to its anti-inflammatory action.[9]

## 3. POLYPHENOLS AND CARBOHYDRATE DIGESTION/ABSORPTION

Prior to being absorbed into the blood stream, all carbohydrates must be hydrolyzed into monosaccharides. The digestion of starch starts with the action of salivary α-amylase and is followed by pancreatic α-amylase in small intestines. The resultant mixture of shorter oligosaccharides crosses the intestinal brush border membrane, where another digestive enzyme, α-glucosidase, degrades them to glucose. The glucose molecules will then enter the bloodstream by a specific means of transport (this will be discussed in the following sections). Therefore, one of the approaches to manage early-stage hyperglycemia is to control the postprandial glycemic response. The inhibition of α-amylase[10] and α-glucosidase[11] can significantly delay the postprandial surge of glucose. Consequently, α-glucosidase inhibitors (AGIs), introduced in 1996, became the only drug class that does not target a pathophysiological defect in T2DM. Acarbose, an example of an AGI, is commonly used in combinatory therapy with sulfonylureas, biguanides or insulin.[12] Therefore, one of the mechanisms by which polyphenols reduce glucose levels may be the inhibitory effect on these digestive enzymes.

### 3.1 Polyphenols and α-Amylase

A recent study evaluated the effect of extract of blueberries from 15 cultivars on α-amylase activity.[13] The cultivars (with polyphenol levels ranging from $0.39 \pm 0.03$ mg GAE/g to $1.00 \pm 0.02$ mg GAE/g) showed a similar inhibitory effect as acarbose on the activities of α-amylase. The wine made from these berries had a similar inhibitory effect on α-amylase activity.[13] Another study demonstrated that raspberry and rowanberry inhibited α-amylase activity *in vitro* at $IC_{50}$ values of 21.0 and 4.5 μg/mL, respectively.[14] The report also proposed that these inhibitory effects were not attributable to anthocyanins but to the proanthocyanidins in the berries. This was further explained by the inhibitory effect of procyanidins from grape seed extract on α-amylase activity.[15] Among all the berries, strawberry and raspberry were found to be the most effective inhibitors of α-amylase.[16] Tiliroside, a glycosidic flavonoid found in strawberry, inhibited porcine pancreatic α-amylase activity *in vitro* more potently than α-glucosidase activity.

Lee *et al.* (2010) investigated the ability of green, black and oolong teas to inhibit α-amylase. Their results revealed that black tea was the most potent in inhibiting α-amylase ($IC_{50}$ 0.42–0.67 mg/ml) and therefore theaflavins (3.7 to 67 μM) appeared to be a better inhibitor, compared to catechins (1.5 to 20 mM).[17] This is in line with the earlier study which reported that 0.05 mg/ml of total polyphenols (catechin, epigallocatechin gallate, epicatechin gallate, epigallocatechin and epicatechin) caused only a mild 61% inhibition of α-amylase activity.[18]

3-Caffeoylquinic acid or CGA at an $IC_{50}$ value of $0.23 \pm 0.03$ mM and its derivatives were shown to inhibit α-amylase activity.[19] Other polyphenols with inhibitory effects on α-amylase activity are luteolin, myricetin and quercetin with $IC_{50}$ values of 0.36, 0.38 and 0.50 mM, respectively. However, other flavonoids like kaempferol, fisetin, apigenin, naringenin, cyanidin and daidzein showed insignificant inhibition. The inhibitory activities of six groups of flavonoids were reported (in decreasing order) as isoflavone > flavone > flavonol > anthocyanidin > flavanone = flavan-3-ol.[20]

### 3.2 Polyphenols and α-Glucosidase

In the same aforementioned study, the authors observed that all blueberry cultivar extracts have higher α-glucosidase inhibitory capabilities than acarbose.[13] Similar inhibition was found in wine fermented from blueberries.[13] Besides blueberry, blackcurrant and strawberry were also shown to be effective in inhibiting α-glucosidase activity.[16] By using rat brush border membrane vesicle and Caco-2 colonic carcinoma cells, Kamiyama *et al.*[21] showed that catechins and gallated catechins inhibited α-glucosidase activity, with gallated catechins appearing to be stronger inhibitors ($IC_{50}$ 16–67 μM), not only on α-amylase, but also α-glucosidase. Theaflavins were found to perform better than catechin in inhibiting α-glucosidase.[17] Interestingly, this finding is in accord with a study which showed that the product of co-fermentation of green tea leaves with loquat leaves exhibited a three-fold increase in inhibitory activity against α-glucosidase, compared to both unfermented constituent leaves. The authors explained that this might be due to the polyphenolic oxidases found abundantly in loquat leaves, which catalyze the oxidation of catechins to theaflavins.[22]



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A study demonstrated that CGA inhibited α-gluocosidase activity, with an $IC_{50}$ value of $2.82 \pm 0.02$ mM.[88] The authors further synthesized ketal and acetal derivatives of CGA and found them to have a remarkable inhibitory effect on α-glucosidase ($IC_{50}$ ranged from $0.1 \pm 0.03$ μM to $154.2 \pm 0.02$ μM).[88] Another study revealed that quercetin, isoquercetin and rutin ($IC_{50}$ of 0.017, 0.185 and 0.196 mM, respectively) were all powerful inhibitors against α-glucosidase.[89] The dose of acarbose ($IC_{50}$ 0.091 mM) was almost five times that of quercetin and half that of isoquercetin and rutin.[89] Out of six groups of flavonoids, most were considerably potent in inhibiting α-glucosidase activity, except for several individual polyphenols such as apigenin and non-gallated catechins. The comparative inhibitory activities of six flavonoid groups (in decreasing order) were concluded to be anthocyanidin ≥ isoflavone ≥ flavonol > flavones > flavanone > flavan-3-ol.[85]

## 3.3 Polyphenols and Intestinal Glucose Absorption

Intestinal absorption of glucose is dependent on luminal glucose concentrations. At lower concentration, glucose is actively transported against its concentration gradient across the intestinal membrane via the sodium-dependent glucose transporter (SGLT) 1. At higher concentrations, glucose is mainly transported by facilitated-transporter glucose transporter (GLUT) 2.[90] The effect of polyphenols on intestinal glucose transporters has been studied using intestinal brush border membrane vesicles or everted sacs and Caco-2 colonic carcinoma cells. A recent report demonstrated the inhibitory effects of apple and strawberry extract on the apical and basolateral uptake of glucose in the Caco-2 cellular model. The authors also resolved that pure polyphenols like phloridzin, quercetin-3-*O*-rhamnoside and pelargonidin-3-*O*-glucoside from the extracts of these two fruits were very effective inhibitors.[91] Tiliroside from strawberry suppressed hyperglycemia induced by oral glucose administration but not by intraperitoneal glucose administration.[81] This indicates that the suppression of postprandial hyperglycemia by tiliroside was not only through inhibition of carbohydrate-hydrolyzing enzymes but also through glucose uptake in intestines. The addition of tiliroside to Caco-2 cells had also been shown to cause a dose-dependent reduction in glucose uptake, with the inhibition more potent under sodium-rich than sodium-free conditions ($IC_{50} = 97$ μM vs. 240 μM), indicating that tiliroside inhibited SGLT 1 more potently than GLUT2.[81] Quercetin, myricetin and fisetin inhibited glucose uptake non-competitively into xenopus oocytes expressed with GLUT2. However, SGLT 1 was unaffected by these flavonoids.[92] In another study, under sodium-free condition, several flavonoids such as myricetin, quercetin and apigenin were similarly found to inhibit glucose uptake in Caco-2 cells, suggesting that they only affected GLUT2. On the other hand, phloridzin caused a marked reduction in glucose uptake under sodium-dependent conditions, thus characterizing its capability to inhibit SGLT 1. Several catechins, such as EGCG, ECG and EGC, were found to inhibit both GLUT2 and SGLT 1.[93] Earlier work by Welsch *et al.*[94] showed that caffeic acid, ferulic acid and CGA inhibited sodium-dependent glucose uptake by rat brush border membrane vesicles. Nevertheless, Johnston *et al.*[95] found that none of these phenolic acids had any significant effects on either sodium-dependent or sodium-free glucose uptake. They believed that it might be due to differences in the weight ratio of test substance to substrate.

## 3.4 Polyphenols and Incretins

Although glucose is the major regulator of insulin secretion, incretins, a group of gastrointestinal hormones, have been estimated to be responsible for as much as 50% of insulin secretion after a glucose challenge.[95] The very first incretin hormone identified was gastric inhibitory polypeptide (GIP), later renamed glucose-dependent insulinotropic polypeptide. It is secreted from intestinal K-cells in response to nutrient ingestion, especially glucose and fat. Its main biological function is to potentiate glucose-dependent insulin secretion.[96] However, it was soon discovered that GIP alone could not account for this stimulatory effect. In humans with ileal resection, the incretin effect was diminished, in spite of normal plasma GIP levels.[97] This search for other stimulatory factors led to the discovery of the second incretin, glucagon-like peptide-1 (GLP-1). GLP-1 is secreted from intestinal L-cells in the distal ileum and colon. Like GIP, the main physiological effect of GLP-1 is to enhance insulin secretion.[98] In addition, GLP-1 increases satiety and decreases food intake,[99] inhibits gastric acid secretion and gastric emptying.[100] However, both endogenous GIP and GLP-1 were rapidly cleaved and deactivated by the enzyme DPP4. DPP-4 inhibitors, like sitagliptin, were introduced as a new class of oral hypoglycemic drugs in 2006.

Acute consumption of decaffeinated coffee (≈2.5 mM CGA) was shown to increase GLP-1 but decrease GIP and thus caused delay in glucose absorption.[101] The same authors also reported that oral administration of apple juice decreased postprandial GIP but increased GLP levels, indicating a suppression of early glucose absorption in the intestines.[102] However, a very recent study showed that although

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the AUC of blood glucose was attenuated in the rat administered CGA, no changes were observed in the GLP-1 level. The finding was similar to that in the human colonic cell line, NCI-H716.[47] In a randomized cross-over trial, decaffeinated coffee slightly increased GLP-1 concentration in overweight men. This change, however, did not correspond with the changes in glucose or insulin levels.[48] Other polyphenols that were shown to increase incretins secretion include resveratrol.[49]

## 4. POLYPHENOLS AND PANCREATIC β-CELL FUNCTION

Over the past few decades, there have been much debate and argument regarding the relative contributions of pancreatic β-cell dysfunction and insulin resistance to the pathogenesis of T2DM. One group of researchers suggested that insulin resistance is the primary abnormality and that β-cell dysfunction is a later event that arises from the chronic hyperglycemia which causes the loss of glucorecognition (glucose toxicity hypothesis).[50,51] In contrast, others have insisted that reduced β-cell function or a decrease in insulin secretion is a prerequisite for the progression of euglycemia to impaired glucose tolerance and T2DM.[52,53] Nonetheless, based on available data, one can conclude that both are typically present very early in the natural history of T2DM.

Insulin secretion is triggered when glucose enters β-cells through the action of glucose transporter, GLUT2. In the glycolysis and respiratory cycles, glucose is oxidized and ATP is produced. An increase in the ATP:ADP ratio closes the ATP-sensitive $K^+$ ($K_{ATP}$) channel which leads to membrane depolarization. Membrane depolarization opens the voltage-gated $Ca^{2+}$ channel and causes calcium influx. The resulting rise in cytoplasmic free $Ca^{2+}$ concentration activates exocytosis of insulin secretory vesicles.[54,55] A defect in any part of this cascade could theoretically impair insulin secretion. Together with some other factors, such as islet amyloidosis[56] and the deleterious effect of non-esterified fatty acids (NEFAs) on β-cell function,[57] they could be potential targets for the action of polyphenols to restore β-cell function.

### 4.1 Observations *in vitro*

In contrast to the results from animal studies, the *in vitro* protective effect of resveratrol against apoptosis was not demonstrated in studies using INS-1E and MIN6 pancreatic insulinoma, with activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase (PARP).[58] This was supported by another study showing that resveratrol (50 μM) exerted antiproliferative or proapoptotic effects on INS-1E β-cells; its negative interference on Akt signaling most likely disrupted insulin signaling.[59] On the other hand, Vetterli *et al.*[60] showed that treatment of INS-1E cells for 24 hours with 25 μM resveratrol resulted in marked potentiation of glucose-stimulated insulin secretion and it was correlated with upregulation of key genes for β-cell function, such as *GLUT2* and glucokinase. They also demonstrated that in human islets isolated from T2DM patients, similar findings were found and glucose responses were eventually restored.[60] This could also be due to the ability of resveratrol to induce membrane depolarization in MIN6 cells, which was comparable to the effect of glibenclamide.[61]

EGCG and rutin enhanced glucose-stimulated insulin secretion and preserved the glucose-sensing ability of RIN-m5F pancreatic β-cells in high-glucose media. Also, EGCG and rutin-activated AMPK, which in turn suppressed lipogenesis and lipid deposition in β-cells.[62] This could have protected β-cells from the lipotoxic effects, thereby preventing defects in insulin secretion.[63] Interestingly, decreased viability by apoptosis in another pancreatic β-cell line, HIT-T15, was observed after treatment with 3–100 μM of EGCG.[64] However, the concentration used to induce 50% inhibition of cell viability was approximately 50 μM while the experimental dose used in the former study was only 10 μM (which did not cause significant cell death). Notwithstanding the difference in the doses of EGCG used in these two studies, one should now question whether EGCG itself, at sufficient dose, can cause β-cell damage before its therapeutic effect on β-cells is established.

In both the INS-1 cell line and isolated human islets, genistein induced β-cell proliferation, mediated through protein kinase A (PKA) or ERK 1/2 and cyclin D1.[65] The insulin-stimulating effect was proposed to be attributed to increased intracellular level of calcium and activation of $Ca^{2+}$/calmodulin kinase II (CAMKII).[66,67] The cinnamic acid derivative, ferulic acid, appeared to be the most effective insulin secretagogue, while isoferulic acid had no effect on insulin secretion in INS-1 β-cells and perfused rat pancreas, suggesting that the hypoglycemic effect of isoferulic acid may not be mediated through stimulation of insulin secretion.[68]

### 4.2 Observations *in vivo*

STZ has widely been used to induce diabetes in animals. It is a β-cell-specific toxin that induces irreversible damage to pancreatic islets through free radical generation and DNA damage.[69,70] Besides, nitroso-containing STZ also releases nitric oxide that causes apoptosis.[71] It was shown that pretreatment with resveratrol protected pancreatic islets from STZ action


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through inhibition of caspase-3 and PARP.[112] However, other studies reported that administration of resveratrol increases insulin secretion in normal rats [115] but not in STZ-diabetic rats.[124] Likewise, in a human trial, despite the improved blood glucose profile, it was thought that the effect was due to improved insulin sensitivity but not β-cell function, as assessed by the $HOMA_\beta$ index.[71] Dietary intake of genistein also significantly improved hyperglycemia and blood insulin levels in STZ-diabetic mice, concomitant with improved islet β-cell proliferation, survival and mass.[64] Similar observations were found in the alloxan-induced diabetic rat, where high-dose genistein decreased β-cell loss and improved insulin secretion.[125] Other polyphenols, quercetin[126] and curcumin,[127] also protected pancreatic islets from degeneration by STZ, thus preserving a higher insulin level compared to control.

### 4.3 Polyphenols and Reactive Oxygen Species-Induced β-cell Damage

Under conditions of supraphysiological levels of blood glucose, excess glucose might be shunted to several other metabolic processes, like glycosylation (Schiff reaction),[128] glucose autoxidation,[129] and the glucosamine pathway.[130] Unfortunately, in these processes, reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and nitric oxide, are formed. In addition, ROS is formed in excess during glycolysis in the hyperglycemic state.[131] The overproduction of ROS has been reported to be associated with defective insulin gene expression and excessive apoptosis of β-cells.[132] Apigenin [133] and kaempferol [134] were shown to attenuate oxidative damage induced by 2-deoxy-ribose in HIT-T15 panceatic β-cells. Pro-inflammatory cytokines like interleukin-1β induce the expression of nitric oxide synthase (iNOS) which leads to the overproduction of nitric oxide that mediates the inhibition of insulin secretion and cytotoxicity to β-cells.[135] Epicatechin[136] and resveratrol[137] efficiently inhibited IL-1β-induced nitric oxide production and iNOS gene expression and restored insulin secretion in RIN-m5F β-cells and isolated rat islets. Similarly, treatment with genistein completely attenuated IL-1β- and interferon (IFN)-γ-induced β-cell damage, through the suppression of ERK 1/2 and JAK/STAT pathways.[138]

### 4.4 Polyphenols and Islet Amyloid Polypeptide

Human islet amyloid polypeptide (IAPP), or amylin, is normally co-localized with insulin within secretory vesicles[139] and is also co-released with insulin in response to glucose or non-glucose secretagogues.[140] Decreased IAPP release but increased islet amyloid deposit have always been found in T2DM.[140] EGCG has been shown to effectively inhibit IAPP amyloid formation and disaggregate preformed amyloid fibrils derived from IAPP.[141] In RIN-5F pancreatic β-cells, oleuropein, the main phenolic component of olive oil, prevented the cytotoxic damage caused by the aggregation of amylin.[142] An *in vitro* study revealed that curcumin reduced IAPP fibrils and aggregate formation. However, in INS-1 cell and IAPP-trangenic rat islets, curcumin failed to protect β-cells from IAPP-induced apoptosis.[143] Overall, more studies in cellular systems and animal models should be conducted to better elucidate the interrelationships between polyphenols, amyloidogenesis and β-cell function.

## 5. POLYPHENOLS AND HEPATIC GLUCOSE HOMEOSTASIS

The liver plays a pivotal role in maintaining glucose homeostasis by controlling uptake and storage of glucose via glycogenesis and release of glucose via glycogenolysis and gluconeogenesis. Several key enzymes regulate these opposing metabolic pathways, as shown in Figure 9.1. For instance, glucogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and fructose-1,6-biphophatase (Fru-1,6-$P_2$ase), are opposed by glycolytic enzymes, pyruvate kinase (PK) and 6-phosphofructo-1-kinase (PFK). Glucose-6-phosphatase (G6Pase), the rate-limiting enzyme in gluconeogenesis and glycogenolysis, is opposed by the glycolytic enzyme glucokinase (GK). Glycogen phosphorylase (GP) is involved in the breakdown of glycogen while glycogen synthase (GS) catalyzes formation and storage of glycogen.[144] Various studies have shown that abnormal or defective expressions of these enzymes disturb glucose metabolism and eventually cause hyperglycemia.[145–147] The hormones, insulin and glucagon, play significant roles in regulating these enzymes.

In post-absorptive and fasting states, euglycemia is maintained by the equilibrium between endogenous glucose production and glucose utilization. Since no nutrients are being absorbed from the gastrointestinal tract, essentially all glucose entering the circulation comes from the liver.[148] In the postprandial state, increased plasma glucose results in insulin secretion and suppression of glucagon release,[149] which in turn abolishes approximately 60% of hepatic glucose output.[150] However, in T2DM patients, this suppression was found to be diminished, and instead hepatic glucose output was observed to be elevated.[151] Therefore, gluconeogenesis and glycogenolysis become major factors in determining hepatic glucose output in both post-absorptive and postprandial states. Impairment of



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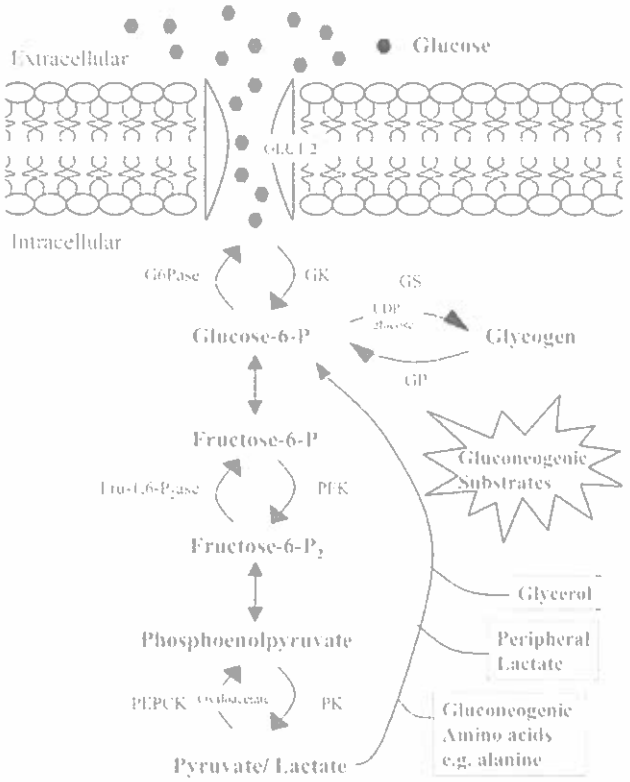



FIGURE 9.1 Gluconeogenic/glycolytic and glycogenolytic/glycogenic pathways that are involved in the regulation of hepatic glucose production. GLUT2, glucose transporter 2; UDP-glucose, uridine diphosphate glucose.

these two pathways is always associated with impaired insulin secretion,[7] insulin resistance, hyperglucagonemia,[8] increased gluconeogenic substrates[9,10] and elevated free fatty acids.[9] Theoretically, polyphenols can act indirectly through these impairments, but like biguanides, they can also act directly on the enzymes regulating hepatic glucose output.[11] Most of the studies we discuss here favor the latter.

## 5.1 Observations *In Vitro*

In primary rat hepatocytes, resveratrol reduced GK expression and activity, via interaction between FoxO1 and HNF-4 on the GK promoter.[12] Likewise, another study showed that resveratrol upregulated the expression of gluconeogenic enzymes, such as PEPCK and G6Pase, by compromising insulin signaling and deacetylating FoxO1.[13] Like the contradictory effects reported *in vivo*, studies *in vitro* also revealed that activation of SIRT1 by resveratrol markedly repressed cytosolic expression of PEPCK in HepG2 hepatoma and AML12 hepatocytes.[14] It is still unclear how to reconcile all these different outcomes; further work is needed to determine whether a differential mechanism is involved.

In primary hepatocytes, EGCG attenuated gluconeogenesis stimulated by cAMP/dexamethasone at a concentration lower than 1 μM.[15] This is important as EGCG is rapidly metabolized through glucuronidation, sulfation, methylation and ring fission,[16] resulting in a peak plasma EGCG concentration of only 1 μM after ingestion of a large amount of green tea.[17] Treatment with STO-609, a CaMKK inhibitor, abolished EGCG-suppression of gluconeogenesis and expression of PEPCK and G6Pase, suggesting that EGCG-mediated inhibition of gluconeogenesis was insulin-independent. This was supported by another study showing similar results but the authors correlated the EGCG-activated CaMKK/AMPK to ROS production.[18] However, Waltner-Law *et al.*[19] disagreed by demonstrating that in H4IIE rat hepatoma cells, suppression of glucose production and expression of the gluconeogenic enzymes, PEPCK and G6Pase, by EGCG was mediated by PI3K/PKB, which is the insulin signaling pathway. Nevertheless, it is noteworthy from this study that the activation of insulin signaling by EGCG at higher concentration resulted in pro-oxidant activity in H4IIE hepatoma, in agreement with the findings from the above study that EGCG caused ROS production. This generation of ROS explained the increase in tyrosine-phosphorylated proteins, triggering the whole insulin-signaling cascade. Hence, at higher concentrations, whether or not there is triggering of the insulin cascade, EGCG may cause toxicity to cells, especially carcinoma cells, as reported in some cancer studies.[3]

The very first anti-gluconeogenic effect of CGA was reported in 1997 where CGA and its synthetic derivatives were found to inhibit glucose-6-phosphate (G6P) translocase, leading to the hypothesis that G6Pase is the locus of interference for inhibition of gluconeogenesis by CGA.[20] Since then, various *in vivo* and *in vitro* experiments involving CGA and gluconeogenesis have been performed. In human liver microsomes, CGA and its derivatives have been shown to inhibit G6Pase activity and the positions of the caffeoyl residue are important for the inhibitory effect.[21] Using microsomes isolated from rat liver, another study showed an inhibitory effect of CGA on its G6Pase activity. However, from liver perfusion experiments, no effect was found on glucose production or alanine catabolism, at different concentrations of CGA.[22]

## 5.2 Observations *In Vivo*

Although resveratrol improves glycemic responses, interestingly, it consistently shows opposing effects on hepatic glucose metabolism *in vivo* and *in vitro*. Resveratrol suppressed GK but enhanced PEPCK


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mRNA and protein expressions in a dose-dependent fashion.[159] In contrast, Knight *et al.*[171] found that cerebral or systemic administration of resveratrol lowered hepatic glucose production by decreasing hepatic glycogenolysis and gluconeogenesis. Also, a significant decrease in G6Pase flux was observed but all these anti-gluconeogenic activities were ablated by SIRT1 inhibition, both pharmacologically and molecularly. This indicates that SIRT1 mediated the resveratrol-induced suppression of glucose production, which is congruent with the inference that activation of SIRT1 reduces gluconeogenesis.[172] Consistent with this, intracerebroventricular infusion of resveratrol normalized hyperglycemia and reduced PEPCK mRNA and protein levels.[68] Besides PEPCK and G6Pase, other enzymes, such as PK, fructose-1,6-bisphosphatase, GS and GP were also shown to be regulated by resveratrol in the STZ-diabetic model.[70] Fisetin restored PEPCK, fructose-1,6-bisphosphatase, G6Pase, PK, GS and GP levels in the livers and kidneys of diabetic animals.[173] Quercitrin[53] and apigenin[56] also inhibited G6Pase activity in STZ-diabetic rats. The isoflavones, genistein and daidzein, lowered hepatic G6Pase and PEPCK activities in non-obese diabetic mice.[174]

Very recently, CGA was tested for its ability to suppress gluconeogenesis in *db/db* mice. Downregulation of G6Pase was observed, accompanied by upregulation of PPAR-$\gamma$ and adiponectin.[175] Although the authors did not further investigate their effects on CGA-mediated gluconeogenesis suppression, we believe that these two factors may impact the suppression of gluconeogenesis by CGA as both PPAR-$\gamma$[176] and adiponectin[177] have been associated with repressed gluconeogenesis. Further studies only showed improved fasting glucose by CGA and elevated expression of PPAR-$\gamma$ but could not elucidate the relationship between CGA-mediated suppression of glucose production and PPAR-$\gamma$.[169] In the liver of rats treated with CGA-derived S4048, strong inhibition of the flux through GK and an increase in the flux through GS were observed, accompanied by a massive glycogen accumulation. mRNA levels of G6P hydrolase, G6P translocase, GS, and PK were increased significantly, whereas GK expression was almost abolished. The authors inferred that inhibition of G6Pase activity by S4048 elicited a repartitioning of newly synthesized G6P into glycogen synthesis without affecting gluconeogenic flux to G6P.[178]

## 6. POLYPHENOLS AND PERIPHERAL GLUCOSE UPTAKE

Glucose uptake in cells, from the simplest single-cell bacterium to the highly specialized mammalian neuron, is facilitated by GLUTs in the plasma membrane. Upon entering the cells, glucose is rapidly phosphorylated by GK and further metabolized via storage and/or oxidation (Figure 9.1). The mammalian *GLUT* is a superfamily of genes, encoding homologous proteins with different functional properties and tissue-specific expressions.[179] The first GLUT cloned and studied was GLUT1, which is ubiquitous in most fetal and adult tissues.[180] Subsequent searches successfully identified 11 other GLUTs, namely GLUT2 to GLUT12.[181] GLUT1 and GLUT4 are the major contributors for glucose disposal in peripheral tissues. The broad distribution of GLUT1 makes it an important transporter to regulate the basal glucose disposal. Its expression has been shown to be altered by sulfonylureas,[182] insulin,[183] hypoxia,[184] insulin-like growth factor-1[185] and a number of other factors. On the other hand, GLUT4 is exclusively expressed in peripheral insulin-sensitive tissues like fat, and skeletal and cardiac muscles. It is the only insulin-responsive GLUT identified so far, with the observation that its level of expression in various muscles and fat cells generally corresponded to the magnitude of insulin-stimulated glucose disposal in the tissues.[186] Later on, it was established that this insulin-stimulated glucose transport was mediated through the redistribution of GLUT4 from the intracellular membrane compartment to the cell surface.[187]

In T2DM, desensitization of peripheral target tissues to insulin action is one of the main pathophysiological characteristics. This is often manifested as fasting hyperinsulinemia together with hyperglycemia. Adipose tissue accounts for 5–20% of the insulin-mediated glucose uptake[188] while skeletal muscle is responsible for the remaining 80–95%.[189] Antidiabetic agents like thiazolidinediones have been shown to upregulate the expression of GLUT4[190] while metformin caused a significant increase in both GLUT1 and GLUT4 contents in plasma membrane, with a corresponding decrease in intracellular membrane fraction.[191] Owing to the lack of *in vivo* models for assessing peripheral glucose uptake and the complicated nature of the hyperinsulinemic-euglycemic clamp procedure, most uptake studies have employed only isolated muscles, fat pad or culture of myocytes and adipocytes.

### 6.1 Polyphenols and GLUT4

Anthocyanidins are absorbed in animals and humans and metabolized rapidly to phenolic acids like protocatechuic acid (PCA).[192] Very recently, Scazzhocchio *et al.*[193] demonstrated that cyanidin-3-*O*-$\beta$-glucoside (C3G) and PCA increase adipocyte glucose uptake and GLUT4 expression and translocation. This was accompanied by increased nuclear PPAR-$\gamma$ activity and



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adiponectin expression, suggesting the role of these two factors in mediating glucose uptake stimulated by C3G and PCA.[77] In differentiated $C_2C_{12}$ muscle cells, curcumin strongly induced glucose uptake in a dose-dependent manner. With pharmacological intervention, it was eventually resolved that the stimulation was mediated through the AMPK/ACC pathway but not the PI3K/PKB pathway.[78] Naringenin, the flavonoid found in citrus fruits, has been reported to stimulate glucose uptake in L6 myotubes in a dose- and time-dependent manner but no experimental work on GLUTs has been conducted. Nevertheless, the authors associated this stimulation of glucose uptake to the activation of the insulin-independent pathway, which is the AMPK pathway.[79] However, in 3T3-L1 adipocytes, at similar concentrations, naringenin and daidzein inhibited both basal and insulin-stimulated glucose uptakes,[80] suggesting their desensitizing role in glucose disposal. EGCG restored insulin sensitivity in L6 myotubes exposed to the desensitizing agent, dexamethasone. This effect was essentially dependent on the concomitant activation of both insulin signaling and AMPK pathways, which in turn caused GLUT4 translocation to the plasma membrane.[81] A very recent study on tangeretin, a flavonoid that is abundant in citrus fruits, revealed its ability to stimulate glucose uptake in $C_2C_{12}$ myotubes, through the activation of AMPK and GLUT4 recruitment.[82]

L6 myotubes incubated with resveratrol were stimulated to uptake glucose in a dose- and time-dependent manner.[83] This finding is in line with a previous study using $C_2C_{12}$ muscle cells.[84] Both studies showed that resveratrol stimulation of glucose uptake is most likely mediated by AMPK activation, with the latter suggesting that AMPK phosphorylation by resveratrol potentiated insulin-induced Akt activation, leading to improvement in insulin sensitivity. The former, however, observed that resveratrol-stimulated AMPK phosphorylation is abolished by nicotinamide and splitomicin, indicating that SIRT-1 is involved in AMPK activation and may be upstream of AMPK. This is supported by a study using genetic inhibition and adenoviral overexpression of SIRT-1 to elucidate its role in hepatic LKB-1/AMPK activation.[85] Most importantly, the authors proposed that resveratrol-stimulated AMPK activation does not lead to GLUT4 translocation but instead enhanced the intrinsic activity of GLUT4, as observed in AICAR-stimulated AMPK activation in 3T3-L1 adipocytes.

Phenolic acids also exhibit potential regulation of glucose uptake in skeletal muscle and adipocytes. Caffeic acid phenethyl ester (CAPE) activated both AMPK and Akt to stimulate glucose uptake in skeletal muscle cells.[86] Caffeic acid but not CGA increased phosphorylation of AMPKα in isolated rat epitrochlearis muscle, leading to increased glucose transport in the absence of insulin. Unfortunately, the exact mechanism of enhanced glucose transport has not been evaluated.[87] On the contrary, our study showed that CGA caused parallel activation of both AMPK and Akt, resulting in GLUT4 translocation to the plasma membrane of both isolated rat soleus muscle and L6 myotubes. We further investigated the importance of AMPK for CGA-mediated glucose transport through pharmacological (compound c) and molecular (AMPK siRNA) interventions, and found that AMPK mediated CGA-induced glucose transport.[87]

However, not all studies report the stimulatory effect of polyphenols on glucose transport. A study involving kaempferitrin, a glycosylated flavonoid, showed its inhibitory effect on insulin-stimulated GLUT4 translocation and glucose transport in 3T3-L1 adipocytes. Interestingly, in the absence of insulin, addition of kaempferitrin did not affect GLUT4 translocation or glucose uptake. Subsequent work resolved that kaempferitrin inhibited insulin signaling through inhibition of Akt.[88,89]

## 6.2 Polyphenols and GLUT1

There is increasing evidence to show that the more widely expressed GLUT1 can be acutely activated by cell stressors such as azides,[90] osmotic stress[91] and glucose deprivation,[92] despite its lack of insulin responsiveness. In addition, the acute activation of GLUT1 by azide, metformin and hypoxia have been attributed to the phosphorylation of AMPK,[90,93,94] establishing a potential role of GLUT1 in diabetes. In L929 fibroblast cells (a cell line that only expresses GLUT1), 10 μM of berberine significantly elevated glucose uptake, achieving a maximum of a five-fold increase at a concentration of 40 μM.[95] However, AMPK, which is known to mediate berberine-stimulated glucose transport in L6 myotubes,[96] did not mediate berberine-stimulated glucose transport in L929 cells, suggesting that AMPK phosphorylation is required for the translocation of GLUT4 but not GLUT1. On the other hand, Zhou *et al.*[97] observed there was no alteration in expression and cellular localization of GLUT4 or GLUT1, though glucose uptake was stimulated by berberine in 3T3-L1 adipocytes. Tyrosine kinase inhibition completely blocked berberine-stimulated glucose uptake, implying that berberine induced glucose transport via increased GLUT1 activity.[97]

# 7. CONCLUSION

While transcriptomics and proteomics are commonly practiced in routine laboratory experiments, metabolomic analyses are also rapidly developing. We


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believe that nutrigenomics/nutriproteomics (which study the ways that specific genes or proteins and bioactive food components interact) will gain momentum in the coming decades. However, the lack of human studies with clinical significance would be a major obstacle to the success of nutrigenomics or nutriproteomics. Mechanistic studies performed *in vitro* at times employ polyphenol concentrations which exceed pharmacological concentrations, hindering the progression to the conduct of follow-up controlled human studies. More disappointingly, human studies usually show contradictory results, compared to cellular and animal studies. The lack of knowledge of the pharmacokinetics of dietary polyphenols is another hurdle to a better understanding of dosage and effects of polyphenols on particular target organs.

Nevertheless, from the studies discussed above, it is evident that the impact of polyphenols on carbohydrate or glucose metabolism is extensive. This is highly significant considering that obesity is positively correlated to metabolic diseases like diabetes mellitus and increased intakes of polyphenol-rich fruits, vegetables and legumes not only have been shown to have beneficial regulatory effects on glucose homeostasis but also combat obesity due to their low-fat nature. For instance, CGA has been shown to improve both fasting and postprandial hyperglycemia, possibly mediated by delaying intestinal glucose absorption, inhibition of hepatic glucose output, and increase in peripheral glucose disposal. At the same time, CGA enhances fat metabolism in the liver[213] that may, in turn, attenuate obesity and further improve glycemic indices.

Although it is premature at this stage to conclude that polyphenols can be used as a therapeutic tool for T2DM, the promotion of polyphenol-rich foods to manage obesity and glucose homeostasis is cost-effective. In addition, such foods might produce greater beneficial effects than a single polyphenol. The synergistic interactions between different polyphenols could be an area for future investigation. In the future, with changes in dietary habits from reduced consumption of high-glycemic and high-caloric diets to an increase in polyphenol-rich diets, we believe that the impact of the growing pandemic of metabolic diseases can be significantly curtailed. It is thus worthwhile to give greater attention to research with polyphenols to provide more evidence for their effectiveness contributing to the management of T2DM.

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CHAPTER

# 10

# Polyphenols in Ayurvedic Medicine and Type 2 Diabetes

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## 1. INTRODUCTION

Globally, more than 220 million people have been diagnosed with diabetes. Not only is diabetes a health concern, but it also puts an immense economic burden on our society.[1] Type 2 diabetes is a heterogeneous disorder that can be altered by environmental and genetic factors. Those with type 2 diabetes can have their health impacted by developing complications such as hyperglycemia, hypertension, and obesity. The American Diabetes Association states that type 2 diabetes is the most common form of diabetes, and it is associated with insulin resistance and insulin deficiency.[2]

Aside from pharmaceutical drugs and conventional treatments for type 2 diabetics, there are a variety of other options for treatment. Ayurvedic medicine is a non-drug holistic approach that incorporates a specific lifestyle in order to bring balance within the physiological components in the body.[3] Ayurveda is a traditional Indian medicine that has been used for centuries.[3] This form of medicine focuses on the patient, rather than the disease itself. With a holistic approach on a disease, ayurvedic medicine uses therapeutic strategies in order to enhance one's quality of life and promote a healthy lifestyle.

A key component in the ayurvedic treatment of diseases is the use of medicinal herbs. There are several thousands of herbs in India which have been known to have medicinal properties. Plants shown to have antidiabetic properties include approximately 45 different species. The few that have been used in current diabetes research include onions, garlic, aloe vera, bitter melon, jambul seeds, fenugreek, tulsi, curry leaf, cinnamon, and Indian mustard leaf.[3] All of these treatments used in ayurveda are pharmaceutical-free options, and are rich in polyphenolic compounds.

Polyphenols are found in phytochemical bearing foods: fruits, vegetables and herbs that are known to have antioxidant effects. Polyphenic structures are found in the plants used to cure diabetes in ayurveda. Melons, legumes, onions, berries, teas, and many vegetables are known to be loaded with polyphenols. For example, a well-accepted natural therapy for diabetes that is rich in polyphenols is cinnamon (*Cinnamomum cassia*).[4] Cinnamon has been used in traditional medicine to treat diabetes in all of the ancient medicine practices. Compared to the other herbs known to assist diabetics, cinnamon has the most insulin enhancing activity.[4] The polyphenols in cinnamon enhance insulin sensitivity, which is key for those suffering with type 2 diabetes.[5]

Studies and research review how ayurvedic substances in asparagus root, bitter melon, cinnamon, green leafy and dark yellow vegetables, and other herbs have therapeutic effects on type 2 diabetics.

## 2. COMPONENTS OF POLYPHENOLS

Polyphenolic compounds are complex ringed molecules that are found in a variety of plant foods. There are many different types of polyphenols with more yet to be discovered.[6] Consumers are sometimes able to identify the concentration of polyphenols found in their foods by the level of bitterness that can be tasted during consumption. Due to polyphenols being distinguishable through tasting, they possess sensory qualities.[6]

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Polyphenols are highly beneficial in many ways, but some of their most important factors give multifaceted medicinal effects. They can aid in the treatment and prevention of many human diseases: cardiovascular disease, diabetes, ulcers, and bacterial infections. Polyphenols are easily accessible through the wide variety of herbs, vegetables, and fruits available. The ability to utilize polyphenols found in foods in order to better one's health is described in many different types of integrated medicines and foods in ayurveda.

## 3. TYPE 2 DIABETES

Type 2 diabetes is a chronic condition that affects the way your body metabolizes glucose. Glucose can be found in muscles and other tissues, and it is a sugar that our bodies use to generate energy. The hormone, insulin, regulates the movement of sugar into the cells. People with type 2 diabetes exhibit issues with resisting the effects of insulin, or do not create enough insulin to regulate a normal glucose level.

The symptoms that come with type 2 diabetes range from increased hunger to blurred vision. To delay or prevent these, a diabetic's lifestyle should completely change as they must be conscientious of many biological, environmental, and psychological factors that affect them. Firstly, a diabetic committed to learning about their health can implement a nutritious diet and exercise in their litestyle. A nutritious diet does not only mean a diet rich in polyphenols, but also the minimization or complete removal of alcohol and tobacco. Stress factors in a diabetic's life must be kept to the minimum. When stress levels are high, the hormones produced in response to stress, will impact the effectiveness of insulin.

Although there is no cure for type 2 diabetes, there are many protocols that must be taken by the individual to minimize the risk of developing more of the symptoms that come with the disease. A diabetic who is motivated to battle the illness will change his or her lifestyle in many ways by staying physically active through regular exercise, daily blood sugar monitoring, medications, and, more importantly, a healthy diet. A healthy diet which consists of vegetables, fruits, and whole grains; all of which are rich in polyphenols

## 4. AYURVEDIC HERBAL REMEDIES FOR DIABETICS THAT CONTAIN POLYPHENOLS

Data was collected from the Women's Health Study between 1993–2003 to identify whether fruits and vegetables affected diabetes. The study was based on whether a high fruit and vegetable diet could protect women against type 2 diabetes. Not all fruits and vegetables contain the same chemical components. Therefore, the study placed these foods into subgroups in an attempt to analyze which types of health foods had a beneficial impact on reducing the risks involved in type 2 diabetes. The vegetables were categorized as follows: cruciferous, green leafy, dark yellow, and other. The two key subgroups involved in playing a role in reducing the risk of type 2 diabetes in overweight women were green leafy and dark yellow. Vegetables that were placed in the green leafy category were kale, lettuce, and spinach. The vegetables that fell under dark yellow were carrots, yellow squash, yams and sweet potatoes. The study concluded that the biochemical mechanisms behind this data are provided by the chemical make-up of these vegetables. Likely active components are antioxidants, phytochemicals, and plant proteins. From this study, polyphenolic structures found in certain herbs and vegetables are likely to contribute to the reduction of risk in people who may have, or are prone to, type 2 diabetes.

A study showed the antioxidant potential and hypercholesteremic potential of asparagus root.[1] The experiment was performed on normal and hypercholesteremic rats. These rats were given a dosage of asparagus root extract during a 4-week period. Asparagus root increased fecal excretion of cholesterol, reduced hepatic and plasma lipid profiles, and improved hepatic antioxidant status. The study concluded that the make-up of asparagus, including polyphenols, could be responsible for these effects. Ayurveda utilizes asparagus root as a tonic, rejuvenator, antispasmodic, anti-ulcerous and anti-inflammatory. It is also used as a dietary supplement for normalizing hyperlipidemic/hypercholesteremic and oxidative stress that is brought on by diets rich in fat and cholesterol.

People tend to avoid acidic-tasting fruits and vegetables such as *Momordica charantia*, bitter melon. Even though these dietary phytonutrients are not the most sweet and succulent foods available, they have immense beneficial health effects, specifically for type 2 diabetics. Ayurveda, as well as Chinese medicine, has traditionally utilized bitter melon as a diabetes therapy. A variety of recipes that used bitter melon as their main ingredient were tested for palatability and acceptability in healthy individuals to determine the most efficient way bitter foods can become part of one's diet.[1] Incorporating bitter foods into food dishes that are most commonly consumed can mask the harsh taste of bitter melon. A fruit so rich in substances that are substantially beneficial in the treatment of diabetes and its complications can easily be incorporated into a typical diet. Thus, health foods not having a pleasing


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taste can be manipulated in the food of a diabetic to reap the health benefits available through the consumption of bitter melon.

### 4.1 *Cinnamomum cassia* (Cinnamon)

Ayurveda utilizes a wide variety of herbs for medicinal purposes. Many of these herbs are suggested to be part of a diabetic's diet due to their strong anti-diabetic agents, which possess hypoglycemic properties. Although many herbal remedies are used in ayurveda, further scientific studies must be performed to increase its usage in other aspects of medicine. α-Amylase inhibition is an effective process where the process of starch degradation is controlled in order to lower the levels of hyperglycemia.[12] Studies where ayurvedic herbs are tested for α-amylase inhibition have been done in order to show how effective these herbs can be.[12] A common ayurvedic herb, known to have these hypoglycemic properties for diabetics is *C. cassia*.

*C. cassia* is commonly referred to as the "bold tasting spice," cinnamon. In 1990, *C. cassia* was identified as an insulin potentiating compound, which could possibly be used to aid or even reverse the symptoms of a type 2 diabetic.[5] The effects of *C. cassia* extracts were investigated in rats. The extract decreased blood glucose levels, and increased the amount of insulin secretion. The rats were administered *C. cassia* extracts and bark, which both further confirmed that the regular use of the herb positively affected the increase of plasma insulin levels. As diabetics generally lack efficient amounts of insulin in their body,[13] a herb which possesses properties that allow the body to generate more insulin is truly a beneficial aspect in the treatment of type 2 diabetes.

A human study on the effects of cinnamon on diabetics was done as a double-blind experiment.[5] Patients with type 2 diabetes who had been administered cinnamon had improved insulin sensitivity, and reduced glucose and cholesterol levels. A significant decrease in body mass was also observed in the group that had been administered the cinnamon extract, compared to the group that received the placebo.

Obesity leads to an increase in oxidative stress. This oxidative stress is a promoter to the development of type 2 diabetes and its tissue damage. The group that had been receiving *Cinnamomum cassia* showed an increase in plasma sulfhydryl groups. Thus, cinnamon serves as an antioxidant that protects lipids and proteins from being oxidized. Cinnamon acts as an antioxidant and positively influences glucose, insulin, and cholesterol levels in diabetics. It also suggests that the use of *Cinnamomum cassia* will reduce the likelihood of an overweight individual developing type 2 diabetes.[5] *Cinnamomum cassia* has positive factors that help regulate individuals who have been diagnosed with type 2 diabetes, and it can also be used as a preventive method to reduce the rapidly growing rate of this chronic disease.

### 4.2 *Curcuma longa* (Turmeric)

*Curcuma longa* (turmeric) is a perennial herb. It is cultivated in tropical regions, including India and China. Ayurveda in India used turmeric for medical purposes. Curcumin is an active ingredient found in turmeric. Based on experimental studies, curcumin is an antioxidant that possesses hypoglycemic qualities. Diabetic rats show that curcumin decreases blood glucose levels, oxidative stress, and thiobarbituric acid reactive substances.[14] The researchers hypothesized that the decrease in these three components may be a result of a decrease of influx of glucose in the polyol pathway.[14] In this metabolic pathway, a decrease of glucose influx results in an elevated NADPH/NADP ratio and an increase in the enzyme's glucose peroxidase activity.[14] Another hypothesis, based on a study of rats fed a curcumin diet, suggests that curcumin lowers cholesterol levels without changing hyperglycemic status. This improves renal lesions that correlate to the symptoms of type 2 diabetics. The diabetic rats that were fed a curcumin-based diet showed a reduction in their excretion of proteins and urinary enzymes that is linked to diabetic nephropathy. The excretion of these large molecules and enzymes provide an added risk to a diabetic's kidneys.[14] Therefore, a diet rich in curcumin can decrease the excretion of these substances, avoiding a chance of affecting the kidneys. The antioxidative effects of curcumin decreases the build-up of advanced glycation end products and cross-linkage of collagen in the skin of diabetics.[14] Research has shown that curcumin can also be used as a wound repair in diabetic animals, and therefore, could be a potential pharmacological agent in diabetic ulcers.[14]

### 4.3 *Trigonella foenum-graecum* (Fenugreek)

*Trigonella foenum-graecum* (fenugreek) is an annual herb cultivated worldwide. It originated from southeastern Europe and western Asia. Various compounds isolated from fenugreek have been shown to be beneficial in the treatment of type 2 diabetes. Galactomannan is a dietary fiber compound of fenugreek, which lowers blood glucose levels in diabetic rats.[14] Fenugreek seeds contain the amino acid, 4-hydroxyisoleucine, which can cause glucose-induced insulin release *in vitro* and *in vivo*. Diabetic rats were given a dose of fenugreek during a 6-day period and glucose tolerance

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was greatly improved. Fenugreek decreases the absorption of glucose from the small intestine and delays gastric emptying. This observation suggests that fenugreek has pancreatic and extra-pancreatic effects. Fenugreek seeds reverse the activities of gluconeogenic, glycolytic, and lipogenic enzymes in the livers and kidneys, which leads to a decrease in fasting blood glucose.[7] The glycolytic enzymes show a decrease of activity in the liver, but an increase of activity in the kidney of diabetic rats. The activity of these glycolytic enzymes reached a controlled level, and there was a reduction of gluconeogenic enzyme activity. These studies show how fenugreek regulates many of the biochemical mechanisms that can aid in the treatment of type 2 diabetes.

### 4.4 *Glymnema sylvestre* (Gurmar)

A commonly used herb in ayurvedic medicine is *Glymnema sylvestre*, nicknamed gurmar. This herb grows in the tropical areas in central and southern India. It is a woody climber plant that has been used for medicinal purposes for centuries. Chewing *G. sylvestre* leaves has been known to eliminate the sweet taste, which is why Hindus believe it to be a "destroyer of sugar." Rabbit and mouse studies have shown that *G. sylvestre* decreases blood glucose levels in animals with residual pancreatic function, and reduces hyperglycemia. GS4, an ethanol leaf extract, has been reported to have a positive impact on diabetic rats and rabbits. The results from experiments using GS4 show a decrease in blood glucose levels and an increase in serum insulin. The mechanism of action is still unknown, although it is assumed that high levels of glucose intake leads to an increase in insulin release due to cell permeability.[7] An increase in β-cells was also observed, meaning an increase in β-cell function could also be a possible explanation to the anti-diabetic properties of *G. sylvestre*. It is definite that an increase in insulin secretion plays a role in the herb's glucose-decreasing effects. Based on two non-randomized clinical trials on *G. sylvestre*'s effects on type 1 and type 2 diabetics, there was an apparent success rate of glycemic control when GS4 is used in conjunction with conventional treatment.[7] There is limited data on how useful *G. sylvestre* is for treating the conditions of a type 2 diabetic, although its use along with a conventional treatment of a physician may be advantageous.

### 4.5 *Aloe vera*

A familiar desert succulent plant, *Aloe vera* is commonly utilized for many skin treatments such as burns and rashes. Its dried sap has been a traditional remedy, used around the Arabian Peninsula for type 2 diabetes. The gel extracted from the inner parts of the leaves contains glucomannan.[7] Glucomannan is a hydrosoluble fiber, thus accounting for its hypoglycemic properties.[7] Animal studies reported that fasting blood glucose improved after a 6-week diet of *Aloe vera* juice. Case reports[7] of type 2 diabetic individuals show a decrease in fasting blood glucose as well. There were no adverse effects reported in animal or human studies. More information in needed regarding *Aloe vera*'s efficacy on type 2 diabetics. It can be concluded that *Aloe vera* is a potential regulator of glycemic control.

### 4.6 *Momordica charantia*

*Momordica charantia* has many names including: bitter melon, karolla and balsam pear. It is a tropical vegetable, native to Africa, Asia, and South America. The herb can be prepared in various ways. Common forms of *M. charantia* are injectable extracts, juice, and fried melon pieces. Animal studies[7] using these variations of *M. charantia* show potential hypoglycemic effects. *M. charantia* contains an active ingredient, polypeptide-p (a protein resembling insulin).[7] Mechanisms theorized by researchers suggest that the herb is involved in an increase in insulin secretion, tissue glucose uptake, liver muscle glycogen synthesis, glucose oxidation, and decreased hepatic gluconeogenesis.[7] Controlled short-term studies reported that type 2 diabetics consuming *M. charantia* notice acute effects on blood glucose.[7] Several uncontrolled studies conclude that after long-term usage of the herb, there is an observable positive impact on glycemic control.

Ancient medicines utilized *M. charantia* in the treatment of type 2 diabetes, and some experiments have shown a decrease in adiposity in rats.[7] Due to the trends of obesity associated with type 2 diabetes, a study researched the use of *M. charantia* inhibitory effects on human adipocyte differentiation.[7] Since there is little research done with alternative medicines and humans, this specific study investigated the effects of bitter melon juice on lipid accumulation and adipocyte differentiation transcription factors. The study by Nerurkar and colleagues[7] was done on primary human differentiating pre-adipocytes and adipocytes. A large decrease in lipid content and a reduction in mRNA expression of adipocyte transcription factors occurred in the pre-adipocytes that were treated with bitter melon juice. Increased lipolysis and a reduction in lipid content suggest that bitter melon juice acts as a potent inhibitor of lipogenesis and stimulates lipolysis activity in human adipocytes.[7] In conjunction with conventional medicine, bitter melon juice is an


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effective complementary therapy that can be used in the treatment of type 2 diabetes.

## 5. CONCLUSION

Based on scientific studies and research, *Cinnamomum cassia*, *Curcuma longa*, *Trigonella foenum-graecum*, *Glymnema sylvestre*, *Aloe vera*, *Momordica charantia*, and other polyphenolic compounds possess beneficial properties that assist with type 2 diabetes complications. Taking these supplements alone may or may not make a difference in insulin and blood glucose levels. The effects will not be the same for each individual. Further research and scientific studies will be helpful in showing the benefits of polyphenolic herbal remedies for type 2 diabetes. This alternative type of non-pharmaceutical therapy is likely to be helpful in treating type 2 diabetes, but it is not meant to replace pharmaceutical diabetic medications and conventional treatment. Ayurvedic remedies with proven effects for the complications of type 2 diabetes are meant to be complementary to the conventional treatments and medications that already exist.

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CHAPTER

# 11

# Antidiabetic and Anticancer Potential of Native Medicinal Plants from Saudi Arabia

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## 1. INTRODUCTION

### 1.1 General Use of Natural Health Products in Saudi Arabia

The Arabian subcontinent is characterized largely by a desert biome with flora and fauna adapted to life in extreme conditions. The rich cultural and scientific traditions of the human inhabitants of this region show both historical usage and scientific study of local medicinal plants. Herbal drugs continue to be used among the common folk, even as modern medicines emerged and spread in usage.[1] Natural health products (NHP) are defined as herbal remedies, homeopathic medicines, traditional medicine systems, probiotics, vitamins and minerals, and other products such as amino acids and essential fatty acids.[2] Rahman *et al.*[3] report that the traditional knowledge-based usage of medicinal plants of the Kingdom of Saudi Arabia (KSA) can complement allopathic medical systems. Persons skilled in traditional healing are called Hakim. They use medicinal plants such as herbs as an alternative, or as a complement, to modern medicine[3] for prevention and treatment of acute and chronic diseases. This chapter will review the scientific evidence in support of several native medicinal plants used in Saudi Arabia as part of the local traditional medicine system to combat two major chronic diseases in Saudi Arabia: type 2 diabetes mellitus and cancer.

### 1.2 Prevalence and Incidence of Diabetes Mellitus and Cancer in Saudi Arabia

Type 2 diabetes mellitus (DM) is a chronic disease caused by a defect in the secretion of pancreatic insulin and/or an insensitivity of target tissues to its action leading to hyperglycemia,[4] contributing to morbidity and mortality. Hyperglycemia can lead to several complications such as chronic microvascular changes and retinopathy, which can in turn, lead to blindness.[5] According to the Centers for Disease Control (CDC), throughout the United States, the prevalence of DM has been increasing for decades: from 1980 through 2010 the number of US Americans with DM increased from 5.6 million to 20.9 million.[6] Due to progressively unhealthy lifestyle choices by an aging population during the last decades of the twentieth century, DM has become a major health problem worldwide. In Saudi Arabia, the number of people who suffer from DM increased from 890,000 in 2000 to a staggering projection of 2,523,000 in 2030.[7] In 2011, Saudi Arabia reported a prevalence of DM at 30% of the total population, with a rate of 27.6% in women and 34.1% in men.[8] According to 2010 data from several sources (WHO, World Bank, UNESCO, CIA and individual country databases), DM is the number three disease-related cause of death in Saudi Arabia.[9]

Currently, cancers are also an important leading disease-associated cause of death in KSA.[8] Cancer is a general term for a variety of chronic diseases which have in common the presence of malignant cells characterized by uncontrolled replication, frequent lack of differentiation, and inability to respond to normal physiological stimuli, contributing to morbidity and mortality. The World Health Organization reports 13 million cancer cases and 7.6 million cancer deaths in 2008 worldwide.[1] The World Health Organization also projects that in 2030 cancer deaths will be 13.1 million. According to Parkin *et al.*,[10] global statistics show that 10.9 million individuals were diagnosed as new cases

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of cancer, 6.7 million died of cancer-related complications, and 24.6 million were living while struggling with cancers in 2002. Lung cancer is the most lethal type of cancer worldwide. According to the KSA Saudi Cancer Registry (SCR), an organization that reports cancer cases based on the Saudi population, in 2007 12,309 cases were reported. Female cancer cases were reported at 51.4%, while male cancer cases were at 48.6% in 2007. The KSA SCR data show that the most common types of cancers among the KSA population for both genders at all ages are: breast, colorectal, non-Hodgkin's lymphoma, thyroid, leukemia, liver, lung, Hodgkin's disease, stomach, and skin cancers. The most prevalent cancer in Saudi children is leukemia (girls 34.2% and boys 34.8% cancer cases). In Saudi adults, the breast cancer rate in women is 27.4% among female cancer cases, and colorectal cancer in men is 12.3% among male cancer cases in 2007. Another common cancer in the KSA is skin cancer (specifically basal and squamous cell carcinomas); however, melanoma, the most deadly type of skin cancer, is uncommon in the KSA. Colorectal cancer is more prominent among young Saudis compared to old Saudis.[7]

## 1.3 Overview of Ethnobotany of Saudi Arabia

The flora of Saudi Arabia provides a rich resource of medicinal plants and endemic species for traditional medicine. Medicinal plants are essential in traditional health systems and are an important economic component of the biodiversity in Saudi Arabia.[8] An inventory of the medicinal plants of KSA is essential to prioritize a scientific assessment of the native ethnomedicine, and to advance new derivative medicines and natural health products. The number of medicinal plants and trees among Saudi Arabia's flora is more than 1200 useful plants out of 2250 species.[9] Seven families of herbal plants from the KSA flora were recently studied, which represent a total number of 254 species in the KSA flora.[9] The majority of these flora species can be found in Asir and Hijaz in the western region of the KSA near the border of the Red Sea.[10] Of these plants reviewed by Rahman *et al.*,[9] *Achyranthes aspera* L., *Acrva lanata* (L.) Juss, *Rhazya stricta* Decne, *Acalypha indica* L., and *Andrachne aspera* Spreng are native to Saudi Arabia, while *Amaranthus viridis* L. is naturalized. Based on some of the reported traditional uses of these herbal plants, it can be seen that many plants in the KSA (native or naturalized) are part of the natural healthcare system and should be scientifically studied.[9] Identification of the bioactive compounds, and delineation of the possible antidiabetes and anticancer properties of native Saudi plants, can further the search for novel therapeutics for such chronic diseases as diabetes and cancer.

## 1.4 Secondary Metabolites/Bioactive Compounds

Plant-derived NHPs are used worldwide for human disease prevention and healing in ethnomedicine practices.[11] Bioactive compounds in plants often are secondary metabolites, and are also known as natural products.[12] Secondary metabolites are derived from primary metabolites (e.g., proteins, lipids, carbohydrates), but are found at much lower concentrations. Natural products are non-nutritive organic compounds found in plants (and other organisms) and may provide the organisms with bioactivities against pathogens and environmental stressors. Secondary metabolites used as NHPs may derive from a variety of plant parts such as the fruits, seeds, flowers, roots, and leaves. They may be used medicinally in the human body to promote health and mitigate disease. The most common classes of secondary metabolites are: terpenes, polyketides/fatty acids, alkaloids, and polyphenols/phenylpropanoids. The first class, terpenes, include hormones, pigments, essential oils, steroids, and rubber. The second class contains fatty acids and compounds derived from malonyl-CoA. The third class is the alkaloids, a large class of structurally unrelated nitrogenous compounds with renowned medicinal or toxic properties. The fourth class contains the phenolic compounds, which include coumarins, flavonoids, lignin, and tannins.[13]

Polyphenols include phenolic acid and flavonoids, which share the same basic phenol structure as the aromatic amino acids.[14] Phenolics are found in most plants and approximately 10,000 phenolic structures have been identified so far. Phenolic acids and flavonoids are found in many fruits, vegetables, coffee, whole grains, bran, and potatoes with skin. Some polyphenols have toxic and mutagenic effects in certain cell culture systems.[15] Duthie *et al.*[16] show that plant polyphenols have beneficial anticancer effects *in vitro* and *in vivo*, including modulation of apoptosis, intercellular signaling, platelet aggregation, gene expression, and dilation of blood vessels. Polyphenols/flavonoids, and other secondary phytochemical compounds, may offer healing properties to provide numerous health benefits in the human body, including anticancer, antimicrobial, and antioxidant effects.[17] Polyphenols are perhaps best known for their role in combating oxidative stress, a risk factor in the development of many chronic diseases including DM and cancer.

## 1.5 Oxidative Stress

During oxidative metabolism, approximately three to ten percent of the oxygen is not completely reduced to water inside the mitochondria by the electron


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transport chain. Oxygen may take up an extra electron, which consequently leads to reactive oxygen species (ROS) formation. ROS is a general term that includes radicals and other oxidizing derivatives, such as the hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide ($H_2O_2$), superoxide ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$).[22] Damage to cellular proteins, lipids, carbohydrates, and DNA can result from interaction of biomolecules with ROS. One of the ROS reactions—the Fenton type reaction—is considered a key reaction in many toxic oxidative processes. This reaction involves the $Fe^{3+}/Fe^{2+}$ redox system, which produces superoxide and subsequently $HO^{\bullet}$ formation in the mitochondria.[22]

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet}$$

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$

Lipid membranes are greatly affected by ROS, such as $HO^{\bullet}$, which cause lipid peroxidation, thereby inciting harmful cell damage.[23] The hydroxyl radical ($HO^{\bullet}$) can attack membrane unsaturated fatty acids, which cause unpaired electrons to bind with carbon atoms after a hydrogen atom is removed from a methylene group ($CH_2^{\bullet}$). As a result, the hydroxyl radical produces a conjugated diene, which then reacts with an oxygen molecule to produce a lipid peroxyl radical ($LOO^{\bullet}$).

$$LH + {}^{\bullet}OH \rightarrow L^{\bullet} + H_2O$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

In addition, ROS can extract hydrogen atoms from carbon in cellular carbohydrates, which can lead to molecular damage and inflammation.[23] Cellular protein may also be oxidized by ROS, resulting in interactions with transition metal ions that can age cells.[23] Reactive oxygen species also affect DNA by extracting a hydrogen atom from the sugar, or by attacking the base. The radicals can attack the sugars of DNA causing oxidative pyrimidine damage such as in thymine glycol, uracil glycol, and urea residue.[23] The hydroxyl radical ($HO^{\bullet}$) also can damage the nucleotide bases themselves.[22]

### *1.5.1 Endogenous and Exogenous Antioxidants*

Within the body, we have endogenous and exogenous factors that work in our defense against ROS-induced oxidative stress. One of the important endogenous antioxidants is superoxide dismutase (SOD) (cofactors: Cu, Zn, and/or Mn) which catalyzes the dismutation of $O_2^{\bullet}$ to $H_2O_2$.[4] There are two enzymatic ways to remove $H_2O_2$ from the cell. First, catalase will reduce $H_2O_2$ to $H_2O$ and $O_2$. Alternatively, Se-dependent glutathione peroxidase (GSH-PX) will reduce $H_2O_2$ to $H_2O$.[4]

There are also exogenous antioxidants provided by the consumption of plant food and products. Some vitamins rich in fruits, vegetables, and whole grains (such as vitamin C, carotenoids, and vitamin E respectively), play important roles as antioxidants. For example, vitamin E (tocopherol) limits lipid peroxidation in membranes by interrupting singlet oxygen reactions ($ROO^{\bullet} \rightarrow ROOH$).[24] Vitamin C then replenishes the vitamin E radical.

Exogenous antioxidants are ROS scavengers that work against oxidative damage to limit free radical-induced damage and thus maintain health.[25] Multiple studies have examined various plant species parts (e.g., the fruit, seeds, leaves), and report the presence of secondary metabolites.[25] The antioxidant potential of secondary metabolite compounds was assessed against biochemically generated ROS *in vitro*.[25] Numerous studies have also focused on examining the antioxidant activity of exogenous compounds in different plant foods—fruits, vegetables, and tea, for example—because the antioxidant compounds in these foods may contribute to a reduction in the incidence of chronic diseases.[26] Many of these dietary antioxidants are polyphenolic compounds.

## 2. DIABETES MELLITUS

### 2.1 Oxidative Stress and Diabetes Complications

Type 2 diabetes mellitus is associated with an increase in ROS formation,[27] and ensuing oxidative stress. Oxidative stress can be defined as an imbalance between oxidizing oxygen derivatives and antioxidant defense mechanisms, favoring the former. Obesity may contribute further to increased oxidative stress and insulin resistance. However, it is unclear whether diabetes is a cause or a result of oxidative stress. It has been known that a primary source of ROS in DM is glucose oxidation.[4] Several studies demonstrate that DM is associated with oxidative stress, which plays a role in the development of atherosclerosis.[27] Hyperglycemia is associated with a superoxide-dependent pathway resulting in the generation of free radicals, which promote lipid peroxidation of low-density lipoprotein (LDL). Also in DM, the interaction of excess blood glucose with cellular proteins leads to free radical formation by producing an Amadori product and then advanced glycation endproducts.[4]



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## 2.2 Use of Natural Health Products in Saudi Arabia in Relation to Diabetes

Many NHPs, especially plant-derived medicines, have been used in the treatment of DM.[1] Natural products (secondary metabolites) are bioactive compounds isolated from a variety of organisms, and are a major source of drugs around the world. About half of the pharmaceutical medicines in use today are derived from natural products. In the Middle East, numerous plant species are used to treat DM.[1] A cross-sectional study conducted on patients with diabetes in Riyadh, KSA found that 51 subjects out of 296 patients with diabetes (17.4%) use herbs in the treatment of diabetes.[1] In the next section the caper (Capparidaceae) family of plants will be discussed in regard to use as a traditional medicine, as well as in regard to the scientific evidence in support of the bioactivities of the plant or its compounds in relation to diabetes in assay and *in vivo* studies.

## 2.3 Capparidaceae (Caper Family)

Capparidaceae is a family of nearly 600 herbs, shrubs, and trees that includes several different species known by common names such as: caper (English), kabbar (Arab), alcaparro (Spain), and gollaro (Pakistan). The Capparidaceae family of plants is found in many places around the world, particularly in the Mediterranean Sea basin, the Atlantic coasts of the Canary Islands, Morocco, the Black Sea, Crimea, Armenia, eastward to the Caspian Sea and Iran. Caper shrubs typically grow 30–100 cm tall, have strong resistance to harsh environmental conditions, are adapted to poor soils, grow in rocky areas, mountains, and in different soil types.

Historically, different members of the Capparidaceae plant family have been used in traditional medicine to treat ailments such as rheumatism, stomach problems, headaches, and toothaches.[2] For example, the leaves of Capparidaceae family plants were used by ancient Arabs to prevent skin disease, to treat earaches, kill worms in the ear, while the buds of the plant were used to guard against spleen disease. There are many species of the Capparidaceae family (e.g., *Capparis sicula*, *C. obcata*, *C. ovata*, *C. decidua*, *C. masaikai*, *C. sicula*, *C. orientalis*, and *C. zoharyii*)[3] that have been used for various purposes, including as culinary additives and ethno-medical treatments. This next section focuses on two species of the Capparidaceae family: *Capparis spinosa* L., and *Capparis decidua* (Forssk.) Edgew., which are plants native to Saudi Arabia and are frequently used for the treatment of diabetes in ethnomedicine.[1,3]

### 2.3.1 *Capparis spinosa*

*Capparis spinosa* L. is considered to be a commercially important species of the Capparidaceae family for the Mediterranean nations where this species is prevalent. *Capparis spinosa* has traditionally been used for several medicinal purposes. For example, researchers in Morocco interviewed patients with diabetes.[4] They found that 80% of the patients used medicinal plants (including fruit of *C. spinosa*) to treat chronic conditions such as diabetes.[4] In Pakistan, traditional medical practitioners have used the leaves of *C. spinosa* as an antirheumatic, analgesic, deobstruent, antihemorrhoid, aperient, depurative, and diuretic agent.

#### 2.3.1.1 CHEMICAL COMPOUNDS OF *CAPPARIS SPINOSA*

In a study conducted by Zhou and colleagues,[5] water was combined with ethanol extracts, and five bioflavonoids were isolated from the *C. spinosa* fruit. The bioflavonoids were isoginkgetin, ginkgetin, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-rutinoside, and sakuranetin (Figures 11.1 and 11.2). Of these, isoginkgetin, ginkgetin and sakuranetin were identified for the first time in *C. spinosa* fruit.[5] Yu and colleagues isolated (from *C. spinosa* fruit) eight different phenolic compounds: 3-sitosterol, vanillic acid, *p*-hydroxybenzoic acid, protocatechuric acid, daucosterol, uracil, butanedioic acid, and uridine by using ether and ethyl acetate extracts.[6] All of these compounds were isolated for the first time from the fruit except 3-sitosterol, which had been isolated previously.[6] Calis and colleagues[7] analyzed the fruit of *C. spinosa*; the researchers extracted the water-soluble part of the fruit by vacuum liquid column chromatography. Various compounds were isolated from the fruit, including (6*S*)-hydroxy-3-oxo-α-ionol glucosides, together with corchoionoside C ((6*S*,9*S*)-roseoside), and a prenyl glycoside.[7] In a study by Khanfar and colleagues,[8] roots from *C. spinosa* were examined

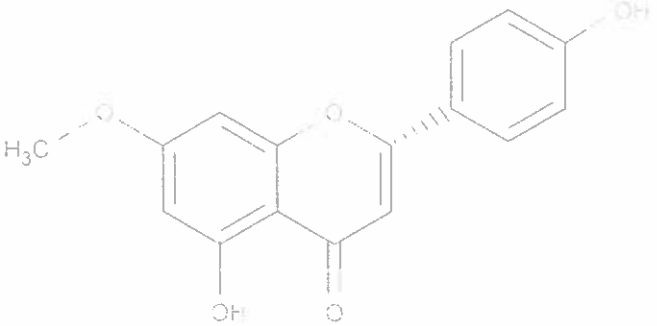



FIGURE 11.1 Sakuranetin: polyphenol compound in *Capparis spinosa*. *Figure courtesy of European Bioinformatics Institute ChEBI Database.*


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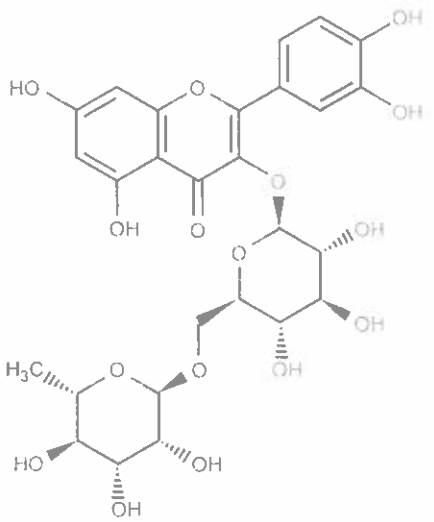


FIGURE 11.2 Quercetin-3-*O*-rutinoside (Rutin): polyphenol compound in *Capparis spinosa*. *Figure courtesy of European Bioinformatics Institute ChEBI Database.*

to identify new compounds in this plant part. The compound *g*-sitosterylglucoside-6′-octadecanoate was isolated from the roots as a pure solid as determined by nuclear magnetic resonance (NMR). A second compound, 3-methyl-2-butenyl-α-glycoside, was isolated as an amorphous solid as determined by NMR spectrometry.[38] From dried aerial plant parts of *C. spinosa*, Sharaf and colleagues[39] used an 80% MeOH extract to identify a flavonol called glycoside quercetin 3-*O*-(6‴-α-L-rhamnosyl-6″-β-D-glucosyl)-3-D-glycoside.

#### 2.3.1.2 *CAPPARIS SPINOSA* AND ANTIDIABETIC EFFECTS *IN VITRO*

In their study of various medicinal plant extracts from Saudi Arabia, Ageel and colleagues[32] identify anti-inflammatory activities. They found that *C. spinosa* exhibited anti-inflammatory properties that guard against edema (*in vivo*). In a study by Bonina and colleagues,[40] they evaluated the *in vitro* antioxidant properties of the methanol extract of the lyophilized flowers and buds of *C. spinosa*. The investigators first identified the phenolic compounds with antioxidant capabilities. Germano and colleagues[41] evaluated the methanolic extracts of *C. spinosa* for antioxidant activity by using liver microsomes. Overall, these findings suggest that there are anti-inflammatory and antioxidant effects from bioactive compounds from *C. spinosa*.

#### 2.3.1.3 *CAPPARIS SPINOSA* AND ANTIDIABETIC EFFECTS *IN VIVO*

Eddouks and colleagues[42,43] conducted two studies evaluating the health benefit of *C. spinosa* fruits in a rodent model. In the first study, an aqueous extract of *C. spinosa* fruits was given orally to two groups of rats: streptozotocin-induced diabetic rats and normal control rats.[42] By testing blood samples of the rats, the researchers found decreased triglycerides and cholesterol levels, and also decreased body weight in the treatment group of rats with diabetes. These findings suggest that treatment with *C. spinosa* fruits may improve clinical parameters and prevent diabetes-associated health complications in rodents. The second study also examined normal and diabetic rats treated with aqueous extracts of *C. spinosa* powdered fruits.[43] The researchers administered a daily oral dosage of the extract for two weeks, and then collected blood samples to test the impact of the extract on blood glucose levels. They found that the glucose level in the diabetic rats normalized, without a significant change in blood insulin concentration. This suggests that *C. spinosa* powdered fruits have antidiabetes properties *in vivo*.[43]

Hashemnia and colleagues[44] studied the effect *C. spinosa* fruit on blood glucose levels in diabetes-induced rats. One group of rats was treated with an extract of garlic root; another group was treated with *C. spinosa* fruit extract, while the untreated control group received no extract. The second group was given daily doses of *C. spinosa* alcoholic extract by intragastric tube over a 12-day period. This study found that the blood glucose of the untreated diabetic rats was significantly higher than that of the diabetic rats treated with *C. spinosa* extract. These results demonstrate the beneficial effect of *C. spinosa* alcoholic extract on the hyperglycemia in diabetic rats.[44]

### 2.3.2 *Capparis decidua*

*Capparis decidua* (Forssk.) Edgew. is a shrub of the Capparidaceae family that grows native in different regions such as the western part of Saudi Arabia and India. Various parts of *C. decidua* have been used throughout the Middle East and Asia for food, fuel, wood, and as a medication. In addition, the fruits of *C. decidua* are used in food preparations—for example, as pickles—due to the belief that the fruits have antidiabetic properties. Other parts of the plant, such as the bark, have been used in the treatment of coughs, asthma and inflammation. Finally, the roots are used in the reduction of fever and the buds are used in the treatment of boils.[33,45,46]

#### 2.3.2.1 CHEMICAL COMPOUNDS OF *CAPPARIS DECIDUA*

A study was done by Zia-Ul-Haq and colleagues to determine the bioactive compounds of *C. decidua*, and to determine their antidiabetic and antioxidant activities.[47] They found that *C. decidua* seeds contain high amount of carbohydrate, lipids (unsaturated fatty acids, tocopherols), and protein (essential and non-essential

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amino acids). They also found that the fruit and leaf *C. decidua* methanol extracts contain high levels of phenolic compounds, as well as glucosinolates, which may contribute to its *in vivo* antidiabetic effects.[1]

Duhan and colleagues[1] assessed the nutritional value of the *C. decidua* fruit. They found that *C. decidua* fruit contains nutrients such as protein, β-carotene, vitamin C, phosphorus, and iron. In another study, the researchers evaluated the fiber content and other properties of an air-dried powder of unripe *C. decidua* fruit.[1] They found that *C. decidua*'s dietary fiber has the ability to lower the blood cholesterol and triglyceride level. Further work should be conducted to determine the type of fiber present in *C. decidua*, as a reduction in the substrates of lipid peroxidation is clearly a desirable outcome in DM.

#### 2.3.2.2 *CAPPARIS DECIDUA* AND ANTIDIABETIC EFFECTS *IN VITRO*

Different aerial parts of *C. decidua*—leaves, flowers, and fruits—were subjected to antioxidant activity screening, using various testing methods by Zia-Ul-Haq and colleagues.[1] The methanol extract of *C. decidua* fruit, flowers, and leaves shows potent antioxidant activity in reducing some radicals. Among the extracts of *C. decidua*, the fruit especially shows satisfactory inhibitory effect on carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase in the digestive tract. This study supports the use of this plant as a dietary supplement for use in diabetes.

#### 2.3.2.3 *CAPPARIS DECIDUA* AND ANTIDIABETIC EFFECTS *IN VIVO*

Yadav and colleagues[1] examined *C. decidua* powder as a hypoglycemic agent adding it to the diet of diabetes-induced rats compared to another group of diabetes-induced rats treated with insulin. Increased blood glucose in diabetic rats was shown to be associated with an increase in the level of $O_2^{\bullet}$ and ROS, which led to oxidative damage to cell membranes. *Capparis decidua* treatment of diabetic rats was associated with lower tissue lipid peroxidation than in insulin-treated rats. The authors speculate that *C. decidua* treatment may lower the $H_2O_2$ level and reduce oxidative stress in diabetes. This study suggests that *C. decidua* may be used as an antidiabetic NHP agent and in lowering oxidative stress in animal models of diabetes.[1]

Sharma and colleagues[1] tested the effects of an alkaloid rich (AR) extract from dried *C. decidua* fruit soaked in methanol as an antidiabetic agent in mice. They treated induced diabetic mice with the AR fraction. Fasting blood glucose levels of the treated mice showed significant improvement after treatment with the AR fraction as compared to untreated mice. In addition, the researchers found reductions of total cholesterol and triglyceride content in the blood of the treated mice. The AR fraction of *C. decidua* fruit shows some antidiabetic effects in diabetic mice.

The unripe fruit of *C. decidua* was used to determine the antihyperlipidemic effects of this fruit, in a study by Goyal and colleagues.[1] They used *C. decidua* dried fruit powder to make baked goods, and served it as a snack to 30 hyperlipidemic volunteers in the age group of 40–60 years for 2.5 months. The results show significant decreases (9.6 to 20.3%) in triglyceride levels in the subjects; also there was a significant negative relationship between levels of dietary fiber from *C. decidua* dried fruit and serum triglycerides. In addition, an 8.56% reduction in plasma phospholipid concentration was associated with consumption of *C. decidua* dried fruit. This study suggests that *C. decidua* fruit consumption is associated with lowering plasma triglyceride and phospholipid levels, which could benefit hyperlipidemic people with diabetes, to reduce the propensity for lipid peroxidation (e.g., atherosclerosis).

### 2.4 Diabetes Summary

Diabetes mellitus is a metabolic disorder affecting approximately 170 million people worldwide and is characterized by hyperglycemia and insufficiency of secretion and/or action of pancreatic insulin. The high level of blood glucose is associated with the formation of ROS by autoxidation of glucose and other oxidative mechanisms. Numbers of people affected by DM are increasing around the world. Saudi Arabia is one of these affected countries, which lead Saudis to use natural health products for DM healing. Different studies show the antidiabetic and/or antioxidative effectiveness of phenolic and other compounds from plants *in vitro* and *in vivo* models. Two Saudi plants from the Capparidaceae family (*Capparis spinosa* and *Capparis decidua*) show antidiabetic promise through *in vitro* and *in vivo* studies. Future clinical studies are needed to ascertain the potential effectiveness of these two plants in humans with diabetes. Thus, bioactive compounds from native plants from Saudi Arabia—with a history of use in traditional medicine—are being studied scientifically for use as natural health products and show promise as compounds for drug design and development in the prevention and treatment of diabetes mellitus.

## 3. CANCER

### 3.1 Biology of Cancer

Cancer can be defined as the unstoppable growth of irregular cells, termed cancerous or malignant cells.[1] Cancer is associated with several mutagenic causes (e.g., exposure to dietary and environmental toxins,


Abdel, P. P., Prasad, V. R., & Zoran, B. (Eds.). (2013). Polyphenols in human health and disease. Retrieved from http://site.ebrary.com/id/10744... (accessed 2013-12-11 12:12:42)

radiation, benzene and other chemicals, genetic problems, and obesity).[52] Abnormal cell growth, also known as hyperplasia, can be benign (non-cancerous) or malignant (cancerous).[53] A major difference between normal and cancer cells is that normal cells can grow and die based on the system's demand. On the other hand, cancer cells grow and divide quickly and abnormally, and are resistant to programed cell death (apoptosis).[52] When malignant tumors develop, they tend to grow over the surrounding tissues, destroy them, and promote further tumor development. Benign tumors are less threatening than malignant ones; however, they can become malignant.[53] Malignant primary tumors can grow in different organs and tissues, such as lungs, liver, breasts, colon, prostate, brain, skin, bones, or nerves. The primary tumor's cells can also spread to different organs in later stages of the disease (termed metastasis), which is usually associated with a poor prognosis.

In metastasis, cancer cells break away and travel through the blood or lymphatic system to replicate in other bodily tissues.[53] Once malignant tumors are initiated, they depend on different tactics to avoid detection by the body's immune and other defense mechanisms to combat aberrant growth. Malignant cells tend to sustain proliferative signals, evade growth suppressors, resist apoptosis, enable replicative immortality, induce angiogenesis, and activate invasion and metastasis mechanisms.[54] These hallmarks are important for understanding neoplastic diseases, as each of these has different roles in the potential of cancer growth. Understanding these signaling processes will therefore assist in the development of specific and potent preventive and therapeutic strategies.[54]

## 3.2 Use of Natural Health Products in Saudi Arabia in Relation to Cancer

With increased mortality among the Saudis due to increased prevalence and/or incidence of various types of cancers in recent decades, there is interest regarding the therapeutic potential of native herbal plants. These alternative and complementary therapies are currently being studied to assess bioactivities relevant to disease prevention or mitigation. The KSA's rich flora may yield herbal medicines to prevent or treat disease, such as cancer.[55] Jazieh and colleagues[56] conducted a cross-sectional survey of Saudi cancer patients. Approximately 85% reported the use of dietary supplements (including herbs). The majority of the patients used these alternative medicines as a treatment for cancer, while others used them for control of symptoms or as complementary treatment.[56] In the next section the milkweed family of flowering plants will be discussed in regard to use as a traditional medicine in the prevention and treatment of cancer, as well as in regard to the scientific evidence in support of the bioactivities of the plant or its compounds in relation to cancer.

## 3.3 Asclepiadaceae (Milkweed Family)

The Asclepiadaceae (milkweed family) is a large family of mostly herbs and shrubs. Most of the approximately 2900 species have a white milky sap that often contains alkaloids or other toxins. The family mainly occurs in tropical and warm areas of the world, with some occurring in temperate regions.

### 3.3.1 *Calotropis procera*

*Calotropis procera* (Aiton) Dryand. is a member of the Asclepiadaceae family (milkweed), which has various therapeutic applications. *C. procera* is a small tree that grows wild in deserts of Africa and Asia, especially in the arid parts of Saudi Arabia and in the northern parts of Egypt. *Calotropis procera* takes the form of a shrub or small tree that measures up to two to three meters in height.[57] Common names of *C. procera* include calotropis, rubber bush or kapock tree, and sodom apple.[58] *C. procera* is known as Ushar or Madar in Greco-Arab. This shrub is also dispersed throughout humid places in Africa and Asia.[59]

According to Kirtikar and Basu,[60] it has been a tradition of Indian groups to heal sicknesses such as leprosy, ulcers, tumors, piles, and diseases of the spleen, liver, and abdomen through the utilization of *C. procera* aerial parts. In addition, its roots are consumed to relieve pain caused by dyspepsia.[61] Samvatsar and Diwanji[62] posited that tribes from central India employed *C. procera*'s root bark and leaves as a health-giving remedy for jaundice. As part of traditional Indian medicine, the plant is boiled to extract its flavor and bioactive components. It is used as an expectorant, purgative, and a remedy for painful muscular spasms, dysentery, fever, rheumatism, and asthma.[63–65] Egyptians were also knowledgeable in employing *C. procera* as traditional medicine. It was discovered that *C. procera* had been used during the Neolithic period, through evidence found in a quarry at Helwan, Egypt.[66] Ebbell[67] stated that in the "Berliner" papyrus, the shrub was described as curing leprosy.

#### 3.3.1.1 CHEMICAL COMPOUNDS OF *CALOTROPIS PROCERA*

Cardenolide compounds were studied from *C. procera* leaves and latex extracts by Seiber and colleagues.[68] Ethanol extract 95% was used to get a total content of cardenolides. They determined the quantity

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of the compounds by using NMR, while thin layer chromatography (TLC) was used to check the quality of the extract and identify the chemical compounds. Results show these compounds in the leaf: uscharidin, uscharin, calotropin, calactin, calotropagerin, calotoxin, unidentified one, and unidentified two. The latex contains: voruscharin, uscharidin, uscharin.[7] The *C. procera* root contains calotropin, according to Gupta *et al.*,[8] who studied a 95% ethanol-based extract using the method of Hesse and Reicheneder.

In a study by Sharma and Sharma[9] the ethanol extracts of flower, bud, and root of *C. procera* were subjected to testing to identify the chemical compounds. The Dragendorff and Mayer's tests were used to identify the alkaloids, Molish and Fehling's tests were used for carbohydrates. In addition, the Keller Killiani test was used to find glycosides, while ferric chloride, lead acetate, and gelatin tests were used to identify phenolic compounds/tannins. Proteins and amino acids were examined by the xanthoprotein test. For flavonoids and saponins, the ammonia test was used. Finally, sterols, acidic compounds, and resins were measured with Liebermann-Burchard, Salkowski, and Hesse assays, respectively. Results show that flower, bud, and root of *C. procera* extracts contain: alkaloids, glycosides, sterols, phenolic compounds/tannins/flavonoids, saponins, carbohydrates, proteins and amino acids, acidic compounds, and resins.

Mossa *et al.*[10] screened a 96% ethanol extract of shade-dried powder from the aerial parts of the *C. procera* to determine the phytochemical components. Results show the following compounds: alkaloids, cardiac glycosides, flavonoids, tannins, coumarins, anthraquinones, saponins, volatile oils, volatile bases, cyanogenic glycosides, glucosinolates sterols, and/or triterpenes.[10] Elgamal *et al.*[11] studied the 70% ethanol extract of dried powder of *C. procera* stem. NMR was used to identify the chemical compounds. Results showed four cardenolide compounds: uzarigenone, uzarigenine, deglucouzarin, and frugoside.[11] Ethanolic extracts of leaf and the latex of *C. procera* were studied by Kawo *et al.*[7] Phytochemical analysis showed the presence of tannins, steroids, saponins, and flavonoids in both leaf and latex.[7] The methanolic extract of *C. procera* root bark was analyzed by successive flash chromatography to identify several cardenolides, such as calotropin, calactin, uscharin, voruscharin, and 2′-oxovoruscharin, by Van Quaquebeke *et al.*[12]

Joshi and colleagues[13] analyzed the total phenols and flavonoids of *C. procera* root, leaf, and latex extracts. The 80% methanol extract of all plant parts was studied. The Follin Ciocalteu reagent method was used to determine the total number of phenols, and the aluminum chloride method was used to determine flavonoids. Results show a high amount of phenols and flavonoids in latex and a lower amount in root and leaf. Another group used methanol, ethanol, and acetone extract from the *C. procera* plant leaves and flowers. Total phenolic and flavonoid content was measured in all samples. Total phenolics and flavonoids in the leaves and flower extract of *C. procera* were from 0.11 to 0.32 g/100 g and 0.01 to 0.10 g/100 g, respectively.[14]

### 3.3.1.2 *CALOTROPIS PROCERA* AND ANTICANCER EFFECTS *IN VITRO*

The antitumor activity of root extracts of *C. procera* has been investigated against Hep2 cancer cell lines.[15] Four different root extracts were used: methanol extract (CM), hexane extract (CH), aqueous extract (CW), and ethyl-acetate extract (CE).[15] Cellular proliferation activities (on tumor Hep2 cells) were tested by microculture tetrazolium (MTT) assay. The extracts CM, CH, and CE show potential effects against cancer growth. Ethylacetate extracts show the strongest growth inhibitor effect by reducing the growth of Hep2 by 98.3% at 48 hours. However, CM and CH show some anticancer effects by inhibiting the cancer cell growth to 72.7 and 60.5%, respectively. On the other hand, CW did not show any botanical inhibitor effect against cancer cell lines at all times and concentrations given.[15]

Van Quaquebeke and colleagues[12] studied the methanol extract of *C. procera* root. Chemical compounds were identified, and they include cardenolide (2′-oxovoruscharin); also, 27 compounds were hemisynthesized. These compounds were tested on five different human cancer cell lines, which were a lung cancer (A549), two glioblastoma (Hs683 and U373), and two colon cancer (HCT-15 and LoVo) lines. The activities of two cancer drugs (taxol and SN-38) were tested and compared to the bioactivity of compound number 23. Results show compound number 23 is the most interesting one due to its antitumor activity against 57 different human cancer cell lines. Compound number 23 shows similar activity to taxol, and is more effective than SN-38, which are two high impact cancer drugs used to treat cancer medically.[12]

In a study by Choedon *et al.*[16] the extract of dried latex (DL) of *C. procera* was studied. The extract was given to X15-myc transgenic mice (hepatocellular carcinoma model), and subsequent investigation of harvested tissues and cell culture studies determined the anticancer potential of the DL extract. Petroleum ether and methanol extracts were applied on cell line cultures to see their cytotoxic effect on hepatoma (Huh-7), non-hepatoma (COS-1), and non-transformed hepatocytes (AML12) by using a proliferation (MTT) assay. The levels of Bcl2, caspase 3, and DNA fragmentation were studied to investigate the machinery of apoptosis.


Vasu, V. R. Reddy, V. R. & Zaato, B. (Eds.) (2011). Polyphenols in human health and disease. Retrieved from http://www.sciencedirect.com
(Date: Tue, 14 Jun 2011 03:09:10 GMT)

The methanolic extract of DL demonstrated widespread cell death in Huh-7 and COS-1 cell lines; however, AML12 cells did not. Canonical markers of apoptosis like Bcl2 and caspase 3 showed no shift in level when observed. This study shows that *C. procera* has potential effect as an anticancer agent in cell line cultures.[79]

Oliveira *et al.*[80] studied the cytotoxic activity of laticifer proteins (LP) found in the latex of *C. procera*. Water extraction was used to collect the latex of *C. procera*. The LP was considered as cytotoxic with $IC_{50}$ values from 0.42 to 1.36 μg/mL for SF295 and MDA-MB-435 cell lines respectively. Four types of cell lines were used: HL-60 (promyelocytic leukemia), HCT-8 (colon), MDA-MB-435 (breast), and SF295 (brain). The HL-60 cells were used in this work; LP showed potential inhibition of DNA synthesis via inhibition of topoisomerase I activity, which resulted in cancer cell lines undergoing apoptosis.[80] Another study was done on the latex of *C. procera*, whereby the water extract was used on HeLa, A549, and BHK21 cell lines.[81] The water extract of *C. procera* latex contains cysteine proteases, and was used to produce copper nanoparticles. From the water extract of *C. procera* latex HeLa, A549, and BHK21 cells displayed high capability even at 120 μM of copper nanoparticles. This work emphasizes that biocompatible copper nanoparticles, synthesized from the latex of *C. procera*, can be used to develop nanoparticle-based platforms to effect drug carriage and produce apoptosis of cancer cells in culture.[81]

Smit *et al.*[82] studied a 70% ethanol extract of *C. procera*.[82] A microculture tetrazolium assay was used to test the effects of the *C. procera* extract on the cytotoxity of COLO 320 tumor cells. The parameter of cytotoxicity used was the $IC_{50}$ value. The extract of *C. procera* flower showed the strongest cytotoxic effect; however, some other extracts of the plants did not show cytotoxic effects, even with high concentration.[82] This study shows the potential effect of *C. procera* flower extract, which may have useful anticancer properties. Further work should be done with animal and human models for study. Currently, there are a few animal model investigations discussed in the next section.

#### 3.3.1.3 *CALOTROPIS PROCERA* AND ANTICANCER EFFECTS *IN VIVO*

Extracted metabolites from *C. procera* latex have shown antitumor effects in both *in vitro* and *in vivo* models. Hemisynthetic cardenolides derived from *C. procera* were shown to interfere with the $Na^+/K^+$ ATPase activity of tumor cell lines.[75] The same cardenolides were shown *in vitro* to stimulate cell death via autophagy,[83] which is different than typical chemical anticancer agents.

*Calotropis procera* latex extract has been studied in both cell culture and animal models. Hepatocellular carcinoma was studied in X15-myc transgenic mice, as previously described.[79] Dried latex (DL) of *C. procera* was used to test for anticancer activity. The extracts of DL were prepared by petroleum ether and methanol extractions. Two groups of young mice were studied. One group was fed bread soaked in an aqueous suspension of DL. The control group was fed bread only. Both groups were fed for 15 weeks. The animals were sacrificed and the livers were collected. Serum vascular endothelial growth factor (VEGF) levels were measured. The results showed that the treatment group had a significantly lower level of serum VEGF compared with the control group. The mice treated with DL thus showed protection against hepatocarcinogenesis with no adverse effects, which suggests that DL of *C. procera* has anticancer therapeutic properties in mice.[79]

In 2007, Oliveira and colleagues[84] focused on proteins secreted by laticifer cells (LP) of *C. procera*, which have been shown to have anti-inflammatory,[84,85] antinociceptive,[86] nitric oxide producing,[87] and anticancer activities. Oliveira and colleagues[84] studied the bioactivity of LP on cancer cell lines. Results showed that LP has cytotoxic activity against cancer in cell line culture. In a more recent study, mice bearing sarcoma 180 were administered either orally or IP (intraperitoneal injection) with LP and fractions, obtained by ion exchange chromatography of the whole protein extract.[88] The research group used fluorouracil (5-FU) (a chemotherapeutic reagent used in treating cancer) as a positive control. Results showed a decrease of tumor size in mice treated with LP compared with tumors from mice treated with 5-FU, and an additive effect when treated with both. Overall low toxicity levels were observed on histological sectioning and assessment of the liver. Oral administration was shown to be more effective than IP injections, suggesting a differing metabolic effect. Oral LP-treated mice had an increase of 23% in life span compared to untreated animals. One of the three LP fractions showed similar effects in comparison to whole LP protein. The researchers demonstrated that the active protein fraction was able to show similar activity after heat treatment of the fraction (100 C). Overall results from this study show the potential to have efficient, less toxic antitumor treatments from *C. procera* latex.[88]

### 3.3.2 *Caralluma tuberculata*

*Caralluma* is a member of the Asclepiadaceae family (milkweed). Generally, the *Caralluma* genus has been found in arid countries, such as Saudi Arabia, and used with ethnomedicinal importance. The two most famous species are *Caralluma edulis* (Edgew.) Hook. f. and



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*Caralluma tuberculata* N.E.Br.[5] The two species *C. edulis* and *C. tuberculata* are disseminated in the northwest Himalayas. *Caralluma tuberculata* also grows widely in Saudi Arabia, United Arab Emirates, Pakistan, India, and the south east of Egypt.[6] The fruits and young shoots of *C. edulis* are used as a vegetable by local Indian residents. *Caralluma tuberculata* grows wild, as well as being cultivated in Pakistan.[7] The *C. tuberculata* stem is described as juicy, and it can be eaten cooked with ground meat, and the root can be consumed either cooked or raw as a vegetable.[8,9] *Caralluma tuberculata* is used in the ethnomedical treatment of rheumatism, diabetes, leprosy, paralysis, joint pains, and fever.[7] *Caralluma tuberculata* is also used as a bitter tonic, febrifuge, stomachic, and carminative uses for rheumatism.[7] Studies of *Caralluma* show potential anti-inflammatory effects and antitumor activities as cited by Waheed and colleagues.[10] The potential effect of the *Caralluma* genus having antitumor and anticancer activities could be related to the chemical compounds found in the plant, which are pregnane glycosides.[11]

### 3.3.2.1 CHEMICAL COMPOUNDS OF *CARALLUMA TUBERCULATA*

The cytotoxic effect of the *C. tuberculata* extract is related to a number of pregnane glycosides that show potential activity against the MRC5 human diploid embryonic cell line.[11] The *Caralluma* genus and other members of the Asclepiadaceae family contain high levels of esterified polyhydroxypregnane glycosides. These are likely responsible for the anticancer and antitumor effects *in vitro*, and they could give interesting hints toward developing new anticancer or antitumor drugs.[11,12] The challenge is to isolate and identify the flavonoid components from *C. tuberculata*, as flavonoids are considered important natural products.

The ethanol extract from fresh whole plants of *C. tuberculata* was studied to identify the chemical compounds. By using the NMR spectrum, the extract shows three flavonoid compounds. The first flavonoid compound has the chemical formula $C_{27}H_{30}O_{15}$ and is identified as luteolin-4′-*O*-β-D-glucopyranosyl-(2→1)-α-L-rhamnopyranoside. The data reported on this compound were based on the method of Mabry *et al.*, Harborne *et al.*,[13] and Nakano *et al.* The extract of *C. tuberculata* was also tested by TLC monitoring. The second identified flavonoid is kaempferol-7-*O*-β-D-glucopyranosyl-(2→1)-α-L-rhamnopyranoside (molecular formula $C_{27}H_{30}O_{15}$). The third compound identified in this study is kaempferol-3-*O*-β-D-glucopyranosyl-(6→1)-α-L-rhamnopyranoside. The third compound has the chemical formula $C_{27}H_{30}O_{15}$ (Figure 11.3). All three compounds were also tested on high performance liquid chromatography (HPLC) plates for their purification.

Waheed *et al.*[10] also studied the chemical compounds of *C. tuberculata*. The dried parts of fresh *C. tuberculata* were used for ethanol extraction. Thin layer chromatography was used to show the quantity of the compounds. Two compounds evident on the TLC were subsequently analyzed using the Liebermann-Burchard test as reported by the methods of Burke *et al.*[14] and Halim and Khalil.[15] To identify the chemical compounds MS, GC-MS, IR, and NMR methodologies were used. The first compound identified was an acylated pregnane glycoside, with the chemical formula $C_{45}H_{72}O_{20}$ (i.e., 12-*O* benzoyl-20-*O*-acetyl-3β,12β,14β,20β-tetrahydroxy-(20*S*)-pregnan-3-yl-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-3-methoxy-β-D-ribopyranoside). The second compound is acylated androstan glycoside, with the molecular formula $C_{65}H_{106}O_{26}$ (i.e., 7-*O*-acetyl-12-*O*-benzoyl-3β,7β,12β,14β-tetrahydroxy-17β-(3-methylbutyl-*O*-acetyl-1-yl)-androstan-3-yl-*O*-β-D-glucopyranosyl-(1→4)-6-deoxy-β-D-allopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymapyranosyl-(1→4)-β-D-cymaropyranoside).[10]

Abdel-Sattar *et al.*[5] studied dried aerial parts of *C. tuberculata* extracted with cold methanol using an ultra turrax homogenizer. Six bioactive compounds were isolated and chemically identified using NMR spectra and HPLC. Some of these compounds contained steroidal skeletons and 2-deoxy sugar moieties in their structures. For example, compound five has the molecular formula $C_{62}H_{88}O_{21}$ (i.e.,12,20-dibenzoyl-3β,12β,14β,20β-tetrahydroxy-(20*S*)-pregn-5-ene-β-D-glucopyranosyl-(1→4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside). Compound six is $C_{57}H_{86}O_{21}$ (i.e., 12-*O*-benzoyl-20-*O* acetyl-3β,12β,14β,20β-tetrahydroxy-(20*S*)-pregn-5-ene-β-D-glucopyranosyl-(1→4)-6-deoxy-

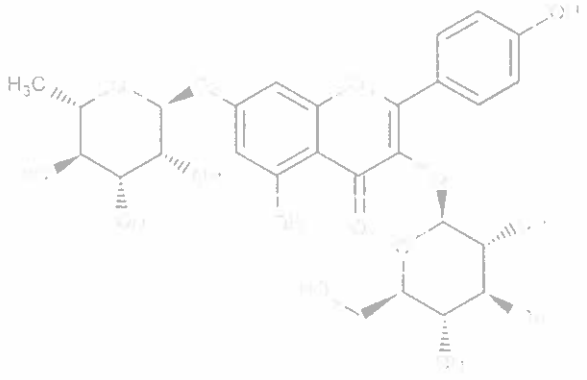



FIGURE 11.3 Kaempferol-3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside: polyphenol compound in *Caralluma tuberculata*. *Figure courtesy of European Bioinformatics Institute ChEBI Database.*


Nabavi, S. M., Habtemariam, S., Ahmed, T., & Sureda, A. (Eds.). (2017). Polyphenols in human health and disease. Retrieved from http://www.sciencedirect.com/science/article/pii/B9780128138427000023

3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside).[96,106]

Ahmad *et al.*[91] studied *C. tuberculata* ethanol extract. Two pregnane glycosides, named caratubenides A and B, were isolated from the whole plant of *C. tuberculata*. Their structures were determined as 3-O-[β-D-glucopyranosyl-(1→4)-β-D-(3-O-methyl-6-desoxy)-galactopyranosyl] -14-hydroxy-14β-pregnane-20-one [I] and 3-O-[β-D-glucopyranosyl-(1→4)-β-D-(3-O-methyl-6-desoxygalactopyranosyl)]-14,20-dihydroxy-14β-pregnane [2] mainly on the basis of spectroscopic studies. The structure of the second sugar was determined as 6-deoxy-3-O-methyl-β-D-galactose on the basis of extensive NMR studies including $^{1}$H NMR (COSY-45-resolved, NOESY, NOE difference) and $^{13}$C-NMR (broad band and DEPT experiments).[91]

#### 3.3.2.2 *CARALLUMA TUBERCULATA* AND ANTI-CANCER EFFECTS *IN VITRO*

As previously discussed, Abdel-Sattar and colleagues[96] studied dried aerial parts of *C. tuberculata* extracted with cold methanol using an ultra turrax homogenizer. Six bioactive compounds were isolated and chemically identified using NMR spectra and HPLC. The bioactivity of these compounds was tested for cytotoxicity against MRC5 human cell lines. Results showed high cytotoxicities of compounds five and six; both were >100 μg/mL followed by compounds three, one, four, and two (64.11 μg/mL, 22.38 μg/mL, 19.20 μg/mL, and 17.75 μg/mL, respectively).

Pregnane glycosides are intoxicating cytotoxic agents that may excel as antitumor drugs. Waheed *et al.*,[95] studied these particularly against breast cancer *in vitro*, because of their structural similarity to estrogenic agonists. Pregnane glycosides are widely found in *Caralluma*. The aim of this study was to test the bioactive components from *C. tuberculata* extract against human breast cancer cell lines. The extract of *C. tuberculata* was tested as an inhibitor of growth on three types of cancer cell lines: MCF-7 estrogen-dependent and MDA-MB-468 estrogen-independent breast cancer cells, Caco-2 human colonic cells, and HUVECs and U937 cells. Western blot of poly-(ADP ribose) polymerase (PARP) was used to detect apoptosis. It was found that the ethyl acetate fraction of *C. tuberculata* was the most potent anti-proliferative fraction against the three cancer cell lines. From *C. tuberculata* extract, two steroidal glycoside compounds were reported on TLC. Both compounds inhibited cancer cell growth and induced apoptosis at <25 μM after 48 hours. The investigators found that the two compounds (steroid glycosides) identified from the extract of *C. tuberculata* showed cytotoxic activity on different cancer cell lines including breast cancer *in vitro*. These results may lead other researchers to design new anticancer drugs,[95] and to further investigate these compounds in animal models.

#### 3.3.2.3 *CARALLUMA TUBERCULATA* CANCER-RELATED EFFECTS *IN VIVO*

In a study by Al-Bekairi *et al.*[108] a 96% ethanol extract from *C. tuberculata* was assessed in male mice (SWR, home bred) in regard to biochemical and cytological changes compared to the standard drug cyclophosphamide (CP). Cyclophosphamide is a drug used to treat cancer (e.g., lymphoma, leukemia, multiple myeloma, neuroblastoma, retinoblastoma, breast cancer).[107] The researchers treated mice with either *C. tuberculata* extract by increasing the concentration of the extract, CP alone, or *C. tuberculata* extract in combination with CP for 7 days.[108] Results showed a dose-dependent increase in micronuclei formation in erythrocytes of animals treated with *C. tuberculata* but slightly lower than the effect of CP alone. Combination treatment significantly decreased the ratio of polychromatic erythrocytes in comparison with CP only treated mice. Cytotoxicity in the mouse bone marrow cells was analogous with that of the standard drug CP. Also, results show a decrease in the amount of RNA in the liver and testes, significantly increased hepatic DNA content, and no effect in brain tissue. These results were comparable with the effects of CP on those tissues. Combination treatment was able to reduce the potential effect of CP on DNA (but not RNA) and cytotoxicity.[108] This study suggests that while *C. tuberculata* may be cytotoxic, it may also have the potential to reduce or counteract the negative effects of certain standard anticancer drugs.

Based on the natural products of *C. tuberculata*, and anticancer activities demonstrated in cell lines, further work is encouraged. Future directions in research should lead to clarification of the role of this medicinal plant in animal studies of cytotoxicity, and the potential for drug interactions.

## 3.5 Cancer Summary

Cancer is a variety of chronic disease affecting approximately 13 million people every year and causing 7.6 million annual cancer deaths worldwide. Cancer is characterized by an uncontrolled growth of abnormal cells. The number of people affected by cancer is increasing around the world. Saudi Arabia is one of these affected countries, which lead Saudis to use natural health products for cancer prevention and healing. Two Saudi plants (*Calotropis procera* and *Caralluma tuberculata*) from the Asclepiadaceae (milkweed) family show anticancer related promise in both *in vitro* and *in vivo* studies. Further animal model and clinical studies are needed to ascertain the potential

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effectiveness of these two plants and/or their compounds against cancer. Thus, the scientific study of bioactive compounds from native plants from Saudi Arabia with a history of use in traditional medicine is encouraged, specifically with a focus on their use as natural health products and on their utility as compounds in drug development for the prevention and treatment of cancer.

## 4. CONCLUSION

The Kingdom of Saudi Arabia is a nation whose flora and fauna are characterized by the desert biome in which it is situated. It is noteworthy that, while plant diversity is not great in such a harsh environment, the plants that do thrive there are likely to be rich in natural bioactive products that may be of therapeutic value to humans. Herbal remedies and derived therapeutics from Saudi plants continue to interest health care practitioners of both traditional and allopathic medicine systems. Today, the KSA cannot escape from the reality that its citizens are not excluded from the worldwide trends of increased prevalence and incidence of DM and various cancers, major contributors of morbidity and mortality. Scientific study of many native and naturalized plants of the Arabian subcontinent is underway, to investigate the preventive and therapeutic value of these plants and their secondary metabolites. Two members of the Capparidaceae family (*Capparis spinosa* and *Capparis decidua*) have been found to have antioxidant and antidiabetic effects; and two members of the Asclepiadaceae family (*Calotropis procera* and *Caralluma tuberculata*) have been found to have antiproliferative and cytotoxic activities. Further study of these plants and their compounds as antidiabetic and anticancer agents, respectively, is warranted.

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# Flavonoids

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## Summary


- Flavonoids are a large family of polyphenolic plant compounds. Six major subclasses of flavonoids, namely anthocyanidins, flavan-3-ols, flavonols, flavanones, flavones, and isoflavones, flavonols are the most widespread in the human diet. *(More information)*
- Dietary flavonoids are naturally occurring in fruit, vegetables, chocolate, and beverages like wine and tea. There has been much interest in the potential health benefits of flavonoids associated with fruit- and vegetable-rich diets. *(More information)*
- The physicochemical properties of flavonoids influence their metabolic fate, i.e., their digestion, absorption, and biotransformation. The bioavailability of these polyphenols *in vivo* is a major determinant in their ability to exert biological activities relevant to human health. *(More information)*
- Many of the biological effects of flavonoids appear to be related to their ability to modulate a number of cell-signaling cascades. Flavonoids have been shown to exhibit antiinflammatory, antithrombogenic, antidiabetic, anticancer, and neuroprotective activities through different mechanisms of action *in vitro* and in animal models. *(More information)*
- Accumulating evidence from randomized controlled trials suggests that consumption of flavan-3-ols and

› Safety
  › Adverse effects
  › Pregnancy and lactation
  › Drug interactions
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anthocyanidins can be beneficial for metabolic and cardiovascular health. *(More information)*

› The results of small-scale randomized controlled trials suggest that consumption of flavonoid-rich food and beverages containing anthocyanins or flavan-3-ols may improve vascular endothelial function. As yet, it is not known whether these acute improvements result in long-term reductions in risk of cardiovascular disease. *(More information)*

› Promising findings in randomized controlled studies indicate that supplementation with flavan-3-ols or anthocyanidins may improve glycemic control in subjects at-risk or diagnosed with type 2 diabetes mellitus. *(More information)*

› Despite promising results in animal studies, only a limited number of observational studies have reported potential cancer preventive effects of flavonoids in humans. Higher intakes of soy isoflavones may be associated with reduced risks of breast cancer in postmenopausal women and prostate cancer in men. *(More information)*

› Evidence suggesting that some flavonoids or flavonoid-rich foods may enhance cognitive function is currently limited, and it is not yet known whether their consumption could lower the risk of cognitive impairments and dementia in humans. *(More information)*

› High intakes of dietary flavonoids are generally regarded as safe, especially because of their low bioavailability. However, flavonoid supplements may affect the action of anticoagulants and increase the toxicity of a wide range of drugs when taken concurrently. *(More information)*

## Introduction

Flavonoids are a large family of over 5,000 hydroxylated polyphenolic compounds that carry out important functions in plants, including attracting pollinating insects; combating environmental stresses, such as microbial infection; and regulating cell growth (1). Their bioavailability and biological activities in humans appear to be strongly influenced by their chemical nature. Since the 1990s, there has been a growing interest in dietary flavonoids due to their likely contribution to the health benefits of fruit- and vegetable-rich diets. This article reviews some of the scientific evidence regarding the role of dietary flavonoids in health promotion and disease prevention in humans; it is not meant to be a comprehensive review on every health topic studied.

## Flavonoid Subclasses

Flavonoids are classified into 12 major subclasses based on chemical structures, six of which, namely anthocyanidins, flavan-3-ols, flavonols, flavones, flavanones, and isoflavones (**Table 1** and **Figures 1-9**) are of dietary significance. Glycosylated flavonols (bound to at least one sugar molecule) are the most widely distributed flavonoids in the diet (2, 3).

Table 1. Common Dietary Flavonoids
(Select the highlighted text to see chemical structures.)

| Flavonoid Subclass | Dietary Flavonoids (aglycones) | Some Common Food Sources (see also Sources) |
|---|---|---|
| Anthocyanidins* | Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin | Red, blue, and purple berries; red and purple grapes; red wine |
| Flavan-3-ols | *Monomers (Catechins)*: (+)-Catechin, (-)-Epicatechin, (-)-Epigallocatechin, (+)-Gallocatechin; and their gallate derivatives | Teas (particularly white, green, and oolong), cocoa-based products, grapes, berries, apples |
| | *Dimers and Polymers*: Proanthocyanidins# | Apples, berries, cocoa-based products, red grapes, red wine |
| | Theaflavins, Thearubigins | Black tea |
| Flavonols | Isorhamnetin, Kaempferol, Myricetin, Quercetin | Onions, scallions, kale, broccoli, apples, berries, teas |
| Flavones | Apigenin, Luteolin, Baicalein, Chrysin | Parsley, thyme, celery, hot peppers |
| Flavanones | Eriodictyol, Hesperetin, Naringenin | Citrus fruit and juices, e.g., oranges, grapefruits, lemons |
| Isoflavones | Daidzein, Genistein, Glycitein, Biochanin A, Formononetin | Soybeans, soy foods, legumes |

*Anthocyanidins with one or more sugar moieties (anthocyanidin glycosides) are called anthocyanins.
#Proanthocyanidin oligomers formed from (+)-catechin and (-)-epicatechin subunits are called procyanidins.

For more detailed information on the health effects of isoflavones, a subclass of flavonoids with estrogenic activity, see the article on Soy Isoflavones.

For more information on the health benefits of foods that are rich in flavonoids, see the articles on Fruit and Vegetables, Legumes, and Tea.

Figure 1. Basic Structures of Flavonoid Subclasses

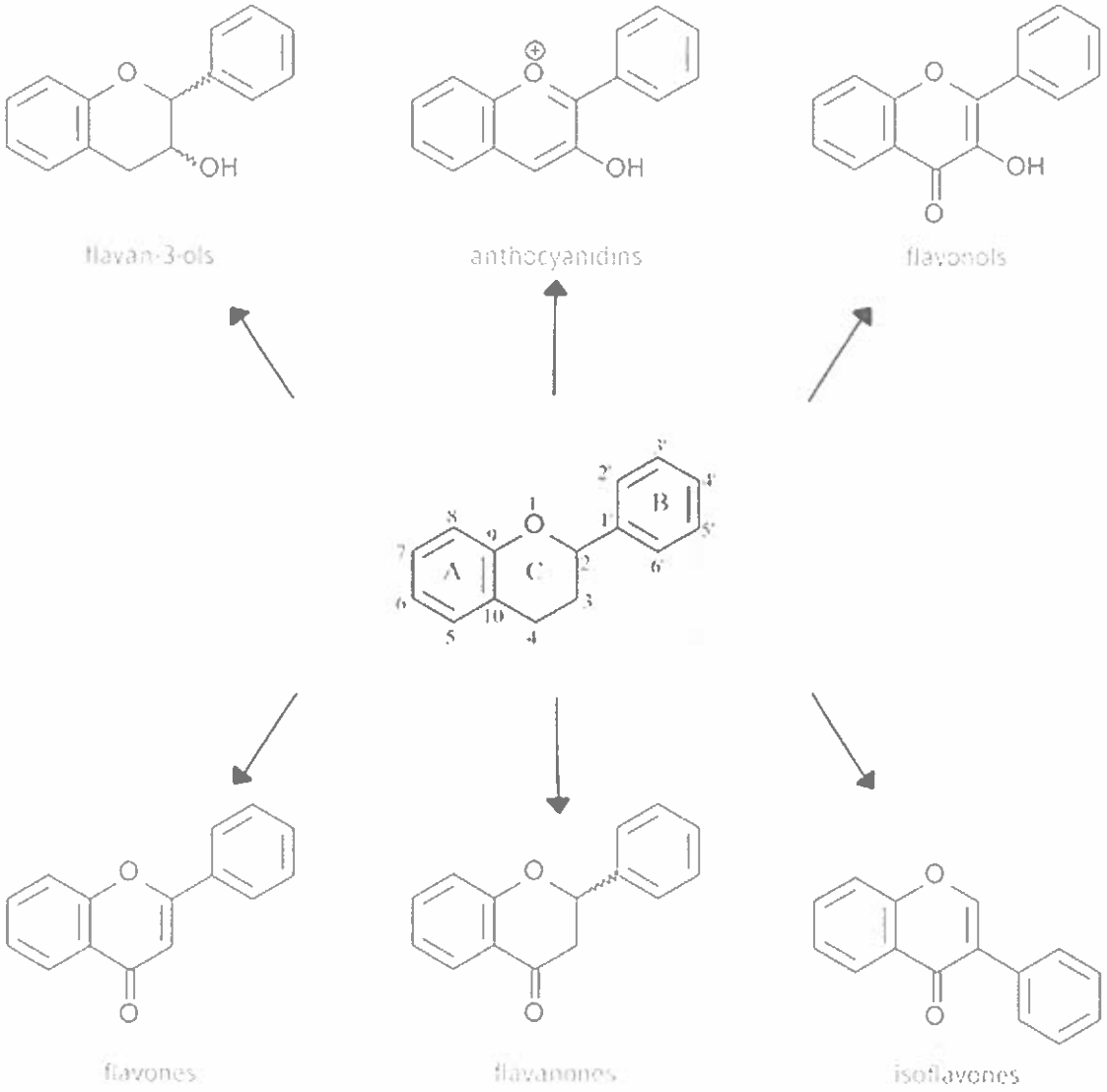
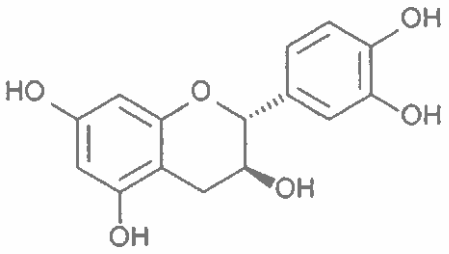
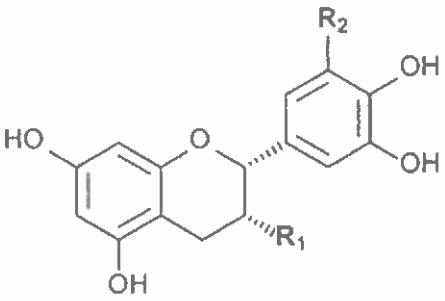


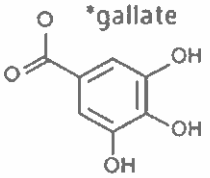
Figure 2. Chemical Structures of Some Flavan-3-ol Monomers (Catechins)

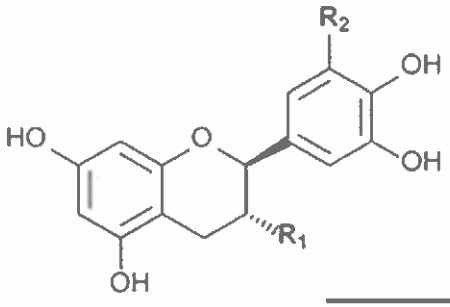


(+)-catechin

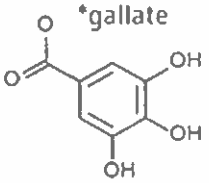


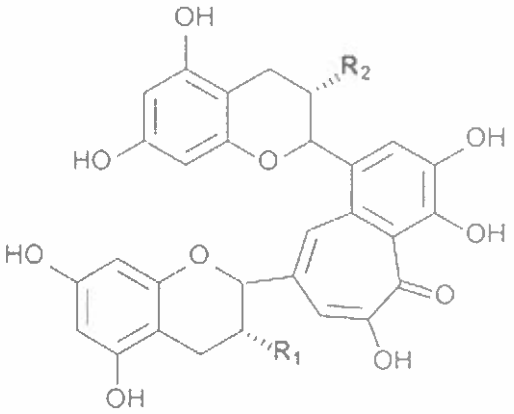
| | $R_1$ | $R_2$ |
|---|---|---|
| (-)-epicatechin | OH | H |
| (-)-epigallocatechin | OH | OH |
| (-)-epicatechin gallate | *gallate | H |
| (-)-epigallocatechin gallate | *gallate | OH |



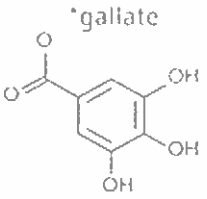



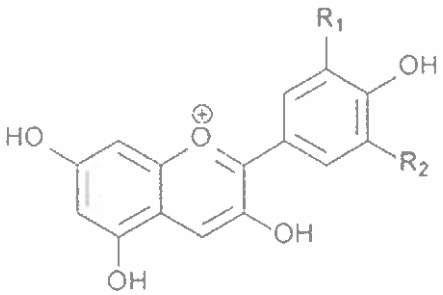
| | $R_1$ | $R_2$ |
|---|---|---|
| (+)-gallocatechin | OH | OH |
| (+)-catechin gallate | *gallate | H |
| (+)-gallocatechin gallate | *gallate | OH |



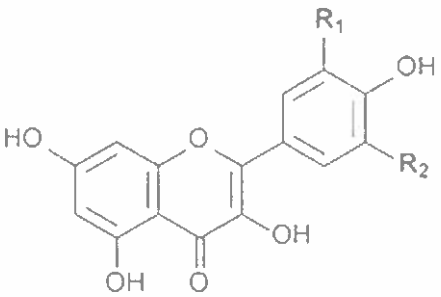



| | $R_1$ | $R_2$ |
|---|---|---|
| Theaflavin | OH | OH |
| Theaflavin-3-gallate | *gallate | OH |
| Theaflavin-3'-gallate | OH | *gallate |
| Theaflavin-3,3'-digallate | *gallate | *gallate |



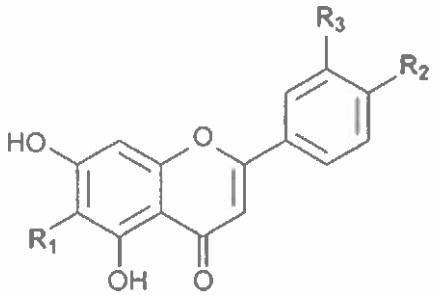




| | $R_1$ | $R_2$ |
|---|---|---|
| Cyanidin | OH | H |
| Delphinidin | OH | OH |
| Malvidin | $OCH_3$ | $OCH_3$ |
| Pelargonidin | H | H |
| Peonidin | $OCH_3$ | H |
| Petunidin | $OCH_3$ | OH |



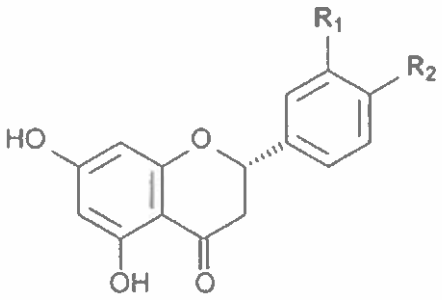

| | $R_1$ | $R_2$ |
|---|---|---|
| Isorhamnetin | $OCH_3$ | H |
| Kaempferol | H | H |
| Myricetin | OH | OH |
| Quercetin | OH | H |

Figure 6. Chemical Structures of Flavones



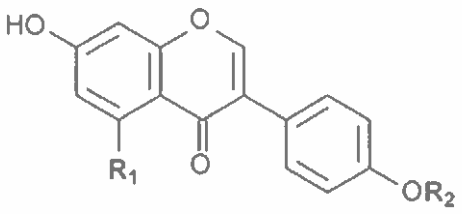

| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Apigenin | H | OH | H |
| Luteolin | H | OH | OH |
| Baicalein | OH | H | H |
| Chrysin | H | H | H |

Figure 7. Chemical Structures of Flavanones



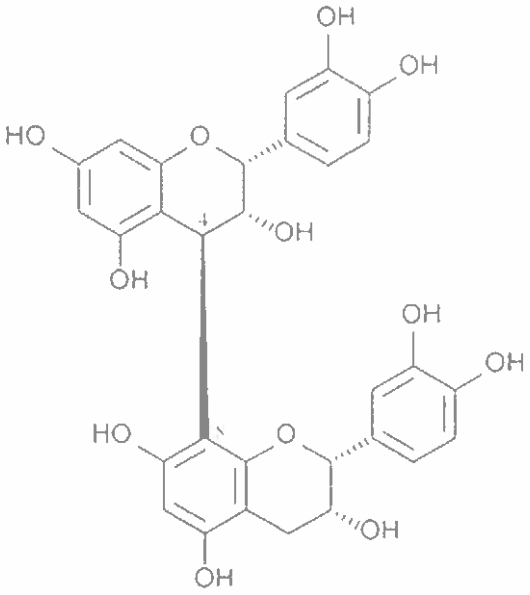

| | $R_1$ | $R_2$ |
|---|---|---|
| Eriodictyol | OH | OH |
| Hesperetin | OH | $OCH_3$ |
| Naringenin | H | OH |

Figure 8. Chemical Structures of Isoflavones

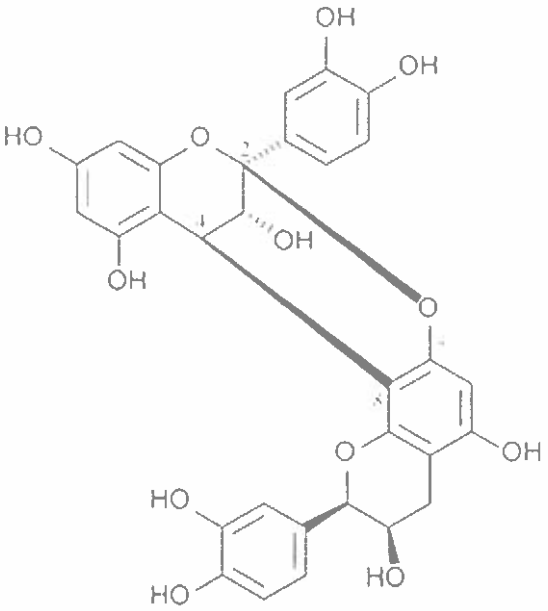



| | $R_1$ | $R_2$ |
|---|---|---|
| Daidzein | H | H |
| Genistein | OH | H |
| Glycitein | $OCH_3$ | H |
| Biochanin A | OH | $CH_3$ |
| Formononetin | H | $CH_3$ |

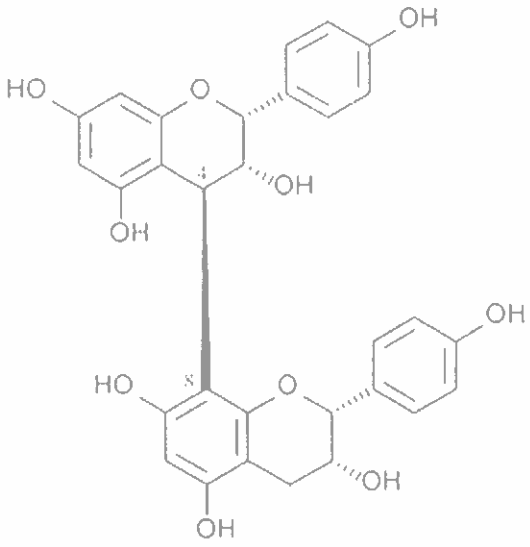
Figure 9. Chemical Structures of Proanthocyanidin Dimers.



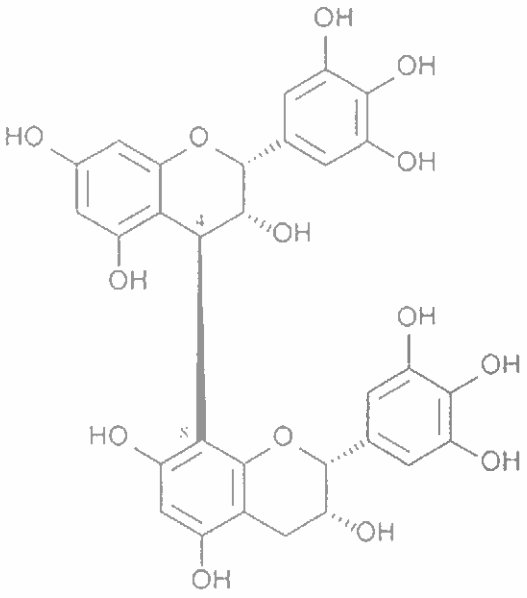
proanthocyanidin B2 dimer



proanthocyanidin A2 dimer



propelargonidin B-type dimer



prodelphinidin B-type dimer

## Metabolism and Bioavailability

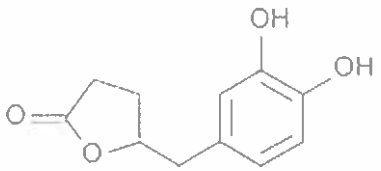
The amount of flavonoids present in ingested food has little importance unless dietary flavonoids are absorbed and become available to target tissues within the body. During and after intestinal absorption, flavonoids are rapidly and extensively metabolized in intestinal and liver cells such that they are likely to appear as metabolites (e.g., phase II metabolites) in the bloodstream and urine (4). Additionally, the biological activities of flavonoid metabolites are likely to be different from those of their parent compounds (5). Some of the factors influencing the metabolic fate and bioavailability of dietary flavonoids are mentioned below.

## Chemical structure of flavonoids

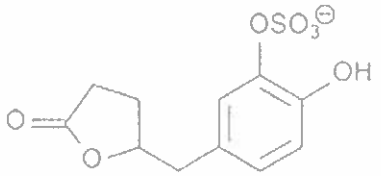
Most flavonoids occur in edible plants and foods as β-glycosides, i.e., bound to one or more sugar molecules (6). Exceptions include flavan-3-ols (catechins and proanthocyanidins) and fermented soy-based products that are exposed to microbial β-glucosidases, which catalyze the release of sugar molecules from glycosylated isoflavones (7). Even after food processing and cooking, most flavonoid glycosides reach the small intestine intact. Only flavonoid aglycones (not bound to a sugar molecule) and a few flavonoid glucosides (bound to glucose) are easily absorbed in the small intestine (8). Glycosylated flavonoids might be able to penetrate the mucus layer of the intestine and be deglycosylated on the cell surface before absorption. Those that cannot be deglycosylated in the small intestine may be hydrolyzed by bacterial enzymes in the colon (7). Nevertheless, colonic bacteria might remove sugar moieties and rapidly degrade aglycone flavonoids, thus limiting their absorption in the colon (9).

In contrast to monomeric flavan-3-ols (catechins), the polymeric nature of proanthocyanidins likely prevents their intestinal absorption. Flavan-3-ol monomers and procyanidins are transformed by the intestinal microbiota to 5-(hydroxyphenyl)-γ-valerolactones which appear in the circulatory system and are excreted in urine as sulfate and glucuronide metabolites (**Figure 10**). Valerolactones may be further degraded by the colonic microbiota to smaller phenolic acids and aromatic compounds. The colonic microbiota also metabolize the gallate esters of flavonoids, generating gallate, which is further catabolized to pyrogallol. Microbe-derived flavonoid metabolites are readily absorbed into the circulatory system and excreted in both free forms and as phase II metabolites in urine (9).

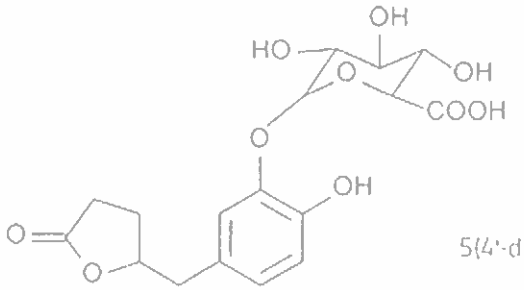
Figure 10. Chemical Structures of Some Flavan-3-ol Metabolites




5(3',4'-dihydroxyphenyl)-γ-valerolactone




5(4'-dihydroxyphenyl)-γ-valerolactone-3'-sulfate




5(4'-dihydroxyphenyl)-γ-valerolactone-3'-O-glucuronide

## Interactions with food matrix

The presence of macronutrients in food influences the bioavailability of co-ingested flavonoids (reviewed in 8, 10, 11). The binding affinity and potential (non-) covalent interactions of flavonoids with food proteins, carbohydrates, and fats are directly associated with the physicochemical properties of flavonoids (reviewed in 8). Proteins in milk might reduce the absorption of polyphenols from cocoa or black tea. The presence of milk proteins bound to flavonoids was shown to weaken the flavonoid antioxidant capacity *in vitro* (12), and milk consumption has been shown to blunt the vascular benefits of tea flavonoids in healthy volunteers (13). Some carbohydrate-rich foods may increase the deglycosylation and absorption of flavonoids by stimulating gastrointestinal motility, mucosal blood flow, and colonic fermentation. Conversely, dietary flavonoids have been shown to interfere with carbohydrate digestion and absorption (see Biological Activities).

## Composition of gut microbiota

In the large intestine, gut microbial enzymes transform flavonoids through deglycosylation, ring fission, dehydroxylation, demethylation, etc. into metabolites that can then be absorbed or excreted (9, 14). The diversity and activity of colonic bacteria, which are partly dependent on a

person's dietary habits, will determine which metabolites can be produced from ingested flavonoids (15, 16). The composition of the colonic microbiota can therefore affect the metabolic fate and bioavailability of dietary flavonoids (17).

## The detoxification pathway

Flavonoids are recognized as xenobiotics by the body such that they undergo extensive modifications first in the intestinal mucosa and then in the liver.

### *Phase II enzymes*

Depending on their structural characteristics, flavonoids can be rapidly transformed by phase II detoxification enzymes to form methylated, glucuronidated, and/or sulfated metabolites (2). This metabolic pathway increases the solubility of phenolic aglycones and facilitates their excretion in the bile and urine (11). Free (unconjugated) aglycones are generally absent from the bloodstream, with the possible exception of trace levels of catechins (17). Catechol-*O*-methyltransferase (COMT) is the detoxifying enzyme responsible for the methylation of the hydroxyl groups of flavonoids, producing *O*-methylated flavonoids. A single nucleotide polymorphism (SNP) in the gene for COMT — known as SNP rs4680 G>A — causes a valine-to-methionine substitution in the sequence of the enzyme. Individuals with the A/A genotype have a form of the enzyme that is three- to four-fold less active than the wild-type variant in G/G genotype carriers (18). It has been suggested that subjects who are less efficient at eliminating green tea flavonoids may be more likely to benefit from their consumption (19).

### *Efflux transporters*

Flavonoid conjugates are excreted via the action of efflux transporters from the ATP-binding cassette (ABC) family, including P-glycoprotein, MRPs (multidrug resistance proteins), or BCRPs (breast cancer-resistant proteins). Depending on their physicochemical properties, some flavonoids may interfere with the activity of ABC transporters (20). This implies that flavonoids can affect their own bioavailability, as well as that of other substrates of these transporters (e.g., pharmacological drugs) (see Drug interactions).

## Binding to plasma proteins

Flavonoid bioavailability may be inversely related to their binding affinity to plasma proteins (21). Greater binding affinity to plasma proteins (and thus, possibly, lower flavonoid bioavailability) has been linked to structural characteristics, such as methylation and galloylation. On the contrary, glycosylation reduced binding affinity to plasma proteins, suggesting that aglycones might have a limited bioavailability compared to glycosylated flavonoids. While glucuronidation is thought to facilitate the excretion of flavonoids from the body, glucuronides show little affinity to plasma proteins and might thus be able to diffuse to target tissues where deglucuronidation can take place (8).

## Summary

In general, the bioavailability of flavonoids is low due to limited absorption, extensive metabolism, and rapid excretion. Isoflavones are thought to be the most bioavailable of all flavonoid subclasses, while anthocyanins and galloylated catechins are very poorly absorbed (8, 22). Yet, given the wide variability in structures within subclasses, it is difficult to generalize the absorbability and bioavailability of flavonoids based only on their structural classification. In addition, when evaluating the data from flavonoid research in cultured cells, it is important to consider whether the flavonoid concentrations and metabolites used are physiologically relevant (23). In humans, peak plasma concentrations of soy isoflavones and citrus flavanones have not been found to exceed 10 micromoles/liter (μM) after oral consumption. Peak plasma concentrations measured after the consumption of anthocyanins, flavan-3-ols, and flavonols (including those from tea) are generally lower than 1 μM (2). A recent quantitative analysis of 88 polyphenolic metabolites (not limited to flavonoids) identified in human blood and urine found median peak concentrations of 0.9 μM and 3.2 μM after food intake and oral supplementation, respectively (4).

## Biological Activities

### Direct antioxidant activity

Flavonoids are effective scavengers of free radicals in the test tube (*in vitro*) (24, 25). However, even with very high flavonoid intakes, plasma and intracellular flavonoid concentrations in humans are likely to be 100 to 1,000 times lower than concentrations of other antioxidants, such as ascorbate (vitamin C), uric acid, and glutathione. Moreover, most circulating flavonoids are actually flavonoid metabolites, some of which have lower antioxidant activity than the parent flavonoid (5). For these reasons, the relative contribution of dietary flavonoids to plasma and tissue antioxidant function *in vivo* is likely to be very small or negligible (26-28).

### Metal chelation

Metal ions, such as iron and copper, can catalyze the production of free radicals. The ability of flavonoids to chelate (bind) metal ions appears to contribute to their antioxidant activity *in vitro* (29, 30). In living organisms, most iron and copper are bound to proteins, limiting their participation in reactions that produce free radicals. Although the metal-chelating activities of flavonoids may be beneficial in pathological conditions of iron or copper excess, it is not known whether flavonoids or their metabolites function as effective metal chelators *in vivo* (26).

### Effects on cell-signaling pathways

Cells are capable of responding to a variety of different stresses or signals by increasing or decreasing the availability of specific proteins. The complex cascades of events that lead to changes in the expression of specific genes are known as cell-signaling pathways or signal transduction pathways. These pathways regulate numerous cell processes, such as proliferation, differentiation, inflammatory responses, apoptosis (programmed cell death), and survival. Although it was initially hypothesized that the biological effects of flavonoids would be related to their antioxidant activity, available evidence from cell culture experiments suggests that many of the effects of flavonoids,

including antiinflammatory, antidiabetic, anticancer, and neuroprotective activities, are related to their ability to modulate cell-signaling pathways (27). Intracellular concentrations of flavonoids required to affect cellular signaling are considerably lower than those required to affect cellular antioxidant capacity. Flavonoid metabolites may retain their ability to interact with cell-signaling proteins even if their antioxidant activity is diminished (31, 32).

Effective signal transduction requires proteins known as kinases that catalyze the phosphorylation of target proteins, which become either activated or inhibited. Cascades involving specific phosphorylations or dephosphorylations of signal transduction proteins ultimately affect the activity of transcription factors — proteins that bind to specific response elements on DNA and promote or prevent the transcription of target genes. Results of numerous studies in cell culture suggest that flavonoids may affect chronic disease by selectively inhibiting kinases (27, 33). Cell growth and proliferation are also regulated by growth factors that initiate cell-signaling cascades by binding to specific receptors in cell membranes. Flavonoids may alter growth factor signaling by inhibiting receptor phosphorylation or blocking receptor binding by growth factors (34).

Each flavonoid subclass contains many types of chemicals with varying biological activities (and potential health benefits) such that the activity of a specific flavonoid cannot easily be generalized. Some examples of major biological activities of flavonoids are highlighted below.

## *Biological activities related to the prevention of cardiovascular disease*

Flavonoids have been shown to (1) reduce inflammation by suppressing the expression of pro-inflammatory mediators (35-37); (2) down-regulate the expression of vascular cell adhesion molecules, which contribute to the recruitment of inflammatory white blood cells from the blood to the arterial wall (38, 39); (3) increase the production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS), thus improving vascular endothelial function (40); (4) inhibit angiotensin-converting enzyme, thus inducing vascular relaxation (41); (5) inhibit platelet aggregation (42); and (6) oppose smooth muscle cell proliferation and migration occurring during atherogenesis (43).

## *Biological activities related to the prevention of diabetes*

Flavonoids have been found to interfere with the digestion, absorption, and metabolism of carbohydrates (reviewed in 44). Each subclass of flavonoids has also demonstrated anti-diabetic properties, including (1) improving insulin secretion and viability of pancreatic β-cells under glucotoxic or pro-inflammatory conditions, (2) increasing insulin-stimulated glucose uptake by target cells, (3) protecting muscle cells against fatty acid-induced insulin resistance, and (4) reducing hyperglycemia and improving glucose tolerance in animal models of obesity and/or type 2 diabetes mellitus (45).

## *Biological activities related to the prevention of cancer*

Flavonoids have been found to (1) scavenge free radicals that can damage macromolecules, including DNA (46, 47); (2) interfere with biotransformation enzymes and efflux transporters, possibly preventing the activation of procarcinogenic chemicals and promoting their excretion from the body (48, 49); (3) regulate proliferation, DNA repair, or activation of pathways leading to apoptosis (programmed cell death) in case of irreversible DNA damage (50); and (4) inhibit tumor invasion and angiogenesis (51, 52).

### *Biological activities related to neuroprotection and cognitive function*

Flavonoids are thought to (1) promote neurogenesis, synaptic growth, and neuron survival in the learning and memory-related brain regions (e.g., hippocampus) by stimulating the production of neurotrophins like BDNF; (2) protect hippocampal cells and striatal dopaminergic cells from cytotoxic molecules (pro-inflammatory mediators and ROS) released by abnormally activated microglia and hypertrophic astrocytes in neurodegenerative disorders; (3) reduce neuroinflammation by inhibiting the generation of pro-inflammatory cytokines, lipid mediators, and reactive oxygen species by astrocytes and microglial cells; (4) stimulate the production of nitric oxide (NO), which improves endothelial function, increases cerebral blood flow, and protects artery walls against the buildup of atherosclerotic plaques (reviewed in 53, 54).

## Disease Prevention

### Cardiovascular disease

Several prospective cohort studies conducted in the US and Europe have examined the relationship between some measure of dietary flavonoid intake and cardiovascular disease (CVD) or mortality. A recent meta-analysis of 14 prospective studies published between 1996 and 2012 reported that higher intakes in each flavonoid subclass were significantly associated with a reduced risk of cardiovascular events (55). Top versus bottom quantiles of intake for each of the flavonoid subclasses were associated with an approximate 10% reduction in the risk of CVD. Another meta-analysis of eight prospective studies found a 14% reduced risk of stroke with the highest versus lowest quintile of flavonol intakes (56). However, several serious limitations highlighted in a recent publication by Jacques et al. suggested caution when interpreting these results (57). In particular, most of the prospective studies in these meta-analyses did not include all flavonoid subclasses nor calculate intakes using the latest and more complete versions of the USDA databases for the flavonoid content of foods (58-60). Another major concern is the lack of adjustment regarding the overall quality of the diet. Consumers with higher flavonoid intakes are likely to have a greater consumption of fruit and vegetables and overall healthier diets than those with poor flavonoid intakes. Additionally, none of the studies excluded potential bias due to constituents of flavonoid-rich foods that are known to either lower (e.g., other phytochemicals, vitamins, dietary fiber) or increase (e.g., sodium, saturated fat) the risk of cardiovascular events (discussed in 57).

In the Framingham Offspring Cohort study that followed 2,880 adults for a mean of 14.9 years, consumption of all flavonoid subclasses except flavones and flavanones was inversely associated with CVD (57). Yet, adjusting for confounding factors, including fruit and vegetable intake and overall diet quality, attenuated these relationships such that they were no longer statistically significant. An analysis of a larger prospective study of the EPIC-Norfolk cohort (24,885 participants) that considered confounding by many dietary factors (vitamin C, dietary fiber, fat, saturated fat, potassium, sodium, and alcohol) found no significant association between flavan-3-ol intake and CVD-related or all-cause mortality (61).

A number of large prospective studies and small-scale, randomized controlled trials have investigated the effects of flavonoids on established biomarkers of CVD, including those involved in oxidative stress, inflammation, abnormal blood lipid profile, endothelial dysfunction, and hypertension; some of these studies are highlighted below.

### *Biochemical markers of cardiovascular disease*

In a cross-sectional analysis of the Framingham Offspring Cohort study, the highest versus lowest intake of anthocyanins (≤3.5 mg/day versus ≥23.5 mg/day) was associated with lower concentrations of acute-phase reactant proteins (-100%), pro-inflammatory cytokines (-75%), and markers of oxidative stress (-52%), even after adjustment for confounding variables (62). Interestingly, a food-based analysis revealed that intakes of foods rich in anthocyanins, e.g., apples, red wine, and strawberries, were also inversely associated with an overall inflammation score based on 12 different biomarkers. Higher intakes of polymeric flavan-3-ols (i.e., theaflavins, thearubigins, and proanthocyanidins) were correlated with lower concentrations of pro-inflammatory cytokines and biomarkers of oxidative stress. Intake levels of total flavonoids and flavan-3-ol monomers (i.e., catechins) were inversely associated with concentrations of the biomarkers of oxidative stress. Although tea is a major source of flavan-3-ols, tea consumption was not correlated with the composite inflammation score or any components of this score in this study (62).

Cocoa is another source of flavan-3-ols, in particular (-)-epicatechin and procyanidins, that may provide cardiovascular benefits (63). Indeed, a recent randomized, double-blind, placebo-controlled study in 100 healthy adults (ages, 35-60 years) suggested that short-term benefits of cocoa flavan-3-ol consumption on cardiovascular health, including improvements in lipoprotein profile (i.e., higher HDL-cholesterol and lower total and LDL-cholesterol) and blood pressure, could be extrapolated to predict a 20%-30% reduced 10-year risk of CVD and CVD-related mortality (64).

An increasing number of trials in which participants were fed with berries (65-67) or juices (68) rich in anthocyanins or with purified anthocyanins (69) also reported reduced levels of inflammatory markers and/or improved antioxidant status, decreased LDL-cholesterol, improved insulin sensitivity, and lowered blood pressure (reviewed in 70). In a randomized, double-blind, placebo-controlled study in 150 individuals with hypercholesterolemia, supplementation with a purified anthocyanin mixture (320 mg/day) for 24 weeks reduced circulating markers of inflammation,

including C-reactive protein (CRP), interleukin-1β (IL-1β), and soluble vascular adhesion molecule-1 (sVCAM-1) (71). Supplementation of dyslipidemic patients for 12 or 24 weeks with a mixture of 17 anthocyanins improved cholesterol clearance via the HDL-mediated reverse cholesterol transport from extra-hepatic tissues back to the liver and lowered LDL-cholesterol compared to a placebo in two randomized controlled trials (72, 73). However, a 12-week, randomized, double-blind, placebo-controlled study in 52 healthy postmenopausal women found that daily consumption of 500 mg of elderberry anthocyanins (as cyanidin-3-glucoside) had no effect on inflammation markers, markers of vascular health, lipid profile, and glycemia; all of these measures were in normal range of concentrations at baseline (74). Whether exposure to high-dose anthocyanins could lower the risk of CVD in subjects with established CVD risk factors and/or help maintain cardiovascular health in apparently healthy individuals remains to be confirmed.

### *Endothelial dysfunction*

The vascular endothelial cells that line the inner surface of all blood vessels synthesize an enzyme, endothelial nitric oxide synthase (eNOS), whose function is essential to normal vascular physiology. Specifically, eNOS produces nitric oxide (NO), a compound that regulates vascular tone and blood flow by promoting the relaxation (vasodilation) of all types of blood vessels, including arteries (75). NO also regulates vascular homeostasis and protects the integrity of the endothelium by inhibiting vascular inflammation, leukocyte adhesion, platelet adhesion and aggregation, and proliferation of vascular smooth muscle cells (76). In the presence of cardiovascular risk factors (e.g., hypertension, hypercholesterolemia, hyperglycemia), early alterations in the structure and function of the vascular endothelium are associated with the loss of normal NO-mediated endothelium-dependent vasodilation. Endothelial dysfunction results in widespread vasoconstriction and coagulation abnormalities and is considered to be an early step in the development of atherosclerosis. Measures of brachial flow-mediated dilation (FMD), a surrogate marker of endothelial function, have been found to be inversely associated with risk of future cardiovascular events (77).

Preclinical studies have demonstrated the benefits of berry fruits, extracts, or purified anthocyanins on vascular function. Anthocyanin supplementation to diabetic mice was found to improve diabetes-induced vascular dysfunction by promoting NO-mediated endothelium-dependent vasodilation through the upregulation of adipocyte-derived adiponectin (78). Supplementation with purified anthocyanins (320 mg/day for 12 weeks) also increased serum adiponectin concentrations and improved FMD in 58 individuals with type 2 diabetes (78). In a randomized trial of 150 participants with hypercholesterolemia, supplemental anthocyanins increased FMD values by 28.4% compared to 2.2% in the placebo group (79).

Several small-scale, intervention studies have also examined the effect of flavan-3-ol-rich food and beverages, including tea, red wine, purple grape juice, cocoa, and chocolate, on endothelium-dependent vasodilation. A meta-analysis of nine intervention studies in a total of 213 participants estimated that the acute ingestion of 2 to 3 cups of tea (500 mL) — containing about 248 mg of flavonoids in green tea and 415 mg in black tea — significantly increased brachial FMD (see also the

article on Tea) (80). Another meta-analysis of 18 randomized controlled studies found that acute (2 h post-ingestion) and chronic (≤18 months) consumption of flavan-3-ol-rich cocoa beverages and chocolate bars significantly increased FMD in participants (81). A small 15-day, cross-over intervention study in hypertensive individuals with endothelial dysfunction found that 100 g/day of flavan-3-ol-rich dark chocolate, but not 90 g/day of flavan-3-ol-free white chocolate, could restore FMD values almost to normal levels (82). Also, using a similar protocol, the authors showed that dark chocolate intake blunted acute endothelial dysfunction-induced by a glucose load challenge in 12 healthy volunteers (83). Other benefits of dark chocolate consumption included reductions in arterial stiffness (measured through pulse wave analysis) and serum concentrations of markers of oxidative stress and vasoconstriction (8-isoprostaglandin F2α and endothelin-1). A randomized controlled trial in overweight and obese participants also reported that the daily consumption of a high-flavan-3-ol cocoa drink (902 mg/day of flavan-3-ols), but not that of a cocoa drink low in flavan-3-ols (38 mg/day), resulted in a sustained increase in FMD during the 12-week study (84). A more recent four-week, randomized, double-blind, cross-over, controlled study in healthy overweight or obese adults found that the consumption of 22 g/day of natural cocoa (in the form of dark chocolate bar and cocoa drink; 814 mg/day of flavan-3-ols) increased arterial diameter and blood flow and lowered peripheral arterial stiffness, but there was no change in FMD (85). Another recent clinical trial found improvements in endothelium-dependent vasodilation in response to acute consumption of one bar (40 g) of dark chocolate (containing 10.8 mg of (+)-catechins and 36 mg of (-)-epicatechins) and daily consumption of two bars (80 g) for up to four weeks in 20 individuals with chronic heart failure (86). Oral administration of pure flavan-3-ol (-)-epicatechin to healthy volunteers showed NO-dependent vasodilatory effects similar to those observed following flavan-3-ol-rich cocoa ingestion (87). Administration of (-)-epicatechin also improved acetylcholine-induced endothelial-dependent vasodilation of thoracic aorta rings from rats with salt-induced hypertension (88).

Endothelial nitric oxide production also inhibits the adhesion and aggregation of platelets, one of the first steps in atherosclerosis and blood clot formation (76). A number of clinical trials that examined the potential for high flavonoid intakes to decrease various measures of platelet function outside of the body (*ex vivo*) have reported mixed results. A recent systematic review of these intervention studies suggested that consumption of flavan-3-ol-rich cocoa and grape seed extract was generally found to improve platelet function by inhibiting platelet adhesion, activation, and aggregation (89). Interestingly, in a cross-over, controlled study, the acute consumption of a flavan-3-ol-rich cocoa beverage (897 mg of total (-)-EC and procyanidins) exhibited additive anti-platelet effects to aspirin (81 mg) in healthy volunteers (90). In contrast, the results of interventions using apigenin-rich soup, quercetin-rich supplements or onion soups, isoflavone-rich soy protein isolates, black tea, wines, berries, or grape juices have given inconsistent results (reviewed in 89).

### *Hypertension*

A meta-analysis of 20 short-term, randomized controlled trials, including a total of 856 mainly healthy participants, found that consumption of flavan-3-ol-rich dark chocolate and cocoa products significantly reduced systolic blood pressure by 2.77 mm Hg and diastolic blood pressure by 2.20 mm Hg. However, heterogeneity across studies was high, and risk of bias was significant (91). A greater blood pressure-reducing effect was observed in a subanalysis of studies using flavan-3-ol-free rather than flavan-3-ol-low control groups (91). Another meta-analysis of 22 trials (highly heterogeneous) found reductions in diastolic blood pressure (-1.60 mm Hg) and mean arterial pressure (-1.64 mm Hg) with chocolate or cocoa intake but no change in systolic blood pressure (81). Additionally, green tea flavan-3-ols have been shown to lower blood pressure especially in (pre-) hypertensive subjects. A pooled analysis of 13 randomized controlled trials in 1,040 subjects found a 2.05 mm Hg reduction in systolic blood pressure and a 1.71 mm Hg reduction in diastolic blood pressure with green tea consumption for at least three weeks (92). The inhibition of angiotensin-converting enzyme (ACE), a key regulator of arterial blood pressure, may partly explain how flavan-3-ol-rich food and beverages might exert blood pressure-lowering effects (93).

Some intervention trials have also examined the effect of the flavonol quercetin on blood pressure in human subjects. In a randomized, double-blind, cross-over, placebo-controlled trial in 96 participants diagnosed with metabolic disorders, supplementation with 150 mg/day of quercetin aglycone significantly reduced systolic blood pressure by 2.6 mm Hg without affecting diastolic blood pressure and other cardiometabolic markers (94). Similar results were found with 730 mg/day of quercetin in hypertensive individuals (95) and with 500 mg/day of quercetin in women with type 2 diabetes mellitus (96). In a recent six-week, cross-over, randomized, double-blind, placebo-controlled trial, daily ingestion of 162 mg of quercetin decreased 24 h-ambulatory blood pressure — but not systolic blood pressure in the resting state — in hypertensive but not in pre-hypertensive participants (97). There was no change in biomarkers of lipid metabolism, inflammation, oxidative stress, or endothelial function, including total, HDL-, LDL-cholesterol, serum CRP, soluble adhesion molecules, plasma oxidized LDL, urinary 8-isoprostaglandin F2α, serum endothelin-1, serum ACE, and plasma endogenous NOS inhibitor.

Additional trials may help establish whether the blood pressure-lowering effect of some flavonoids could be translated into long-term benefits for cardiovascular health.

## Type 2 diabetes mellitus

The association between flavonoid consumption and risk for type 2 diabetes mellitus has been examined in a recent European, multicenter, nested case-control study — the "EPIC-InterAct" project — that included 16,835 diabetes-free participants and 12,043 diabetics. In this study, participants in the highest quintile of total flavonoid intake (>608.1 mg/day) had a 10% lower risk of diabetes than those in the lowest quintile (<178.2 mg/day) (98). Specifically, the risk of diabetes was inversely correlated with the intake of flavan-3-ols (monomers and dimers only) and flavonols (98, 99). Recent meta-analyses of randomized controlled trials have examined the possible health effects of green tea flavan-3-ol monomers (catechins) on glucose metabolism and have provided

conflicting results. A meta-analysis of seven trials in pre-diabetic and diabetic patients found no effect of green tea or green tea extracts on fasting plasma glucose, fasting serum insulin, or measures of glycemic control (glycated hemoglobin, HbA1c) and insulin sensitivity (HOMA-IR) (100). Conversely, another meta-analysis of 17 trials in pre-diabetic, diabetic, or overweight/obese subjects found that administration of green tea extracts for 4 to 16 weeks improved fasting plasma glucose and HbA1c level (101). The effect on fasting glucose was observed only with high doses of catechins (≥457 mg/day) and when the confounding effect of caffeine was removed. Finally, a third meta-analysis of 25 trials found that ingestion of green tea extracts for at least two weeks could lower fasting blood glucose in both the presence or absence of caffeine (102).

Dark chocolate is another good source of flavan-3-ols such that the effects of cocoa flavan-3-ols have been examined in individuals at-risk or with established type 2 diabetes. In a 15-day, cross-over, randomized controlled study, the daily consumption of 100 g of dark chocolate bars containing 110.9 mg of (-)-EC and 36.1 mg of (+)-C significantly improved measures of pancreatic β-cell function and insulin sensitivity, along with cardiometabolic markers in glucose-intolerant and hypertensive subjects (103). Daily supplementation with flavonoid-enriched chocolate containing 850 mg of flavan-3-ols and 100 mg of isoflavones for one year significantly improved insulin sensitivity and reduced a predicted risk of coronary heart disease (CHD) at 10 years in 93 postmenopausal women treated for type 2 diabetes (104).

The EPIC-InterAct study did not find any association between dietary anthocyanin intake and risk of diabetes (98, 99). Yet, a 10-fold increase in anthocyanin consumption was correlated with a 15% lower risk of diabetes in the pooled analysis of three large US prospective cohorts (120,003 participants) (105). Of note, this pooled analysis also reported a moderately higher risk of diabetes (+6%) in individuals in the highest versus lowest quintiles of flavone and flavanone intakes. Moreover, the consumption of berries, rich in anthocyanins, has been shown to trigger favorable glycemic responses in type 2 diabetics (reviewed in 70). In recent intervention studies, anthocyanins demonstrated beneficial effects on metabolic abnormalities in patients at-risk or diagnosed with diabetes. In an eight-week, randomized, double-blind, placebo-controlled trial in 38 healthy overweight and obese subjects, the consumption of 2 g/day of grape polyphenols rich in proanthocyanidins and anthocyanins prevented increases in oxidative stress and insulin resistance induced by a six-day, high-fructose challenge (106). Another six-week randomized trial in individuals with diabetes showed that daily supplementation with Cornelian cherry (*Cornus mas*) extracts containing 600 mg of anthocyanins significantly lowered serum levels of HbA1c and triglycerides and increased serum insulin concentrations (107). The administration of 320 mg/day of anthocyanins for 24 weeks also improved serum lipid and lipoprotein profile, decreased markers of oxidative stress and inflammation, elevated antioxidant capacity, and reduced insulin resistance compared to a placebo in patients with diabetes (108). Further, supplemental anthocyanins up-regulated adiponectin expression and improved nitric oxide-mediated endothelium-dependent vasodilation within 12 weeks of treatment (see also Cardiovascular disease) (78).

These promising findings warrant additional randomized controlled trials to confirm preventive and/or therapeutic benefits of (cocoa) flavan-3-ols and anthocyanins in type 2 diabetes.

## Cancer

Although various flavonoids have been found to inhibit the development of chemically-induced cancers in animal models of lung (109), oral (110), esophageal (111), gastric (112), colon (113), skin (114), prostate (115, 116), and mammary cancer (117), observational studies do not provide convincing evidence that high intakes of dietary flavonoids are associated with substantial reductions in human cancer risk (reviewed in 118). A meta-analysis of 13 case-control and 10 prospective cohort studies found little-to-no evidence to support a preventive role of dietary flavonoid intake in gastric and colorectal cancer (119). In addition, a recently published analysis of two large prospective studies (the Health Professionals Follow-up Study [HPFS] and the Nurses' Health Study [NHS]) — using the most up-to-date flavonoid food composition databases — found no association between the risk of colorectal cancer and intakes of each subclass of flavonoids or flavonoid-rich foods (tea, blueberries, oranges) (120). A meta-analysis of 19 case-control studies and 15 cohort studies found that total flavonoid intake and intakes of specific flavonoid subclasses (i.e., flavonols, flavones, flavanones) were inversely correlated with the risk of smoking-sensitive cancers of the aerodigestive tract (mouth, pharynx, larynx, esophagus, and stomach) in smokers but not in nonsmokers (121). The risk of lung cancer was not significantly associated with high flavonoid intakes (121), although an earlier meta-analysis of eight prospective studies (with substantial heterogeneity across them) suggested a protective role of flavonoids against lung cancer in smokers only (122). Further, a prospective analysis of over 45,000 postmenopausal women from the Multiethnic Cohort Study found a reduced risk of endometrial cancer with the highest intakes of total isoflavones, daidzein, and genistein (123). Additionally, limited evidence from observational studies suggests no relationship between total flavonoid intake and ovarian cancer (124-127). To date, there is little evidence that flavonoid-rich diets might protect against various cancers, but larger prospective cohort studies are needed to address the association.

### *Hormone-dependent cancers*

Because isoflavones are phytoestrogens, it is thought that they may interfere with the synthesis and activity of endogenous hormones, eventually influencing hormone-dependent signaling pathways and protecting against breast and prostate cancers (128). A meta-analysis of 14 observational studies that examined breast cancer incidence in 369,934 women found an overall 11% reduced risk of breast cancer with the highest versus lowest intake of soy isoflavones (129). Subgroup analyses revealed a 24% lower risk of cancer in Asian but not in European or US women, and the risk was 22% lower in postmenopausal but not lower in premenopausal women. In addition to the ethnicity and menopausal status, polymorphisms for hormone receptors (130) and phase I biotransformation enzymes (131) have been found to modify the association between isoflavone intake and breast cancer. Another recent meta-analysis of 12 observational studies (six prospective cohort studies, one nested case-control study, and five case-control studies) investigated the chemopreventive effects of flavonoids (except isoflavones) (132). The results

suggested that intakes of flavonols and flavones may also be inversely associated with the risk of breast cancer. Further, a pooled analysis of four case-control studies that stratified by menopausal status showed inverse associations between breast cancer and intakes of flavonols, flavones, or flavan-3-ols in postmenopausal women only. Finally, a meta-analysis of four prospective cohort studies found an overall 16% reduced risk of breast cancer recurrence in women with high versus low isoflavone intakes (129).

A meta-analysis of 13 observational studies also suggested an inverse relationship between prostate cancer risk and consumption of soy products, especially tofu (133). Yet, further analyses supported a protective role of soy food based only on case-control studies, which have inherent flaws such that associations may often be overestimated or underestimated. In a recent 12-month, multicenter, randomized, double-blind, placebo-controlled phase II clinical trial in 158 Japanese men (aged ≥50 years) with elevated risk of prostate cancer, oral isoflavone (60 mg/day) resulted in a significant decrease in prostate cancer incidence in participants aged 65 years and older (134). In this study, no changes were reported in sex hormone concentrations in blood, suggesting that isoflavones may reduce prostate cancer incidence without interfering with hormone-dependent pathways.

Additional investigations will be necessary to determine whether supplementation with specific flavonoids could benefit cancer prevention or treatment.

For more information on flavonoid-rich foods and cancer, see articles on Fruit and Vegetables, Legumes, and Tea.

## Cognitive function

Inflammation, oxidative stress, and transition metal accumulation appear to play a role in the pathology of several neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease (135). Therefore, the various properties of flavonoids, including their role in protecting vascular health, could have beneficial effects on the brain, possibly in the protection against cerebrovascular disorders, cognitive impairments, and subsequent stroke and dementias. Dietary flavonoids and/or their metabolites have been shown to cross the blood-brain barrier (54) and exert preventive effects towards cognitive impairments in animal models of normal and pathological aging (53).

The cross-sectional data analysis of 2,031 participants (ages, 70-74 years) from the Hordaland Health Study in Norway indicated that, when compared to non-consumers, consumers of flavonoid-rich chocolate, tea, and wine had better global cognitive function, assessed by a battery of six cognitive tests (136). The risk of poor performance in all tests was estimated to be 60 to 74% lower in consumers of all three flavonoid-rich foods compared to non-consumers. An early prospective cohort study in 1,367 older French men and women (aged ≥65 years; free from dementia at baseline) found that those with the lowest flavonoid intakes (<11.5 mg/day) had a 50%

higher risk of developing dementia over the next five years than those with higher intakes (137). In addition, those with higher dietary flavonoid intakes at baseline experienced significantly less age-related cognitive decline over a 10-year period than those with the lowest flavonoid intakes (138).

The effect of cocoa flavan-3-ols have been investigated in an eight-week, randomized, double-blind trial — the Cognitive, Cocoa, and Aging (CoCoA) study — in 90 individuals (ages, 64-82 years) with mild cognitive impairments (MCI); participants were given dairy-based cocoa drinks with either high (993 mg/day) or low (48 mg/day) levels of flavan-3-ols (139). The daily consumption of the cocoa drink high in flavan-3-ols improved some, but not all, measures of cognitive process speed and flexibility and verbal fluency compared to baseline test scores and scores following low flavan-3-ol drink consumption. A composite test score reflecting overall cognitive performance was found to be significantly greater in those given cocoa drinks high rather than low in flavan-3-ols. The study also reported reductions in cardiovascular risk markers (i.e., systolic and diastolic blood pressure, total and LDL-cholesterol, insulin resistance), and these changes were proposed to partly contribute to ameliorate cognitive performance in those who consumed the flavan-3-ol-rich cocoa drink (139). The data could be replicated in cognitively healthy older people (ages, 61-85 years), suggesting that cocoa flavan-3-ols might enhance some aspects of cognitive function during healthy aging (140). Interestingly, a two-week, randomized, double-blind, controlled study has reported an increase in blood flow velocity in the middle cerebral artery of 21 healthy subjects (mean age, 72 years) following the daily intake of a flavan-3-ol-rich cocoa drink (900 mg/day of flavan-3-ols) (141). Because cerebral blood flow is correlated with cognitive function in humans, these preliminary data suggest that cocoa flavan-3-ol consumption could exert a protective effect against dementia (54).

Yet, in other randomized controlled trials (142-144), the lack of an effect of cocoa flavan-3-ols on blood pressure, cerebral blood flow, mental fatigue, and cognitive performance in healthy young and old adults suggested that benefits may only be seen in very demanding cognitive exercises (145).

Some randomized controlled studies also reported improvements in measures of cognitive function in healthy and cognitively impaired subjects with other flavonoid subclasses, including anthocyanins (146), flavanones (147, 148), and isoflavones (149, 150). Although some flavonoids and flavonoid-rich foods may enhance cognitive function in the aging brain, it is not yet clear whether their consumption could lower the risk of cognitive impairments and dementia in humans.

For more detailed information on flavan-3-ol-rich tea and cognitive function, see the article on Tea.

## Sources

### Food sources

Recent data analyses of the National Health and Nutrition Examination Survey (NHANES) estimated flavonoid intakes in US adults (aged ≥19 years) average between 200 and 250 mg/day, with 80% being flavan-3-ols, 8% for flavonols, 6% for flavanones, 5% for anthocyanidins, and ≤1% for soflavones and flavones (151, 152). The main dietary sources of flavonoids include tea, citrus fruit, citrus fruit juices, berries, red wine, apples, and legumes. Individual flavonoid intakes may vary considerably depending on whether tea, red wine, soy products, or fruit and vegetables are commonly consumed (reviewed in 2). Information on the flavonoid content of some flavonoid-rich foods is presented in **Tables 2-8**. These values should be considered approximate since a number of factors may affect the flavonoid content of foods, including agricultural practices, environmental conditions, ripening, storage, and food processing. For additional information about the flavonoid content of food, the USDA provides databases for the content of selected foods in flavonoids (60) and proanthocyanidins (58). For more information on the isoflavone content of soy foods, see the article on Soy Isoflavones or the USDA database for the isoflavone content of selected foods (59).

Table 2. Anthocyanidin Content of Anthocyanidin-rich Foods (mg/100 g or 100 mL*)

| | Anthocyanidins | | | | | |
|---|---|---|---|---|---|---|
| Food | Cyanidin | Delphinidin | Malvidin | Pelargonidin | Peonidin | Petunidin |
| Blackberries, raw | 100 | 0 | 0 | <1 | <1 | 0 |
| Blood orange juice | 5.5 | <1 | - | - | <1 | - |
| Blueberries, raw | 8.5 | 35.4 | 67.6 | 0 | 20.3 | 31.5 |
| Currants, black, raw | 62.5 | 89.6 | - | 1.2 | <1 | 3.9 |
| Elderberries, raw | 485.3 | 0 | - | <1 | - | 0 |
| Grapes, red | 1.2 | 2.3 | 39 | <1 | 3.6 | 2 |
| Onions, red, raw | 3.2 | 4.3 | - | <1 | 2.1 | - |
| Plums, raw | 5.6 | 0 | 0 | 0 | <1 | 0 |
| Radishes, raw | 0 | 0 | 0 | 63.1 | 0 | 0 |
| Raspberries, raw | 45.8 | 1.3 | <1 | 1 | <1 | <1 |
| Red cabbage, raw | 209.8 | <1 | - | <1 | - | - |
| Strawberries, raw | 1.7 | <1 | <1 | 24.9 | <1 | <1 |

*per 100 g (fresh weight) or 100 mL (liquids); 100 grams is equivalent to about 3.5 ounces; 100 mL is equivalent to about 3.5 fluid ounces.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Wine, red, Shiraz | - | 9.3 | 121.6 | - | 7.8 | 14.2 | - |

*per 100 g (fresh weight) or 100 mL (liquids); 100 grams is equivalent to about 3.5 ounces; 100 mL is equivalent to about 3.5 fluid ounces.

Table 3. Flavan-3-ol Content of Flavan-3-ol-rich Foods (mg/100 g or 100 mL*)

| | Flavon-3-ol Monomers[#] and Thearubigins | | | | | | |
|---|---|---|---|---|---|---|---|
| Food | C | GC | EC | ECG | EGC | EGCG | Thearubigins |
| Apples, Red Delicious, raw, with skin | 2 | 0 | 9.8 | 0 | <1 | <1 | - |
| Apricots, raw | 3.7 | 0 | 4.7 | 0 | 0 | 0 | - |
| Chocolate, dark | 24.2 | - | 84.4 | - | - | - | - |
| Tea, black, brewed | 1.5 | 1.2 | 2.1 | 5.9 | 8 | 9.4 | 81.3 |
| Tea, green, brewed | 4.5 | 1.5 | 8.3 | 17.9 | 29.2 | 70.2 | 1.1 |
| Tea, oolong, brewed | <1 | - | 2.5 | 6.3 | 6.1 | 34.5 | - |
| Tea, white, brewed | - | - | - | 8.3 | 18.6 | 42.4 | - |
| Wine, red, Shiraz | 6.8 | - | 10 | - | - | - | - |

*per 100 g (fresh weight) or 100 mL (liquids); 100 grams is equivalent to about 3.5 ounces; 100 mL is equivalent to about 3.5 fluid ounces.
[#]Catechins: C, (+)-catechin; GC, (-)-gallocatechin; EC (-)-epicatechin; ECG, (-)-epigallocatechin; ECG, (-)-epicatechin gallate; EGCG, (-)-epigallocatechin gallate.

Table 4. Proanthocyanidin Content of Flavan-3-ol-rich Foods (mg/100 g or 100 mL*)

| | Proanthocyanidins (Flavon-3-ol Polymers) | | | |
|---|---|---|---|---|
| Food | Monomers | Dimers | Trimers | ≥4mers |
| Apples, Red Delicious, raw, with skin | 8.3 | 15.1 | 10.1 | 94.2 |
| Baking chocolate, unsweetened | 198.5 | 206.5 | 130.9 | 1,100 |
| Cocoa, dry powder, unsweetened | 316.6 | 183.5 | 159.5 | 713.4 |
| Cranberries, raw | 7.3 | 25.9 | 18.9 | 385.6 |
| Currants, black, raw | <1 | 2.9 | 3 | 142.9 |
| Grapes, red, raw | 1.4 | 2.4 | 1 | 56.9 |
| Nuts, pecan | 17.2 | 42.1 | 26 | 408.6 |
| Nuts, pistachio | 10.9 | 13.3 | 10.5 | 202.6 |
| Peaches, yellow, with peel, raw | 4.5 | 12.2 | 4.4 | 50.6 |
| Plums, with peel, raw | 10.9 | 38.5 | 22.2 | 149.1 |
| Spices, cinnamon, ground | 23.9 | 256.3 | 1252.2 | 6,576 |
| Strawberries, raw | 3.7 | 5.3 | 4.9 | 127.8 |
| Wine, table, red | 16.6 | 20.5 | 1.8 | 22.7 |

*per 100 g (fresh weight) or 100 mL (liquids); 100 grams is equivalent to about 3.5 ounces; 100 mL is equivalent to about 3.5 fluid ounces.

Table 5. Flavonol Content of Flavonol-rich Foods (mg/100 g or 100 mL*)

| | Flavonols | | | |
|---|---|---|---|---|
| Food | Isorhamnetin | Kaempferol | Myricetin | Quercetin |
| Blueberries, raw | - | 1.7 | 1.3 | 7.7 |
| Broccoli, raw | - | 7.8 | <1 | 3.3 |
| Chili peppers, green, raw | - | 0 | 1.2 | 14.7 |
| Cowpea, black seeds, raw | - | 1.9 | 2.7 | 17.2 |

*per 100 g (fresh weight) or 100 mL (liquids); 100 grams is equivalent to about 3.5 ounces; 100 mL is equivalent to about 3.5 fluid ounces.

| | | | | |
|---|---|---|---|---|
| Kale, raw | 23.6 | 46.8 | 0 | 22.6 |
| Onions, red, raw | 4.6 | <1 | 2.2 | 39.2 |
| Parsley, fresh | 0 | 1.5 | 14.8 | <1 |
| Rocket, wild, raw | <1 | 1.8 | - | 66.2 |
| Scallions, raw | - | 1.4 | 0 | 10.7 |
| Spinach, raw | - | 6.4 | <1 | 4 |
| Tea, black, brewed | - | 1.4 | <1 | 2.2 |
| Tea, green, brewed | - | 1.3 | 1 | 2.5 |
| Watercress, raw | 0 | 23 | <1 | 30 |

*per 100 g (fresh weight) or 100 mL (liquids); 100 grams is equivalent to about 3.5 ounces; 100 mL is equivalent to about 3.5 fluid ounces.

Table 6. Flavone Content of Flavone-rich Foods (mg/100 g or 100 mL*)

| | Flavones | |
|---|---|---|
| Food | Apigenin | Luteolin |
| Celery hearts, green | 19.1 | 3.5 |
| Celery, raw | 2.8 | 1 |
| Chili peppers, green, raw | 1.4 | 3.9 |
| Oregano, fresh | 2.6 | 1 |
| Parsley, fresh | 215.5 | 1.1 |
| Peppermint, fresh | 5.4 | 12.7 |
| Thyme, fresh | 2.5 | 45.2 |

*per 100 g (fresh weight) or 100 mL (liquids); 100 grams is equivalent to about 3.5 ounces; 100 mL is equivalent to about 3.5 fluid ounces.

Table 7. Flavanone Content of Flavanone-rich Foods (mg/100 g or 100 mL*)

| | Flavanones | | |
|---|---|---|---|
| Food | Eriodictyol | Hesperetin | Naringenin |
| Grapefruit juice, white, fresh | <1 | 2.3 | 18.2 |
| Grapefruit, white, raw | - | <1 | 21.3 |
| Lemon juice, fresh | 4.9 | 14.5 | 1.4 |
| Lemon, raw | 21.4 | 27.9 | <1 |
| Orange juice, fresh | <1 | 12 | 2.1 |
| Orange, raw | - | 27.2 | 15.3 |
| Pummelo juice, fresh | 2.9 | 1.8 | 25.3 |

*per 100 g (fresh weight) or 100 mL (liquids); 100 grams is equivalent to about 3.5 ounces; 100 mL is equivalent to about 3.5 fluid ounces.

Table 8. Isoflavone Content of Isoflavone-rich Foods (mg/100 g or 100 mL*)

| | Isoflavones | | |
|---|---|---|---|
| Food | Diadzein | Genistein | Glycitein |
| Black bean sauce | 6 | 4 | <1 |
| Natto | 33.2 | 37.7 | 10.5 |
| Soybeans, mature seeds, raw | 62.1 | 81 | 15 |
| Soymilk, low-fat | 1 | 1.5 | <1 |
| Tofu, firm, cooked | 10.3 | 10.9 | 1.3 |

*per 100 g (fresh weight) or 100 mL (liquids); 100 grams is equivalent to about 3.5 ounces; 100 mL is equivalent to about 3.5 fluid ounces.

## Supplements

### *Anthocyanins*

Bilberry, elderberry, black currant, blueberry, red grape, and mixed berry extracts that are rich in anthocyanins are available as dietary supplements without a prescription in the US. The anthocyanin content of these products may vary considerably. Standardized extracts that list the amount of anthocyanins per dose are available.

### *Flavan-3-ols*

Numerous tea extracts are available in the US as dietary supplements and may be labeled as tea catechins or tea polyphenols. Green tea extracts are the most commonly marketed, but black and oolong tea extracts are also available. Green tea extracts generally have higher levels of catechins (flavan-3-ol monomers), while black tea extracts are richer in theaflavins and thearubigins (tea flavan-3-ol dimers and polymers, respectively). Oolong tea extracts fall somewhere in between green and black tea extracts with respect to their flavan-3-ol content. Some tea extracts contain caffeine, while others are decaffeinated. Flavan-3-ol and caffeine content vary considerably among different products, so it is important to check the label or consult the manufacturer to determine the amounts of flavan-3-ols and caffeine that would be consumed daily with each supplement (for more information on tea flavan-3-ols, see the article on Tea).

### *Flavanones*

Citrus bioflavonoid supplements may contain glycosides of hesperetin (hesperidin), naringenin (naringin), and eriodictyol (eriocitrin). Hesperidin is also available in hesperidin-complex supplements, with daily doses from 500 mg to 2 g (153).

### *Flavones*

The peels and tissues of citrus fruit (e.g., oranges, tangerines, and clementines) are rich in polymethoxylated flavones: tangeretin, nobiletin, and sinensetin (2). Although dietary intakes of these naturally occurring flavones are generally low, they are often present in citrus bioflavonoid complex supplements. Several dietary supplements may also contain various amounts of baicalein (aglycone) and/or baicalin (glycoside). Some tea preparations may also include baicalein-7-glucuronide (153).

### *Flavonols*

The flavonol aglycone, quercetin, and its glycoside rutin are available as dietary supplements without a prescription in the US. Other names for rutin include rutoside, quercetin-3-rutinoside, and sophorin (153). Citrus bioflavonoid supplements may also contain quercetin or rutin.

### *Isoflavones*

A 50-mg soy isoflavone supplement usually includes glycosides of the isoflavones: genistein (genistin; 25 mg), daidzein (daidzin; 19 mg), and glycitein (glycitin; about 6 mg). Smaller amounts of daidzein, genistein, and formononetin are also found in biochanin A-containing supplements (derived from red clover) (153).

## Safety

### Adverse effects

No adverse effects have been associated with high dietary intakes of flavonoids from plant-based food. This lack of adverse effects may be explained by the relatively low bioavailability and rapid metabolism and elimination of most flavonoids.

### *Quercetin*

Oral supplementation with quercetin glycosides at doses ranging between 3 mg/day-1,000 mg/day for up to three months has not resulted in significant adverse effects in clinical studies (reviewed in 154). A randomized, placebo-controlled study in 30 patients with chronic prostatitis reported one case of headache and another of tingling of the extremities associated with supplemental quercetin (1,000 mg/day for one month); both issues resolved after the study ended (155). In a phase I clinical trial in cancer patients unresponsive to standard treatments, administration of quercetin via intravenous infusion resulted in symptoms of nausea, vomiting, sweating, flushing, and dyspnea (difficulty breathing) at doses ≥10.5 mg/kg body weight (~756 mg of quercetin for a 70 kg individual) (156). Higher doses up to 51.3 mg/kg body weight (~3,591 mg of quercetin) were associated with renal (kidney) toxicity, yet without evidence of nephritis, infection, or obstructive uropathy (reviewed in 154).

### *Cocoa flavan-3-ols*

In a recent randomized, double-blind, controlled study in healthy adults, the daily intake of 2 g of cocoa flavan-3-ols for 12 weeks was found to be well tolerated with no adverse side effects (157).

### *Tea extracts*

In clinical trials employing caffeinated green tea extracts, cancer patients who took 6 g/day in three to six divided doses reported mild-to-moderate gastrointestinal side effects, including nausea, vomiting, abdominal pain, and diarrhea (158, 159). Central nervous system symptoms, including agitation, restlessness, insomnia, tremors, dizziness, and confusion, have also been reported. In one case, confusion was severe enough to require hospitalization (158). In a systematic review published in 2008, the US Pharmacopeia (USP) Dietary Supplement Information Expert Committee identified 34 adverse event reports implicating the use of green tea extract products (containing 25%-97% of polyphenols) as the likely cause of liver damage (hepatotoxicity) in humans (160). In a four-week clinical trial that assessed the safety of decaffeinated green tea extracts (800 mg/day of EGCG) in healthy individuals, a few of the participants reported mild nausea, stomach upset, dizziness, or muscle pain (161). In the Minnesota Green Tea Trial (MGTT), 1,075 postmenopausal women were randomized to receive green tea extracts (1,315±116 mg/day of catechins; the equivalent of four 8-ounce mugs of brewed decaffeinated green tea) or a placebo for one year. The total number of adverse events and the number of serious adverse events were not different between the treatment and placebo groups (162). However, the use of green tea extracts was directly associated with abnormally high liver enzyme levels in 7 out of the 12 women who experienced serious adverse events. Also, the incidence of nausea was twice as high in the green tea arm as in the placebo group (162).

## Pregnancy and lactation

The safety of flavonoid supplements in pregnancy and lactation has not been established (153).

## Drug interactions

### *Inhibition of ABC drug transporters*

ATP-binding cassette (ABC) drug transporters, including P-glycoprotein, multidrug resistance protein (MRP), and breast cancer-resistant protein (BCRP), function as ATP-dependent efflux pumps that actively regulate the excretion of a number of drugs limiting their systemic bioavailability (8). ABC transporters are found throughout the body, yet they are especially important in organs with a barrier function like the intestines, the blood-brain barrier, blood-testis barrier, and the placenta, as well as in liver and kidneys (163). There is some evidence that the consumption of grapefruit juice inhibits the activity of P-glycoprotein (164). Genistein, biochanin A, quercetin, naringenin, hesperetin, green tea flavan-3-ol (-)-CG, (-)-ECG, and (-)-EGCG, and others have been found to inhibit the efflux activity of P-glycoprotein in cultured cells and in animal models (163). Thus, very high or supplemental intakes of these flavonoids could potentially increase the toxicity of drugs that are substrates of P-glycoprotein, e.g., digoxin, antihypertensive agents, antiarrhythmic agents, chemotherapeutic (anticancer) agents, antifungal agents, HIV protease inhibitors, immunosuppressive agents, $H_2$ receptor antagonists, some antibiotics, and others (reviewed in 165).

Many anthocyanins and anthocyanidins, as well as some flavones (apigenin, chrysin), isoflavones (biochanin A, genistein), flavonols (kaempferol), and flavanones (naringenin), have been identified as inhibitors of BRCP-mediated transport, theoretically affecting drugs like anticancer agents (mitoxantrone, topotecan, thyrosine kinase inhibitors), antibiotics (fluoroquinolones), β-blockers (prazosin), and antiarthritics (sulfasalazine). Finally, flavonols (quercetin, kaempferol, myricetin), flavanones (naringenin), flavones (apigenin, robinetin), and isoflavones (genistein) have been reported to inhibit MRP, potentially affecting MRP-mediated transport of many anticancer drugs, e.g., vincristin, etoposide, cisplatin, irinotecan, methotrexate, camptothecin, anthracyclines, vinca alkaloids (reviewed in 163).

### *Anticoagulant and antiplatelet drugs*

High intakes of flavonoids from purple grape juice (500 mL/day) and dark chocolate (235 mg/day of flavan-3-ols) have been found to inhibit platelet aggregation in *ex vivo* assays (166-169). Theoretically, high intakes of flavonoids (e.g., from supplements) could increase the risk of bleeding when taken with anticoagulant drugs, such as warfarin (Coumadin), heparin, dalteparin (Fragmin), enoxaparin (Lovenox), and antiplatelet drugs, such as clopidogrel (Plavix), dipyridamole (Persantine), non-steroidal anti-inflammatory drugs (NSAIDs: diclofenac, ibuprofen, naproxen), aspirin, and others (170).

### *Inhibition of CYP 3A4 by flavonoid-rich grapefruit*

Cytochrome P450 (CYP) enzymes are phase I biotransformation enzymes involved in the metabolism of a broad range of compounds, from endogenous molecules to therapeutic agents. The most abundant CYP isoform in the liver and intestines is cytochrome P450 3A4 (CYP3A4); the CYP3A family catalyzes the metabolism of about one-half of all marketed drugs in the US and Canada (171). One grapefruit or as little as 200 mL (7 fluid ounces) of grapefruit juice have been found to irreversibly inhibit intestinal CYP3A4 (164). The most potent inhibitors of CYP3A4 in grapefruit are thought to be furanocoumarins, particularly dihydroxybergamottin, rather than flavonoids. All forms of the fruit — freshly squeezed juice, frozen concentrate, or whole fruit — can potentially affect the activity of CYP3A4. Some varieties of other citrus fruit (Seville oranges, limes, and pomelos) that contain furanocoumarins can also interfere with CYP3A4 activity.

Specifically, the inhibition of intestinal CYP3A4 by grapefruit consumption is known or predicted to increase the bioavailability and the risk of toxicity of more than 85 drugs. Because drugs with very low bioavailability are more likely to be toxic when CYP3A4 activity is inhibited, they are associated with a higher risk of overdose with grapefruit compared to drugs with high bioavailability. Some of the drugs with low bioavailability include, but are not limited to, anticancer drugs (everolimus); anti-infective agents halofantrine, maraviroc); statins (atorvastatin, lovastatin, and simvastatin); cardioactive drugs (amiodarone, clopidogrel, dronedarone, eplenorone, ticagrelor); HIV protease inhibitors (saquinavir), immunosuppressants (cyclosporine, sirolimus, tacrolimus, everolimus); antihistamines (terfenadine); gastrointestinal agents (domperidone); central nervous system agents (buspirone, dextromethorphan, oral ketamine, lurasidone, quetiapine, selective serotonin reuptake inhibitors [sertraline]); and urinary tract agents (darifenacin) (reviewed in 171). Because of the potential for adverse drug interactions, some clinicians recommend that people taking medications with low bioavailability (i.e., undergoing extensive metabolism by CYP3A4) avoid consuming grapefruit and grapefruit juice altogether during the treatment period (171).

## Nutrient interactions

### *Iron*

Flavonoids can bind nonheme iron, inhibiting its intestinal absorption (172, 173). Nonheme iron is the principal form of iron in plant foods, dairy products, and iron supplements. The consumption of one cup of tea or cocoa with a meal has been found to decrease the absorption of nonheme iron in that meal by about 70% (174, 175). Flavonoids can also inhibit intestinal heme iron absorption (176). Interestingly, ascorbic acid greatly enhances the absorption of iron (see the article on Iron) and is able to counteract the inhibitory effect of flavonoids on nonheme and heme iron absorption (173, 176, 177). To maximize iron absorption from a meal or iron supplements, flavonoid-rich food and beverages and flavonoid supplements should not be consumed at the same time.

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# Advanced research on the antioxidant and health benefit of elderberry (*Sambucus nigra*) in food – a review



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ABSTRACT

Elderberry plant (*Sambucus nigra*) is a good source of protein, free and conjugated forms of amino acids, unsaturated fatty acids, fibre fractions, vitamins, antioxidants and minerals. Analysis of elderberry showed that it contains high biological activity components, primarily polyphenols, mostly anthocyanins, flavonols, phenolic acids and proanthocyanidins, as well as terpenes and lectins. In folk medicine, elderberry has been used in the treatment of many diseases and ailments. Elderberry has medicinal properties associated with the presence of polyphenols, which are compounds with potential antioxidant properties. They can greatly affect the course of disease processes by counteracting oxidative stress, exerting beneficial effects on blood pressure, glycaemia reduction, immune system stimulation, antitumour potential, increase in the activity of antioxidant enzymes in the blood plasma, including also glutathione, and the reduction of uric acid levels.

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## 1. Introduction

Recent years have seen a surge in research concerning bioactive compounds in food. The plant world is a source of many substances affecting functionality of the human organism and its status (Costa, Garcia-Diaz, Jimenez, & Silva, 2013). One of the relatively little known sources of bioactive compounds is elderberry, the consumption of which provides prevention and therapy for a number of diseases.

## 2. Compounds in elderberry that affect human health

The composition of elderberry (*Sambucus nigra*) depends on many factors: the variety, degree of ripeness, as well as environmental and climatic conditions (Kader & Barrett, 2005). Elderberry is a good source of protein, with content in fruits at 2.7–2.9%, in flowers at 2.4%, while in the leaves at 3.3%. Amino acids in fruits, leaves and flowers occur in the free or conjugated form. The protein of elderberry is a complete one; among sixteen amino acids found in fruits seven belong to the group of exogenous (the human body cannot synthetize, so they must be supplied in the diet) and relative exogenous amino acids (can be synthetized within the body from other amino acids), while in flowers and leaves there are nine. Considering the amino acid composition of elderberry the chemical score of a protein (CS) was established at 0.9 (the nutritional value of a protein, calculated as the ratio of the amount of amino acid in a product to the amount of the corresponding amino acid in a reference egg albumin) (Akbulut, Ercisli, & Tosun, 2009; Kislichenko & Vel'ma, 2006). Lipids in elderberry fruits are located primarily in the seeds. The oil content in seeds was

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estimated at 22.4% (Dulf, Oroian, Vodnar, Socaciu, & Pinteа, 2013) and in meal produced from seeds at 15.9% (Fazio, Plastina, Meijerink, Witkamp, & Gabriele, 2013). The fatty acid profile of lipids in total is dominated by unsaturated fatty acids, namely linolenic (40.7 g/100 g oil), linoleic (34.3 g/100 g oil) and oleic acid (13.8 g/100 g oil) (Dulf et al., 2013). Oil derived from seeds and meal produced from seeds are characterized by a very favourable n-6/n-3 ratio, at 0.84 and 1.19 respectively (Dulf et al., 2013; Fazio et al., 2013). The carbohydrate content of elderberry fruits amounts to 18.4%, of which 7.4% is dietary fibre (Bender, 2006). Fibre fractions present in fruit include pectin, pectic acid (polygalacturonic acid), protopectin (hemicellulose) and calcium pectinate (Vulić, Vračar, & Sumić, 2008). Sugars constitute 6.8–11.5% in the fruit, of which over 95% are reducing sugars in the form of glucose and fructose. Glucose and fructose are present in similar amounts. Sucrose accounts for less than 0.33% of the fruits (Mratinić & Fotirić, 2007; Veberic, Jakopic, Stampar, & Schmitzer, 2009). Vitamins present in elderberry are B-group vitamins, A-group vitamins, tocopherols and vitamin C. The content of vitamin C in fresh elderberry fruits is 6–35 mg/100 g (Akbulut et al., 2009; Kaack & Austed, 1998). Among tocopherols in elderberry seed oil, $\alpha$-tocopherol and $\gamma$-tocopherol were detected at 0.49 and 2.63 μg/g oil (Fazio et al., 2013), while the residues after pressing of oil from seeds contained $\alpha$-, β-, $\gamma$- and $\delta$-tocopherols (0.96, 0.31, 12.44 and 0.16 mg/100 g respectively) and $\alpha$-tocotrienol (0.89 mg/100 g) (Helbig, Böhm, Wagner, Schubert, & Jahreis, 2008). Tocopherols determined in leaves were α-, β-, $\gamma$-tocopherols (313, 8.1, 2.9 μg/g DW respectively) (Barros, Cabrita, Boas, Carvalho, & Ferreira, 2011). Ash in elderberry fruit constituted about 0.99%, and consisted of the following minerals: K, Ca, Fe, Mg, P, Na, Zn, Cu, Mn, Se, Cr, Ni, Cd (Kołodziej, Maksymiec, Drożdżal, & Antonkiewicz, 2012; Rupasinghe & Clegg, 2007).

Elderberry has components with high biological activity, primarily polyphenols, such as flavonols, phenolic acids, proanthocyanidins and anthocyanins, which give the fruit its characteristic black-purple colour (Anton et al., 2013). Elderberry anthocyanins are cyanidin derivatives, with other anthocyanins present in trace amounts. *S. nigra* contains cyanidin-3-glucoside (C3G), cyanidin-3-sambubioside (C3S), cyanidin 3-sambubioside-5-glucoside (C3S5G), cyanidin 3,5-diglucoside (C3,5-DiG), cyanidin 3-rutinoside (C3R), pelargonidin 3-glucoside (P3G), pelargonidin 3-sambubioside (P3S) and delfinidine-3-rutinoside (D3R). The composition of anthocyanins from *Sambucus canadensis* is more diversified due to the presence of acylated anthocyanins, namely cyanidin-3-(Z)-(p-coumarin)-sambubioside-5-glucoside, cyanidin-3-(p-coumarin)-glucoside, cyanidin-3-(E)-(p-coumarin)-sambubioside-5-glucoside and cyanidin-3-(p-coumarin)-sambubioside. In addition, petunidin 3-rutinoside in the absence of cyanidin 3-rutinoside and pelargonidin 3-glucoside (Łabun, Fejér, Šalamon, & Ragác, 2011; Lee & Finn, 2007; Wu, Gu, Prior, & McKay, 2004) were reported in *S. canadensis*. The presence of individual anthocyanins in elderberry fruits is variable. The predominant anthocyanin of *S. nigra* is cyanidin 3-glucoside, depending on the variety and fruition (204.6–481.4 mg CGE/100 g fruits). In turn, the second anthocyanin in terms of the content was cyanidin 3-sambubioside (122.2–269.1 mg CGE/100 g fruits) (Lee & Finn, 2007). The fraction of cyanidin 3-glucoside prevailed in twelve of the thirteen varieties of *S. nigra* under study, with var. Allesoe being exceptional due to the presence of the largest quantities of cyanidin 3-sambubioside (Kaack & Austed, 1998). Veberic et al. (2009) determined that predominant anthocyanins in *S. nigra* were cyanidin 3-sambubioside (630.8 mg CGE/100 g) and cyanidin 3-glucoside (586.4 mg CGE/100 g fruit). Ochmian, Oszmiański, and Skupień (2009) determined the content of C3G and C3S on an equal level of 225 mg/100 g. Other reports indicate that the main anthocyanin in *S. nigra* fruits was cyanidin 3-sambubioside-5-glucoside representing 55.5% of total cyanidin content, whereas the content of cyanidin 3-glucoside was at only 10.4% of the total cyanidins (Bridle & García-Viguera, 1997). The anthocyanin profile in *S. canadensis* was dominated by p-coumarin acylated cyanidins representing 69.8% of the total anthocyanins (Johansen et al., 1991; Osamu, Tamura, Kikuzaki, & Nakatani, 1996).

Elderberry is also a good source of flavonols and phenolic acids. The predominant flavonols were quercetin, kaempferol and isorhamnetin. Flavonols derived from elderberry occur as glycosides of rutin and glucose; moreover, acylated quercetins were also present (Christensen, Kaack, & Frette, 2008). Polyphenolic compounds are present in the leaves, fruits and flowers. The flowers of elderberry contained tenfold more flavonols (214.25 mg/100 g) than fruits (20.18 mg/100 g) and several times more than the leaves (17.01 mg/100 g) (Dawidowicz, Wianowska, & Baraniak, 2006). Elderberry flowers and fruits, apart from flavonols, contain large amounts of phenolic acids. Christensen et al. (2008) isolated from flowers nine phenolic acids, derivatives of caffeic and *p*-coumaric acid, and two phenolic acids with no established structures. Phenolic acids in the elderberry fruits were less diverse than those present in flowers. Fruits contained chlorogenic, crypto-chlorogenic and neochlorogenic acids, while additionally small amounts of ellagic acid were also determined (0.04 mg/100 g seeds) (Fazio et al., 2013; Lee & Finn, 2007).

Proanthocyanidins from *S. nigra* are present at 23.3 mg/100 g fruits, which is a small amount as compared to 663.7 mg/100 g present in black chokeberry fruits, and several times lower than those in blackcurrant fruits (120.6–165.8 mg/100 g). The degree of polymerization may be considered as a characteristic feature of proanthocyanidins, which have been reported only in the forms ranging from dimeric to hexameric (Wu et al., 2004). The content and structural formulas of polyphenolic compounds in fruits and flowers of elderberry (*S. nigra*) are presented in Table 1 and Fig. 1.

It was found that the processing and storage of elderberry-derived products cause changes in the composition of polyphenols. Blanching of fresh fruits generally reduced the polyphenol content, with an increase in anthocyanin contents, which was also observed in the juice (Galić et al., 2009). Storage of elderberry juice resulted in a reduction of polyphenol contents at about 40% during the first 30 days. The amount of polyphenols in the subsequent days of storage was unchanged (Casati et al., 2012). Studies on the storage of wine from elderberry must showed that the anthocyanin content decreased, while the content of phenolic acids and flavonols increased. During wine ageing the level of anthocyanins was reduced to a considerable extent (up to 94%) and the total loss of polyphenols reached 21% (Schmitzer, Veberic, Slatnar, & Stampar, 2010). Phenolic acids and flavonols in teas from elderberry flowers were relatively stable compounds during their

**Table 1 – Composition of polyphenolics in fruits, leaves and flowers of elder (*Sambucus nigra*).**

| Compound | Fruits | Flowers |
|---|---|---|
| Total polyphenols | 371–432 mg GAE/100 g FW[1]; 364–582 mg GAE/100 g[8]; 513.6 mg/100 g FW[11]; 19.5 mg GAE/g FW[14]; | |
| Anthocyanins total/sum | 242–283 mg CGE/100 g FW[1]; 272.87 mg/100 g FW[2]; 664–1816 mg CGE/100 g FW[6]; 8.33–101.40 mg CGE/g DW[7]; 1.9–20.2 g CGE/kg[7]; 170–343 mg CGE/100 g[8]; 465.1 mg/100 g FW[11]; 602.9–1265.3 mg CGE/100 g FW[12]; 1374.4 mg CGE/100 g[14]; | |
| Cyanidin total/sum | 48,4568–52,8869 g/100 g extr[5]; 3316 mg/kg FW[9]; | |
| Delphinidin | Nd[9]; | |
| Petunidin | Nd[9]; | |
| Peonidin | Nd[9]; | |
| Malvidin | Nd[9]; | |
| Pelargonidin 3-glucoside | Tr[8]; 1.8 mg CGE/100 g[14]; | |
| Pelargonidin 3-sambubioside | Tr[8]; Tr[14]; | |
| Cyanidin 3-sambubioside-5-glucoside | 23.34 mg/100 g FW[2]; 14–47 mg CGE/100 g FW[6]; 0.86–11.5 mg CSE/g DW[7]; 16.0–59.2 mg CGE/100 g FW[8]; 19.52–53.49 mg CGE/100 g FW[12]; 82.6 mg CGE/100 g[14]; | |
| Cyanidin 3,5-diglucoside | 47.1 mg/100 g FW[2]; 5–36 mg CGE/100 g FW[6]; 0.12–5.22 mg CSE/g DW[7]; 8.2–19.5 mg CGE/100 g FW[8]; 14.39 mg/100 g FW[11]; 7.41–23.29 mg CGE/100 g FW[12]; | |
| Cyanidin 3-sambubioside | 134.94 mg/100 g FW[2]; 27.0453–27.7027 g/100 g extr[5]; 269–656 mg CGE/100 g FW[6]; 4.62–40.30 mg CSE/g DW[7]; 122.2–269.1 mg CGE/100 g FW[8]; 225.45 mg/100 g FW[11]; 270.8–630.8 mg CGE/100 g FW[12]; 3500–3907 µg CGE/g FW[13]; 545.9 mg CGE/100 g[14]; | Nd[5]; |
| Cyanidin 3-glucoside | 44.83 mg/100 g FW[2]; 21.4115–25.1842 g/100 g extr[5]; 361–1266 mg CGE/100 g FW[6]; 2.74–49.5 mg CSE/g DW[7]; 204.6–481.4 mg CGE/100 g FW[8]; 225.26 mg/100 g FW[11]; 221.4–586.4 mg CGE/100 g FW[12]; 739.8 mg CGE/100 g[14]; | Nd[5]; |
| Cyanidin 3-rutinoside | Tr[8]; 1.49–9.63 mg CGE/100 g FW[12]; 4.40 mg CGE/100 g[14]; | |
| Delphinidin 3-rutinoside | Tr[8]; | |
| Petunidin 3-rutinoside | Nd[8]; | |
| Flavonols total/sum | 38.26 mg/100 g FW[2]; 13.6978–20.1836 g/100 g extr[5]; 57.0–102.7 mg QRE/100 g[8]; | 20.5 mg/100 g DW[3]; 139.3422–214.2503 g/100 g extr[5]; |
| Quercetin total/sum | 73.43–51.94 mg/100 g FW[12]; | |
| Quercetin | 0.55 mg/100 g FW[2]; 331 mg/kg FW[9]; 29–60 mg/100 g FW[6]; 2.70–4.50 mg/100 g FW[12]; | |
| Kaempferol | Tr[9]; | |
| Myricetin | Nd[9]; | |
| Isorhamnetin | Nd[9]; | |
| Quercetin 3-O-rutinoside | 29.02 mg/100 g FW[2]; 10.8567–15.3875 g/100 g extr[5]; 1.94–6.31 mg QRE/g DW[7]; 42.6–95.6 mg QRE/100 g[8]; 397.3–493.1 mg/kg FW[10]; 21.95 mg/100 g FW[11]; 35.59–52.02 mg/100 g FW[12]; 1197.0–1313.2 µg QRE/g FW[13]; | 14.93 mg/g DW[3]; 11.6–42.3 mg/g DW[4]; 132.6892–202.0781 g/100 g extr[5]; |
| Quercetin 3-O-glucoside | 8.69 mg/100 g FW[2]; 1.7863–3.0097 g/100 g extr[5]; 0.11–1.08 mg QRE/g DW[7]; 3.9–14.9 mg QRE/100 g[8]; 19.6–46.2 mg/kg FW[10]; 2.30 mg/100 g FW[11]; 6.38–26.52 mg/100 g FW[12]; 26.1–33.4 µg QRE/g FW[13]; | 2.39 mg/g DW[3]; 0.4–1.9 mg/g DW[4]; 5.3684–9.6749 g/100 g extr[5]; |
| Quercetin 3-O-galactoside | 1.1–1.5 mg/kg FW[10]; | |
| Quercetin 3-O-vicianoside | 2.3–3.3 mg/kg FW[10]; | |
| Quercetin 3-O-(6″-acetyl)glucoside | | 0.9–2.8 mg/g DW[4]; |
| Quercetin 3-O-(6″-acetyl)galactoside | 5.1–12.0 mg/kg FW[10]; | |
| Kaempferol 3-O-rutinoside | 0.7–1.2 mg QRE/100 g[8]; 2.9–4.4 mg/kg FW[10]; 1.47 mg/100 g FW[11]; | 1.57 mg/g DW[3]; 0.2–3.0 mg/g DW[4]; |
| Kaempferol 3-O-glucoside | 1.0548–1.7864 g/100 g[5]; 1.04 mg/100 g FW[11]; | 1.2846–2.4973 g/100 g extr[5]; |
| Isorhamnetin 3-O-rutinoside | 0.3–2.2 mg QRE/100 g[8]; 8.0–8.1 mg/kg FW[10]; | 0.2–1.0 mg/g DW[4]; |
| Isorhamnetin 3-O-glucoside | Tr–0.3 mg QRE/100 g[8]; 2.7–3.2 mg/kg FW[10]; | 1.58 mg/g DW[3]; 2.0–7.5 mg/g DW[4]; |
| Myricetin 3-O-rutinoside | 3.2–3.7 mg/kg FW[10]; | |

*(continued on next page)*

**Table 1 – (*continued*)**

| Compound | Fruits | Flowers |
|---|---|---|
| Flavan-3-ols | | |
| (–)-Catechin | Nd[9]; | |
| (–)-Epicatechin | Nd[9]; | |
| Phenolic acids total/sum | 20.00 mg/100 g FW[11]; | |
| 3-O-Caffeoylquinic acid (neochlorogenic acid) | 0.05–0.40 mg ChAE/g DW[7]; 0.7–4.4 mg ChAE/100 g[8]; 5.30 mg/100 g FW[11]; | 0.8–2.4 mg/g DW[4]; |
| cis-3-O-Caffeoylquinic acid | | 0.23 mg/g DW[3]; |
| 4-O-Caffeoylquinic acid (cryptochlorogenic acid) | 1.2–2.5 mg ChAE/100 g[8]; | 0.6–1.5 mg/g DW[4]; |
| 5-O-Caffeoylquinic acid (chlorogenic acid) | 0.53–1.22 mg ChAE/g DW[7]; 26.4–35.9 mg ChAE/100 g[8]; 14.69 mg/100 g FW[11]; 1033–1296 μg ChAE/g FW[13]; | 3.38 mg/g DW[3]; 10.1–20.7 mg/g DW[4]; |
| 1,5-Di-O-caffeoylquinic acid | | 8.0–13.9 mg/g DW[4]; |
| 3,5-Di-O-caffeoylquinic acid | | 1.06 mg/g DW[3]; 0.5–3.2 mg/g DW[4]; |
| 3,4-Di-O-caffeoylquinic acid | | 0.4–1.2 mg/g DW[4]; |
| 4,5-Di-O-caffeoylquinic acid | | 0.1–1.5 mg/g DW[4]; |
| 3-O-p-Coumaroylquinic acid | | 0.15 mg/g DW[3]; 0.4–1.9 mg/g DW[4]; |
| 5-O-p-Coumaroylquinic acid | | 0.5–1.2 mg/g DW[4]; |
| cis-5-O-p-Coumaroylquinic acid | | 0.33 mg/g DW[3]; |
| trans-5-O-p-Coumaroylquinic acid | | 0.23 mg/g DW[3]; |
| Proanthocyanidins total/sum | 23.3 mg/100 g FW[14]; | |
| Monomers | 1.44 mg/100 g FW[14]; | |
| Dimers | 10.62 mg/100 g FW[14]; | |
| Trimers | 5.63 mg/100 g FW[14]; | |
| 4–6- mers | 10.30 mg/100 g FW[14]; | |
| 7–10- mers | Nd[14]; | |
| >10- mers | Nd[14]; | |
| low molecular weight proanthocyanidins | <1 mg/kg FW[9]; | |
| low molecular weight proanthocyanidins | <1 mg/kg FW[9]; | |
| Procyanidin (PC): prodelphinidin (PD) | 100:0[9]; 100:0[11]; | |

CGE, cyanidin 3-glucoside equivalents; ChAE, chlorogenic acid equivalents; CSE, cyanidin 3-sambubioside equivalents; DW, dry weight; FW, fresh weight; GAE, gallic acid equivalents; QRE, quercetin 3-rutinoside equivalents.
1. Akbulut, Ercisli, and Tosun (2009); 2. Anton et al. (2013); 3. Barros et al. (2011); 4. Christensen, Kaack and Fretté (2008); 5. Dawidowicz, Wianowska and Baraniak (2006); 6. Kaack and Austed (1998); 7. Kaack et al. (2008); 8. Lee and Finn (2007); 9. Määttä-Riihinen et al. (2004); 10. Mikulic-Petkovsek et al. (2012); 11. Ochmian, Oszmiański and Skupień (2009); 12. Veberic et al. (2009); 13. Wibisono et al. (2009); 14. Wu et al. (2004).

21-months of storage (Kaack & Christensen, 2010). The stability of anthocyanins from elderberry was higher in the presence of glucose, but significantly lower in the presence of fructose and ascorbic acid (Sadilova, Stintzing, Kammerer, & Carle, 2009).

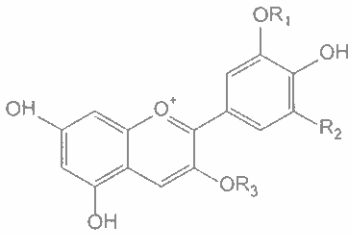
## 3. The effect of elderberry-derived compounds on the organism

In folk medicine elderberry has been used in the treatment of many diseases and ailments. Most folk tales relate to the activity of elderberry in respiratory diseases, mainly in colds and flu, but also in conditions such as burns, swelling, cuts, sprains, dislocations, skin rashes, haemorrhoids, insect bites and stings, rheumatic symptoms, toothaches, kidney and eye problems, in hepatitis and dyspepsia (Allen & Hatfield, 2004; Barnes, Anderson, & Phillipson, 2007; Duke et al. 2002; Roxas & Jurenka, 2007). Traditional medicine recommends consumption of elderberry primarily in respiratory diseases such as influenza, colds and catarrh, and as a diaphoretic, diuretic, laxative and anti-inflammatory agent. Elderberry has medicinal properties associated mainly with the presence of polyphenols, which are compounds of potential antioxidant properties (Gramza-Michałowska, Sidor, & Hes, 2011; Kmiecik et al., 2011; Kobus-Cisowska, Flaczyk, Rudzińska, & Kmiecik, 2014). They can greatly affect the course of disease processes by counteracting oxidative stress (Gollücke, Peres, Odair, & Ribeiro, 2013), in which the amount of generated reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS) exceeds the antioxidant defence capability of the organism (Xiang et al., 2013).

A diet that includes a large intake of plant materials, particularly fruits and vegetables, provides antioxidants of exogenous origin, such as polyphenols, tocopherols, carotenoids and ascorbic acid (Lee, 2013; Li, 2011), which play an important role in maintaining proper health status, acting in the prevention and treatment of many diseases (Fernandes, Faria, Calhau, de Freitas, & Mateus, 2014; Gramza-Michałowska, 2014; Norberto et al., 2013; Yahia, 2010).

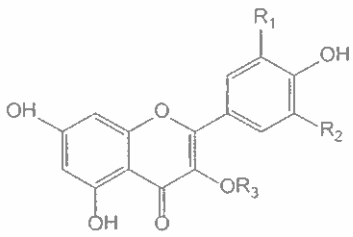
### 3.1. *Antioxidant activity*

Elderberry is considered to be a rich source of polyphenols, therefore the diet involving fruits, flowers and leaves may



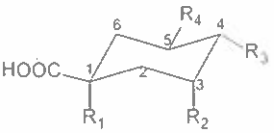

**Anthocyanins**

| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Cyanidin 3-glucoside | OH | H | glucose |
| Cyanidin 3-sambubioside | OH | H | sambubiose |
| Cyanidin 3-sambubioside-5-glucoside | OH | H | sambubiose |
| Cyanidin 3,5-diglucoside | OH | H | glucose |
| Cyanidin 3-rutinoside | OH | H | rutinose |
| Pelargonidin 3-glucoside | OH | H | glucose |
| Pelargonidin 3-sambubioside | H | H | sambubiose |
| Delphinidin 3-rutinoside | OH | OH | rutinose |

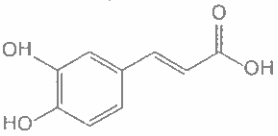



**Flavonols**

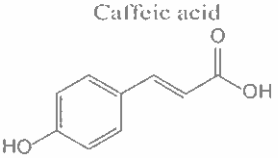
| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Quercetin 3-*O*-rutinoside | OH | H | rutinose |
| Quercetin 3-*O*-galactoside | OH | H | galactose |
| Quercetin 3-*O*-vicianoside | OH | H | vicianose |
| Quercetin 3-*O*-glucoside | OH | H | glucose |
| Quercetin 3-*O*-(6''-acetyl)galactoside | OH | H | 6-acetyl galactose |
| Quercetin 3-*O*-(6''-acetyl)glucoside | OH | H | 6-acetyl glucose |
| Kaempferol 3-*O*-rutinoside | H | H | rutinose |
| Kaempferol 3-*O*-glucoside | H | H | rutinose |
| Isorhamnetin 3-*O*-rutinoside | $OCH_3$ | H | rutinose |
| Isorhamnetin 3-*O*-glucoside | $OCH_3$ | H | glucose |
| Myricetin 3-*O*-rutinoside | OH | OH | rutinose |




Quinic acid




Caffeic acid




*p*-Coumaric acid

**Phenolic acids**

| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| 3-*O*-Caffeoylquinic acid | OH | Caffeic acid | OH | OH |
| 4-*O*-Caffeoylquinic acid | OH | OH | Caffeic acid | OH |
| 5-*O*-Caffeoylquinic acid | OH | OH | OH | Caffeic acid |
| 1,5-Di-*O*-caffeoylquinic acid | Caffeic acid | OH | OH | Caffeic acid |
| 3,5-Di-*O*-caffeoylquinic acid | OH | Caffeic acid | OH | Caffeic acid |
| 3,4-Di-*O*-caffeoylquinic acid | OH | Caffeic acid | Caffeic acid | OH |
| 4,5-Di-*O*-caffeoylquinic acid | OH | OH | Caffeic acid | Caffeic acid |
| 3-*O*-*p*-Coumaroylquinic acid | OH | *p*-Coumaric acid | OH | OH |
| 5-*O*-*p*-Coumaroylquinic acid | OH | OH | OH | *p*-Coumaric acid |

**Fig. 1 – Chemical structures of polyphenolics in fruits and flowers of elder (*Sambucus nigra*).**

constitute a potential protective agent against growth and unfavourable effects of oxidative stress in the human body. Antioxidant activity of elderberry and elderberry-derived products was confirmed during antiradical activity assays *in vitro*. Studies have shown that the antioxidant activity is exhibited by fruits, flowers and leaves of elderberry (Dawidowicz et al., 2006). In other studies the antiradical activity of aqueous extracts of elderberry flowers was determined in relation to DPPH radical, which was established at 60.2 mg ascorbic acid equivalents (AAE)/g and was intermediate in comparison with other analysed plant raw materials (Bufićová & Réblová, 2003). The extract of elderberry flowers scavenged hydroxyl radicals (HO•) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), and inhibited lipid peroxidation in linoleic acid emulsion (conjugated dienes and thiobarbituric acid reactive substances – TBARS) (Stoilova, Wilker, Stoyanova, Krastanov, & Stanchev, 2007). Nakajima, Tanaka, Seo, Yamazaki, and Saito (2004) found that elderberry had a slightly lower DPPH radical scavenging potential as compared to the most active extract of bilberry. Other studies showed comparable activity of elderberry and raspberry fruit extracts, although lower as compared to chokeberry (Viskelis, Rubinskienė, Bobinaitė, & Dambrauskienė, 2010). Another very popular method for antioxidant activity analysis is ORAC – oxygen radical absorbance capacity (Gramza-Michałowska & Korczak, 2013). Wu et al. (2004) evaluated the antioxidant potential in different fruits using the ORAC assay for hydrophilic (H-ORAC) and lipophilic (L-ORAC) compounds. Among fifteen fruit samples, the best antiradical activity was reported for chokeberry and elderberry fruits. H-ORAC values were repeatedly higher as compared to L-ORAC and were estimated at 145.0 and 1.97 μmol Trolox equivalent (TE)/g of elderberry fresh weight. The ability to scavenge the peroxyl radical ($ROO^{\bullet}$) in the ORAC assay was 5783 μmol TE/g extract and was 10.7% higher than the activity of black chokeberry. The $ROO^{\bullet}$ radical in the TRAP assay (total radical-trapping antioxidant parameter) was more effectively scavenged by the fruits of chokeberry (4051 μmol TE/g) as compared to elderberry (3230 μmol TE/g). No statistically significant difference was observed in the activity of scavenging hydroxyl radical (HO•) by the fruits of chokeberry, elderberry and blueberry.

The elderberry extracts scavenged nitric oxide radical (NO) very effectively and inhibited the peroxidation of α-linolenic acid (Denev et al., 2010). In turn, the studies on the ability to scavenge ABTS radical cation (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) showed a high activity of extracts from blueberry and elderberry, which was also confirmed using the ferric reducing ability of plasma assay (FRAP) (Jabłońska-Ryś, Zalewska-Korona, & Kalbarczyk, 2009). In other studies, elderberry fruits were subjected to extraction under different conditions. It was found that an increase in the extraction temperature significantly affected the antioxidant activity of black elderberry fruit extracts in ORAC assay, which was not confirmed in the FRAP assay (Wibisono, Zhang, Saleh, Stevenson, & Joyce, 2009).

The extract from elderberry fruits scavenged $O_2^{\bullet-}$ radical anion very effectively in the photochemiluminescence (PCL) assay (Bratu, Doroftei, Negreanu-Pirjol, Hostina, & Porta, 2012). Studies carried out by Cejpek, Malousková, Konečný, and Velíšek (2009) showed that other products from elderberry such as syrup, juice, tea and jam showed reducing ability during electrochemical measurements. Juices from coloured fruits were also subjected to the analyses in the DPPH• assay. It was found that the antioxidant potential of elderberry juice was lower than black chokeberry juice (Jakobek, Šeruga, Medvidović-Kosanović, & Novak, 2007). Data on the antioxidant activity of fruits, flowers, leaves and elderberry-derived products are presented in Table 2. Rupasinghe and Clegg (2007) determined the antioxidant activity in different wines in the FRAP assay, in which they confirmed the highest activity for cabernet, elderberry, blueberry and blackcurrant wines (>1590 mg AAE/L), while the lowest activity values were determined for ice wine, chardonnay, pear and Riesling wines (<AAE 300 mg/L). The activity of elderberry wine was dependent on the quantities of sugar and water used in their production and the degree of fineness of fruits (Garofulić, Kovačević Ganić, Galić, Dragović-Uzelac, & Savić, 2012). Other studies have reported a decrease in antiradical activity in the DPPH• assay for stored wine produced from elderberry (Schmitzer et al., 2010).

Studies evaluating the impact of elderberry constituents on the antioxidant status of the organism showed no significant effect of consumption of multifruit juice containing elderberry on the increased content of polyphenols, antioxidant potential of plasma and blood serum in the PCL assay (Böhm et al., 2004). A statistically significant increase in the antioxidant potential was observed for plasma in the FRAP assay, and for serum in FRAP and TRAP assays. In another study Netzel et al. (2005) pointed at the antioxidant effect of elderberry juice supply *in vivo*. The antioxidant potential measured by $ABTS^{\bullet+}$ and TRAP assays an hour after consuming 400 mL of juice reached the maximum values, corresponding to an increase of 17 and 28% respectively, although after 4 hours the antioxidant potential returned to the initial level. Time required to achieve the highest value for the antiradical assays was similar to the maximum concentration of total polyphenols (TP) and total anthocyanins (TA) in the plasma. The amount of anthocyanins excreted in unchanged form was independent of the dose. Anthocyanins excreted in the urine were at a comparable level (±0.04%) despite the increase of supply. Similar levels of urinary excretion of anthocyanins was estimated to be between 0.03 and 0.08% after the supply of much higher doses as reported by other authors (Bitsch, Janßen, Netzel, Straß, & Frank, 2004a; Bitsch, Netzel, Frank, Strass, & Bitsch, 2004b; Bitsch et al., 2004c; Milbury, Cao, Prior, & Blumberg, 2002; Murkovic, Mülleder, Adam, & Pfannhauser, 2001; Wu, Cao, & Prior, 2002). Anthocyanins derived from elderberry appear in the blood and urine fairly quickly and their amounts are low, which indicates their metabolism before the absorption (Frank et al., 2005). Data on the absorption of polyphenols derived from elderberry are presented in Table 3.

### 3.2. *Cardiovascular diseases*

Results of many studies suggest beneficial effects of the elderberry components on blood pressure, reduction in oxidative stress, increase in the activity of antioxidant enzymes in the blood plasma, including glutathione (GSH), and the reduction of uric acid (UA) levels (Ciocoiu, Badescu, Badulescu, & Badescu, 2012a; Ciocoiu, Badescu, Badulescu, Tutunaru, & Badescu, 2012b; Ciocoiu et al., 2010; Moroşanu, Ciocoiu, Bădescu, & Bădescu, 2011). An elevated UA level is associated with

**Table 2 – Antioxidant activity of elder (*Sambucus nigra*) plant and its preparations.**

| Sample | Sample extraction conditions | Test | Antioxidant activity | References |
|---|---|---|---|---|
| Wild grown elderberry genotypes | 10 g of fresh samples were extracted using 40 mL of 80% EtOH. The mixture was extracted (in water bath at 80 °C), kept for 20 min in inert atmosphere and filtered. Extraction of the residue was repeated under the same conditions. The filtrates were combined. | FRAP [mmol TE/100 g FW] | Genotype AR2 6.37 > genotype AR1 6.09 > genotype AR3 5.31 > genotype AR4 5.04 | Akbulut et al. (2009) |
| Medicinal plants | A fine dried powder (20 mesh; 1 g) was stirred with 50 mL of methanol at 25 °C and 150 rpm for 1 h and filtered. The residue was then extracted with one additional 50 mL portion of methanol. Extracts were combined. | DPPH [$EC_{50}$ mg TE/mL] | *Filipendula ulmaria* 0.05 > *Sambucus nigra* 0.57 > *Cytisus multiflorus* 2.02 | Barros et al. (2011) |
| | | Reducing power [$EC_{50}$ mg TE/mL] | *Filipendula ulmaria* 0.06 > *Sambucus nigra* 0.27 > *Cytisus multiflorus* 0.64 | |
| | | β-Carotene bleaching test [inhibition; $EC_{50}$ mg TE/mL] | *Filipendula ulmaria* 0.09 > *Sambucus nigra* 0.16 > *Cytisus multiflorus* 0.35 | |
| | | TBARS [inhibition peroxidation; $EC_{50}$ mg TE/mL] | *Filipendula ulmaria* 0.05 > *Sambucus nigra* 0.12 > *Cytisus multiflorus* 0.30 | |
| | | TEAP [mg AAE/g DW] | *Filipendula ulmaria* 13.7 > *Sambucus nigra* 12.2 > *Cytisus multiflorus* 9.64 | |
| Elderberry extract | Extract provided by BerryPharma AG (Leichlingen, Germany) solution 0.05 g/dL | PCL [nmol TE/μL] | 30 μL 9.241 > 20 μL 3.299 > 10 μL 0.747 > 5 μL 0.048 | Bratu et al. (2012) |
| Medicinal plants | Water extracts: 50 mL of deionized water was added to 1 g of dry ground plant material. The water temperature was 98 °C, infusion time was 20 min. | Reduction ability (FIA with amperometric detection) | Sweet balm tops > thyme tops > oregano tops > strawberry leaves > mint tops > elder flowers > raspberry leaves > nettle flowers > blackberry leaves > chamomile flowers > linden flowers > blueberry fruits > rose hip > rowan fruits > buckthorn fruits > chicory roots and tops > fennel fruits | Buřičová and Réblová (2008) |
| | | DPPH [mg AAE/g DW] | Strawberry leaves 121.6 > oregano tops 118.2 > blackberry leaves 114.8 > thyme tops109.4 > raspberry leaves 102.7 > sweet balm tops 100.00 > mint tops 72.2 > nettle flowers 65.8 > linden flowers 63.0 > rosehip 62.7 > elder flowers 60.8 > chamomile flowers 34.8 > blueberry fruits 34.2 > rowan fruits 25.0 > buckthorn fruits 24.6 > chicory roots and tops 12.4 > fennel fruits 4.2 | |
| | Ethanol extracts: 50 mL of ethanol (96%) was added to 1 g of dry ground plant material at laboratory temperature. The mixture was left in a dark place. Infusion time was 24 h. | DPPH [mg AAE/g DW] | Linden flowers 36.7 > strawberry leaves 33.6 > blackberry leaves 30.1 > thyme tops 21.4 > mint tops 21.3 > raspberry leaves 17.1 > blueberry fruits 14.9 > nettle flowers 14.3 > elder flowers 13.5 > oregano tops 11.8 > rowan fruits 9.4 > sweet balm tops 6.5 > rose hip 6.3 > chamomile flowers 5.5 > chicory roots and tops 4.0 > fennel fruits 1.3 > buckthorn fruits 0.9 | |
| Elderberry foods and supplements | – | Electrochemical activity (amperometric detection) [g AAE/kg lub g AAE/L] | Tea FT 21 > macerate FM 8.8 > juice BJ 0.71 > syrup S2 0.090 > syrup S3 0.054 > syrup S1a 0.045 > syrup S5 0.045 > syrup S4 0.041 > syrup S1b 0.040 > syrup S1c 0.033 ≥ spread BS 0.033 | Cejpek et al. (2009) |

*(continued on next page)*

**Table 2 –** *(continued)*

| Sample | Sample extraction conditions | Test | Antioxidant activity | References |
|---|---|---|---|---|
| Elderberry flowers, berries and leaves extracts | ASE 200 (Accelerated Solvent Extractor), 1 g of the flowers, berries or leaves was mixed with the neutral glass and placed into a 22 mL stainless steel extraction cell. Extraction conditions were: solvent EtOH 80%, static extraction time equals 10 min, pressure 60 bar, temperature range 20–200 °C, solvent flush volume, 60% of the extraction cell volume; purge 120 s using pressurized nitrogen (150 psi). | DPPH [inhibition %] | flowers 100 °C 94.15 > flowers 20 °C 91.95 > leaves 200 °C 84.04 > leaves 180 °C 71.74 > leaves 160 °C 69.61 > berries 100 °C 67.69 > leaves 140 °C 57.12 > leaves 120 °C 56.10 > berries 20 °C 50.25 > leaves 100 °C 48.52 > leaves 80 °C 41.34 > leaves 60 °C 35.29 > leaves 40 °C 27.18 > leaves 20 °C 16.76 | Dawidowicz et al. (2006) |
| | | β-Carotene method [antioxidant activity coefficient] | Leaves 200 °C 25.93 > leaves 180 °C 25.51 > leaves 160 °C 23.28 > leaves 140 °C 22.16 > leaves 120 °C 20.07 > leaves 100 °C 19.13 > leaves 80 °C 15.99 > leaves 60 °C 14.60 > leaves 40 °C 8.59 > berries 100 °C 6.63 > flowers 100 °C 5.79 > flowers 20 °C 5.15 > leaves 20 °C 4.25 > berries 20 °C 3.87 | |
| Berry fruits | 50 g homogenized fruits were transferred into extraction tubes and mixed with 150 mL 1% citric acid in water. Extraction was conducted on an orbital shaker 60 °C for 1 h | ORAC [μmol TE/g extract] | Elderberry 5783 ≥ blueberry 5646 > chokeberry 5165 > blackberry 4042 ≥ blackcurrant 3949 | Denev et al. (2010) |
| | | TRAP [μmol TE/g extract] | Chokeberry 4051 > elderberry 3230 > blueberry 2860 ≥ blackberry 2771 > blackcurrant 2132 | |
| | | HORAC [μmol GAE/g extract] | Blueberry 1293 ≥ elderberry 1264 ≥ chokeberry 1265 > blackcurrant 374 ≥ blackberry 834 | |
| | | TBARS [inhibition of induced lipid peroxidation nmol/mL] | Chokeberry > elderberry ≥ blackcurrant ≥ blueberry > blackberry | |
| | | NO scavenging activity time [s] | Blueberry 183 ≥ chokeberry 215 ≥ blackcurrant 280 ≥ elderberry 290 > blackberry 363 | |
| Elderberry flower extracts | 2 g of dry flowers was extracted 50% EtOH, 10–120 min | DPPH [$IC_{50}$] | Extract 90 min 6.58 > extract 120 min 6.97 > extract 60 min 8.03 > extract 40 min 11.39 > extract 30 min 11.85 > extract 20 min 14.32 > extract 10 min 13.47 | Diankov and Parlapanska (2013) |
| Elderberry fruit, elderberry wines | 5 g of sample using 20 mL 80% EtOH. The mixture was extracted with reflux for 20 min and filtered. Extraction of the residue was repeated using the same conditions. The filtrates were combined. | FRAP [mmol TE/L; mmol TE/kg] | Wine A 146.41 > fruits 135.83 > wine E > wine B > wine C > wine F > wine D | Garofulić et al. (2012) |
| Tea infusions | 15 g of ground material was boiled for 5 min in 100 mL distilled water, 5 g sucrose was dissolved | DPPH [scavenging effect %] | Elderberry leaves + carob + doum 89.72 > elderberry leaves + carob 81.12 > elderberry leaves + doum 75.9 > carob + doum 71.58 > carob 58.29 > doum 49.62 > elderberry leaves 42.7 | Hussein et al. (2011) |
| Wild edible fruits | – | ABTS [μM TE/g FW] | Dog rose 38.75 > blueberry 16.87 > elderberry 15.88 > blackberry 9.55 > rowan 5.94 ≥ wild strawberry 5.61 ≥ blackthorn 5.35 | Jabłońska-Ryś et al. (2009) |
| | | FRAP [mM Fe/100 g FW] | Dog rose 127.78 > blueberry 30.48 ≥ elderberry 29.56 > blackberry 15.17 ≥ blackthorn 14.17 ≥ wild strawberry 10.99 ≥ rowan 10.75 | |
| Fruit juices | – | DPPH [μmol TE/mL] | Chokeberry 72.44 > elderberry 62.14 > black currant 30.15 > sour cherry 12.52 > blackberry 8.75 > red raspberry 8.20 > strawberry 4.39 > sweet cherry 4.07 | Jakobek et al. (2007) |

| | | | | |
|---|---|---|---|---|
| Frozen berries, commercial juice concentrates | Commercial juice concentrates and frozen fruits were homogenized in 450 mL of 90% ethanol (0.1% $H_2SO_4$) and stirred overnight at room temperature. After centrifugation at 3000 rpm for 5 min, the supernatants were filtered and applied to a column of nonionic polymeric absorbent (Amberlite XAD-7) followed by washing with water. Anthocyanin fraction was eluted with aqueous EtOH (0.05% citric acid) | DPPH (scavenging activity %) | Trolox > bilberry>rabbiteye > blackcurrant>chokeberry > elderberry | Nakajima et al. (2004) |
| Medicinal herbs | 6 g of each herb was homogenized with 18 g of 2% metaphosphoric acid (in water) | DPPH [mmol ChAE/g FW] | Plantain leaf ≥ thyme flower > barberry flower > linden flower > rowan berry > coltsfoot flower > milfoil flower > elder berry | Pogačnik and Poklar Ulrih (2011) |
| | | Chemiluminescence [mmol ChAE/L FW; mmol ChAE/g FW] | Barberry ≥ thyme flower > linden flower ≥ plantain leaf > milfoil flower > elder berry > rowan berry ≥ coltsfoot flower | |
| | 6 g of each herb was homogenized with 18 g of 2% metaphosphoric acid (in MeOH) | Chemiluminescence [mmol ChAE/L FW] | Linden flower > thyme flower ≥ barberry > rowan berry > plantain leaf > milfoil flower > coltsfoot flower > elder berry | |
| | 6 g of dried herb was poured over by 18 g of boiling water and let 5 min to extract | Chemiluminescence [mmol ChAE/g FW] | Barberry > milfoil flower ≥ coltsfoot flower > rowan berry ≥ thyme flower > linden flower > elder berry ≥ plantain leaf | |
| Fruit wines | – | FRAP [mg AAE/L] | Cabernet (grapes, red wine) 2447 > elderberry 1911 > blueberry 1655 > black currant 1595 > cherry 1102 > raspberry 1067 > cranberry 875 > plum 618 > apple 404 > peach 395 > ice wine (grapes) 383 > chardonnay (grapes, white wine) 276 > pear 271 > riesling (grapes) 219 | Rupasinghe and Clegg (2007) |
| Elderberry must and wine | – | DPPH [mM TE/L] | Elderberry wine 9.95 > elderberry must 8.15 > aged wine 6.13 | Schmitzer et al. (2010) |
| Medicinal plants and fruits from two localities: Tuzia Canton (TC) and Brcko District (BD) | Medicinal plants were dried at 105 °C, chopped to size of 2–8 mm. 1 g sample was transferred into a 100 mL flask and supplemented with distilled water at a temperature of 95 °C. Crude extracts from berries were prepared by weighing fresh fruits (100 g), mixed with 100 mL of distilled water or appropriate solvent and then milled using a commercial mini-processor. | FRAP [μmol Fe/L] | Lemon balm TC 21070 > lemon balm BD 20443 > sage TC 17520 > dog rose TC 16784 > dog rose BD 16761 > sage BD 16752 > elder TC 14734 > hawthorn TC 14125 > mountain germander BD 10975 > elder BD 8906 > silver birch TC 8247 > hawthorn BD 7234 > milfoil TC 6179 > silver birch BD 5897 > milfoil BD 5397 > small leave lime BD 4629 > Mountain germander TC 3961 > small leave lime TC 2538 > pot marigold TC 2097 > pot marigold BD 1538 | Srabovic et al. (2011) |
| Elder flower extract | Extract provided by GfN GmbH & Selco GmbH, Germany. The extract contained min. 0.5% rutin and min. 1.0% tannic acid | DPPH ($IC_{50}$ μg/mL) | Elder flower 0.152 > BHA 1.120 > BHT 4.407 > rutin 14.65 | Stoilova et al. (2007) |
| | | Hydroxyl radical scavenging activity by deoxyribose assay ($IC_{50}$) | Elder flower 0.0122 > rutin 0.505 > quercetin 4.61 | |
| | | Conjugated dienes formation [reduction peroxidation %] | BHT (C = 0.01%) 58.69 > elder flower (C = 0.0025%) 57,6 > elder flower (C = 0.0025%) 45.65 | |
| | | TBARS [reduction peroxidation %] | BHT (C = 0.01%) 76.47 > elder flower (C = 0.01%) 76.42 > elder flower (C = 0.05%) 74.70 | |

(continued on next page)

**Table 2** – (*continued*)

| Sample | Sample extraction conditions | Test | Antioxidant activity | References |
|---|---|---|---|---|
| Berry fruits | 5 g of ground berries with 25 mL of MeOH at ambient temperature for 2 h with constant shaking. The solution was filtered, and the residue was repeatedly extracted with 20 mL of MeOH for 2 h. Finally extracts were combined. | DPPH [inhibition %] | Chokeberry 'Cleata' ≥ chokeberry 'Aron' ≥ chokeberry 'Viking' ≥ raspberry 'Bristol' > raspberry 'Meeker' > elderberry 'Lacimiata' > raspberry 'Poranna Rosa' ≥ elderberry 'Aurea' | Viskelis et al. (2010) |
| Different fruit extracts | Samples for extraction were prepared by mixing the freeze-dried sample powder (4 g) with an equal weight of diatomaceous earth, which was then packed into the (34 mL) extraction cells. Extraction conditions were: 3 static cycles of either 10 min at 40 °C or 2 min at 100 °C, using 95% MeOH, 90 s purging of $N_2$ and 60% flush volume. | FRAP [μmol TE/g FW] | Elderberry 100 °C ≥ elderberry 40 °C > sour cherry 100 °C ≥ sour cherry 40 °C > 'Red Delicious' apple puree 100 °C > 'Red Delicious' apple puree 40 °C > swede leaf 100 °C > turnip leaf 100 °C > turnip leaf 40 °C ≥ swede leaf 40 °C | Wibisono et al. (2009) |
| | | ORAC [μmol TE/g FW] | Elderberry 40 °C > elderberry 100 °C > 'Red Delicious' apple puree 100 °C > 'Red Delicious' apple puree 40 °C > sour cherry 40 °C ≥ turnip leaf 40 °C > sour cherry 100 °C > swede leaf 40 °C > swede leaf 100 °C ≥ turnip leaf 100 °C | |
| Berry fruits | ASE 200 extraction, 1 g of each sample was mixed with 5 g of sea sand, transferred to a 22 mL extraction cell, and extracted with hexane/dichloromethane (1:1) followed by acetone/water/acetic acid (70:29.5:0.5; Ace/$H_2O$/AcAc) extraction. The extracts from hexane/dichloromethane were used to measure lipophilic $ORAC_H$. Ace/$H_2O$/AcAc extracts were used to measure the hydrophilic $ORAC_H$. | L-ORAC [μmol TE/g FW] | Chokeberry 2.42 > elderberry 1.97 > red currant 1.27 > blackcurrant 'Titania' 1.15 > blackcurrant 'Ben Alder' 0.84 > blackcurrant 'Ben Nevis' 0.75 = blackcurrant 'Ban Tirran' 0.75 > blackcurrant 'Ben Lomond' 0.68 = blackcurrant 'Ukraine' 0.68 > gooseberry 'Marigold' 0.45 > gooseberry 'Leveller' 0.43 > gooseberry 'Careless' 0.35 > gooseberry 'Dan's Mistake' 0.28 > gooseberry 'Whinham' 0.15 = gooseberry 'Lancashine' 0.15 | Wu et al. (2004) |
| | | H-ORAC [μmol TE/g FW] | Chokeberry 158.2 > elderberry 145.0 > blackcurrant 'Ben Alder' 100.6 > blackcurrant 'Ben Lomond' 92.3 > blackcurrant 'Ben Nevis' 90.6 > blackcurrant 'Ban Tirran' 86.6 > blackcurrant 'Ukraine' 53.7 > blackcurrant 'Titania' 49.0 > gooseberry 'Lancashine' 41.3 > gooseberry 'Whinham' 39.2 >gooseberry 'Dan's Mistake' 37.1 > gooseberry 'Marigold' 33.7 > red currant 32.6 > gooseberry 'Leveller' 26.4 > gooseberry 'Careless' 20.4 | |

AAE, ascorbic acid equivalents; ChAE, chlorogenic acid equivalents; DW, dry weight; FW, fresh weight; GAE, gallic acid equivalents; TE, Trolox equivalents.

Table 3 – Data on the absorption of polyphenols derived from elderberry.

| Species | n | Source | Dose (total intake) | Duration | Urinary excretion | $C_{max}$ (maximal serum/plasma concentration) | $t_{max}$ (time to reach $C_{max}$) | Reference |
|---|---|---|---|---|---|---|---|---|
| Humans | 6 | 30 mL elderberry extract | 147.3 mg | 7 h | 0.37% | – | – | Bitsch et al. (2004a) |
| Humans | 7: 6F, 1M | 150 mL of concentrated elderberry juice | 3569 mg | 5 h | 0.053% | – | – | Bitsch et al. (2004c) |
| Humans | 4F | 12 g elderberry extract dissolved in 500 mL water | 720 mg | 24 h | 0.055% | 97.4 nmol/L | 1.19 h | Cao et al. (2001) |
| Humans | 6 | 30 mL elderberry extract | 278 mg | 7 h | 0.39% | – | – | Frank et al. (2007) |
| | | 200 mL elderberry extract | 1852 mg | 7 h | 0.27% | | | |
| Humans | 7 | 150 mL of concentrated elderberry juice | 3570 mg | 24 h | 0.06% | – | – | Frank et al. (2005) |
| Humans | 4F | 12 g elderberry extract dissolved in 500 mL water | 720 mg | 24 h | 0.055% | 97.4 nmol/L | 1.19 h | Milbury et al. (2002) |
| Humans | – | Spray dried elderberry juice | 180 mg | – | – | 35 ng/mL | 1 h | Murkovic, Adam, and Pfannhauser (2000) |
| Humans | 7: 4M, 3F | 4 g spray-dried elderberry juice (equivalent 50 mL fresh juice) containing 12.5% anthocyanins | 500 mg | 6 h | 0.05% | – | – | Murkovic et al. (2001) |
| Humans | 16: 8M, 8F | 11 g elderberry concentrate (equivalent 235 mL fresh juice) | 1900 mg | 6 h | 0.003–0.012% | – | – | Mülleder, Murkovic, and Pfannhauser (2002) |
| Humans | 8 | 200 mL elderberry juice | 361 mg | 7 h | 0.033% | – | – | Netzel et al. (2005) |
| | | 300 mL elderberry juice | 541 mg | 7 h | 0.038% | | | |
| | | 400 mL elderberry juice | 722 mg | 7 h | 0.040% | | | |
| Humans | 4 F | 12 g elderberry extract blended with 500 mL water | 720 mg | 24 h | 0.077% | – | – | Wu et al. (2002) |
| Pigs | 9 | Freeze dried powder | 228 μg/kg body weight | 24 h | 0.131% | – | – | Wu et al. (2005) |

F, female; M, male.

hypertension accompanied with advanced vascular changes (Perlstein et al., 2006; Takayama, Kawamoto, Kusunoki, Abe, & Onji, 2012). A positive aspect of uric acid activity is associated with antioxidant activity, which is exhibited at a sufficiently high concentration. Uric acid has the ability to scavenge ROS and chelate transition metal such as Cu and Fe, which makes it an important compound for living organisms (Li, 2011). The results of Moroşanu et al. (2011) indicate higher total antioxidant activity (TAA) values and a more favourable lipid profile in the serum of hypertensive rats fed with food containing polyphenols from elderberry. A decrease in total cholesterol and triacylglycerol contents as well as LDL lipoproteins and malonic dialdehyde content was confirmed (Ogino & Wang, 2007). The above-mentioned results were not confirmed in the group of healthy volunteers (Ciocoiu et al., 2012a, 2012b). Frank et al. (2002) established that the administration of elderberry extract in the amount corresponding to a daily intake does not significantly affect the lipid profile in healthy rats.

The protective effect of polyphenols towards tocopherols during the oxidation of $Cu^{2+}$-catalysed LDL in the presence of elderberry extract was shown by Abuja, Murkovic, and Pfannhauser (1998). The addition of elderberry extract caused a delay in the oxidation of LDL, although depending on the reaction time, in which the extract was added, in some cases the prooxidative effect was observed. Histological examination of kidneys and heart muscle of rats administered black elderberry specimen proved its protective effect (Ciocoiu et al., 2010; Moroşanu et al., 2011).

One of mechanisms responsible for the protective effect of elderberry against vascular diseases is the ability of polyphenols to penetrate into endothelial cells. Incubation of endothelial cells in the presence of polyphenols resulted in its occurrence in cell structures (Youdim, Martin, & Joseph, 2000). Despite their comparable amounts in the initial extracts anthocyanins used in the studies to a different extent penetrated into endothelial cells with structure-bound polyphenols, which however had a significantly longer life when subjected to the action of ROS in the form of $H_2O_2$.

### 3.3. *Diabetes and obesity*

Diabetes is a disease occurring along with other components of the metabolic syndrome such as hypertension, dyslipidaemia and obesity (Eckel, Grundy, & Zimmet, 2005). Diabetes leads to pathological changes in the vascular system, resulting in damages to the internal organs. Treatment of diabetes is mainly based on the change of lifestyle, proper diet and physical exercise (Fowler, 2010; Regula & Gramza-Michałowska, 2013; Song et al., 2014). The diet appropriate in the treatment of diabetes should include increased contents of foods rich in polyphenols, such as fruits and their preserves. Results showed a reduction in the increase of glycaemia in diabetic rats administered with elderberry extract (Ciocoiu et al., 2012a, 2012b), as well as maintaining the glycaemic index at the same level from 10th to 16th week from the disease induction (Badescu, Badulescu, Badescu, & Ciocoiu, 2012). The administration of elderberry extract significantly reduced the glycosylation rate of haemoglobin (HbA1c) in diabetic rats, and the decreased level indicates a reduced risk of complications related to the course of the disease. Elderberry may reduce the risk of concomitant diseases in diabetes by reducing the concentration of fibrinogen, increasing antioxidant protection of the organism, reducing lipid oxidation processes and improving the lipid profile of the organism. Type 2 diabetes is associated with an impaired secretion and action of insulin. In the initial stage of diabetes, insulin resistance, i.e. an impaired response of tissues to insulin, is observed. An organism with insulin resistance, in order to maintain normal glucose levels, increases the amount of secreted insulin by β-cells of the pancreas (hyperinsulinaemia). Prolonged hyperinsulinaemia leads to the loss of insulin production by β-cells of the pancreas, resulting in the development of diabetes (Kashyap & DeFronzo, 2007). The role of elderberry in the prevention and treatment of diabetes may involve the increase of muscle glucose uptake (GU) metabolism and increase in the secretion of insulin (Bhattacharya et al., 2013).

It has been found that the ability to stimulate GU of selected polyphenolic compounds occurring and related to the compounds present in the elderberry flowers (5-O-cafeoylquinic acid, naringenin, kaempferol, ferulic acid, caffeic acid) varied depending on the concentration. An aqueous extract of elderberry flower (1 g/L) increased GU (70%), glucose oxidation (50%) and glycogenesis (70%) *in vitro* in abdominal muscles of mice (Gray, Abel-Wahab, & Flatt, 2000). Secretion of insulin through clonal pancreatic β-cells improved under the activity of the extract and the secretion increase was followed by its increased concentration. Selected compounds in elderberry flowers such as lectin, rutin, lupeol, β-sitosterol, tannic acid and choline at a concentration ranging from 0.5 to 50 μg/mL did not improve insulin secretion (Swanston-Flatt, Day, Flatt, Gould, & Bailey, 1998).

Obesity is one of the main factors that plays a role in the development of insulin resistance (Golay & Ybarra, 2005). Compounds present in elderberry flower extract decreased fat accumulation (FAc-fat accumulation) in the model system *in vivo* in the form of the *Caenorhabditis elegans* nematode. A significant reduction in FAc was observed at a concentration of 200 μg/mL of the extract (Bhattacharya et al., 2013; Christensen et al., 2010). In other studies, the diet of volunteers was enriched in specimens of elderberry, and a decrease in body weight and improvement of body mass index (BMI) indicators were reported. The fat content in the organisms of studied individuals was reduced by 1% on the average, the physical and mental well-being of diet-subjected individuals was also improved (Chrubasik et al., 2008).

### 3.4. *Antiviral, antibacterial and antifungal activity*

Elderberry is a well-known plant which supports treatment of influenza and colds, exhibiting antiviral and antibacterial activity (Kong, 2009). Elderberry flower extract at a concentration of 252 μg/mL inhibited the influenza A virus (H1N1)-induced Madin–Darby canine kidney (MDCK) cell infection (Roschek, Fink, McMichael, Li, & Alberte, 2009). The mechanism of antiviral activity of analysed polyphenolic compounds was based on direct binding to the H1N1 virus, resulting in the prevention of virus penetration into cells, inhibiting the infection *in vitro*. Studies on antiviral activity against H1N1 virus and cytotoxicity of elderberry juice concentrate with the use of MDCK cells have also been conducted (Kinoshita, Hayashi,

Katayama, Hayashi, & Obata, 2012). The results showed the effect of the juice in the early stages of replication, binding and viral penetration into cells. Monitoring of body weight in rats infected with the H1N1 virus indicated a protective role of elderberry juice concentrate against weight loss in the course of the disease. Moreover, elderberry juice concentrate stimulated the systemic and local immune response. It was found that the most active fraction was the high molecular fraction (HM), and the highest replication inhibition of viruses and stimulation for the production of antibodies were caused by HM II fraction, consisting mainly of acidic polysaccharides. The medium molecular weight fraction rich in polyphenols (288 mg/g) did not show high activity.

Krawitz et al. (2011) studied the antibacterial and antiviral activities of *Rubini* – commercial product of elderberry extract, and found the inhibition of propagation of influenza viruses A and B. In other studies it was found that the extract from elderberry bark showed strong inhibitory properties against FIV (feline immunodeficiency virus) in CrFK (Crandell feline kidney cells). Phenolic compounds in the form of flavonoids present in elderberry extract also exhibited the ability to block the penetration and infection of GHOST cells by HIV-1 virus (Fink, Roschek, & Alberte, 2009). Antibacterial activity of *Rubini* extract was also demonstrated in relation to gram-positive *Streptococcus pyogenes*, *Streptococcus* group G and *Streptococcus* group C, and gram-negative *Branhamella catarrhalis* bacteria causing frequent infections of the upper respiratory tract (Krawitz et al., 2011). All of the analysed extract concentrations exhibited an inhibitory effect on bacterial growth and their addition at 10 and 20%, respectively, inhibited bacterial growth by 70 and 99%. In other studies it was found that infusion from elderberry leaves exhibited an inhibitory effect on growth of bacteria (*Bacillus subtilis*, *Bacillus megaterium*, *Escherichia coli* and *Staphylococcus aureus*) and yeasts (*Debaryomyces hansenii*, *Zygosaccharomyces rouxii*, *Rhodotorula rubra*, *Candida shehatae* and *Candida tropicalis*) (Hussein, Shedeed, Abdel-Kalek, & El-Din, 2011).

Elderberry extracts exhibited antibacterial properties in relation to nosocomial pathogens, including methicillin-resistant *S. aureus* (Hearst et al., 2010). The antimicrobial activity of flower extracts was higher as compared to fruit extracts. Leaf extracts had a moderate activity against *Bacillus cereus* and *Serratia marcescens*, but did not inhibit the development of any key nosocomial pathogens. A different effect was reported by Pehlivan Karakaş, Yildirim, and Türker (2012), who showed no inhibitory activity of extracts of elderberry leaves and fruits against any tested pathogenic bacteria.

### 3.5. *Impact on the immune system*

Elderberry has the potential to regulate defence mechanisms both in healthy organisms and in the case of disease. It was found that the extract of elderberry stimulated the production of proinflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α (tumour necrosis factor) as well as anti-inflammatory cytokine IL-10 (Barak, Birkenfeld, Halperin, & Kalickman, 2002). Depending on the type of the extract (leaf, flower, solvent type) biosynthesis of IL-1α, IL-1β and TNFα or its inhibition was observed (Yeşilada et al., 1997). Elderberry extract increased the production of IFN-β interferon associated with upregulation of TLR-3 (toll-like receptor 3) and changes in the amount of produced IL = 12, IL = 6, IL-1β cytokines and TNF-α by *Lactobacillus acidophilus* in murine derived dendritic cells (Frøkiær et al., 2012). Elderberry extract increased the production of TNF-α and IFN-γ in healthy and diabetic rats (Groza, Ciocoiu, Bădescu, Bădulescu, & Bădescu, 2010). The level of IL-1β responsible for long-term inflammation in chronic diseases, leading to internal organ damage, decreased by more than 50% under the influence of elderberry derived polyphenols (Ciocoiu et al., 2012a, 2012b). In other research elderberry polyphenols increased the capability of immune defence in diabetic rats by increasing the lymphocyte number (Groza, Jitaru, Bădescu, Ciocoiu, & Bădescu, 2011).

### 3.6. *Antitumour activity*

Studies on the anticancer properties of elderberry have not confirmed any significant interaction. It was found that elderberry fruit and leaf extracts moderately inhibited tumour growth in potato disk tumour induction assay (Pehlivan Karakaş et al., 2012), and leukaemia (Goun et al., 2002). Thole et al. (2006) determined antitumour activity of various fractions of elderberry extract and found that polyphenolics (e.g., quercetin, proanthocyanidins) and probably sesquiterpenes, phytosterols as well as iridoid glycoside monoterpenes are responsible for the above-mentioned activity.

### 3.7. *Protection against UV radiation*

Polyphenols have the ability to protect against UV radiation and its negative consequences (Nichols & Katiyar, 2010). The extract from elderberry fruits was used to produce an emulsion that protects against solar radiation, which was characterized by the favourable value of sun protection factor, SPF = 9.88, UVA protection factor, PF-UVA = 5.18, UVA/UVB = 0.73; critical wavelength, λc = 376.5 nm and photostability expressed in % SPF = 87.07 and % PF-UVA = 92.05. The effectiveness of this preparation was higher as compared to filters used in commercial products available on the market; additionally, synergism of elderberry extract with extracts of *Helichrysum arenarium* and *Crataegus monogyna* was also demonstrated (Jarzycka, Lewińska, Gancarz, & Wilk, 2013). Elderberry flowers are potential sources of active substances, which in contrast to the antioxidants commonly added to cosmetics, are not subjected to degradation under the influence of UVR and exhibit a high biological activity (Chen, Hu, & Wang, 2012).

### 3.8. *Laxative and diuretic activity*

Clinical trials on the effect of plant mixtures with medicinal properties indicate a laxative effect and safety when using the infusion from elderberry flowers (Picon et al., 2010). Consumption of the mixture prepared from dried fruits of green anises (*Pimpinella anisum*), fennel (*Foeniculum vulgare*), sabugueiro flower (*S. nigra*) and senna flower (*Cassia angustifolia*) leads to an increase in the daily number of defecations, and subjective feeling of improved intestinal function. Other studies have shown that extracts from five plants considered as herbal diuretics, involving the extract of elderberry's flowers, caused increased urine excretion in rats. Aqueous extracts of elderberry (50 mg/

kg) acted similar to diuretic properties of hydrochlorothiazide as a reference substance. Moreover, elderberry extract caused increased sodium excretion in the urine (Beaux, Fleurentin, & Mortier, 1999).

## 4. Side effects and contraindications to elderberry consumption

Elderberry is not a toxic plant; however, its leaves, stems, bark, roots, flowers and unripe fruits contain cyanogenic glycosides, mainly sambunigrin, prunasin, holocain and zierin, which are converted to hydrogen cyanide during digestion (Jensen & Nielsen, 1973). The consumption of immature plants or high quantities of fruits may cause nausea, vomiting and diarrhoea, but elderberry is most widely consumed in the processed form. Thermal processing of raw material leads to the decay of sambunigrin, so the consumption of fruits subjected to heating processes does not cause symptoms of poisoning (Williamson, Driver, & Baxter, 2009). There was no interaction between elderberry, food components and other medicinal plants. However, it is worth remembering that medicinal plants contain many components which can act in the same way as conventional medicines or counteract their activity, therefore they cannot be considered as completely safe. There are no clinical trials on the interaction of elderberry components with medicines. Only concerns associated with the use of elderberry together with antidiabetic drugs, morphine, phenobarbital, diuretics, and immunoactive drugs are considered (Barnes et al., 2007; NCCAM, 2012). Förster-Waldl et al. (2003) determined the presence of a homologue of RIP (ribosomal inactivating proteins) which may cause allergic symptoms after inhalation (conjunctivitis and asthma) in a selected group of patients. Due to the lack of sufficient information on its toxicity, it is recommended to avoid eating of elderberry by pregnant and lactating women as well as children and adolescents under 18 years of age (EMA/HMPC European Medicines Agency, 2012).

Analysis of the results from studies evaluating the effects of anthocyanin mixture doses (reaching 9 g/kg of body mass) extracted from currants, blueberries or elderberries does not confirm adverse effects observed in experimental animals. The acute oral toxicity values for the anthocyanin mixture were determined to be 25 g/kg in mice and 20 g/kg of body weight in rats. Tested anthocyanins did not cause toxicity in three different doses at 1.5, 3, or 9 g/kg month/day in three consecutive generations (EFSA, 2013, Pourrat, Bastide, Doner, & Tronche, 1967).

## 5. Conclusions

Plants are rich sources of diverse bioactive compounds with high antioxidant potential, significantly affecting the health status of consumers. Results from many studies point to the beneficial effect of consumption of elderberry (*S. nigra*) preparations. Their antioxidant and protective potential has been established in respiratory ailments, colds, cardiovascular diseases, diabetes and obesity. Moreover, a significant impact on the immune system, antiviral and antibacterial activity, as well as protection against UV radiation has been confirmed. Despite the promising results, additional studies are needed to understand interactions with other compounds that may affect the activity of elderberry components. So far, literature sources have not provided any answers to questions concerning the interaction mechanism of elderberry components, their stability during storage and usage in complex systems, e.g. in food.

## Conflict of interest

The authors declare no conflict of interest.

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# Elderberry Supplementation Reduces Cold Duration and Symptoms in Air-Travellers: A Randomized, Double-Blind Placebo-Controlled Clinical Trial

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**Abstract:** Intercontinental air travel can be stressful, especially for respiratory health. Elderberries have been used traditionally, and in some observational and clinical studies, as supportive agents against the common cold and influenza. This randomized, double-blind placebo-controlled clinical trial of 312 economy class passengers travelling from Australia to an overseas destination aimed to investigate if a standardised membrane filtered elderberry (*Sambucus nigra* L.) extract has beneficial effects on physical, especially respiratory, and mental health. Cold episodes, cold duration and symptoms were noted in a daily diary and assessed using the Jackson score. Participants also completed three surveys containing questions regarding upper respiratory symptoms (WURSS-21) and quality of life (SF-12) at baseline, just before travel and at 4-days after travel. Most cold episodes occurred in the placebo group (17 *vs.* 12), however the difference was not significant ($p = 0.4$). Placebo group participants had a significantly longer duration of cold episode days (117 *vs.* 57, $p = 0.02$) and the average symptom score over these days was also significantly higher (583 *vs.* 247, $p = 0.05$). These data suggest a significant reduction of cold duration and severity in air travelers. More research is warranted to confirm this effect and to evaluate elderberry's physical and mental health benefits.

**Keywords:** elderberry; travel; cold symptoms; clinical trial; nutritional supplements; complementary medicines; sambucus; physical health

## 1. Introduction

Air travel, especially on intercontinental flights can be stressful, thus putting extra strain on passenger's physical and psychological health [1]. Studies have shown that passengers' well-being is influenced by the cabin environment such as cabin ozone concentration and oxygen pressure, motion or vibration and oil additives used in aircraft engines [2,3]. Fatigue, impairment of immunity as well as increased stress and mental changes has been reported during and after long-distance flights [4–6]. A recent review of medical in-flight events lists respiratory symptoms among the most common medical complaints reported [7]. Not surprisingly, many studies have investigated the occurrence of nasal dryness and the increased risk of developing upper respiratory disorders such as allergic rhinitis and attracting virus or bacteria induced respiratory infections during long-haul air travel [8,9]. Moreover, an added risk of spreading respiratory diseases, including influenza, aboard commercial flights exists [10].

Recently, for the first time a herbal medicine, echinacea, was trialled in travellers and showed protective effect against the development of respiratory symptoms during travel involving long-haul

flights [11]. Following travellers return from overseas, participants using echinacea displayed a lower respiratory symptom score and the overall percentage of participants affected by respiratory disease symptoms was marginally lower in the Echinacea group compared to placebo.

However, there are other herbs that have traditionally been used to treat respiratory symptoms and aid in the recuperation from a cold. Black elderberries for example, are well known to be supportive agents against common cold and flu like symptoms and have been used for centuries [12]. Interestingly, a non-travel related clinical trial just revealed that a combination of echinacea herb and root extract supplemented with elderberry (*Sambucus nigra* L.) can be as effective as the conventional antiviral medicine oseltamivir for the early treatment of influenza [13].

Elderberries have shown antibacterial [14] and antiviral activities in *in vitro* [15]. Two clinical trials using a liquid elderberry extract (Sambucol®, Israel) showed a reduction in symptoms and duration of influenza infection [16]. A pilot trial with elderberry extract lozenges (HerbalScience, Singapore) also confirmed a beneficial effect on severity and duration of cold and flu like symptoms [17].

In recent times, elderberry has gained popularity in research and the wider community due to its reported antioxidant [18], antidiabetic [19], anti-inflammatory and immune-modulating [20], as well as antidepressant [21] properties. The berries are dark violet-black drupes which grow in clusters and owe their colour to the anthocyanins; a group of phenolic compounds which, amongst flavonoids, are abundant in elderberries and considered the active constituents of the fruits [22]. However, elderberries also contain a variety of nutrients ranging from various vitamins (A, $B_1$, $B_2$, $B_6$, $B_9$, C and E), trace elements such as Cu, Zn, Fe and minerals such as K, Ca and Mg to phytochemicals such as carotenoids, phytosterols and polyphenols. These additional constituents and activities make elderberries a likely candidate for beneficial nutritional and/or medical supplementation not only for respiratory, but also for cardiovascular and mental health, all of which may be affected during travel.

Given that elderberry supplementation has never been investigated so far for its possible beneficial effects in air-travelers, we conducted a randomized controlled trial aimed at identifying whether capsules containing a proprietary membrane filtered Elderberry extract (Iprona, Italy), standardized to polyphenols, are effective in preventing respiratory symptoms, but also if they positively impact on the overall physical and mental health of travelers when using long-haul, commercial flights as means of transport.

## 2. Experimental Section

### 2.1. Study Participants and Randomisation

Participants were recruited through travel agencies; radio, newspaper and TV advertisements, and emails circulated to all staff and students at a university on the Gold Coast, Australia. All participants gave written informed consent before participating in the study. Volunteers were included if they were a minimum of 18 years of age and in good general health. Volunteers were excluded if they were currently in another clinical trial or were in one less than 30 days ago, had a known plant allergy, were suffering from respiratory diseases (e.g., asthma, chronic obstructive pulmonary disease), had any other condition that could compromise the study or the participants health (e.g., autoimmune disease, cystic fibrosis), had received flu vaccination less than 10 days of starting the trial, were lactating, pregnant or planning to become pregnant or were on regular treatment with antibiotics, corticosteroids, antihistamines, antivirals, non-steroidal anti-inflammatory drugs, anticancer drugs and immune-suppressants.

Three hundred and twenty five volunteers met the inclusion criteria and were randomly assigned to trial capsules. The random allocation sequence provided by an independent consultant was computer generated using a randomisation plan from www.randomization.com with randomisation in blocks of 10. A list of consecutive study numbers was generated. Treatment groups were allocated by trial staff, but the allocation was concealed by assigning each participant with a unique number. Participants, chief investigators and trial staff were blinded to group allocation.

### 2.2. Study Design

A randomised, double-blind placebo controlled clinical trial was conducted between April 2013 and December 2014 in Australia with economy class passengers travelling to an overseas destination, on a minimum of 7 h flight, *i.e.*, Australia-Asia, less than a 12-h stopover and a minimum of four days stay at the destination.

The clinical trial received ethical approval from the institutional Human Research Ethics Committee (PHM/08/12/HREC, January 2013) and was registered with the Australian New Zealand Clinical Trials Registry http://www.anzctr.org.au (ACTRN12612001301853).

Figure 1 outlines the study design. For all participants treatment would commence 10 days before flying overseas and would be completed five days after arriving at the travel destination. The actual treatment time varied between participants depending on their actual travel duration ranging from 15 to 16 days.

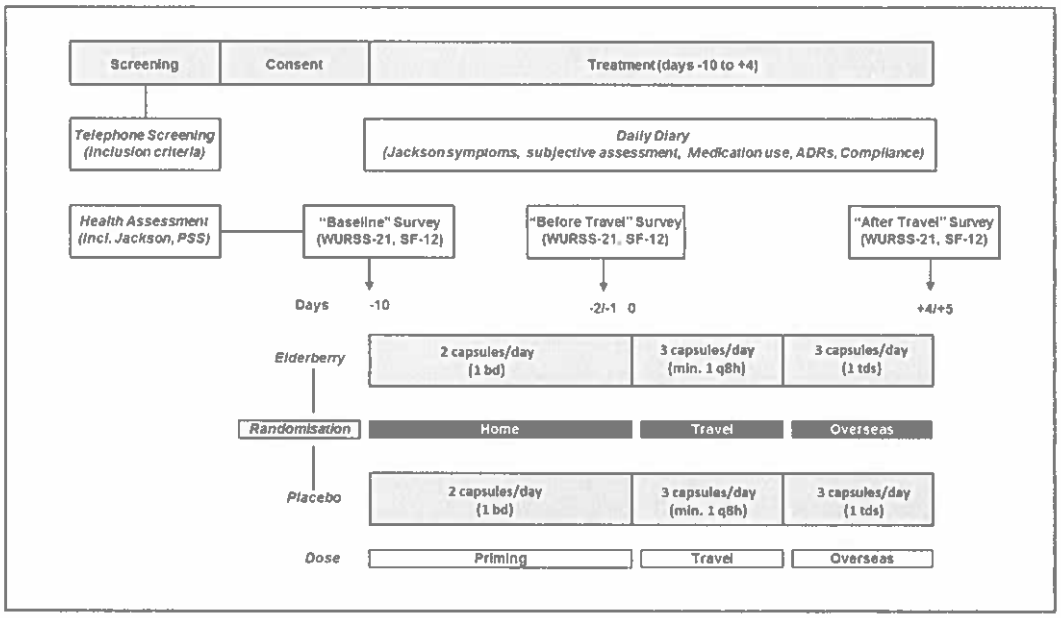



**Figure 1.** Study design.

Participants completed surveys at baseline (−10 days), before travel (−2 days) and after travel (+4/5 days), as well as recording cold symptoms in a daily diary. The health assessment included the Perceived Stress Scale (PSS). Surveys included assessments of general well-being (SF-12 acute) and quality of life related to respiratory symptoms (Wisconsin Upper Respiratory Symptom Survey, WURSS-21) (Figure 1). Cold diagnosis was assessed by measuring the Jackson Score.

### 2.3. Elderberry Supplementation

The elderberry extract used in this trial is a proprietary membrane filtered elderberry (*Sambucus nigra* L., Haschberg variety, Steiermark region in Austria) extract produced by Iprona AG, Lana (BZ), Italy, under their BerryPharma® brand, and has been shown to possess antimicrobial activity against human respiratory bacterial pathogens (Gram-positive bacteria of *Streptococcus pyogenes* and group C and G Streptococci, and the Gram-negative bacterium *Branhamella catarrhalis*), and also displays an inhibitory effect on the propagation of human pathogenic influenza viruses [14].

This extract is currently used in the production of various products marketed in Asia, the US and Europe. The elderberry capsules used in this trial were produced by Plantafood in Germany in

accordance with the principles and guidelines of Good Manufacturing Practice. They contain: 300 mg of elderberry extract (22% polyphenols (*i.e.*, quercetin and its glycosides, rutin), 15% anthocyanins (*i.e.*, cyanidin and pelargonidin glycosides) and 150 mg of rice flour. The 300 mg elderberry extract also contain several mineral, trace elements and vitamins including relatively high levels of magnesium 1.19 mg (Mg: 3.97 mg/g).

Placebo capsules were manufactured to match the elderberry capsules in size, excipients and appearance. Capsules were supplied in identical blister packs with identical labelling. Labelling only identified the participant number.

Travellers took elderberry capsules or placebo from 10 days before travel (2 capsules/day, priming dose (−10 to −2 days) and from 1 day before leaving home until 4/5 days after arriving at the destination (3 capsules/day, overseas dose (−1 to +5 days) (Figure 1). The dose selection was based on dosages used by popular elderberry products which range from 650 mg to 1500 mg per day). The trial dose used here is similar with participants required to take two capsules per day (600 mg) during the priming phase (before travel, −10 until −2 days) and three capsules per day (900 mg) while travelling and overseas (−1 until +4 days).

Compliance was assessed by calculating the percentage of capsules taken against total capsules expected to be taken during the treatment period.

### *2.4. Clinical Outcome Measurements and Statistical Analysis*

Jackson Score: Over the entire study period, all participants maintained a diary. This was to record cold symptoms, as well as additional health issues or disease symptoms and additional medication taken. The diary helped participants with recalling information when completing the surveys and allowed researchers to identify possible inconsistencies in data documentation.

The main question the participant answered in the diary, "Do you believe you have a cold today?" was answered yes or no. During acute colds, the symptoms "headache", "chilliness", "sneezing", "nasal obstruction", "nasal discharge", "sore throat", "cough", and "malaise" were rated on a 4-point Likert scale with 0 or no entry = absence, 1 = mild, 2 = moderate, and 3 = severe symptoms. In addition, the participant indicated in the diary the daily intake of concomitant medication and/or therapy. This matrix was based on the work by Jackson and colleagues, who described the clinical features and symptoms of a virally induced common cold [23]. Their definition is currently accepted as the most valid method for differentiating a cold from isolated symptoms (like hay fever or allergies) that do not develop into the clinical picture of a cold. Thus, a cold episode was defined as a minimal total symptom score of 14 (summed over a minimum of six consecutive days), and the participants believed they had a cold and/or reported rhinorrhoea that lasted for $\geq$ 3 days. A set of three predefined prophylactic variables were analyzed in a confirmatory manner: (i) the total number of cold episodes; (ii) cumulative episode days; and (iii) co-medicated cold episodes. The three parameters were analyzed individually with a Chi-square test or Fisher's exact test to determine whether the proportions of cumulated events (*i.e.*, cold episodes) in the treatment groups deviated from expectation under the null hypothesis of equal proportions among groups.

WURSS-21: In this study the upper respiratory symptom-related Quality of life (QoL) was measured using the questions from the 21-item Wisconsin Upper Respiratory Symptom Survey (WURSS-21) [24]. The WURSS-21 is a responsive, reliable and valid instrument for evaluating QoL outcomes related to respiratory illness, measuring all significant health-related dimensions that are negatively affected by the common cold [24,25]. It includes 10 items assessing symptoms, nine items assessing functional impairments and one item each assessing global severity and global change over the last 24 h, all of which are based on 7-point Likert-type severity scales. A previous validation of the instrument showed that a cumulative score should be calculated by summing the severity scores of the first 20 items with high severity scores indicating high symptom load. The mean WURSS-21 score was calculated and analyzed and compared between groups using ANOVA.

In addition, the minimal important difference (MID) for the WURSS-21 was calculated. The MID is the term generally used to quantify the minimum amount of positive change that patients perceive, and would accept an associated treatment as being beneficial or worth taking—a clinically significant effect [26]. For the WURSS-21 score, a MID of 10.3 was determined [27]. Therefore, individuals that presented with a respiratory disorder symptom score of 10.3 and above (RDS+) were compared in both groups at baseline, return and follow-up. Difference in the proportion of RDS+ individuals between groups at follow-up was statistically compared using a Chi-squared test and Fisher's exact test. The amount of missing data was different among variables but on average was less than 10%. We analyzed observed data only, *i.e.*, did not impute data or conduct missing at random analyses.

SF-21: The SF-21 was utilised to assess general Quality of Life (QoL). The SF-12 is a generic measure and has been developed to provide a shorter, yet valid alternative to the SF-36, which has been seen by many health researchers as too long to administer [28]. The SF-12 is weighted and summed to provide easily interpretable scales for physical and mental health. Physical and Mental Health Composite Scores (PCS and MCS) are computed using the scores of twelve questions and range from 0 to 100, where a zero score indicates the lowest level of health measured by the scales and 100 indicates the highest level of health. All SF-21 variables were statistically analyzed using ANOVA to compare means.

PSS-SCALE: The Perceived Stress Scale (PSS) is the most widely used psychological instrument for measuring the perception of stress, the scale is based on feelings and thoughts during the last month. It has been validated to correlate with cold episodes [29]. It will be used to identify a possible sub-group of heightened stress participants and help analysing a possible correlation between increased stress and the effects of elderberry for this sub-group as opposed to the overall participant group. Individuals scoring > 14 points were considered "stressed" and as such selected for sub-group analysis.

### *2.5. Sample Size Calculation*

The primary outcome measure for this study is the total number of cold episode days measured for the previous six days. A cold episode is defined by a Jackson score $\geqslant$14 and either (i) the participant believed they had a cold or (ii) reported rhinorrhea. In the placebo group we assumed a prevalence of cold episode days of 35% (235 days) over the 6-day survey period. This estimate is based on similar previous studies conducted by Jawad *et al.*, 2012 [30] and Tiralongo *et al.*, 2011 [11]. We also expected a prophylactic effect size ($\delta$) of 0.5 (or a 2-fold decrease), this is an estimate based on the findings of an echinacea trial by Tiralongo *et al.*, 2011 [11]. With an anticipated 2-fold prophylactic effect ($\delta = 0.5$) we expected to observe a statistically significant reduction in episode days from 35% to 18% in the treatment group. Considering these parameters we estimated that a sample size of 280 ($n = 140$ in each arm) entering the study (ITT) would be required to achieve at least 80% power ($\beta \geqslant 0.8$) to detect a $\delta \geqslant 0.5$ as statistically significant ($\alpha \leqslant 0.05$).

## 3. Results

The flow of participants through the trial between April 2013 and December 2014 is summarised in Figure 2. Six hundred and twelve people were screened, with a number deemed ineligible by inclusion criteria *i.e.*, plant allergies, inappropriate destination and/or extended stopovers during travel (>12 h). Reasons for declining participation included not wanting to be on placebo, travel cancellation and personal circumstances. Of the 325 trial participants, 13 participants did not receive the trial medication due to various reasons (e.g., trial pack lost in mail, diagnosed with pneumonia, change of travel plans, change of mind and family emergency).

However, 312 completed the first survey. Thus Intention to treat (ITT) analysis was performed on 312 participants. Of the 312 participants analysed, 158 were assigned elderberry capsules and 154 were assigned placebo. The majority of the participants were women, non-smoker, on average 50 years old, travelled for holiday with a travel time of over 16 h (Table 1). The majority of participants indicated to be stressed (PSS > 14). About half of the participants received the flu vaccination. Participants in the

placebo and active groups did not differ significantly at baseline. Thus, the two treatment groups can be considered reasonably well balanced at baseline.

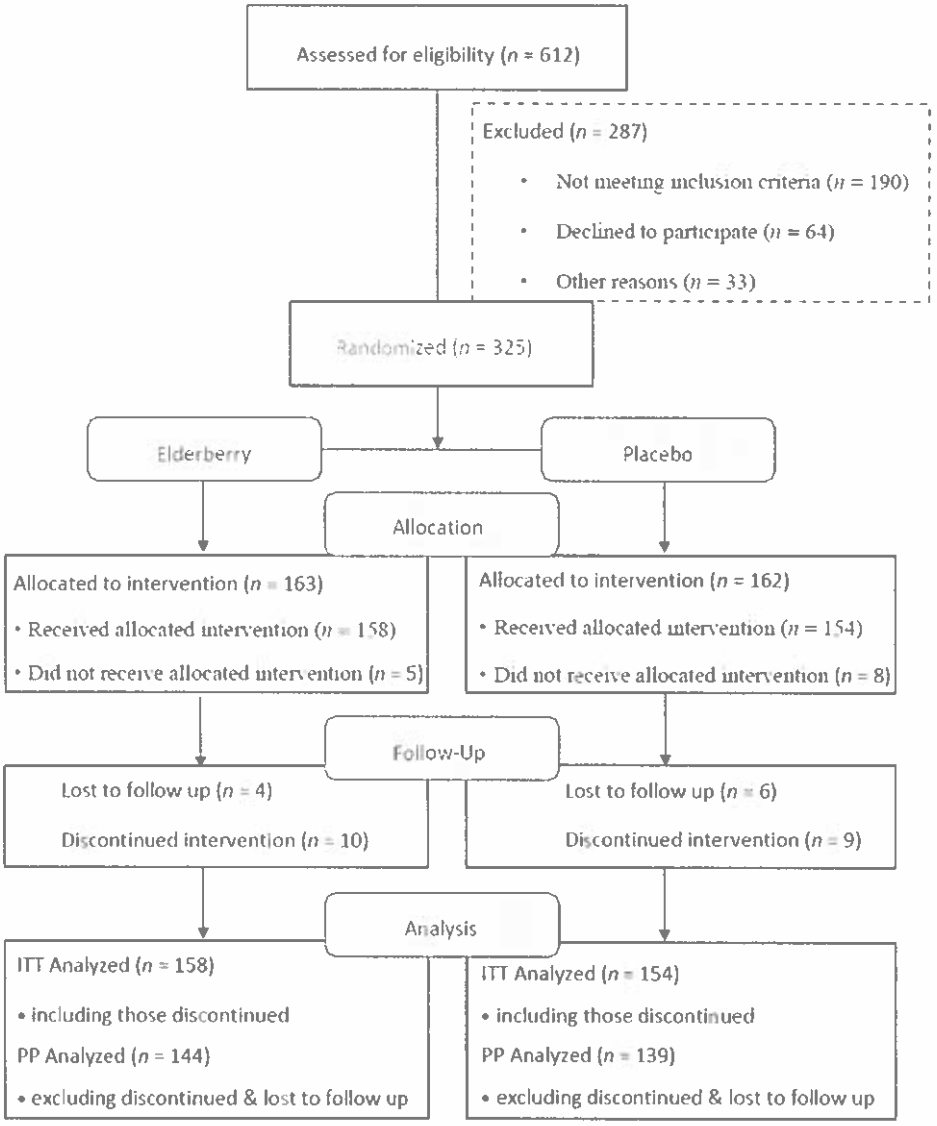



Figure 2. Flowchart of trial participants (ITT—Intention to treat analysis, PP—Per protocol analysis).

To confirm that blinding was effective, all participants were asked to speculate whether they were taking elderberry or placebo. 80 participants (52%) identified themselves correctly in the placebo and 73 (46%) in the elderberry group. Thus, there was an even distribution of mismatches in the placebo and treatment group ($p = 0.5$) providing evidence of effective randomisation.

Analysis of the Jackson Score identified that 29 of 312 participants (~9%) suffered from a well-defined cold—17 of those were on placebo and 12 on elderberry. Although more cold episodes occurred in the placebo group, the difference between the groups was not significant ($p = 0.2$). The cold symptoms presented for approximately one third of participants ($n = 11$) before travel, for three during travel and for the majority of participants ($n = 15$) when they arrived overseas.

**Table 1.** Demographics and characteristics of the overall sample.

| Variable | Total ($n$ = 312) | Elderberry ($n$ = 158) | Placebo ($n$ = 154) |
|---|---|---|---|
| Age in years—mean (SD) [a] | 51 (16) | 52 (16) | 50 (17) |
| Females (%) | 206 (66) | 108 (68) | 98 (64) |
| Weight in kg (SD) | 72 (14) | 72 (15) | 71 (14) |
| BMI (SD) | 25 (4) | 25(4) | 25 (4) |
| Non-Smoker (%) | 299 (96) | 153 (98) | 146 (95) |
| Perceived to be Stressed (%) | 266 (85) | 135 (86) | 131 (85) |
| Received Flu Vaccination (%) | 168 (54) | 86 (54) | 82 (53) |
| [b] RDS+ (%) | 20 (6) | 9 (6) | 11 (7) |
| Travel time > 16 h (%) | 218 (70) | 112 (71) | 106 (69) |
| Travel Reason—Holiday (%) | 255 (82) | 131 (83) | 124 (81) |

[a] Values are either means with standard deviations (SD) or frequencies with percentages (%); [b] RDS + indicates a Respiratory Disease Symptoms score of 10.3 and above.

However, the placebo group had a collective duration of 117 episode days; in comparison, the elderberry group had a significantly lower number of cold episode days (57, $p$ = 0.05) (Figure 3A). Moreover, the symptom score in the placebo group over these days was 583, whereas in the elderberry group it was 247 (Figure 3B). The difference in symptom score between both groups was also significant ($p$ = 0.02).

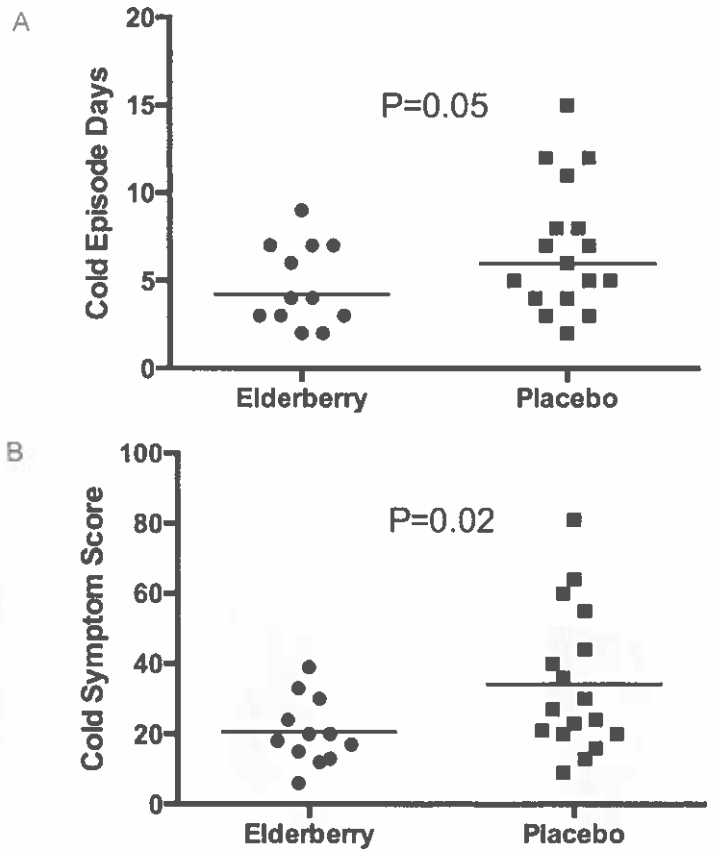



**Figure 3.** Cold episode days (A) and cold symptom score (B) of participants with a well-defined cold established from the Jackson Score.

This indicates that a participant suffering from a cold episode while taking elderberry would on average experience a 2-day shorter duration of the cold (4.75 days *vs.* 6.88 days), and would experience a lower symptom severity (21 *vs.* 34) in comparison to participants suffering from a cold and taking placebo.

In both groups half the participants with a defined cold used co-medication to relieve symptoms. On average participants co-medicated for 1.5 days; the difference was not significant between groups ($p = 0.9$). The participants suffering from a cold took in total 25 conventional medicines, belonging to 10 different categories, and one complementary medicine. Some took one medicine, others up to four. The medicines used were: one nasal spray, four cold and flu tablets, seven analgesics, one antibiotic, three decongestants, one inhaler, one gargle/mouthwash, five lozenges, one cough syrup and one antihistamine. The complementary medicine used was an Indonesian product called Tolak angin.

The analysis of the WURSS data showed an increase of the mean WURSS-21 scores for elderberry and placebo participants from baseline to the end of the trial. However, when comparing both groups with each other at each individual time point (baseline, just before travel and four days after travel), the WURSS-21 scores did not differ significantly at any time point, *i.e.*, the difference in profiles was not statistically significant ($p = 0.18$). The same analysis was conducted just for the participants with a defined cold, $n = 29$. Whilst, there was a tendency for increasing mean WURSS scores for elderberry and placebo participants across the three time points, the difference between both groups was not significant ($p = 0.35$).

When comparing the percentage of participants from the whole sample considering themselves to be affected by respiratory illness (RDS+, WURSS-21 score > 10.3) there was no significant difference between elderberry and placebo participants at any survey point (Figure 4). In both groups there is an increase of participants with significant symptoms (MID > 10.3). However, the survey completed immediately before travel and overseas showed a trend for more placebo participants reporting significant symptoms than elderberry participants, a trend that approaches significance at least for the before travel survey. When analysing only the cold sufferers who had a WURSS-21 score of >10.3 the numbers were too small to give reliable statistical data.

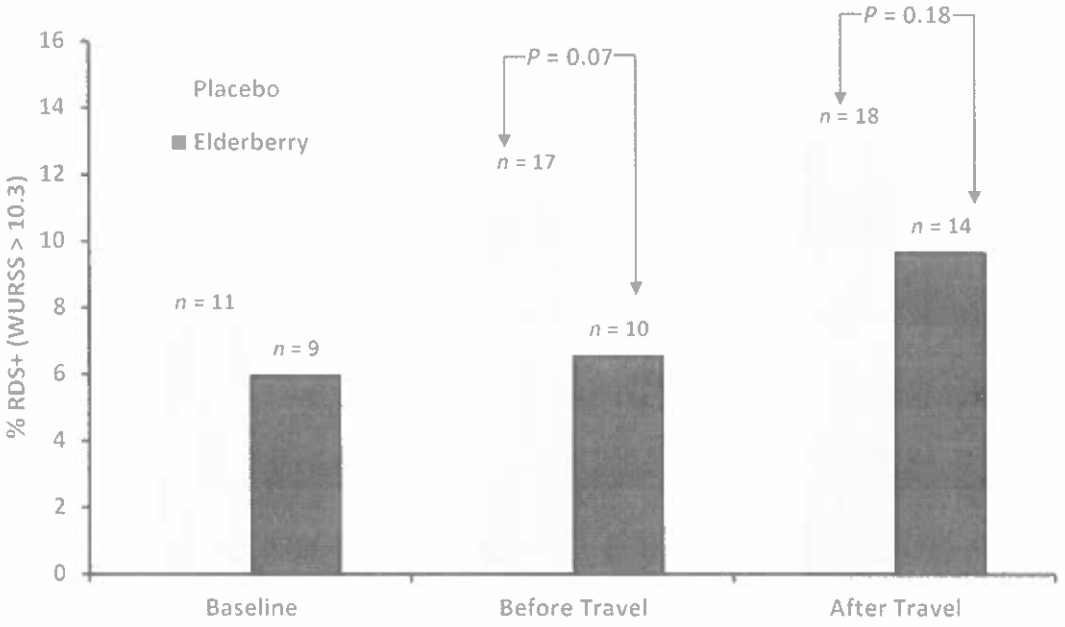



Figure 4. Effect of elderberry supplementation on the prevalence of Respiratory Disease Symptom positive (RDS+) participants.

With regards to the secondary outcome measures, Quality of Life (QoL) measures, the individual scales of SF12 and their component summaries (physical and mental) were analyzed.

Figure 5 shows the average change in physical health (reduction in mean Physical Component Score) for placebo and active group between baseline (−10 days) and overseas (+4/5 days).

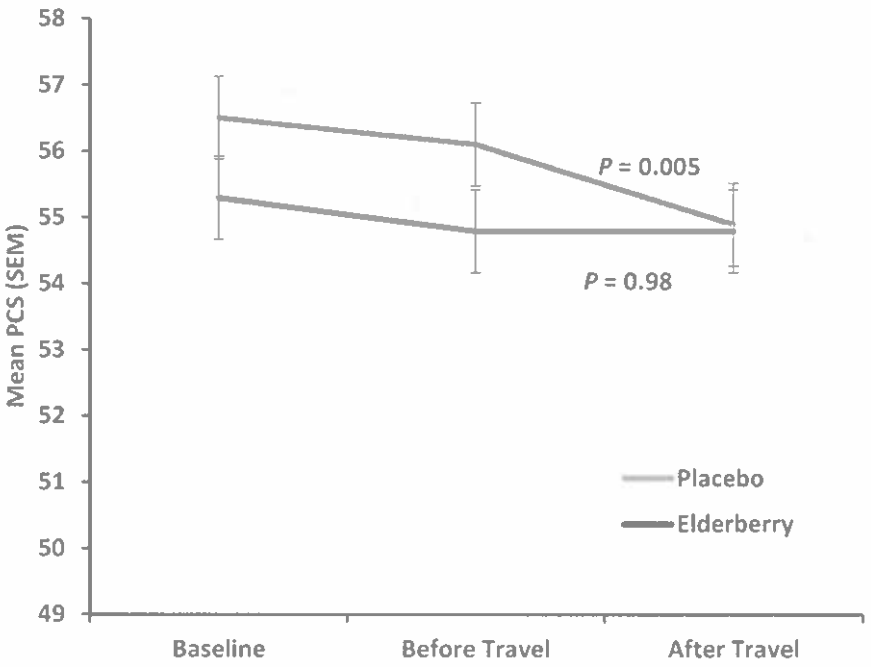



**Figure 5.** Y axis shows average Quality of Life (QoL) scores for physical health Physical Health Composite Scores (PCS) for each treatment group at the three time points surveyed (X-axis) for all participants.

The difference between placebo and active was minor and not statistically significant at overseas time point ($p = 0.27$), indicating that there is no treatment effect on change in physical health over the entire trial period. However, for the time of actual travel and arriving in a potential different climate and time zone (change observed from two days before until 4–5 days after travel) physical health declined significantly in the placebo group ($p = 0.005$), but was stable in the elderberry group ($p = 0.9$). Subdomain analysis for the PCS score (Physical Functioning (PF), Role Physical (RP), Body Pain (BP) and General Health (GH)) was performed to understand which subdomain(s) contribute(s) to this significant elderberry effect. There was a trend of PCS score rise for the three subdomains PF, RP and BP, but not GH. However, the change was not significant for any of the subdomains (data not shown).

Figure 6 shows an overall improvement of mental health (increase in mean Mental Component Summary score (MCS) for both, the placebo and active group from baseline (−10 days) to after travel (+4 days) with a non-significant trend for the active group to have a higher average mental health score compared to placebo ($p = 0.07$). Of note, the improvement in mental health was significant for both groups from travel to after travel (placebo, $p = 0.004$; elderberry group, $p = 0.03$). This indicates that mental health is strongly positively affected by travel whether people take elderberry or not.

Even though only 60% of participants were 100% compliant, *i.e.*, took all of their trial medication, more than 90% of participants were over 90% compliant. This means that 90% of trial participants did not miss more than three elderberry capsules throughout the whole trial period.

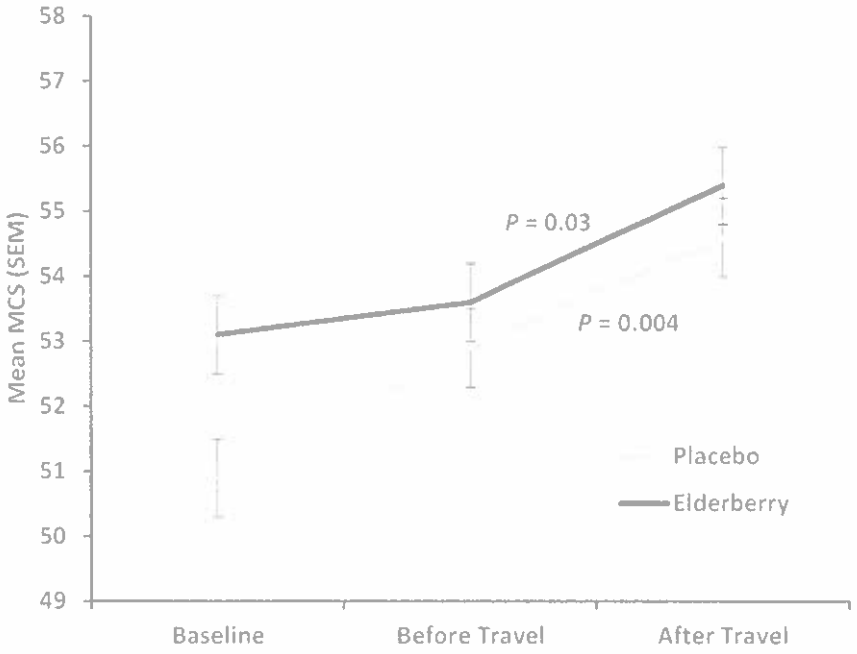



Figure 6. Y axis shows average QoL scores for mental health Mental Health Composite Scores (MCS) for each treatment group at the three time points surveyed (X-axis) for all participants.

Moreover, no significant differences in participant's compliance to the study medication were observed between the placebo and elderberry group (90% compliance: elderberry: $n = 125$; 92% vs. Placebo: $n = 124$; 92.5%, $p = 0.6$).

Overall, treatment was well tolerated. Five participants reported adverse events such as (i) itchy throat and cold-like symptoms (2 participants); (ii) fatigue (2 participants) and (iii) kidney pain (1 participant), however, a causal relationship between elderberry and the events could not be established. The two participants who reported itchy throat and cold-like symptoms were later unblinded as taking placebo and elderberry capsules, respectively. The two participants who reported fatigue were also later un-blinded as taking placebo and elderberry capsules, respectively. And, the participant who reported kidney pain was unblinded as taking placebo.

Individual sub-group analysis for participants that had no flu vaccination, travelled for more than 16 h, were more than 50 years old, fully complied with study medication schedule and were stressed, showed no significant differences between placebo and elderberry for any of the QoL indices for any of the survey time points (data not shown).

## 4. Discussion

In this study, participants in both, the placebo and elderberry group tended to experience an increase in respiratory symptoms from the time the trial started (baseline) to four days after arriving overseas. This was indicated by an increase in the prevalence of Respiratory Disease Symptom positive (RDS+) participants in both study groups. This result was expected as research has reported increased medical issues especially respiratory symptoms following commercial air travel independent of aircraft types [1].

Based on previous travel research we estimated the prevalence of cold episode days as 35% in the placebo group and hypothesized a ~50% prophylactic effect of elderberry (18%). Whilst we did observe a ~50% effect the prevalence of cold episode days in the overall study population was lower than expected (17% in the placebo and 8% in the active group). This is due to a lower than expected incidence of colds and reflects recent research that estimates the incidence of respiratory problems as a

common complaint from air-travelers as ~11% [31], with in flight emergencies based on respiratory problems ranging from 2% to 14% [7]. Our trial is consistent with these recent studies identifying 29 participants (9%) as having a cold, defined by the Jackson score.

In our trial elderberry supplementation decreased the symptom load (mean 21 *vs.* 34) and shortened the cold duration by approximately two days (mean 4.75 days *vs.* 6.88 days). This supports the results from, previous limited research conducted with two other elderberry products, and shows for the first time that elderberry may be effective for decreasing respiratory symptoms during travel on long-haul flights. A reduction in the duration of symptoms has previously been reported for another elderberry formulation, Sambucol®, manufactured in Israel in two independent, but not travel related trials. Patients with either influenza A or B virus infection took 15 mL of elderberry syrup (38% extract equivalent to approx. 5.7 mg extract) four times a day for 5–6 days [16,32]. This formulation however, also contains honey and raspberry extract. A pilot trial with elderberry extract (175 mg) lozenges taken by patients with flu like symptoms four times for two days also confirmed a beneficial effect on severity and duration of cold and flu like symptoms [17].

Similarly, other complementary or nutritional medicines have shown to decrease cold duration. For example, a recent randomised clinical trial showed that supplementation with 1 g of vitamin C daily reduces cold duration by approximately three days [33]. Also, echinacea has been reported to reduce severity and cold duration [30,34], however more trials report a preventative effect on cold occurrences and re-occurrences rather than a conclusive treatment effect for echinacea [34,35]. In contrast, although our trial with elderberry also showed a lower incident of cold occurrences (*i.e.*, detected episodes) between the elderberry group and placebo, this difference was not significant. However, it may be that elderberry also has a weak preventative effect on the common cold which may have not been fully detected in our study due to sample size.

Similar to the Echinacea travel trial conducted by our group, the WURSS questions were used to detect impact on quality of life due to respiratory symptoms. In contrast to the Echinacea trial, the WURSS-21, which was not available then, was used in the elderberry study [11] to decrease survey completion times for participants as the WURSS-21 was reported to exhibit similar performance to WURSS-44 in terms of reliability, responsiveness, importance-to-patients, and convergence with other measures [25,27]. The WURSS-21 analysis of the cold sufferers ($n$ = 29) did not show any significant differences between groups, probably due to sample size. However, it detected a weak elderberry effect in the overall sample on the prevalence of Respiratory Disease Symptom positive (RDS+) participants who are participants suffering from treatment worthy respiratory disorder symptoms (scores of 10.3 and above are RDS+). The difference between groups was not significant, but showed a trend especially at the "just before travel" and "overseas" time point. It would mean that travellers who take elderberry are less likely to suffer from respiratory symptoms that are worth treating.

Although there was a significant difference in symptom score (assessed with Jackson) and a slight difference in numbers of participants reporting treat worthy respiratory symptoms (assessed with WURSS-21) between both groups, this was not reflected by significantly increased co-medication of cold episodes or cold episode days in the placebo group. Nevertheless, most cold episodes in the elderberry group were co-medicated with one item, whereas most participants in the placebo group who suffered from a cold and co-medicated, used two and more medicines. More distinct was the result in a previously reported trial with Echinacea; a significant difference in co-medicated cold episodes was detected between the active and placebo group participants with a cold [30].

Intercontinental air travel can be stressful, adding extra strain on passenger's physical health in general [1]. What effect elderberry has on physical health needs to be further investigated in larger studies, as so far the clinical evidence is very limited and diverse [36].

In our trial it seemed as if Elderberry "stabilizes" physical health (PCS score) from before travel until four days overseas, whereas the physical health in placebo participants significantly declined further. The effect was only noticeable when all PCS domains were considered in a holist (or additive) manner. When looking at individual PCS subdomains the effect was not detectable.

The slight effect of elderberry on physical health could be due to its reported antioxidant properties [37,38]. Antioxidant polyphenols are present in elderberries [22], bioavailable [39] and can increase serum antioxidant capacity [18]. In a recent randomised clinical trial it was shown that vitamin C supplementation improved physical activity levels in a population with adequate-to-low vitamin C status. The authors related this effect to vitamin C's antioxidant properties since oxidative stress is related to fatigue [33].

Dosages recommended by popular elderberry products range from 650 mg to 1500 mg. In the three beneficial influenza trials patients took 60 mL of elderberry syrup (38% extract equivalent to approx. 22.8 mg extract standardised to flavonoids) daily for 5–6 days [16] or lozenges with an equivalent of 700 mg elderberry extract daily for two days with no indication on the anthocyanin content [17]. The minimum of anthocyanin doses for the treatment of metabolic syndrome disorders was recently estimated as 110 mg per day and as 3.5 g per day for influenza [40]. In our trial we used 600–900 mg of elderberry extract with 90–135 mg of anthocyanins daily and we recorded significant effects on severity and duration of respiratory symptoms and a possible stabilising effect on physical health.

While this study did not detect elderberry effects on mental health, it did show that going on holiday improves mental health. Over 80% of the participants in this trial travelled for leisure and this improved mental health (measured as MCS in SF-12) significantly, thus confirming psychological health effects of a vacation [41].

While raw and unripe fruit contain toxic cyanogenic glycosides, elderberries can be safely consumed when processed. Previous human studies and clinical trials using orally administered elderberry fruit extract reveal a very good safety profile [16,17,32]. Moreover, no adverse reaction reports were existent at the Advisory Committee on the Safety of Medicines (ACSOM, part of the Therapeutic Goods Administration (TGA) in Australia) prior to starting this trial [42].

In this trial, elderberry was generally well tolerated. For the adverse events reported by five participants no causal relationship between elderberry and the events could be established mainly due to the fact that participants unblinded to taking placebo. Due to only limited information for its use during pregnancy [43] we excluded any women who were pregnant or wanting to become pregnant. Moreover, we excluded travellers with plant allergies, due to one report on the possible allergenic potential of elder flowers and elderberry tree dietary products [44]. However, to our knowledge no allergic reactions to products using elderberry extract have been reported from any clinical trial, cohort study or government authority thus far. If this continues to be the case, further travel studies with elderberry products could include people with pre-existing plant allergies and respiratory conditions such as allergic rhinitis as this would mean that the trial population better represent the general traveller's population and as such be more generalizable to a wider community.

As with all RCTs, this study had several limitations. Whilst diaries were used during the trial to help document cold symptoms and duration, as well as relief medication, a certain amount of recall bias has to be expected when the surveys were completed just before travel and after travel which contained the WURSS-21 and SF-12 questions. This study used travel including long haul intercontinental flights, time zone and climate change as a model to see whether elderberry is beneficial for respiratory and physical health overall. Generalisability of these results can only be extended to similar populations and season. In addition, this trial excluded travellers suffering from allergy and respiratory diseases (e.g., COPD, asthma, pneumonia) or immune disorders. As such effects and safety in these specific populations would need to be established in further studies.

## 5. Conclusions

Although the occurrence of the common cold for this trial in travellers was low overall, a significant effect of elderberry on cold duration and cold associated symptoms was detected. Travellers using elderberry from 10 days before travel until 4–5 days after arriving overseas on average experienced a 2-day shorter duration of the cold and also noticed a reduction in cold symptoms. Physical health may stabilise during air travel due to elderberry, but further studies are needed to

confirm this. No elderberry effect was observed on mental health of the participants. The incidence of adverse events was low overall and no adverse effects could be directly attributed to elderberry. Based on these results it seems worthwhile to undertake more clinical research with high quality elderberry preparations to better understand beneficial health implications of this nutritional traditional medicine.

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**Author Contributions:** Evelin Tiralongo designed the trial, analysed the data and wrote the manuscript. Shirley Wee participated in the design of the trial, conducted the research and participated in the interpretation of data and manuscript writing. Rod Lee participated in the design of the trial, mainly conducted data analysis and interpretation of data and participated in manuscript writing.

**Conflicts of Interest:** The study was funded by Iprona AG, Italy. The sponsor provided the elderberry and placebo capsules and was partially involved in the design of the study. The randomisation of participants, collection, analysis, interpretation and publication of the data was conducted by the researchers. The decision to publish the results was made by the researchers and supported by the sponsor.

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# Elderberry and Elderflower Extracts, Phenolic Compounds, and Metabolites and Their Effect on Complement, RAW 264.7 Macrophages and Dendritic Cells

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**Abstract:** Modulation of complement activity and inhibition of nitric oxide (NO) production by macrophages and dendritic cells may have therapeutic value in inflammatory diseases. Elderberry and elderflower extracts, constituents, and metabolites were investigated for their effects on the complement system, and on NO production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages and murine dendritic D2SC/1 cells. The EtOH crude extracts from elderberry and elderflower and the isolated anthocyanins and procyanidins possessed strong complement fixating activity and strong inhibitory activity on NO production in RAW cells and dendritic cells. Phenolic compounds in the range of 0.1–100 μM showed a dose-dependent inhibition of NO production, with quercetin, rutin, and kaempferol as the most potent ones. Among the metabolites, caffeic acid and 3,4-dihydroxyphenylacetic acid showed the strongest inhibitory effects on NO production in both cell lines, without having cytotoxic effect. Only 4-methylcatechol was cytotoxic at the highest tested concentration (100 μM). Elderberry and elderflower constituents may possess inflammatory modulating activity, which increases their nutritional value.

**Keywords:** *Sambucus nigra*; elderberry; elderflower; complement system; nitric oxide; anti-inflammatory; metabolites; polyphenols; LPS; RAW 264.7 cells; dendritic D2SC/1 cells

## 1. Introduction

Inflammation is one of the organism's attempts at self-protection, with the aim to remove harmful stimuli, including damaged cells, irritants, or pathogens. There is a growing research interest concerning anti-inflammatory natural products that will prevent and clarify the pathogenesis of various diseases such as cardiovascular diseases, diabetes, and chronic inflammatory diseases [1]. Numerous epidemiological studies indicate that an increase in the consumption of polyphenol-rich food is associated with a decrease in the incidence of cardiovascular diseases [1–4]. A large number of dietary polyphenols is consumed, and their anti-inflammatory activities have been reported [5]. This protective effect has been attributed in part to anti-inflammatory properties of polyphenols. At the level of bioactive compounds occurring in plants, substances such as polyphenols, polysaccharides, peptides, proteins, triterpenoids, lipid derivatives, and glycoproteins seem to modulate inflammatory as well as immunological processes [6,7].

The complement system plays an important role in the first-line defense against infections and holds important effector functions of the innate and the adaptive immune system. Complement participates in diseases with an immune component, such as autoimmune heart disease, multiple sclerosis, Alzheimer's disease, rheumatoid arthritis, and severe bacterial infections [8,9]. The inflammation

process involves the activation of monocytes and/or macrophages. The activation of macrophages releases many inflammatory mediators such as interleukins (IL-1β, IL-6), tumor necrosis factor (TNF)-α, and inflammatory mediators including reactive oxygen species (ROS), nitric oxide (NO), and prostaglandin $E_2$ ($PGE_2$). NO formed by inducible NO synthase (iNOS) plays a vital role in host defense as it possesses cytotoxic effects on bacteria, virus, and tumor cells [10]. On the other hand, in high concentrations it can lead to tissue damage and inflammatory diseases such as rheumatoid arthritis, cardiovascular diseases, type 2 diabetes, chronic hepatitis, and pulmonary fibrosis [11]. For this reason, NO is a well-established marker of inflammation, and inhibition of its production might be a useful therapeutic strategy in inflammatory diseases [10].

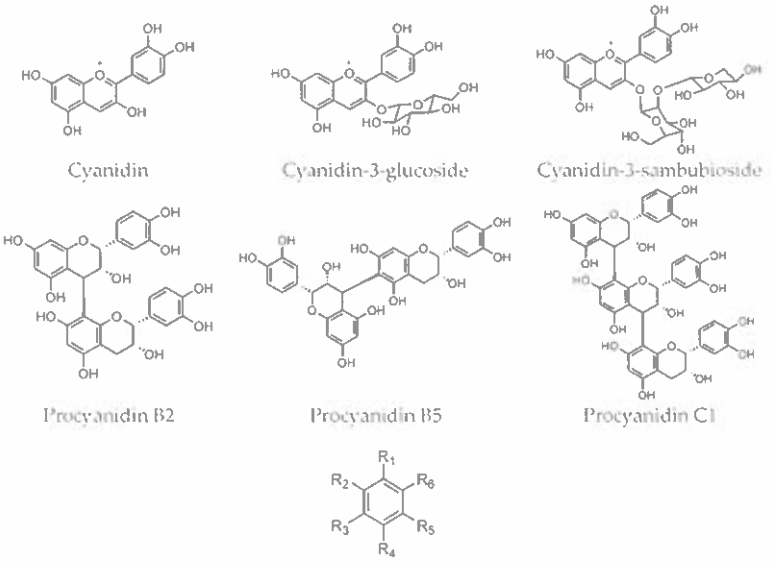
*Sambucus nigra* L., also called black elder, is a deciduous tree that can grow up to 10 m high and has blue-black elderberries and cream-white elderflowers [12]. Both the elderberries and elderflowers have a long tradition in herbal medicine of being used to reduce inflammation and diabetic symptoms, as diuretics, and in the treatment of colds and flu. Studies have shown that elderberry and elderflower extracts possess diverse biological activities such as anti-inflammatory, antioxidant, and antidiabetes effects [12,13]. *S. nigra* has components with high biological activity, primarily flavonoids, such as flavonols, proanthocyanidins and anthocyanins, and simpler phenolic acids [12]. However, due to the complex bioavailability of polyphenols, it is difficult to know what the most relevant substance is after intake of elderberries and elderflowers in humans. Due to the fact that polyphenols are metabolized to simpler phenolic substances [14], it was also of interest to investigate whether these metabolites could contribute to an effect. In human intervention studies involving feeding elderberry extracts and anthocyanins, many potential metabolites have been identified in urine, serum, and feces [15,16], and selected metabolites were included in this study.

In the present study, we systematically investigated the effects of the elderberry and elderflower extracts, constituents, and metabolites on the complement fixating activity and on the NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages and murine dendritic D2SC/1 cells.

## 2. Results and Discussion

### 2.1. Extraction, Fractionation, and Chemical Characterization

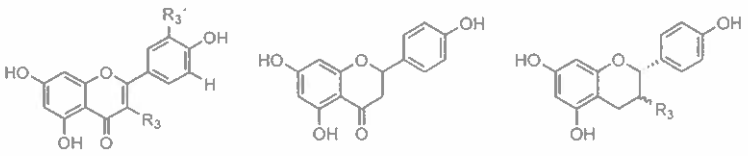
Freeze-dried elderberries and elderflowers were extracted with dichloromethane (DCM), 96% EtOH, 50% EtOH, and water (50 and 100 °C) on an accelerated solvent extraction instrument (ASE). The phenol–sulfuric acid test [17] and $^1$H NMR analyses revealed that carbohydrates were present in the 50% EtOH and 50 and 100 °C water extracts. $^1$H and $^{13}$C NMR analyses of the EtOH elderberry and elderflower extracts showed signals from aromatic compounds, organic acids, and carbohydrates. The 96% EtOH elderflower extract showed signals which could be attributed to rutin [18] and caffeoyl moieties [19], structures previously reported to be major constituents in the elderflowers [20]. From the acidic elderberry MeOH extract (800 g), cyanidin-3-glucoside (340 mg, 0.043% yield) and cyanidin-3-sambubioside (250 mg, 0.031% yield), the major anthocyanins in elderberry, were isolated, in addition to the aglycone cyanidin (270 mg, 0.034% yield). The substances were identified by one- and two-dimensional NMR spectroscopy ($^1$H, $^{13}$C, COSY, APT, HSQC, and HMBC) and the spectroscopic data were compared with those reported in the literature [21,22]. Chemical structures of the phenolic constituents and their metabolites from elderberries and elderflowers employed in this study are shown in Figures 1 and 2.





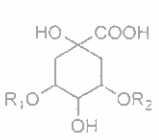

| Chemical Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ |
|---|---|---|---|---|---|---|
| *p*-Coumaric acid | CH=CH-COOH | H | H | OH | H | H |
| Homovanillic acid | $CH_2$-COOH | H | H | OH | $OCH_3$ | H |
| Phloroglucinol aldehyde | CHO | OH | H | OH | H | OH |
| 4-Hydroxybenzoic acid | COOH | H | H | OH | H | H |
| Hippuric acid | CO-NH-$CH_2$-COOH | H | H | H | H | H |
| Ferulic acid | CH=CH-COOH | H | $OCH_3$ | OH | H | H |
| 4-Hydroxybenzaldehyde | CHO | H | H | OH | H | H |
| Protocatechuic acid | COOH | H | H | OH | OH | H |
| Caffeic acid | CH=CH-COOH | H | H | OH | OH | H |
| Vanillic acid | COOH | H | H | OH | $OCH_3$ | H |

**Figure 1.** Structures of elderberry constituents and their metabolites.

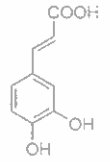



| Flavonoid | Structure | $R_3$ | $R_3'$ |
|---|---|---|---|
| Quercetin | A | OH | OH |
| Quercetin-3-glucoside | A | O-Glucose | OH |
| Quercetin-3-rhamnoside | A | O-Rhamnose | OH |
| Rutin (quercetin-3-rutinoside) | A | O-Rutinose | OH |
| Kaempferol | A | OH | H |
| Kaempferol-3-rutinoside | A | O-Rutinose | H |
| Isorhamnetin | A | OH | $OCH_3$ |
| Isorhamnetin-3-rutinoside | A | O-Rutinose | $OCH_3$ |
| Naringenin | B | - | - |
| Catechin | C | OH (β) | - |
| Epicatechin | C | OH (α) | - |

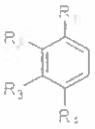
**Figure 2.** *Cont.*




Chlorogenic acid ($R_1$ = Caffeoyl; $R_2$ = H)
Neochlorogenic acid ($R_1$ = H; $R_2$ = Caffeoyl)




Caffeic acid



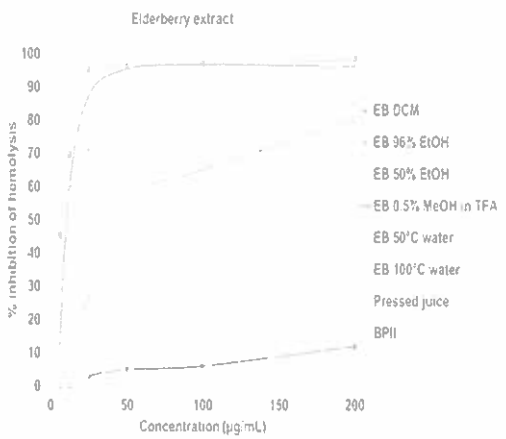

Structure D

| Metabolites | Structure | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|
| Benzoic acid | D | COOH | H | H | H |
| 3-Hydroxyphenylacetic acid | D | $CH_2$–COOH | H | OH | H |
| 3,4-Dihydroxyphenylacetic acid | D | $CH_2$–COOH | H | OH | OH |
| 4-Methylcatechol | D | OH | OH | H | $CH_3$ |

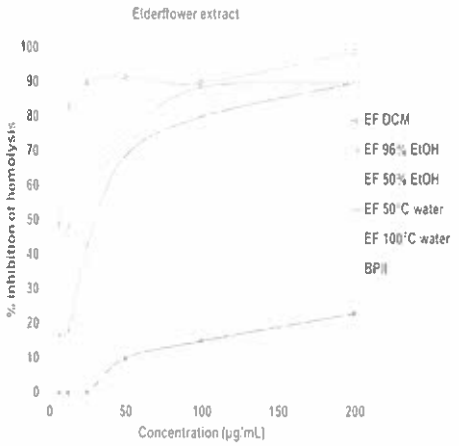
Figure 2. Structures of elderflower constituents and their metabolites.

## 2.2. Complement Fixating Activity

Due to the important role of the complement system in the immune system, complement modulation is involved in various diseases and considered as an interesting target for inflammatory diseases [23]. Elderberry and elderflower crude extracts, constituents, and metabolites were tested for complement fixating activity (Figure 3). The inhibitory concentration to give 50% effect ($IC_{50}$) values are presented in Table 1 for the crude extracts and in Table 2 for the constituents and metabolites. A pectic polysaccharide from *Biophytum umbraculum* Welw. (syn. *B. petersianum* Klotzsch) was used as positive control [24,25].




(A)




(B)

Figure 3. *Cont.*

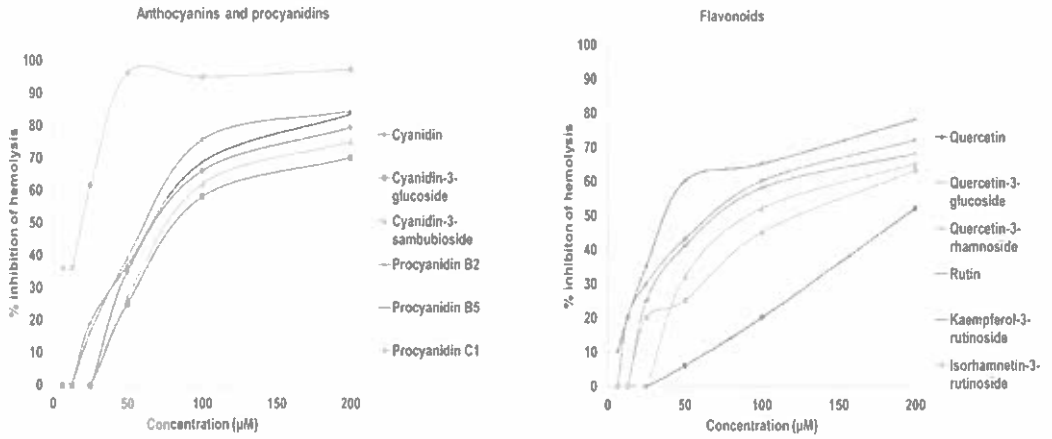



**Figure 3.** Complement fixating activity, expressed as % inhibition of hemolysis of sensitized sheep erythrocytes. Inhibition of hemolysis by (**A**) crude extracts from elderberry; (**B**) crude extracts from elderflower; (**C**) isolated anthocyanins and procyanidins; and (**D**) flavonoids. The results represent the mean of three independent experiments.

**Table 1.** Complement fixating activity of crude extracts from elderberry (EB) and elderflower (EF). DCM, dichloromethane; TFA, trifluoroacetic acid.

| Test Compounds | $IC_{50}$ (μg/mL) [a] |
|---|---|
| **Crude extracts from elderberry** | |
| EB DCM | >200 |
| EB 96% EtOH | 7.8 ± 2.3 |
| EB 50% EtOH | 13.4 ± 2.9 |
| EB 0.5% TFA in MeOH | 12.3 ± 1.9 |
| EB 50 °C water | 44.9 ± 5.3 |
| EB 100 °C water | 23.3 ± 3.5 |
| Pressed juice | 142.4 ± 13.1 |
| **Crude extracts from elderflower** | |
| EF DCM | >200 |
| EF 96% EtOH | 6.5 ± 1.5 |
| EF 50% EtOH | 8.9 ± 2.2 |
| EF 50 °C water | 32.3 ± 5.2 |
| EF 100 °C water | 14.4 ± 3.1 |
| BPII (positive control) | 17.7 ± 1.5 |

[a] $IC_{50}$: Concentration to give 50% inhibition of hemolysis.

**Table 2.** Complement fixating activity of flavonoids and metabolites from elderberries and elderflowers.

| Test Compounds | $IC_{50}$ (μM) [a] |
|---|---|
| **Flavonoids** | |
| Cyanidin | 74.2 ± 3.4 |
| Cyanidin-3-glucoside | 87.1 ± 5.6 |
| Cyanidin-3-sambubioside | 82.8 ± 3.9 |
| Procyanidin B2 | 70.6 ± 4.5 |

Table 2. *Cont.*

| Test Compounds | $IC_{50}$ (μM) [a] |
|---|---|
| **Flavonoids** | |
| Procyanidin B5 | 65.0 ± 3.1 |
| Procyanidin C1 | 19.4 ± 2.1 |
| Quercetin | 193.8 ± 5.6 |
| Quercetin-3-glucoside | 76.5 ± 2.6 |
| Quercetin-3-rhamnoside | 95.1 ± 3.1 |
| Rutin | 40.0 ± 2.6 |
| Kaempferol | >200 |
| Kaempferol-3-rutinoside | 70.6 ± 3.8 |
| Isorhamnetin | >200 |
| Isorhamnetin-3-rutinoside | 127.8 ± 4.8 |
| Naringenin | >200 |
| Catechin | >200 |
| Epicatechin | >200 |
| **Metabolites** | |
| *p*-Coumaric acid | >200 |
| Homovanillic acid | >200 |
| Phloroglucinol aldehyde | >200 |
| 4-Hydroxybenzoic acid | >200 |
| Hippuric acid | >200 |
| Ferulic acid | >200 |
| 4-Hydroxybenzaldehyde | >200 |
| Protocatechuic acid | >200 |
| Caffeic acid | >200 |
| Vanillic acid | >200 |
| Benzoic acid | >200 |
| 3-Hydroxybenzoic acid | >200 |
| 3,4-Dihydroxyphenylacetic acid | >200 |
| 4-Methylcatechol | >200 |

[a] $IC_{50}$: Concentration to give 50% inhibition of hemolysis.

Among the elderberry crude extracts, the acidic MeOH ($IC_{50}$ 12.3 ± 1.9 μg/mL), the 96% EtOH ($IC_{50}$ 7.8 ± 2.3 μg/mL), and the 50% EtOH ($IC_{50}$ 13.4 ± 2.9 μg/mL) extracts showed the highest complement fixating activity. Thus, the acidic MeOH extract and the EtOH extracts have the highest selectivity for both polyphenols and components with high complement fixating activity. The 50 °C water and 100 °C water extracts containing polysaccharides also possessed complement fixating activities, in accordance with a previous report [26]. The $IC_{50}$ values of cyanidin-3-glucoside and cyanidin-3-sambubioside, on a weight basis, are 18.0 μg/mL and 13.4 μg/mL, which is higher compared to the acidic elderberry MeOH extract ($IC_{50}$ 12.3 μg/mL). There might be other strong complement fixating compounds in the acidic extract, or the effect is caused by synergism. The aglycone cyanidin showed somewhat higher activity compared to cyanidin-3-glucoside and cyanidin-3-sambubioside. The anthocyanins seem to have a lower inhibition of hemolysis compared to the corresponding aglycone. Proanthocyanidins that are present in small amounts in elderberries also possessed strong inhibitory activity on the hemolysis. Trimeric procyanidin C1 ($IC_{50}$ 19.4 ± 2.1 μM) possessed stronger complement fixating activity compared to the dimeric procyanidins B2 ($IC_{50}$ 70.6 ± 4.5 μM) and B5 ($IC_{50}$ 65.0 ± 3.1 μM), while the monomers epicatechin and catechin were almost inactive (>200 μM). These results are in agreement with those reported previously [27,28]. Due to the fact that anthocyanins are metabolized to simpler phenolic substances, it was also of interest to investigate whether these metabolites that are detected in urine, feces and plasma after consumption of elderberries could have complement fixating activity. However, the metabolites shown in Figure 1 did not show any particular complement fixating activity from 0.1 to 200 μM.

The 96% EtOH and 50% EtOH elderflower extracts were the most potent inhibitors of hemolysis in the complement assay, followed by the water extracts. Pectic polysaccharides present in the water extracts might be a reason for the high complement fixating activity observed, in accordance with previous results [26,29,30]. Rutin ($IC_{50}$ 40.0 ± 2.6 μM), quercetin-3-rhamnoside ($IC_{50}$ 95.1 ± 3.1 μM), and quercetin-3-glucoside ($IC_{50}$ 76.5 ± 2.6 μM) showed a higher complement fixating activity compared to the aglycone quercetin ($IC_{50}$ 193.8 ± 5.6 μM), which is also reported previously [27,31]. Quercetin has even been reported to be inactive in a study [32]. The same trend is observed for kaempferol-3-rutinoside ($IC_{50}$ 70.6 ± 3.8 μM) and its aglycone kaempferol (>200 μM) and for isorhamnetin-3-rutinoside ($IC_{50}$ 127.8 ± 4.8 μM) and its aglycone isorhamnetin ($IC_{50}$ > 200 μM). An earlier study showed that kaempferol and quercetin-3-rhamnoside had weak complement fixating activity with $IC_{50}$ values 730 and 440 μM, respectively [32,33]. It appeared that the flavonol glycosides were more active than their aglycones in this system. These results are opposite to the activity of anthocyanins versus their aglycone. The presence of sugars seems to be important for complement fixating activity of the flavonoids included in this study. The metabolites from the elderflower phenolics (Figure 2) showed no particular complement fixating activity at the highest concentration tested ($IC_{50}$ > 200 μM).

### *2.3. Inhibition of NO Production in LPS-Stimulated RAW 264.7 Macrophages and Dendritic D2SC/I Cells, and Cell Viability*

During inflammation, bacterial products and proinflammatory cytokines induce the formation of large amounts of nitric oxide (NO) by inducible nitric oxide synthase (iNOS), and compounds that inhibit NO production can be inflammatory modulators. Exposure of lipopolysaccharides (LPS) to macrophages triggers several disadvantageous cellular responses and causes responses similar to inflammation, sepsis, and stroke. Macrophages and dendritic cells play a major role in the initiation and modulation of immune responses [34]. An induction of iNOS in dendritic cells or macrophages via stimulation with LPS has been described in the literature [35]. The dendritic cells may function as the most potent antigen-presenting cells for T cell activation, and increased levels of NO may inhibit T cell proliferation and apoptosis in the dendritic cells [34]. The proposed mechanism associated with the inhibition of NO production in macrophages is scavenging of NO radicals, direct inhibition of iNOS enzyme activity, and/or inhibition of iNOS gene expression [11]. The inhibitory effect of elderberry and elderflower extracts, constituents, and metabolites on NO production in RAW 264.7 cells and dendritic D2SC/I cells exposed to LPS are shown in Figure 4. The NO concentration after exposure to the LPS control was 10.3 ± 2.1 μM for the RAW 264.7 cells and 14.6 ± 2.4 μM for the dendritic D2SC/I cells. Quercetin, which is a constituent in elderflower, was used as a positive control as well [11].

Among the crude extracts, the 96% EtOH elderflower extract (100 μg/mL) showed the highest NO inhibition in RAW 264.7 and dendritic cells (75.6% ± 3.1% and 80.3% ± 2.9%, respectively) followed by the 96% EtOH elderberry extract (57.3% ± 5.1% and 47.8% ± 8.7%, respectively) (Figure 4A,B). A high content of phenolic compounds, revealed by $^1$H NMR spectroscopy, in the 96% EtOH elderberry and elderflower extracts could be the reason for the high NO inhibitory activity. The DCM elderberry and elderflower extracts exhibited little effect on NO production in LPS-activated RAW 264.7 and dendritic D2SC/I cells. The water extracts induced the production of NO, explained by the presence of pectic polysaccharides, which previously have shown macrophage stimulation [26,29,30]. The elderberry and elderflower crude extracts did not show any cytotoxic effects against RAW 264.7 macrophages and dendritic D2SC/I cells at 100 μg/mL (81.2%–105.6% cell viability). Also, cold-pressed raw juice possessed moderate NO inhibition on LPS-activated RAW 264.7 and dendritic cells.

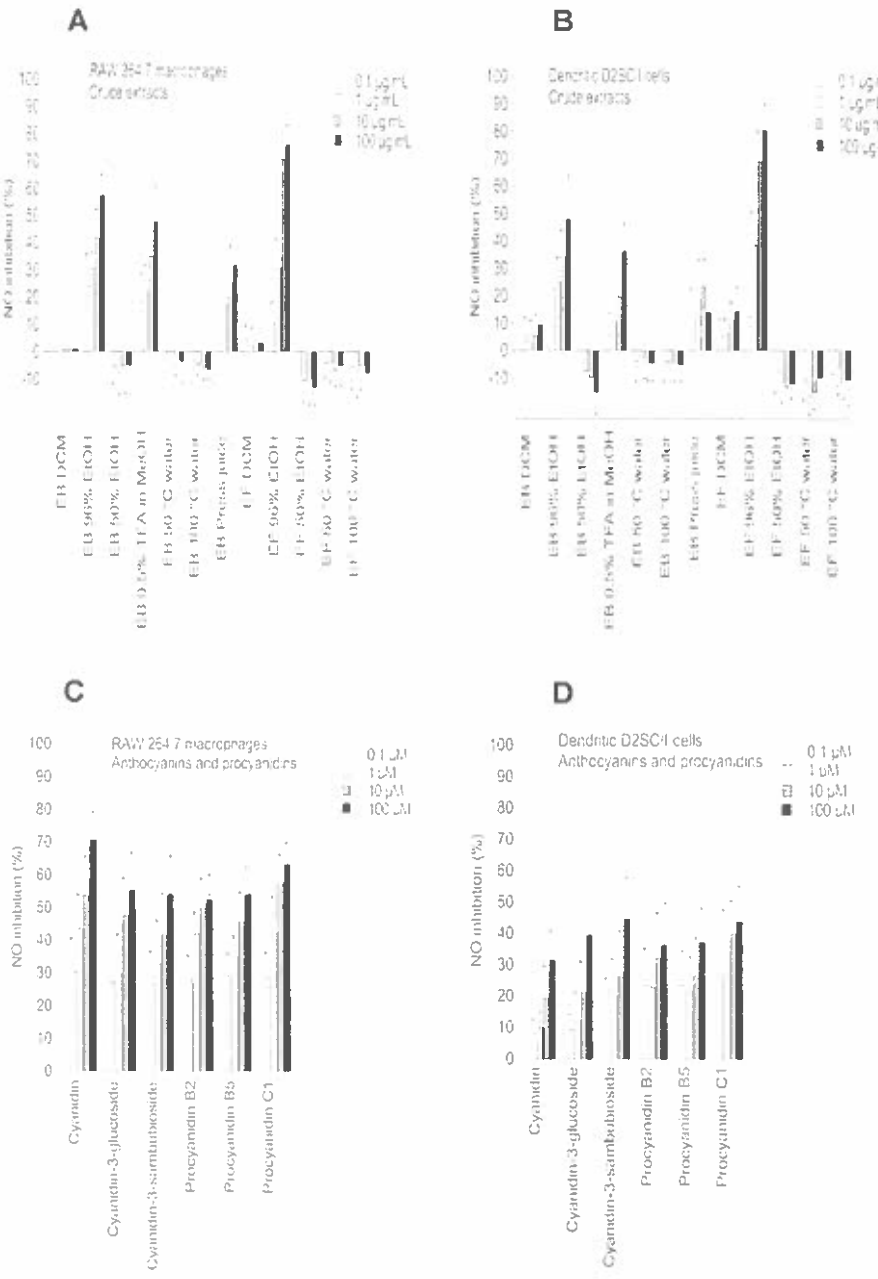



Figure 4. *Cont.*

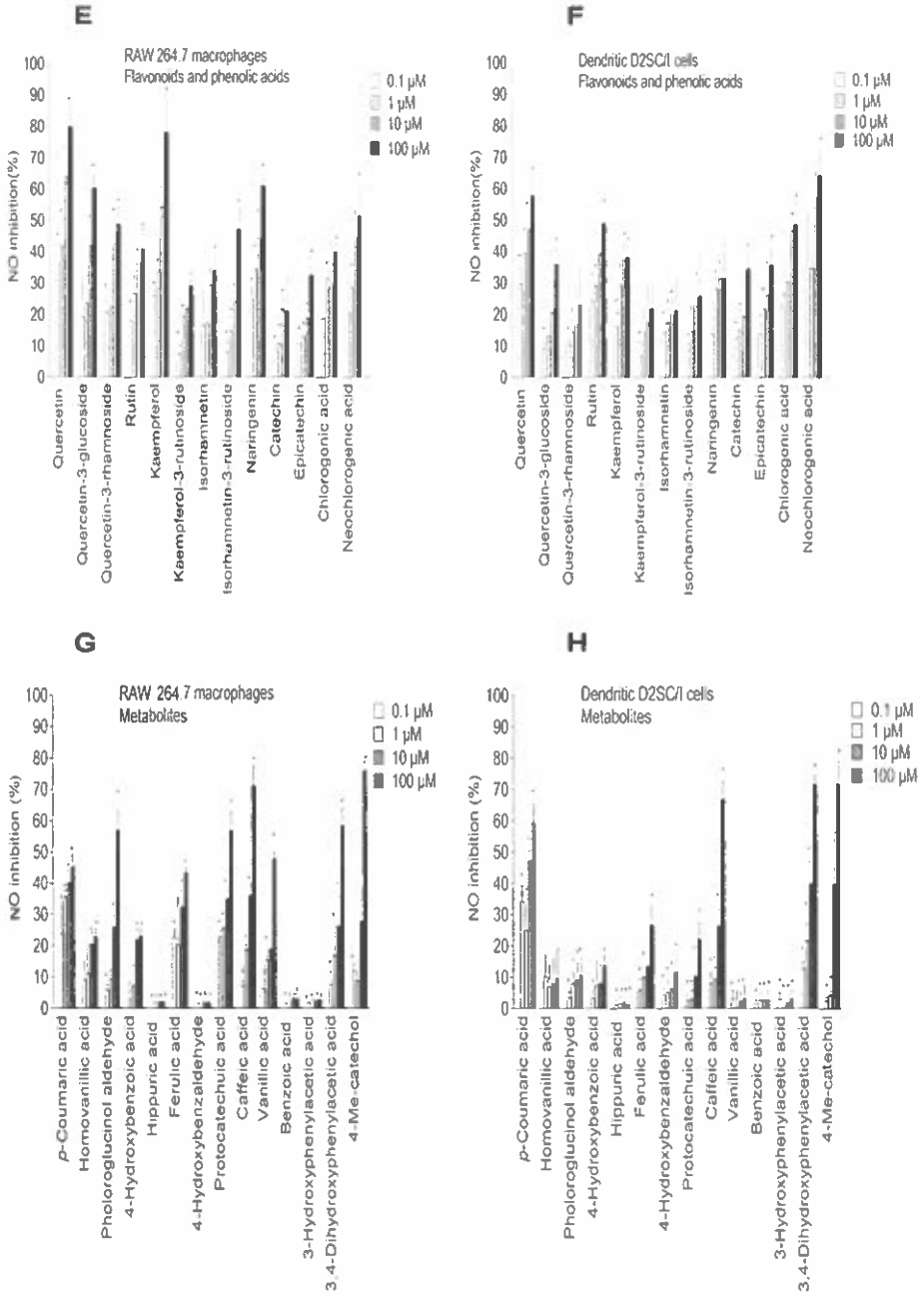



**Figure 4.** Inhibitory effects of elderberry (EB) and elderflower (EF) crude extracts (A,B); anthocyanins and proanthocyanidins (C,D); flavonoids and phenolic acids (E,F); and metabolites (G,H) on NO production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages (A,C,E,G) and dendritic D2SC/I cells (B,D,F,H). The experiments were repeated independently three times, and results shown are expressed as the average ± SEM. * $p < 0.05$ as compared to response of lipopolysaccharide (LPS) (0.1% DMSO) alone.

The elderberry anthocyanins and procyanidins showed high inhibitory effects on NO production (Figure 4C,D) and no toxicity towards the RAW 264.7 and dendritic D2SC/I cells (Figure 5C,D). The aglycone cyanidin showed a stronger inhibitory effect when compared to cyanidin-3-glucoside

and cyanidin-3-sambubioside in the RAW 264.7 cells, and the effect shown was dose-dependent (Figure 4C). Cyanidin-3-glucoside and cyanidin-3-sambubioside, however, showed a higher inhibitory effect on NO production compared to its aglycone cyanidin in the dendritic cells at 100 µM (Figure 4D). The sugar moiety attached to cyanidin aglycone seems in any case to influence the NO inhibitory effect in both the RAW 264.7 cells and the dendritic D2SC/1 cells. Cyanidin glycosides are present in high amounts in elderberries and may therefore be the most important contributor to the NO inhibition in the RAW 264.7 cells and the dendritic D2SC/1 cells. The trimeric procyanidin C1 showed somewhat higher inhibitory activity compared to the dimers procyanidin B2 and procyanidin B5.

Quercetin-3-glucoside, quercetin-3-rhamnoside, and rutin (quercetin-3-rutinoside) showed NO inhibition of 60.4% ± 4.0%, 48.8% ± 5.1%, and 40.9% ± 6.5% at 100 µM in RAW 264.7 cells, respectively (Figure 4E), while the aglycone quercetin had a NO inhibition of 79.9% ± 6.9% at 100 µM. Glycosylated forms seemed to have a weaker NO inhibition in the RAW 264.cells. The same trend was also found for kaempferol and kaempferol-3-rutinoside. Glycosylation of the flavonoids might lead to increased hydrophilicity and steric hindrance, which again results in reduced absorption or penetration into the cells. This might influence the NO inhibitory effect in the RAW 264 7 cells. Similar results have also been reported previously [2,11]. NO inhibition by flavonoids in dendritic D2SC/1 cells (Figure 4F) showed almost the same pattern as in RAW 264.7 cells, but generally the activity in dendritic cells was somewhat lower. Kaempferol showed a strong NO inhibition in the RAW 264.7 cells and weaker inhibition in the dendritic cells. Rutin (glycosylated with a disaccharide), however, was a more potent NO inhibitor in the dendritic cells than in the macrophages, and was also more active than the other quercetin-glycosides tested. Dendritic cells have many of the same properties as macrophages, but their surface phenotype and functional characteristics indicate that they belong to a unique lineage. The polyphenols might act on different cellular targets in the RAW 264.7 cells and dendritic cells. Epicatechin and catechin showed a small but significant inhibitory effect compared to the LPS control in both the RAW 264.7 cells, and showed similar inhibitory effect in dendritic D2SC/1 cells. Chlorogenic acid has previously been reported to have no inhibitory effect in RAW 264.7 cells and in J774 macrophages [2,11]. In our study, chlorogenic acid showed NO inhibitory activity in the RAW 264.7 and dendritic D2SC/1 cells, with the highest activity in dendritic cells. At the highest concentration tested (100 µM), neochlorogenic acid inhibited NO production with 64.3% ± 6.8% in the dendritic D2SC/1 cells, and had somewhat lower activity 51.5% ± 8.8% in RAW cells. Both rutin and chlorogenic acid are present in high amounts in elderflower [20] and may therefore be the most important contributors to modulation of inflammatory effects.

The polyphenols are absorbed rather poorly from the gut and are extensively metabolized in vivo, suggesting that the accumulation of multiple phenolic metabolites may be responsible for some of the reported bioactivity of the polyphenols. The metabolites showed somewhat similar effects of NO inhibition in macrophages and dendritic cells. However, there were some variations in activity from one compound to the other. Phloroglucinol aldehyde, protocatechuic acid, and vanillic acid showed a considerably higher inhibitory effect on NO production in the RAW 264.7 cells compared to the dendritic cells. Caffeic acid, 3,4-dihydroxyphenylacetic acid, protocatechuic acid, and phloroglucinol aldehyde were the most active metabolites, which inhibited the NO production in LPS-activated RAW 264.7 cells by 71.3% ± 6.2%, 58.5% ± 5.1%, 57.1% ± 6.2%, and 56.9% ± 5.8%, respectively, at 100 µM. Protocatechuic acid, the major metabolite from cyanidin-3-glucoside, showed the same inhibitory effect on NO production compared to cyanidin 3-glucoside in RAW 264.7 cells at 100 µM (56.9% ± 5.9% and 55.3% ± 4.0%, respectively). Previous studies have reported that protocatechuic acid and caffeic acid have strong anti-inflammatory properties [36,37]. In LPS-activated dendritic D2SC/1 cells, 3,4-dihydroxyphenylacetic acid, caffeic acid, and *p*-coumaric acid were the most potent metabolites that inhibited NO production by >50% at 100 µM without showing cytotoxicity. 4-Methylcatechol was toxic at 100 µM against RAW 264.7 cells and dendritic D2SC/1 cells (45.3% ± 3.6% and 51.2% ± 8.1% cell viability, respectively) and the strong NO inhibition observed for 4-methylcatechol at 100 µM could be caused by cell cytotoxicity (Figure 5G,H).

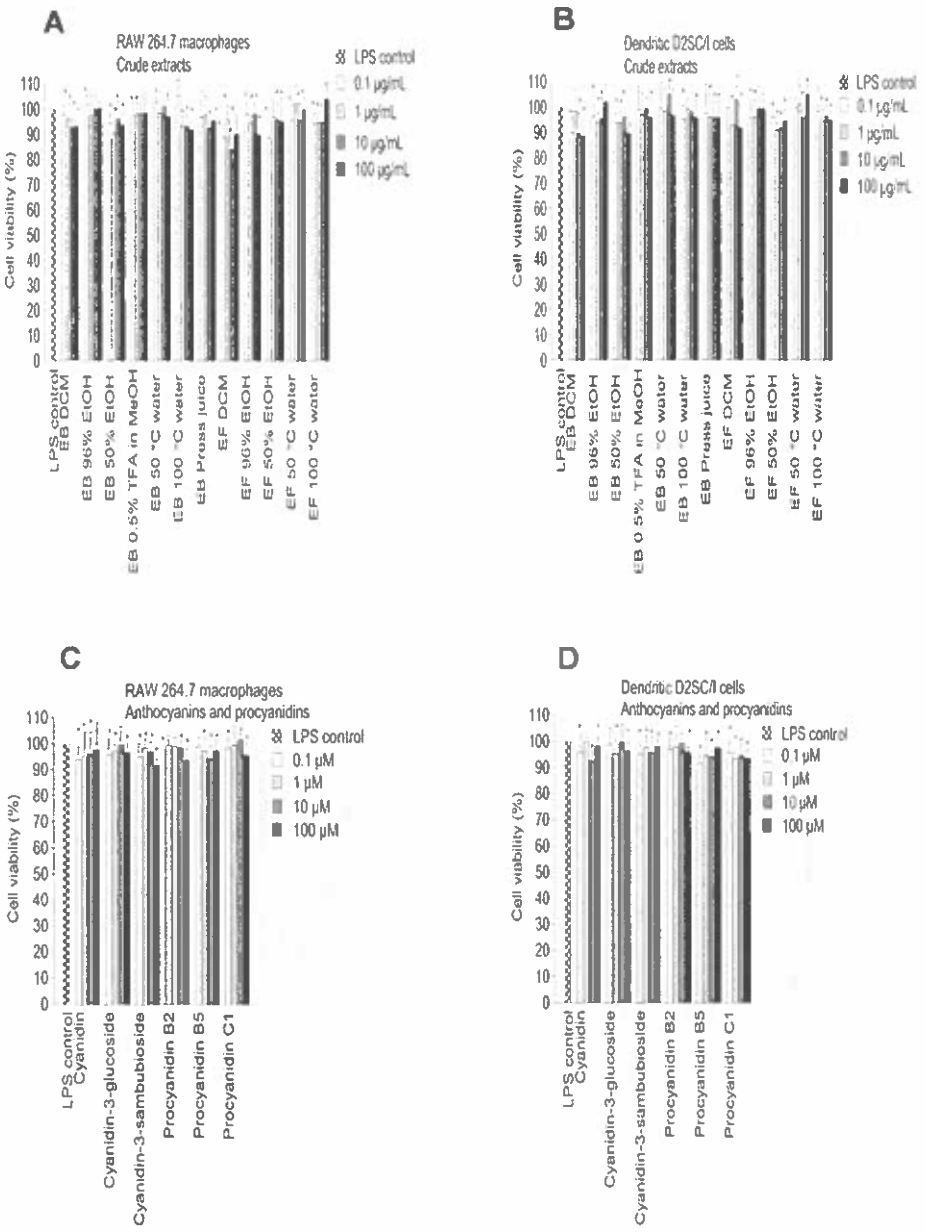



Figure 5. *Cont.*

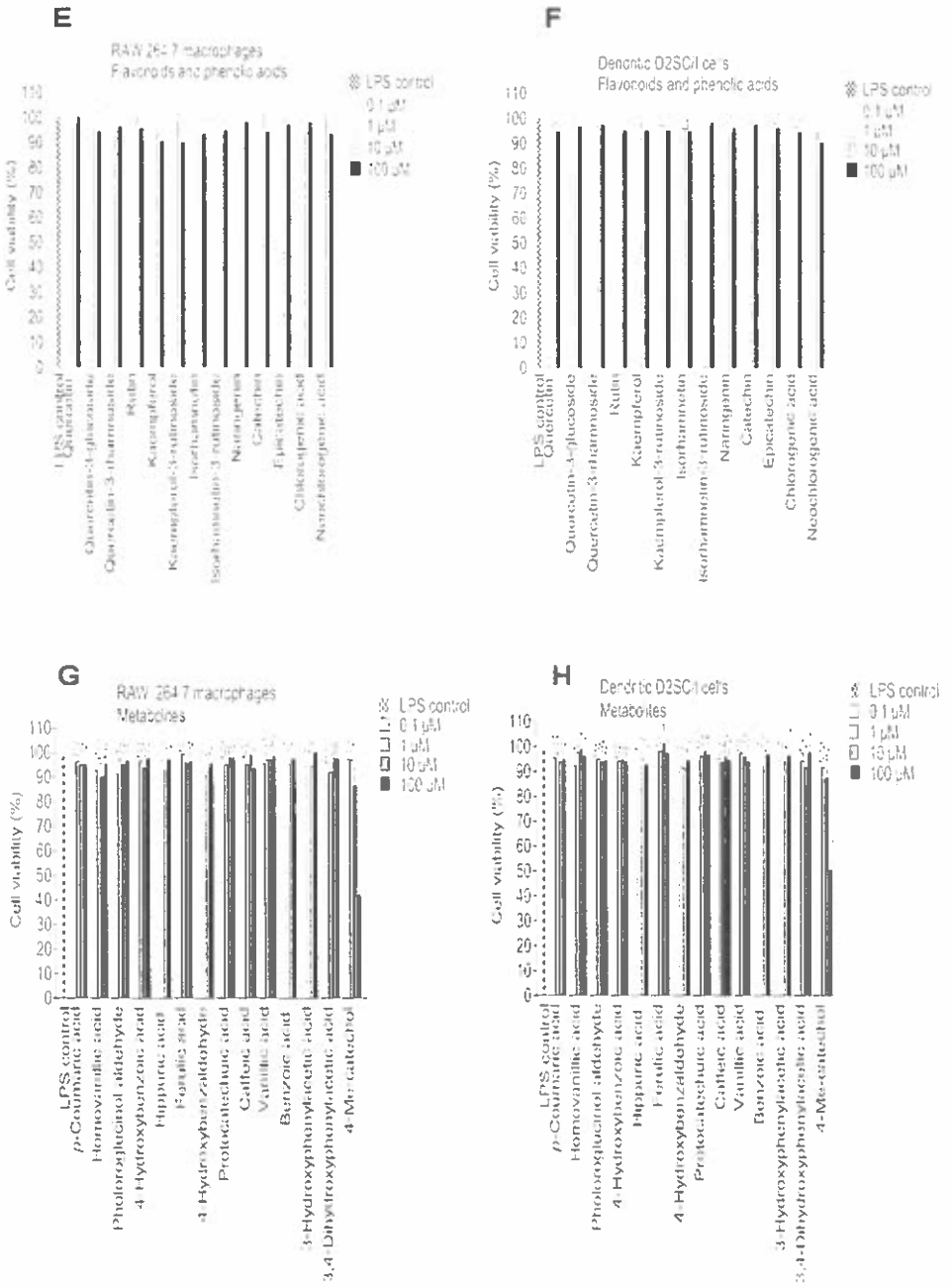



Figure 5. Effect of elderberry (EB) and elderflower (EF) crude extracts (A,B); anthocyanins and proanthocyanidins (C,D); flavonoids and phenolic acids (E,F) on cell viability in LPS-activated RAW 264.7 cells (A,C,E,G) and dendritic D2SC/1 cells (B,D,F,H). Cell viability was assessed using the MTT assay. The experiments were repeated independently three times, and results shown are expressed as the average ± SEM. * $p < 0.05$ vs. LPS (0.1% DMSO) as control.

The bioavailability of elderberry anthocyanins is reported to be low; less than 0.1% of the ingested dose has been detected in humans [38]. Concentrations of 0.1–0.4 µM have been detected for the elderberry anthocyanins in blood and urine after intake of elderberry extract (500–700 mg anthocyanins) [15,16]. The bioavailability of the anthocyanin metabolites was reported to be 60-

and 45-fold higher than their parent compounds based on concentrations in urine and plasma, respectively [15,16]. Bioavailability studies in humans of elderflower and their phenolic compounds are still missing. However, Williamson and Manach [39] reported that polyphenol concentrations of less than 10 μM may have physiological effects. This indicates that concentrations used in this study might have a clinical relevance.

As far as we know, this is the first study comparing the inhibitory effects on NO production in LPS-activated RAW 264.7 cells and dendritic D2SC/1 cells of elderberry and elderflower extracts. In addition, this study gives a comprehensive overview of complement fixating activity and NO inhibition in RAW 264.7 and dendritic cells of the major phenolic compounds present in elderberry and elderflower. Moreover, for the first time we showed that the metabolites in physiological concentrations inhibited the NO production in macrophages and dendritic cells. In sum, these results bring new and supportive data to the inflammatory modulating properties of the phenolic compounds present in elderberries and elderflowers. Thus, intake of elderberry and elderflower might help to regulate inflammatory diseases.

## 3. Materials and Methods

### 3.1. Plant Material

Fresh elderberries (cultivar "Sampo") and raw elderberry juice (made by cold-pressing followed by filtering to remove peels and seeds and heating to 68 °C) were gifts from cultivator Rune Hatleli, Fresvik in Sogn, Norway. The berries were harvested in October 2014 and stored at −20 °C until extraction. Certified ecologically cultivated flowers of *Sambucus nigra* L., harvested in Norway October 2012, were purchased from Odins Marked, Oslo, Norway (org. No: 876905892) in November 2012. Voucher specimens of elderberries (No. EB1010) and elderflowers (No. EF1020) are deposited in the Pharmacognosy section, School of Pharmacy, University of Oslo, Norway.

### 3.2. Chemicals

4-Hydroxybenzaldehyde, 4-hydroxybenzoic acid, caffeic acid, homovanillic acid, vanillic acid, protocatechuic acid, phloroglucinol aldehyde, hippuric acid, ferulic acid, *p*-coumaric acid, quercetin, chlorogenic acid, neochlorogenic acid, epicatechin, catechin, kaempferol, kaempferol-3-rutinoside, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, isorhamnetin-3-rutinoside, naringenin, isorhamnetin, rutin, quercetin, quercetin-3-rhamnoside, quercetin-3-glucoside, 4-methylcatechol, benzoic acid, DMSO-$d_6$, $CD_3OD$, Griess reagent A, Griess reagent B, cell proliferation kit I (MTT), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Proanthocyanidins B2, B5, and C1 were obtained from Plantchem (Klepp, Norway). Dulbecco's modified Eagle's medium (DMEM-Glutamax™, 5.5 mM), fetal bovine serum, and penicillin–streptomycin–amphotericin B were obtained from Gibco Life Technologies (Paisley, UK). Corning CellBIND tissue culture plates were obtained from Corning Life-Sciences (Schiphol-Rijk, The Netherlands). All other reagents were of the highest purity available.

### 3.3. Extraction and Isolation of Anthocyanins

Elderberries and elderflowers were extracted according to Ho et al. [40]. For extraction, 30 g freeze-dried berries or flowers were mixed with diatomaceous earth (Dionex, Sunnyvale, CA, USA) (4:1) and loaded in a 100 mL steel cartridge. The elderberries and elderflowers were then extracted successively with dichloromethane (DCM) (~900 mL for elderberries and ~700 mL for elderflowers), followed by 96% EtOH (~950 mL for elderberries and ~800 mL for elderflowers), 50% EtOH (~650 mL for elderberries and ~500 mL for elderflowers), 50 °C water (~450 mL for elderberries and ~300 mL for elderflowers), and 100 °C water (~450 mL for elderberries and ~300 mL for elderflowers). The anthocyanins from elderberries were isolated in accordance to Bräunlich et al. [41]. Briefly, freeze-dried elderberries were extracted by maceration with MeOH (0.5% trifluoroacetic acid (TFA) *v*/*v*) for 24 h. The dried extract was

applied on an Amberlite XAD-7HP column (5 cm × 50 cm) with water followed by MeOH (0.5% TFA) as mobile phase. The anthocyanin-enriched fraction was then fractionated on a Sephadex LH-20 column (5 cm × 100 cm) (water–MeOH (0.5% TFA) as gradient), and purified by preparative HPLC using a Microsorb 60-8 $C_{18}$ (250 mm × 21.4 mm) column (Varian, Palo Alto, CA, USA) with a water-acetonitrile (0.5% TFA) gradient as eluent.

### 3.4. NMR Spectroscopy

$^1$H and $^{13}$C nuclear magnetic resonance (NMR) was conducted on a Bruker DPX 300 or a Bruker AVII 400 instrument (Bruker, Rheinstetten, Germany) with $CD_3OD$, $CD_3OD$:TFA (95:5) or DMSO-$d_6$ as solvents and tetramethylsilane (TMS) as reference.

### 3.5. Complement Fixating Assay

The complement fixating test is based on inhibition of hemolysis of antibody-sensitized sheep red blood cells (SRBC) by human sera, as described by Michaelsen et al. [42] (Method A). Samples were dissolved in 0.1% DMSO or veronal buffered saline (VBS) pH 7.2 containing 0.2 mM $CaCl_2$ and 0.8 mM $MgCl_2$ with 2 mg/mL bovine serum albumin (BSA). A pectic polysaccharide, BPII, from *Bioplytum umbraculum* Welw. (syn. *B. petersianum* Klotzsch) was used as a positive control [25]. A dose–response curve was constructed to calculate the concentration of the test sample that gave 50% inhibition ($IC_{50}$) of hemolysis. The experiments were repeated independently three times.

### 3.6. RAW 264.7 and Dendritic D2SC/1 Cells

The mouse macrophages cell line RAW 264.7 and dendritic cell line D2SC/1 were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% 100 mM sodium pyruvate, 1% Pen Strep (10,000 U/mL penicillin and 10,000 µg/mL streptomycin), and $5 \times 10^{-5}$ M 2-mercaptoethanol and maintained in a 37 °C humidified incubator containing 5% $CO_2$.

### 3.7. Nitrite Assay

The nitrite assay was carried out as previously described with minor modifications [28]. Macrophages and dendritic cells at a density of $5 \times 10^5$ cells/mL were seeded into 96-well flat-bottomed plates and pre-incubated with the test samples for one hour before the addition of 500 ng/mL LPS (*Escherichia coli* O55:B5, Sigma-Aldrich). Samples were dissolved in DMSO (0.1% final concentration) or medium. The cells were incubated for 24 h in duplicates containing 0.1, 1, 10, and 100 µg/mL (extracts) or 0.1, 1, 10, and 100 µM (pure compounds) of test samples (final concentrations). NO was determined by measuring the amount of nitrite, which can be measured in a colorimetric assay using the Griess reagents. The supernatant (50 µL) was mixed with 50 µL of Griess reagent A (1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature in the dark for 10 min. Then 50 uL Griess reagent B (0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in water) was added, and the absorbance was measured at 540 nm using a Bio-Rad reader. Quercetin was used as a positive control. A serial dilution of $NaNO_2$ in medium was used to construct the standard reference curve. Percentage NO inhibition was expressed as the percentage decrease in NO production as: $100 - [NO]^a/[NO]^b \times 100$, where $[NO]^a$ represents the NO concentration for test samples and $[NO]^b$ represents the NO concentration from LPS-activated control. The experiments were repeated independently three times.

### 3.8. Cell Viability Assay

Cell viability was determined by the MTT assay. The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. The method was done according to the manufacturer's procedure [43]. Briefly, RAW 264.7 and dendritic D2SC/1 cells were

seeded into 96-well plates at a density of $5 \times 10^5$ cells/well and incubated with test samples (0.1, 1, 10, and 100 µg/mL (extracts) or 0.1, 1, 10, and 100 µM (pure compounds)) for 24 h in a humidified 6.5% $CO_2$ atmosphere at 37 °C. After the incubation period, 10 µL of MTT reagent was added to each well. After 4 h, 100 µL of solubilization solution was added. The quantity of formazan (presumed to be directly proportional to the number of viable cells) is measured by recording changes in absorbance at 595 nm using a microplate spectrophotometer (iMark Microplate reader, Bio-Rad, 'Hercules, CA, USA). Medium was used as negative control and 20% DMSO as positive control. The experiments were repeated independently three times.

### 3.9. Statistical Analysis

Each assay was performed in duplicate or triplicate and results are given as mean (±SEM) from three separate experiments. Comparisons of different treatments were evaluated by two-tailed Student's *t*-test. Statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

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**Author Contributions:** Helle Wangensteen and Hilde Barsett conceived and designed the experiments; Giang Thanh Thi Ho performed the experiments, analyzed the data and wrote the paper. All authors read and approved the final manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

| | |
|---|---|
| DCM | Dichloromethane |
| DMSO | Dimethylsulfoxide |
| EB | Elderberry |
| EF | Elderflower |
| EtOH | Ethanol |
| $IC_{50}$ | Inhibitory concentration to give 50% effect |
| LPS | Lipopolysaccharide |
| MeOH | Methanol |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NO | Nitric oxide |
| NMR | Nuclear magnetic resonance |
| TMS | Tetramethylsilane |
| TFA | Trifluoroacetic acid |

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# European elderberry (*Sambucus nigra* L.) rich in sugars, organic acids, anthocyanins and selected polyphenols

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ABSTRACT

Sugars and organic acids in the fruit of two cultivars and three selections of black elderberry (*Sambucus nigra* L.): 'Haschberg', 'Rubini', 'Selection 13', 'Selection 14' and 'Selection 25' were quantified. The anthocyanin as well as quercetin profiles of this plant material were also established by the use of HPLC/MS. Significant differences in the concentration of sugars and organic acids were detected between the widely spread cultivar 'Haschberg' and all other cultivars/selections; 'Haschberg' was the richest in organic acids (6.38 g kg $^{-1}$ FW), and it contained the least sugar (68.5 g kg $^{-1}$ FW). The following major cyanidin based anthocyanins were identified in the fruit of black elderberry: cyanidin 3-sambubioside-5-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-sambubioside, cyanidin 3-glucoside and cyanidin 3-rutinoside. The most abundant anthocyanin in elderberry fruit was cyanidin 3-sambubioside, which accounted for more than half of all anthocyanins identified in the berries. The 'Rubini' cultivar had the highest amount of the anthocyanins identified (1265 mg/100 g FW) and the lowest amount was measured in berries of the 'Selection 14' (603 mg/100 g FW). The 'Haschberg' cultivar contained a relatively low amount of anthocyanins in ripe berries (737 mg/100 g FW). From the quercetin group, quercetin, quercetin 3-rutinoside and quercetin 3-glucoside were identified; the latter prevailing in black elderberry fruit. The cultivar with the highest amount of total quercetins was 'Selection 25' (73.4 mg/100 g FW), while the 'Haschberg' cultivar contained average amounts of quercetins (61.3 mg/100 g FW). The chemical composition of the 'Haschberg' cultivar, the most commonly planted, conforms to the standards for sugars, anthocyanins and quercetins and exceeds them in the content levels of organic acids, the most important parameter in fruit processing.
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## 1. Introduction

Black or common elder (*Sambucus nigra* L.), also called elderberry, is a widespread species, that grows on sunlight-exposed locations in most parts of Europe, Asia, North Africa and the USA. It is a deciduous shrub reaching up to 6 m in height, developing small, white hermaphrodite flowers in large corymbs, and flowering in early summer. Umbels consist of dark purple individual berries, with a diameter up to 6 mm; the fruit ripen in late summer. Elderberry cultivars are planted for ornamental purposes, but elderflower extracts are used as beverage and food flavourings (Christensen, Knaack, & Frette, 2007), and elderberry berries have been globally utilised as a medicine or a source of dietary supplements (Dawidowicz, Wianowska, & Baraniak, 2006; Lee & Finn, 2007). Many cultivars of *S. nigra* L. are planted in Europe, the most common being the 'Haschberg' cultivar with several selections, which are mainly harvested for fruit. This cultivar is seldom used for fresh consumption; mostly it is processed to concentrates and juices. The juice pressed from black elderberry fruit contains many primary metabolites including various sugars and organic acids. High concentrations of organic acids are important in processing, since, unlike sugars, they cannot be added to the final product. Among secondary metabolites, elderberry juice is predominantly characterised by high amounts of the anthocyanins.

Anthocyanins are a class of flavonoid compounds responsible for the attractive orange to blue colour of flowers, as well as an important fruit quality indicator, since they influence fruit appearance and flavour (Del Caro & Piga, 2008; Lee & Finn, 2007). They have gained interest as functional compounds in food colorants and as potent agents against oxidative stress (Stintzing, Stintzing, Carle, Frei, & Wrolstad, 2002), reducing oxidative damage to the human body (Dawidowicz et al., 2006). Anthocyanins, as well as other flavonoids (e.g. quercetins), exhibit antioxidant, anticarcinogenic, immune-stimulating, antibacterial, antiallergic and antiviral properties; therefore, their consumption may contribute to prevention of several degenerative diseases such as cardiovascular disease, cancer, inflammatory disease and diabetes (Bonerz, Würth, & Dietrich, 2006; Brambilla, Lo Scalzo, Bertolo, & Torreggiani, 2008; Dawidowicz et al., 2006; Lata & Tomala, 2007; Nakajima, Tanaka, Seo, Yamazaki, & Saito, 2004). These compounds are well known

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free radical scavengers, reported as potential chemo-preventive agents. Therefore, elderberry cultivars containing higher concentrations of anthocyanins are considered most important.

Several studies were made on anthocyanin and other polyphenolic content levels of various *S. nigra* L. cultivars with little information on the content of various classes of primary metabolites (Nakajima et al., 2004; Wu, Gu, Prior, & Mckay, 2004; Zakay-Rones, Thom, Wollan, & Wadstein, 2004; Lee & Finn, 2007); however, research papers on the overall chemical composition of elderberry fruit are scarce.

The objective of this study was to compare the content levels of three sugars (sucrose, fructose and glucose) and four organic acids (malic acid, citric acid, fumaric acid and shikimic acid) in two cultivars and three selections of *S. nigra* L. Moreover, the anthocyanin as well as quercetin profiles of the selected plant material were established. Since common elderberry is an important fruit species for processing, internal quality parameters were compared to other fruit cultivated for processing as well as those for fresh consumption.

## 2. Material and methods

### 2.1. Plant material

The fruit of two elderberry cultivars ('Rubini', 'Haschberg') and three elderberry selections ('Selection 13', 'Selection 14' and 'Selection 25') were picked at their optimum fruit maturity at the end of August 2006 in Austria, on a laboratory research field near Graz. All plants were mature and growing in similar, sunny conditions. Umbels were harvested from several representative plants of each cultivar/selection, and approximately 1 kg of elderberry berries (with stalks) were packed in plastic bags and stored at −18 °C until further analysis. Various compounds (sugars, organic acids, anthocyanins and quercetins) were analysed from whole umbels (berries, including stalks). For each cultivar/selection five replicates were carried out ($n = 5$).

### 2.2. Analysis of individual carbohydrates and organic acids

Elderberry fruit samples were analysed for their content levels of carbohydrates (sucrose, glucose and fructose) and organic acids (malic, citric, fumaric and shikimic). In the laboratory, umbels of each cultivar/selection were mashed to a pulp and 10 g of the fresh mass was immersed in 50 ml of twice distilled water and homogenised with the T-25 Ultra-Turrax (Ika-Labortechnik). The fruit samples were left for extraction for half an hour at room temperature, with frequent stirring, and the extracted samples were afterwards centrifuged at 10,000*g* for 7 min at 10 °C (Eppendorf Centrifuge 5810R, Hamburg, Germany). The supernatants were filtered through a 0.45 μm filter (Macherey-Nagel), transferred to a vial, and analysed according to the method described by Sturm, Koron, and Stampar (2003) using high-performance liquid chromatography (HPLC; Thermo Scientific, Finnigan Spectra System, Waltham, MA, USA). For each analysis, 20 μl of sample was used. Analysis of sugars was carried out using a Rezex-RCM-monosaccharide column (300 × 7.8 mm; Phenomenex, Torrance, CA) with a flow rate of 0.6 ml min$^{-1}$ and with column temperature maintained at 65 °C. For the mobile phase, twice distilled water was used, and an RI detector for identification. Organic acids were analysed in the Aminex-HPX-87 H column (300 × 7.8 mm; Bio-Rad, USA), and a UV detector set at 210 nm with a flow rate of 0.6 ml min$^{-1}$ maintaining the column temperature at 65 °C. For the mobile phase, 4 mM sulphuric acid was used. The concentrations of carbohydrates and organic acids were calculated with the help of corresponding external standards.

### 2.3. Extraction and HPLC analysis of anthocyanins and quercetins

For the analysis of individual anthocyanins and quercetins, 1 g of berries was extracted with 20 ml methanol containing 1% HCl and 1% 2,6-di-*tert*-butyl-4-methylphenol (BHT) in an ultrasonic bath for 30 min. After extraction, the treated samples were centrifuged for 7 min at 12,857 rcf and the supernatant was filtered through a Chromafil AO-45/25 polyamide filter produced by Macherey-Nagel (Düren, Germany) to a vial prior to injection into the HPLC system. HPLC analysis was performed using a Surveyor HPLC system with a diode array detector (DAD), controlled by ChromQuest 4.0 software (Thermo Finnigan, San Jose, CA). The anthocyanins were analysed at 530 nm and the quercetins at 350 nm. The column used was a Gemini $C_{18}$ (150 × 4.6 mm 3 μm; Phenomenex) operated at 25 °C. The elution solvents were 1% formic acid in twice distilled water (A) and 100% acetonitrile (B). The samples were eluted according to the linear gradient described by Marks, Mullen, and Crozier (2007), with the injection amount of 20 μl and a flow rate 1 ml min$^{-1}$. The phenolics were identified by comparing their UV–Vis spectra from 220 to 550 nm and retention times. Quantification was achieved according to concentrations of the corresponding standard and was confirmed using the Thermo Scientific LCQ Deca XP$^{+}$ mass spectrometer with an electrospray interface (ESI) operating in positive (anthocyanins) or negative (quercetins) ion mode. The concentrations of the identified anthocyanins cyanidin 3-sambubioside-5-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-sambubioside, cyanidin 3-glucoside and cyanidin 3-rutinoside were assessed from peak areas and calculated as equivalents of cyanidin. The content of total anthocyanins was expressed in mg cyanidin equivalents (CGE) per 100 g of elderberry fruit. The concentrations of the identified quercetins were calculated from peak areas of the corresponding standards – quercetin-glucoside, quercetin-rutinoside and quercetin.

### 2.4. Chemicals

The following standards were used to determine the chemical compounds in elderberry fruit: sucrose, fructose, glucose; malic, citric, fumaric and shikimic acid; cyanidin chloride; rutin, Q-glucoside, Q-xyloside, phloretine-xylosilglucoside and quercetine.

The chemicals for mobile phases and sample preparation were methanol and acetonitrile from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and formic acid from Fluka Chemie GmbH (Buchs, Switzerland).

The water used in sample preparation, solutions and analysis was twice distilled and purified with a Milli-Q water purification system by Millipore (Bedford, MA).

### 2.5. Statistical analysis

Statistical analysis was conducted with the Statgraphics Plus 4.0 programme (Statgraphics, Herndon, VA). One-way analysis of variance (ANOVA) was used for the analysis of the effect of cultivar/selection on the content level of sugars, organic acids, anthocyanins and quercetins. Differences between cultivars/selections were estimated with the HSD test and were considered statistically significant when $p < 0.05$.

## 3. Results and discussion

### 3.1. Sugars

Two cultivars and three selections of black elderberry were examined in this study and the concentrations of individual sugars were assessed. The most abundant sugars in black elderberry fruit

Table 1
Concentrations of individual sugars in fruits of two cultivars and three selections of black elderberry (*S. nigra* L.) in g kg$^{-1}$ FW

| *S. nigra* L. cultivar/selection | Sucrose | Fructose | Glucose | Sum sugars |
|---|---|---|---|---|
| 'Haschberg' | 1.21 ± 0.06 b | 33.99 ± 0.93 a | 33.33 ± 0.67 a | 68.53 ± 1.55 a |
| 'Selection 13' | 0.47 ± 0.04 a | 44.14 ± 1.02 b | 42.42 ± 0.84 b | 87.03 ± 1.86 b |
| 'Selection 14' | 0.48 ± 0.05 a | 45.30 ± 1.02 b | 45.17 ± 1.13 c | 90.96 ± 2.16 b |
| 'Selection 25' | 1.68 ± 0.16 c | 52.25 ± 1.43 c | 50.23 ± 0.53 d | 104.16 ± 1.84 c |
| 'Rubini' | 1.38 ± 0.03 b | 44.12 ± 1.63 b | 41.93 ± 1.26 b | 87.44 ± 2.88 b |
| Mean | 1.04 ± 0.10 | 43.96 ± 1.29 | 42.62 ± 1.18 | 87.62 ± 2.48 |

Average values ± standard error are presented. Different letters (a–d) in rows mean statistically significant differences between individual sugar contents among cultivars/selections at $\alpha < 0.05$.

were fructose and glucose, sucrose was detected only in small amounts. The individual and total sugar content in elderberry fruit was highest in 'Selection 25', with average values of 104.2 g kg$^{-1}$ fresh weight (FW); significant differences were present between this selection and all other cultivars/selections. Lowest concentrations of total carbohydrates were detected in the fruit pulp of the 'Haschberg' standard cultivar, with average values of 68.53 g kg$^{-1}$ (Table 1). Elderberry fruit contains moderate amounts of sugars compared to apple, which contains 115–183 g kg$^{-1}$ total analysed sugars (Hofer et al., 2005) and significantly lower amounts of total sugars than sweet cherry, which averagely contains 150–230 g kg$^{-1}$ (Usenik, Fabcic, & Stampar, 2008). The content level of sugars in elderberry fruit is comparable to sour cherry fruit (*Prunus cerasus* L.), which contain approximately 90 g l$^{-1}$ of total sugars and are also mainly used in processing (Bonerz et al., 2006; Füzfai, Katona, Kovács, & Molnár-Perl, 2004). The amount of sugar in elderberry fruit, however, is not the focal chemical compound for technological processing, as fructose and sucrose can easily be added to the final products (i.e. juices, concentrates, beverages).

### 3.2. Organic acids

Four organic acids were identified in the elderberry berries: citric acid, malic acid, shikimic acid and fumaric acid (Table 2). Citric acid was the most abundant organic acid in all cultivars/selections, followed by malic acid and smaller concentrations of shikimic and fumaric acid. The content level of citric acid in the fruit of black elderberry ranged from 3.11 g kg$^{-1}$ FW in 'Selection 13' to 4.81 g kg$^{-1}$ FW in cultivar 'Haschberg'. Compared to apple, which contains between 0.07 and 0.52 g kg$^{-1}$ FW citric acid (Hofer et al., 2005), sweet cherry, which contains between 0.11 and 0.54 g kg$^{-1}$ FW citric acid (Usenik et al., 2008) and sour cherry, which contains between 0.08 and 0.14 g kg$^{-1}$ FW citric acid (Bonerz et al., 2006), elderberry fruit is exceptionally rich in this organic acid. This is an important parameter for processing purposes and justifies the leading role of the 'Haschberg' elderberry cultivar in European plantations, since it is particularly rich in citric acid. Moreover, the most significant statistical differences were observed in the concentration of shikimic acid among different cultivars/selections with values ranging from as low as 0.14 g kg$^{-1}$ FW in 'Selection 14' to 0.93 g kg$^{-1}$ FW in 'Selection 25'. The lowest amount of total organic acid was detected in fruits of 'Selection 13, which contained 4.52 g kg$^{-1}$ FW and the highest in the cultivar 'Haschberg' with 6.38 g kg$^{-1}$ FW. The total organic acid concentration of all cultivars/selection of elderberry is lower than in apple, which contains, on average, between 6.00 and 14.00 g kg$^{-1}$ FW organic acids (Hofer et al., 2005) and sweet cherry, which contains, on average, between 3.50 and 8.20 g kg$^{-1}$ FW organic acids (Usenik et al., 2008).

### 3.3. Anthocyanins

High performance liquid chromatography analysis of black elderberry (*S. nigra* L.) revealed the presence of five anthocyanin peaks: cyanidin 3-sambubioside-5-glucoside ($[M+H]^+$ at *m/z* 743), cyanidin 3,5-diglucoside ($[M+H]^+$ at *m/z* 611), cyanidin 3-sambubioside ($[M+H]^+$ at *m/z* 581), cyanidin 3-glucoside ($[M+H]^+$ at *m/z* 449) and cyanidin 3-rutinoside ($[M+H]^+$ at *m/z* 595). Other anthocyanins occured in lower amounts. According to Inami, Tamura, Kikuzaki, and Nakatani (1996), Wu et al. (2004) and Kaack, Fretté, Christensen, Landbo, and Meyer (2008) the two major anthocyanins in *S. nigra* L. are cyanidin 3-glucoside and cyanidin 3-sambubioside, and this is consistent with our results (Table 3). The major anthocyanin in all berries from these cultivars, cyanidin 3-sambubioside, accounted for up to half of all determined anthocyanins, approximately. The only exception was 'Selection 13', where the major anthocyanin was cyanidin 3-glucoside, accounting for more than 60% of all analysed anthocyanins. The lowest concentration of cyanidin 3-sambubioside was detected in 'Selection 13' (271 mg CGE/100 g FW) and the highest in cultivar 'Rubini' (631 mg CGE/100 g FW). Significant differences in concentration of cyanidin 3-sambubioside were present between 'Selection 13' and all cultivars/selections analyzed. The lowest amount of second major anthocyanin, cyanidin 3-glucoside, was measured in the berries of 'Selection 14' (221 mg CGE/100 g FW) and the highest in 'Selection 13' (456 mg CGE/100 g FW). Significant differences were detected between each cultivar/selection, with the exception of 'Haschberg' cultivar and 'Selection 25', which contained similar amounts of cyanidin 3-glucoside. Separation and peak identification of cyanidin 3-sambubioside and cyanidin 3-glucoside were

Table 2
Concentrations of organic acids in fruits of two cultivars and three selections of black elderberry (*S. nigra* L.) in g kg$^{-1}$ FW

| *S. nigra* L. cultivar/selection | Citric acid | Malic acid | Shikimic acid | Fumaric acid | Sum organic acids |
|---|---|---|---|---|---|
| 'Haschberg' | 4.81 ± 0.19 b | 1.10 ± 0.04 b | 0.18 ± 0.04 ab | 0.29 ± 0.001 d | 6.38 ± 0.26 d |
| 'Selection 13' | 3.11 ± 0.10 a | 1.10 ± 0.03 b | 0.16. ± 0.03 a | 0.14 ± 0.001 b | 4.52 ± 0.13 ab |
| 'Selection 14' | 3.39 ± 0.09 a | 1.31 ± 0.03 c | 0.14 ± 0.02 a | 0.10 ± 0.001a | 4.95 ± 0.12 bc |
| 'Selection 25' | 3.09 ± 0.05 a | 0.97 ± 0.01 a | 0.93 ± 0.02 c | 0.18 ± 0.01 c | 5.17 ± 0.07 c |
| 'Rubini' | 3.08 ± 0.07 a | 1.02 ± 0.04 ab | 0.24 ± 0.01 b | 0.13 ± 0.004 b | 4.48 ± 0.12 a |
| Mean | 3.50 ± 0.14 | 1.10 ± 0.03 | 0.33 ± 0.06 | 0.17 ± 0.01 | 5.10 ± 0.15 |

Average values ± standard error are presented. Different letters (a–d) in rows mean statistically significant differences between individual organic acids among cultivars/selections at $\alpha < 0.05$.

Table 3
Concentrations of five anthocyanins in fruits of two cultivars and three selections of black elderberry (*S. nigra* L.) in mg CGE/100 g FW

| *S. nigra* L. cultivar/selection | Cy 3-sam-5-glu | Cy 3,5-diglu | Cy 3-sam | Cy 3-glu | Cy 3-rut | Sum anthocyanins |
|---|---|---|---|---|---|---|
| 'Haschberg' | 33.29 ± 1.30 c | 9.47 ± 1.03 ab | 352.7 ± 25.6 b | 331.7 ± 13.0 b | 9.63 ± 0.45 c | 736.7 ± 40.2 b |
| 'Selection 13' | 19.52 ± 0.98 a | 7.41 ± 0.31 a | 270.8 ± 18.6 a | 456.2 ± 26.4 c | 2.98 ± 0.52 b | 756.9 ± 45.3 b |
| 'Selection 14' | 21.91 ± 0.64 ab | 11.35 ± 0.23 b | 346.7 ± 10.5 b | 221.4 ± 6.0 a | 1.49 ± 0.59 a | 602.9 ± 15.5 a |
| 'Selection 25' | 53.49 ± 3.55 d | 23.29 ± 1.63 d | 592.8 ± 42.0 c | 285.1 ± 15.8 b | 2.52 ± 0.21 b | 957.3 ± 63.0 c |
| 'Rubini' | 25.63 ± 0.73 b | 20.18 ± 0.84 c | 630.8 ± 9.00 c | 586.4 ± 12.1 d | 2.25 ± 0.30 ab | 1265.3 ± 21.0 d |
| Mean | 30.77 ± 2.61 | 14.34 ± 1.33 | 438.8 ± 31.2 | 376.2 ± 27.4 | 3.77 ± 0.62 | 863.8 ± 49.9 |

Anthocyanins in black elderberry fruit: cy 3-sam-5-glu: cyanidin 3-sambubioside-5-glucoside; cy 3,5-diglu: cyanidin 3,5-diglucoside; cy 3-sam: cyanidin 3-sambubioside; cy 3-glu: cyanidin 3-glucoside; cy 3-rut: cyanidin 3-rutinoside. Average values ± standard error are presented. Different letters (a–d) in rows mean statistically significant differences between individual anthocyanins among cultivars/selections at $p < 0.05$.

not possible given the chosen method because of identical retention times. The identification was therefore done with the help of mass analysis (Fig. 1). Three minor peaks were also identified in the berries of analysed cultivars/selections of elderberry: cyanidin 3-sambubioside-5-glucoside was lowest in 'Selection 13' (195 mg kg$^{-1}$ FW) and highest in 'Selection 25' (53.5 mg CGE/100 g FW), cyanidin 3,5-diglucoside was lowest in 'Selection 13' (7.41 mg CGE/100 g FW) and highest in 'Selection 25' (23.3 mg CGE/100 g FW) and cyanidin 3-rutinoside was lowest in 'Selection 14' (1.49 mg CGE/100 g FW) and highest in cultivar 'Haschberg' (9.60 mg CGE/100 g FW). The concentration of total anthocyanins ranged from 603 mg CGE/100 g FW in 'Selection 14' to 1265 mg CGE/100 g FW in berries of cultivar 'Rubini', which was similar to the results of Kaack et al. (2008). The standard 'Haschberg' cultivar contained moderate amounts of total anthocyanins (737 mg CGE/100 g FW); however, we must take into account that a higher content of anthocyanins does not automatically mean a darker colour of the fruit, and 'Haschberg' is, therefore also suitable for processing. In comparison to black figs, which contain approximately 95 mg CGE/100 g FW total anthocyanins (Del Caro & Piga, 2008) and sweet cherry, which contains, on average, 100–120 mg CGE/100 g FW (Usenik et al., 2008), elderberry fruit has significantly higher anthocyanin content levels (see Table 4).

### 3.4. Quercetins

In the group of quercetins, we detected the following: quercetin ([M−H]$^-$ at *m/z* 301), quercetin 3-rutinoside ([M−H]$^-$ at *m/z* 609) and quercetin 3-glucoside ([M−H]$^-$ at *m/z* 463). The main quercetin in these elderberry cultivars/selections was quercetin 3-rutinoside (rutin), with values ranging from 35.6 mg CGE/100 g FW in 'Selection 14' to 52.0 mg CGE/100 g FW in the 'Haschberg' cultivar. These results are comparable to the concentration of quercetin 3-rutinoside in fruit of black figs, which according to Del Caro and Piga (2008) contain approximately 53 mg CGE/100 g FW of this compound. The other two quercetins were in considerably lower amounts, especially the aglicon quercetin; the concentration of this was approximately 1/10 of the amount of quercetin 3-rutinoside. Total quercetins were lowest in 'Selection 14' (51.9 mg CGE/100 g FW) and highest in 'Selection 25' (73.4 mg CGE/100 g FW), the 'Haschberg' cultivar containing moderate amounts of total quercetins (61.3 mg CGE/100 g FW).

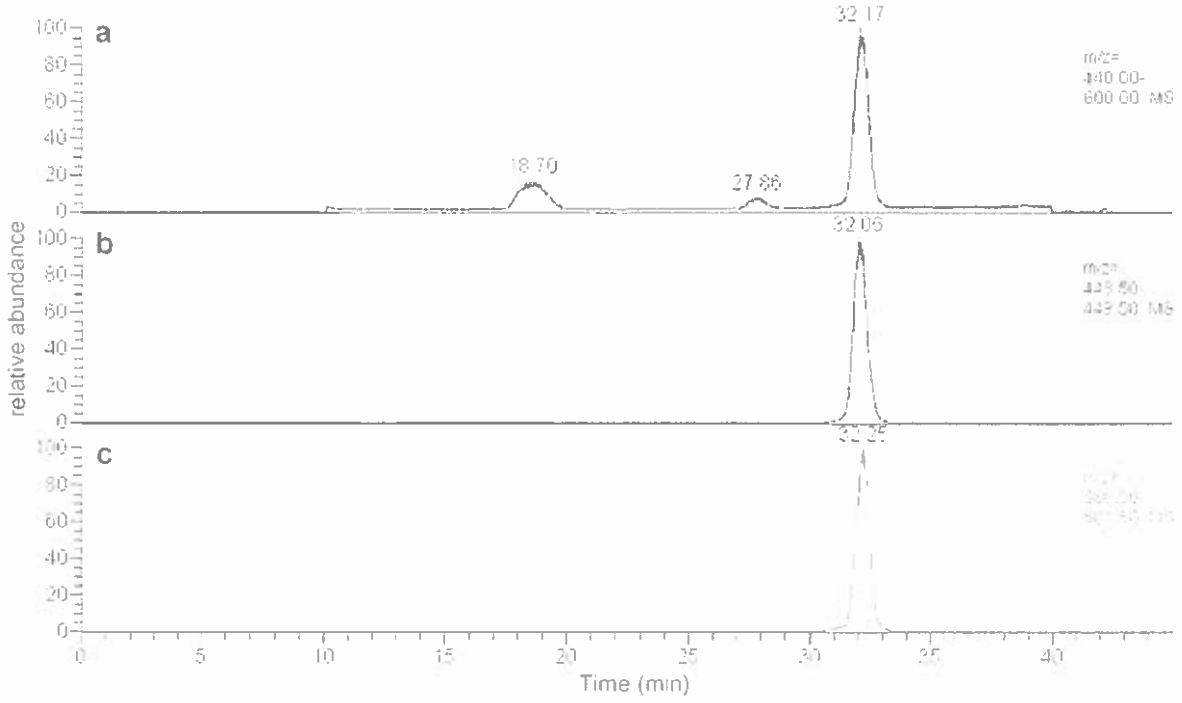



Fig. 1. Mass chromatogram of elderberry extract selected by m/z. Mass chromatogram revealed that elderberry extract in the peak at 32.17 min (a) contains two anthocyanins, namely cyanidin 3-glucoside (b) and cyanidin 3-sambubioside (c).

Table 4
Concentrations of quercetins in fruits of two cultivars and three selections of black elderberry (*S. nigra* L.) in mg CGE/100 g FW

| *S. nigra* L. cultivar/selection | Q | q-rut | q-glu | Sum quercetins |
|---|---|---|---|---|
| 'Haschberg' | 2.89 ± 0.46 a | 52.02 ± 2.48 b | 6.38 ± 0.37 a | 61.29 ± 2.79 bc |
| 'Selection 13' | 3.22 ± 0.21 ab | 35.82 ± 2.24 a | 17.42 ± 1.33 c | 56.44 ± 3.52 ab |
| 'Selection 14' | 2.70 ± 0.22 a | 35.59 ± 1.37 a | 13.64 ± 0.57 b | 51.94 ± 1.92 a |
| 'Selection 25' | 4.06 ± 0.46 bc | 50.04 ± 0.65 b | 19.32 ± 0.41 c | 73.43 ± 1.20 d |
| 'Rubini' | 4.50 ± 0.13 c | 35.88 ± 0.89 a | 26.52 ± 0.57 d | 66.90 ± 1.49 cd |
| Mean | 3.47 ± 0.19 | 41.87 ± 1.68 | 16.67 ± 1.37 | 62.00 ± 1.82 |

Quercetins in fruit of black elderberry: Q: quercetin; q-rut: quercetin 3-rutinoside; q-glu: quercetin 3-glucoside. Average values ± standard error are presented. Different letters (a–d) in rows mean statistically significant differences between individual quercetins among cultivars/selections at $\alpha < 0.05$.

## 4. Conclusions

Elderberry fruit is predominately used for processing; therefore cultivars with higher concentrations of organic acids, anthocyanins and other phenolics are particularly suitable for commercial growing. The leading European cultivar 'Haschberg' has significantly higher amounts of total organic acids than other cultivars/selections analysed but had lower concentrations of secondary metabolites than some selections. All cultivars of black elderberry contained high amounts of anthocyanins especially of cyanidin 3-sambubioside and cyanidin 3-glucoside compared to other fruit varieties (sweet cherry, fig). The cultivar with the highest amounts of total analysed anthocyanins was 'Rubini'; it also contained high amounts of quercetins, especially quercetin 3-rutinoside, the predominant quercetin in our study. Analysis of the combined data of primary and secondary metabolites can be utilised for further selection of *S. nigra* L. cultivars/selections, used for processing elderberry juices and extracts with health-promoting properties.

## Acknowledgement

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

# Characterization of Anthocyanins and Proanthocyanidins in Some Cultivars of *Ribes*, *Aronia*, and *Sambucus* and Their Antioxidant Capacity

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Anthocyanins and proanthocyanidins were characterized by HPLC-ESI-MS/MS coupled with a diode array and/or fluorescent detector in seven cultivars of *Ribes nigrum* (black currant) and *Ribes rubrum* (red currant, Red Lake), six cultivars of *Ribes grossularia* (gooseberries), *Aronia melanocarpa* (chokeberry), and *Sambucus nigra* (elderberry). Thirty-one different anthocyanins were detected in these berries, but not every anthocyanin was observed in each berry. A number of minor anthocyanins were identified from these berries for the first time. The concentrations of individual anthocyanins in all of the berries were quantified using relevant anthocyanidin 3-glucoside standards. Among the berries studied in this paper and in berries in general, chokeberry has the highest total anthocyanin concentrations [1480 mg/100 g of fresh weight (FW)], whereas the lowest total anthocyanin concentration in the berries studied was found in the gooseberry cv. Careless, which contained only 0.07 mg/100 g of FW. Two cultivars of gooseberries (Marigold and Leveller) did not contain any anthocyanins. Total proanthocyanidin concentrations in the berries studied ranged from 23 to 664 mg/100 g of FW in elderberry and chokeberry, respectively. Procyanidin or prodelphinidin polymers were the predominant components (>65% w/w) in most of the berries. The lipophilic and hydrophilic antioxidant capacities were measured by the oxygen radical absorbance capacity ($ORAC_{FL}$) procedure. The total antioxidant capacity varied from 21 μmol of TE/g of FW in Careless gooseberry to 161 μmol of TE/g of FW in chokeberry. Total phenolics in the berries in general paralleled hydrophilic antioxidant capacity.

**KEYWORDS: Anthocyanin; proanthocyanidin; antioxidant; ORAC; phenolics; black currant; gooseberry; chokeberry; elderberry; red currant**

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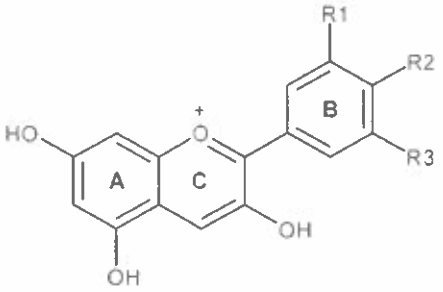
## INTRODUCTION

Considerable interest has developed over the years in fruits and vegetables containing high concentrations of flavonoids, due to their potential biological and health-promoting effects (*1*−*3*). These biological effects are in part due to their antioxidant capacity (*4*, *5*). Among all common fruits and vegetables in the diet, berries, especially those with dark blue or red colors, have the highest antioxidant capacities (*6*).

The targeted berries in this project are sometimes referred to as the "purple berries" (except for gooseberries and red currants), which are known for their high antioxidant capacity and potential health benefits. These berries are under consideration for commercialization in the northeastern United States. As in other berries, anthocyanins and proanthocyanidins are important polyphenolic components in *Ribes*, *Aronia*, and *Sambucus*. Anthocyanins are water-soluble glycosides and acylglycosides of anthocyanidins, which are polyhydroxy and polymethoxy derivatives of the 2-phenylbenzopyrylium (flavylium) cation (**Figure 1**) (*7*). Proanthocyanidins (PAs) are oligomeric and polymeric flavan-3-ols. The size of proanthocyanidin molecules can be described by their degree of polymerization (DP) (*8*). PAs containing (epi)catechin or (epi)gallocatechin as subunits are named procyanidins or prodelphinidins, respectively. Anthocyanins and PAs are among the major phytochemicals in berries (*7*, *9*), both of which have been shown to be effective antioxidants (*10*, *11*). These two classes of flavonoids account for a major fraction of the total flavonoids ingested in the Western diet and are of interest in nutrition and medicine because of potential protective effects against diseases (*12*, *13*). The average daily intake of anthocyanins per person has been estimated to be ~200 mg in the United States (*14*). Our studies have suggested that the average intake of proanthocyanidins is 58 mg/day in the United States. (*9*). In animal studies, consumption of dark-colored berries has been shown to protect against peroxynitrite-induced endothelial dysfunction and chemically induced cancer (*15*−*17*).

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| Anthocyanidin | $R_1$ | $R_2$ | $R_3$ | MW |
|---|---|---|---|---|
| Pelargonidin | H | OH | H | 271 |
| Cyanidin | OH | OH | H | 287 |
| Delphinidin | OH | OH | OH | 303 |
| Peonidin | OMe | OH | H | 301 |
| Petunidin | OMe | OH | OH | 317 |
| Malvidin | OMe | OH | OMe | 331 |

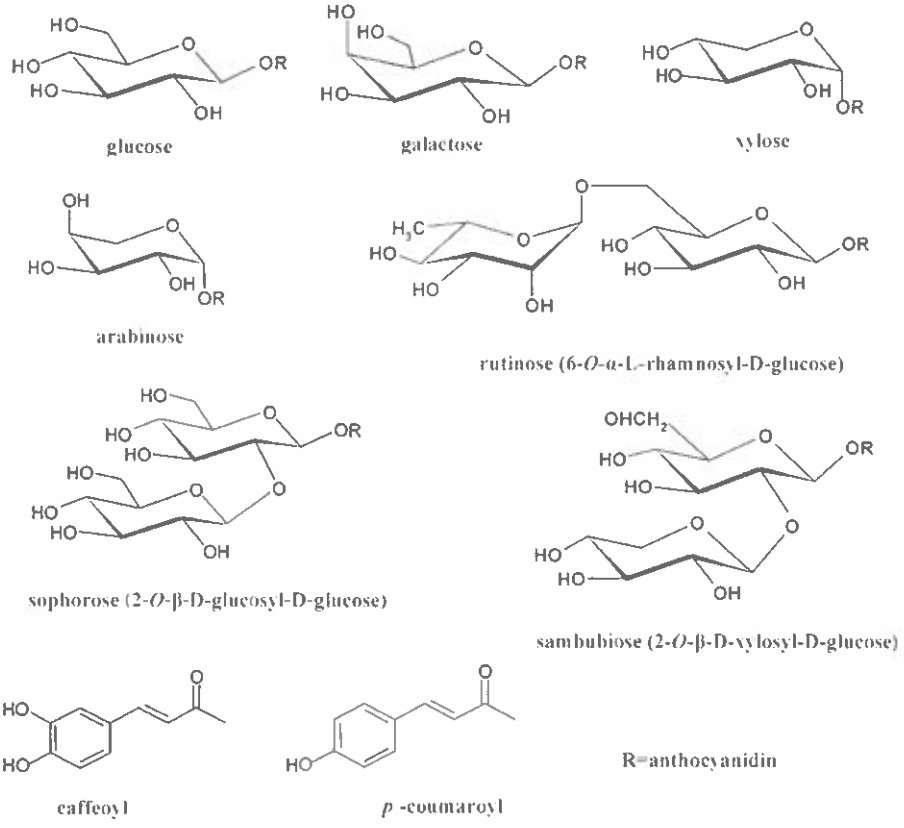



Figure 1. Structures of anthocyanidins, sugar moieties, and acylated substitutes that occur in the berries considered in this paper

Although some studies have been conducted on *Ribes*, *Aronia*, and *Sambucus* in terms of phenolic compounds and their antioxidant capacities (*18*−*23*), most studies have focused on only one or two of the major classes of compounds, and the minor compounds have been neglected. No data are available on the PA content of *Ribes*, *Aronia*, or *Sambucus*. Additionally, anthocyanins and antioxidant capacity can significantly vary across species and cultivars of berries (*24*).

The objective of this study was to characterize individual anthocyanins, especially the minor anthocyanins, and PAs in different cultivars of black currant, gooseberry, red currant, chokeberry, and elderberry, by HPLC-ESI-MS/MS. Quantitative analysis of individual anthocyanins and PAs was performed as well. Furthermore, lipophilic and hydrophilic antioxidant capacities and concentrations of total phenolics were measured.

## MATERIALS AND METHODS

**Chemicals and Apparatus.** 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich (Milwaukee, WI). Randomly methylated β-cyclodextrin (Trappsol) (Pharm grade) (RMCD) was obtained from Cyclodextrin Technologies Development Inc. (High Springs, FL). Fluorescein (Na salt) was obtained from Aldrich (Milwaukee, WI). Folin−Ciocalteu's phenol reagent, sodium carbonate, and gallic acid were all purchased from Sigma (St. Louis, MO). All

other solvents were purchased from Fisher (Fair Lawn, NJ). Extraction of samples was performed on an ASE 200 accelerated solvent extractor (Dionex Corp., Sunnyvale, CA). $ORAC_{FL}$ analyses were carried out on a FLUOstar Galaxy plate reader (BMG Labtechnologies, Durham, NC). Fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used. Microplates (48 wells) were purchased from VWR (St. Louis, MO). Total phenolics were analyzed on the ANALETTE model 9006 chemistry analyzer (Precision Systems Inc., Natick, MA).

**Standards.** The 3-*O*-$\beta$-glucoside standards of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six mixed anthocyanin standards, HPLC grade) and cyanidin 3-glucoside (HPLC grade) were obtained from Polyphenols Laboratories (Sandnes, Norway). A composite proanthocyanidin oligomer standard containing monomers through decamers was purified from cocoa. A polymer proanthocyanidin fraction with a mean DP of 36.1 was purified from blueberries and used as a polymer standard (*9*).

**Plant Materials and Sample Preparation.** Gooseberries (*Ribes grossularia*), black currants (*Ribes nigrum*), and red currants (*Ribes rubrum*) were obtained from Wisley Gardens, Royal Horticultural Society, England. Of the gooseberry varieties, cvs. Leveller (yellow variety), Careless (green variety), and Whinham (red variety) are the leading varieties in England, Europe, and Holland, respectively. The cv. Marigold (green/yellow) may be a potential dessert variety. Varieties of black currant studied were selected because of recognized characteristics in the industry. Cv. Titania is the major cultivar grown in the United States, whereas Titania and cv. Ben Nevis are common in Canada. Cv. Ben Alder is perhaps one of the better flavored varieties, and cv. Ben Lomond is recognized as having good processing characteristics. Elderberry (*Sambucus nigra*) and chokeberry (*Aronia melanocarpa*) were provided by Artemis International, Fort Wayne, IN. Fresh berry samples were frozen and stored frozen at −20 °C until transferred to the Arkansas Children's Nutrition Center, at which time the frozen samples were freeze-dried and ground into powder. The powder was kept at −70 °C until analyzed.

Extraction of dried berries was performed on an ASE 200 accelerated solvent extractor as previously reported (*6*, *25*). Briefly, 1 g of each sample was mixed with 5 g of sea sand (Unimin Corp., Le Sueur, MN), transferred to a 22 mL extraction cell, and extracted with hexane/dichloromethane (1:1) followed by acetone/water/acetic acid (70:29.5:0.5; Ace/$H_2O$/AcAc) extraction. The extracts from hexane/dichloromethane were used to measure lipophilic $ORAC_{FL}$ capacity after further dilution. Ace/$H_2O$/AcAc extracts were used to measure the hydrophilic $ORAC_{FL}$ value and total phenolics after dilution into the concentration range of the standards.

For anthocyanin analysis, 1 g of sample was weighed and put into a 15 mL screw-cap tube. The sample was extracted with 15 mL of methanol/water/acetic acid (85:15:0.5; v/v, MeOH/$H_2O$/AcAc). The sample was then vortexed for 30 s followed by sonication for 5 min. The tube was shaken twice during sonication to resuspend the sample. The tube was then kept at room temperature for 10 min, being vortexed for 30 s after 5 min. The tube was then centrifuged at 4550*g* for 10 min and the supernatant removed. The sample was extracted one more time with 10 mL of MeOH/$H_2O$/AcAc using the same procedure, and the supernatants were combined. The combined supernatant was transferred into a 25 mL volumetric flask, and MeOH/$H_2O$/AcAc was added to make up the final volume to exactly 25 mL. The solution was further diluted appropriately and filtered using a 0.22 $\mu$m Teflon syringe filter (Cameo 25F, Micron Separations Inc., Westboro, MA) for analysis.

To compare the solvent influence on anthocyanin composition, 4 mL of Ace/$H_2O$/AcAc extracts of black currant (Ben Alder) and elderberry were put into a SpeedVac (ThermoSavant, Holbrook, NY) to remove acetone. Then the remaining solution was diluted appropriately with acidic methanol and filtered using a 0.22 $\mu$m Teflon syringe filter (Cameo, 25F, Micron Separations Inc., Westboro, MA) for anthocyanin analysis.

Extraction and purification of PAs were performed according to a published method (*26*, *27*).

**HPLC-MS/MS Analyses of Anthocyanins.** Analyses were performed on an HP 1100 series HPLC (Hewlett-Packard, Palo Alto, CA) equipped with a diode array detector. A 250 × 4.6 mm i.d. Zorbax SB-$C_{18}$ column was used for separation. Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (methanol). The flow rate was 1 mL/min, and detection was at 520 nm. Two gradient systems that were used were as follows: gradient 1, 5% B, 0−2 min; 5−20% B, 2−10 min; 20% B, 10−15 min; 20−30% B, 15−30 min; 30% B, 30−35 min; 30−45% B, 35−50 min; 45% B, 50−55 min; 45−5% B, 55−65 min, 5% B, 65−68 min; gradient 2, 5% B, 0−2 min; 5−24% B, 2−10 min; 24% B, 10−15 min; 24−35% B, 15−30 min; 35% B, 30−35 min; 35−45% B, 35−50 min; 45% B, 50−55 min; 45−5% B, 55−65 min, 5% B, 65−68 min. Gradient 1 was used to separate anthocyanins in black currant, gooseberry, and chokeberry, whereas gradient 2 was used to separate elderberry and red currant. Low-resolution electrospray mass spectrometry was performed with an Esquire-LC mass spectrometer (MS) (Bruker Daltoniks, Billerica, MA), an ion trap instrument equipped with an electrospray interface. Column effluent was monitored in positive mode of the MS. Major MS parameters were as follows: capillary exit, 3500 V; capillary offset, 500 V; skim 1, 25.4 V; nebulizer, 45 psi; dry gas, 11 L/min; temperature, 345 °C.

For quantification and identification purposes, a mixture of six anthocyanin standards (3-*O*-$\beta$-glucosides of delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin) was used to create a calibration curve of the individual anthocyanins. The results were expressed as anthocyanidin glucoside equivalents.

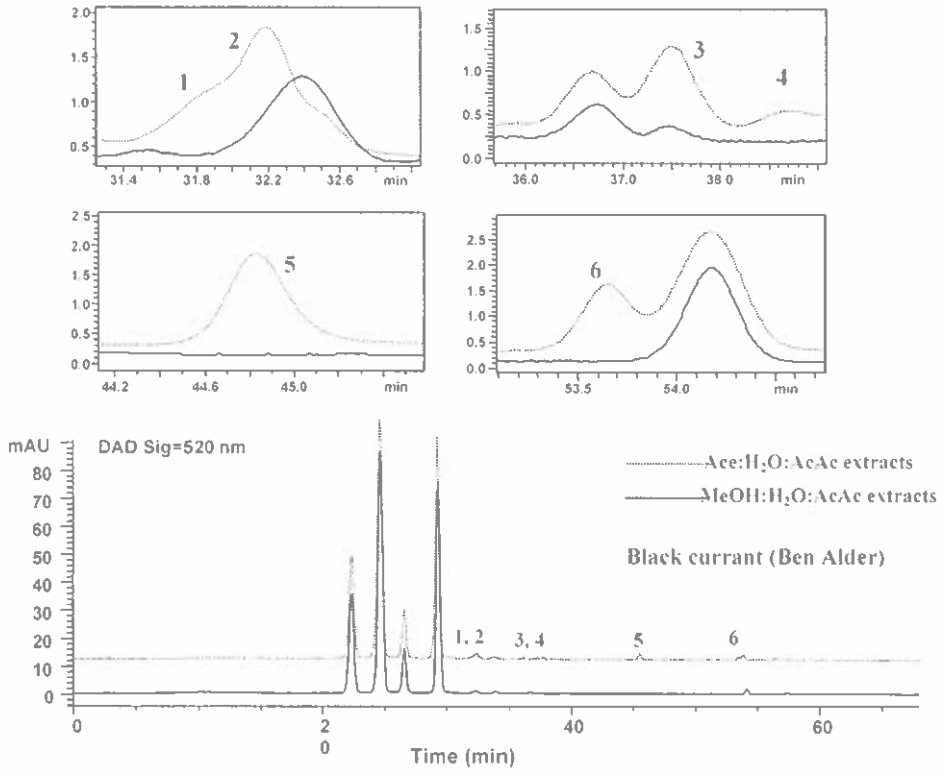
**HPLC-MS/MS Analyses of Proanthocyanidins.** Methods of quantification and identification of PAs have been described previously (*9*).

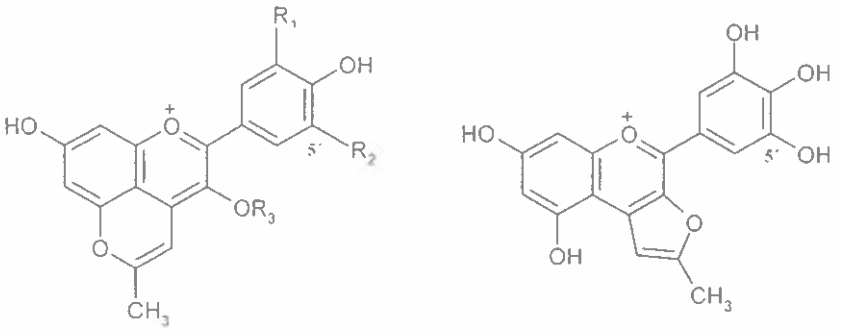
**$ORAC_{FL}$ Assay.** Both hydrophilic and lipophilic $ORAC_{FL}$ assays were carried out on a FLUOstar Galaxy plate reader, which was equipped with a temperature-controlled incubation chamber and two injection pumps. The temperature of the incubator was set at 37 °C. The procedures were based on our modified $ORAC_{FL}$ method (*28*) and on that reported by Ou and co-workers (*29*, *30*). The data were expressed as micromoles of Trolox equivalents per gram of fresh weight ($\mu$mol of TE/g of FW).

**Total Phenolic Analysis.** The Ace/$H_2O$/AcAc extracts were subjected to total phenolics measurement by Folin−Ciocalteu reagent according to the method of Wu et al. (*6*). The results were expressed as milligrams of gallic acid equivalents per 100 g of fresh weight (mg of GAE/100 g of FW).

## RESULTS AND DISCUSSION

**Polyphenol Extraction Strategies.** It is difficult to find one solvent system that is universal for all different compounds. Normally different solvent systems are chosen on the basis of the specific analytical task. In this study, the two most commonly used solvent systems for polyphenol analysis and antioxidant capacity measurement were used. One is an acetone/water/acid system, which has been demonstrated to be a good solvent system to extract proanthocyanidins (*31*). The other solvent system was a methanol/water/acid system, which was used to extract anthocyanins. Traditionally, both acetone and methanol have been widely used for anthocyanin analysis. However, two recent papers (*32*, *33*) demonstrated that anthocyanins may undergo unusual facile reactions with acetone to give rise to pyranoanthocyanins. Pyranoanthocyanins can further undergo hydrolysis and rearrangement to generate two furoanthocyanidins. These findings were verified in this investigation by analyzing anthocyanins from black currant and elderberry using Ace/$H_2O$/AcAc extractions. **Figure 2** shows the six artificial anthocyanins from black currant (Ben Alder) extracts using Ace/$H_2O$/AcAc. Among them, four pyranoanthocyanins (peaks 1−4) were formed either from cyanidin or from delphinidin glycoside; two furoanthocyanidins (peaks 5 and 6) from delphinidin were the predominant artificial compounds. It is of interest that we could detect only furoanthocyanidins from delphinidin. From elderberry extracts using Ace/$H_2O$/AcAc, in which cyanidin-based anthocyanins are predominant




Compound 1-4, 5 Compound 6

| Compound | $R_1$ | $R_3$ | $R_5$ | MS | MS/MS |
|---|---|---|---|---|---|
| 1 | OH | OH | Rutinose | 649 | 341 |
| 2 | OH | OH | Glucose | 503 | 341 |
| 3 | OH | H | Rutinose | 633 | 325 |
| 4 | OH | H | Glucose | 487 | 325 |
| 5 | OH | OH | H | 341 | --- |
| 6 | --- | --- | --- | 341 | --- |

Figure 2. Comparison of anthocyanin profiles of black currant (cv. Ben Alder) using acetone/water/acetic acid (Ace/$H_2O$/AcAc, 70:29.5:0.5, v/v) and methanol/water/acetic acid (MeOH/$H_2O$/AcAc, 85:15:0.5, v/v) extractions. Six artificial anthocyanins (a–f) were found with Ace/$H_2O$/AcAc extraction. Type of extraction solvent is represented by a solid line (MeOH/$H_2O$/AcAc extraction) and dotted line (Ace/$H_2O$/AcAc extraction) in chromatograms.

and their concentrations are much higher, we could not detect any furoanthocyanidin from cyanidin. This meant that the hydrolytic reaction and rearrangement from pyranoanthocyanin to furoanthocyanidin might be determined by the hydroxyl groups in B-ring of the flavylium structure. It appears that the hydroxyl group at the 5′-position significantly increased the reactivity. Although the quantities of these products formed are small, our results suggest that acetone may not be a good solvent for anthocyanin extraction and analysis.

**Characterization and Quantification of Anthocyanins.** Identification and peak assignment of anthocyanins was based on the comparison of their retention time and MS data with standards and published data (**Figure 1**; **Table 1**). Under current separation conditions, the elution order of six mixed anthocyanins was delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, pelargonidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside. Quantification of anthocyanins was performed by utilizing the corresponding anthocyanidin 3-glucoside as a standard (**Tables 2** and **3**).

*Black Currant.* All six cultivars of black currant had similar anthocyanin profiles. A representative chromatogram of anthocyanins from one cultivar of black currant, Ben Alder, is shown

Table 1. Identification of Anthocyanins from Berries Using HPLC-ESI/MS/MS

| berry sample | peak | $t_R$[a] (min) | $[M]^+$ (*m/z*) | MS/MS | compound |
|---|---|---|---|---|---|
| black currant | 1 | 22.4 | 465 | 303 | delphinidin 3-glucoside |
| | 2 | 24.7 | 611 | 465/303 | delphinidin 3-rutinoside |
| | 3 | 26.6 | 449 | 287 | cyanidin 3-glucoside |
| | 4 | 29.4 | 595 | 449/287 | cyanidin 3-rutinoside |
| | 5 | 30.0 | 479 | 317 | petunidin 3-glucoside |
| | 6 | 30.9 | 433 | 271 | pelargonidin 3-glucoside |
| | 7 | 32.4 | 625 | 479/317 | petunidin 3-rutinoside |
| | 8 | 33.8 | 463 | 301 | peonidin 3-glucoside |
| | 9[c] | 33.9 | 435 | 303 | delphinidin 3-xyloside |
| | 10 | 34.0 | 579 | 433/271 | pelargonidin 3-rutinoside |
| | 11 | 36.6 | 609 | 463/301 | peonidin 3-rutinoside |
| | 12[c] | 39.5 | 419 | 287 | cyanidin 3-xyloside |
| | 13 | 54.2 | 611 | 303 | petunidin 3-(6-coumaroyl)-glucoside |
| | 14 | 57.4 | 595 | 287 | cyanidin 3-(6-coumaroyl)-glucoside |
| gooseberry | 18[c] | 24.0 | 449 | 287 | cyanidin-3-galactoside |
| | 3 | 26.3 | 449 | 287 | cyanidin 3-glucoside |
| | 4 | 29.2 | 595 | 449/287 | cyanidin 3-rutinoside |
| | 8[c] | 33.4 | 463 | 301 | peonidin 3-glucoside |
| | 11[c] | 35.7 | 609 | 463/301 | peonidin 3-rutinoside |
| | 12[c] | 38.9 | 419 | 287 | cyanidin 3-xyloside |
| | 15[c] | 44.2 | 595 | 287 | cyanidin glycoside |
| | 16[c] | 50.4 | 611 | 287 | cyanidin 3-(6-caffeyl)-glucoside |
| | 17[c] | 52.0 | 595 | 287 | cyanidin glycoside |
| | 14[c] | 56.1 | 595 | 287 | cyanidin 3-(6-coumaroyl)-glucoside |
| chokeberry | 18 | 24.0 | 449 | 287 | cyanidin 3-galactoside |
| | 3 | 26.6 | 449 | 287 | cyanidin 3-glucoside |
| | 19[c] | 27.9 | 433 | 271 | pelargonidin 3-galactoside |
| | 20 | 29.2 | 419 | 287 | cyanidin 3-arabinoside |
| | 21[c] | 31.1 | 517 | 355 | NI[b] |
| | 22[c] | 31.6 | 565 | 287 | cyanidin + rhamnose + pentose |
| | 23[c] | 33.2 | 403 | 271 | pelargonidin 3-arabinoside |
| | 12 | 39.5 | 419 | 287 | cyanidin 3-xyloside |
| elderberry | 24 | 14.6 | 743 | 581/449/287 | cyanidin 3-sambubioside-5-glucoside |
| | 25 | 14.6 | 611 | 449/287 | cyanidin 3,5-diglucoside |
| | 26 | 21.1 | 581 | 287 | cyanidin 3-sambubioside |
| | 3 | 21.6 | 449 | 287 | cyanidin 3-glucoside |
| | 4[c] | 24.0 | 595 | 449/287 | cyanidin 3-rutinoside |
| | 6[c] | 25.6 | 433 | 271 | pelargonidin 3-glucoside |
| | 27[c] | 26.1 | 565 | 271 | pelargonidin 3-sambubioside |
| red currant | 28[c] | 16.1 | 597 | 303 | delphinidin 3-sambubioside |
| | 29 | 17.4 | 611 | 287 | cyanidin 3-sophoroside |
| | 30 | 18.7 | 757 | 287 | cyanidin 3-glucosylrutinoside |
| | 26 | 20.2 | 581 | 287 | cyanidin 3-sambubioside |
| | 3 | 20.7 | 449 | 287 | cyanidin 3-glucoside |
| | 31 | 21.4 | 727 | 581/287 | cyanidin 3-xylosylrutinoside |
| | 4 | 22.8 | 595 | 449/287 | cyanidin 3-rutinoside |

[a] Retention times of elderberry and red currant were based on gradient 2, and the retention times of all others were based on gradient 1. [b] Not identified. [c] Identified in this berry for the first time.

in **Figure 3A**. A total of 14 anthocyanins were detected across all black currant cultivars. By comparing their MS data with those recently published (*34*), 12 of them were identified (peaks 1−8, 10, 11, 13, 14). MS data of peak 9 showed a molecular ion of 435 ($[M]^+$ *m/z* 435) and a fragment ion of 303 (MS/MS, *m/z* 303). The fragment loss of 132 revealed this anthocyanin to be a delphinidin 3-pentoside. Under our current separation conditions, the retention time of delphinidin 3-arabinoside should be shorter than that of cyanidin 3-glucoside. However, the retention time of peak 9 (33.9 min) was longer than that of peak 3 (26.6 min) (cyanidin 3-glucoside). Thus, peak 9 was identified as delphinidin 3-xyloside. Peak 12 had the same MS data ($[M]^+$ *m/z* 419, MS/MS, *m/z* 287) as cyanidin 3-arabinoside published previously (*34*), but the $t_R$ of peak 12 was 39.5 min, which is longer than that of cyanidin 3-arabinoside. The sequence of elution of anthocyanidin glycosides under our conditions is galactose, glucose, arabinose, and xylose. This indicated that peak 12 was cyanidin 3-xyloside. Xylosides of delphinidin and cyanidin were not identified earlier (*34*), whereas malvidin 3-glucoside and malvidin 3-rutinoside, which were identified earlier (*34*), were not detected in this group of berries. In the cultivars of black currant studied, individual anthocyanin concentrations varied slightly (**Table 2**). Our data on the concentrations of anthocyanins in black currant are much higher than those in a recently published paper that utilized the pH differential method (*22*).

*Gooseberry*. Among the six cultivars of gooseberries, Whinham, Lancashine, and Dan's Mistake had either pink or light red berry color and were found to have similar anthocyanin profiles containing a total of nine anthocyanins. Because there are few published data available on the anthocyanin composition of gooseberry, the peak identification and assignment were primarily based on the comparison of their MS data and retention times with those of standards and the anthocyanins in black currant cultivars. A representative chromatogram of anthocyanins in the gooseberry cv. Whinham is shown in **Figure 3B**. Black currant and Whinham gooseberry share six anthocyanins, which are peaks 3, 4, 8, 11, 12, and 14 (**Table 1**).

Table 2. Anthocyanin Concentrations in Six Cultivars of Black Currants (Milligrams per 100 g of FW)

| peak | anthocyanin | black currant cultivars | | | | | |
|---|---|---|---|---|---|---|---|
| | | Ben Alder | Ben Nevis | Ben Lomond | Ben Tirran | Titania | Ukraine |
| 1 | delphinidin 3-glucoside | 112.54 | 108.74 | 113.21 | 74.34 | 63.12 | 54.20 |
| 2 | delphinidin 3-rutinoside | 275.17 | 311.42 | 286.69 | 215.85 | 204.72 | 180.30 |
| 3 | cyanidin 3-glucoside | 28.56 | 21.26 | 26.76 | 22.12 | 12.43 | 12.50 |
| 4 | cyanidin 3-rutinoside | 134.94 | 138.83 | 136.69 | 113.11 | 73.32 | 70.44 |
| 5 | petunidin 3-glucoside | T[a] | T | T | T | T | T |
| 6 | pelargonidin 3-glucoside | ND[b] | ND | T | ND | ND | ND |
| 7 | petunidin 3-rutinoside | 2.90 | 3.08 | 4.15 | 4.15 | 2.73 | 1.88 |
| 8 | peonidin 3-glucoside | T | ND | ND | ND | ND | ND |
| 9 | delphinidin 3-xyloside | T | ND | T | ND | T | ND |
| 10 | pelargonidin 3-rutinoside | 2.22 | 2.31 | 2.15 | 2.13 | 1.27 | 1.26 |
| 11 | peonidin 3-rutinoside | 0.77 | 0.93 | 1.87 | 1.38 | 0.69 | 0.40 |
| 12 | cyanidin 3-xyloside | T | T | T | T | T | ND |
| 13 | petunidin 3-(6-coumaroyl)-glucoside | 3.60 | 2.26 | 1.58 | 14.41 | 1.92 | 1.34 |
| 14 | cyanidin 3-(6-coumaroyl)-glucoside | 1.23 | T | 0.65 | 4.43 | T | 0.43 |
| | total | 561.8 | 526.6 | 573.5 | 452.0 | 360.4 | 322.7 |

[a] Trace. [b] Not detected.

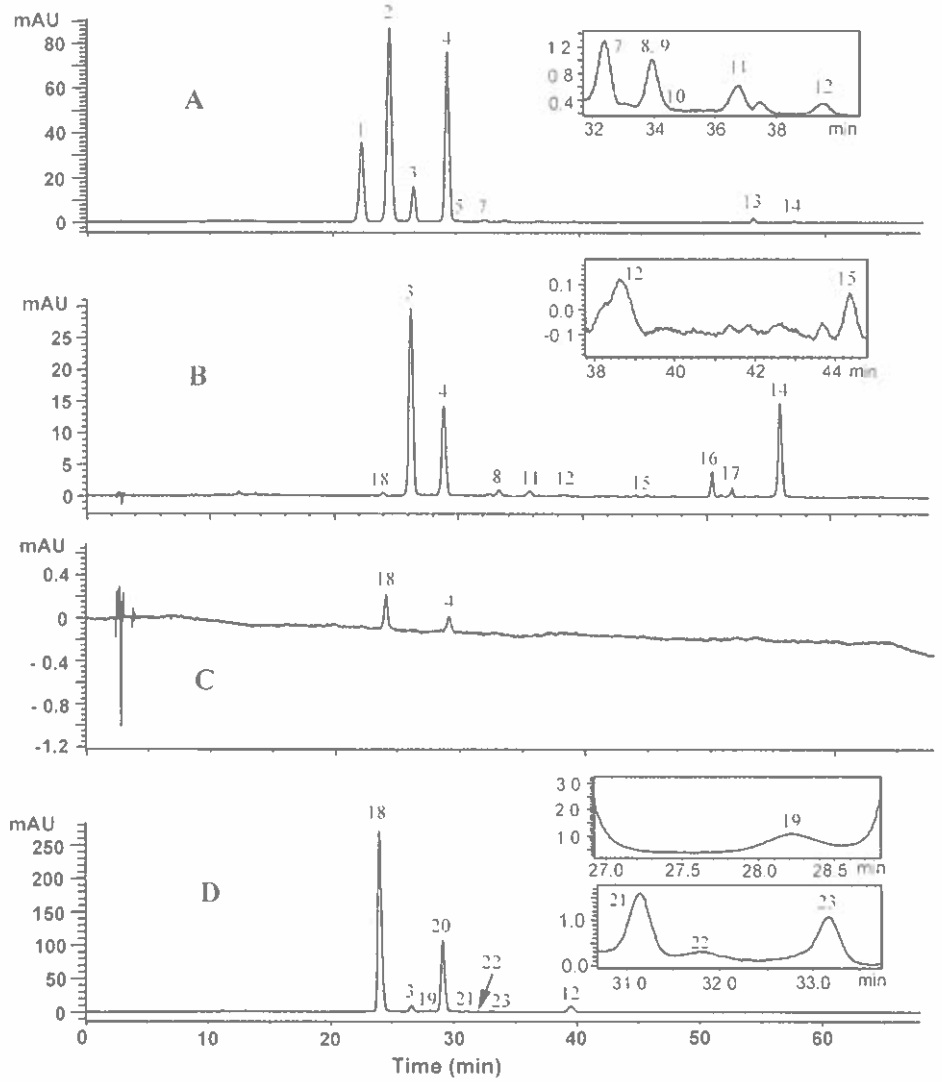



Figure 3. Reverse-phase HPLC chromatograms of anthocyanins detected at 520 nm from (A) black currant (cv. Ben Alder), (B) gooseberry (cv. Whinham), (C) gooseberry (cv. Careless), and (D) chokeberry. Elution gradient 1 was used to separate anthocyanins. Refer to Table 1 for the identification of each numbered peak.

Peak 16 in gooseberry had a molecular weight of 611 ([M]$^+$ $m/z$ 611) and a fragment ion $m/z$ of 287. The fragment loss was 324, which could be accounted for by a caffeoyl glucoside. The UV spectrum of this peak showed that it had a weak absorption maximum at 327 nm, which agrees with the MS data indicating a caffeic acid residue (35). Thus, this anthocyanin was tenta-

Table 3. Anthocyanin Concentrations in Different Cultivars of Gooseberry, Chokeberry, Elderberry, and Red Currant (Milligrams per 100 g of FW)

| | | gooseberry cultivars | | | | | | |
|---|---|---|---|---|---|---|---|---|
| peak | anthocyanin | Whinham | Lancashine | Dan's Mistake | Careless | chokeberry | elderberry | red currant |
| 3 | cyanidin 3-glucoside | 4.91 | 5.42 | 1.44 | 0.049 | 37.6 | 739.8 | 0.16 |
| 4 | cyanidin 3-rutinoside | 2.32 | 3.36 | 0.46 | | | 4.40 | 1.61 |
| 6 | pelargonidin 3-glucoside | | | | | | 1.80 | |
| 8 | peonidin 3-glucoside | 0.13 | 0.07 | 0.05 | | | | |
| 11 | peonidin 3-rutinoside | 0.15 | 0.13 | 0.05 | | | | |
| 12 | cyanidin 3-xyloside | T[a] | ND[b] | ND | | 51.5 | | |
| 15 | cyanidin glycoside | T | ND | ND | | | | |
| 16 | cyanidin 3-(6-caffeoyl)-glucoside | 0.45 | 0.24 | ND | | | | |
| 17 | cyanidin glycoside | 0.15 | 0.07 | 0.07 | | | | |
| 14 | cyanidin 3-(6-coumaroyl)-glucoside | 2.26 | 1.18 | 0.13 | | | | |
| 18 | cyanidin 3-galactoside | | | | 0.025 | 989.7 | | |
| 19 | pelargonidin 3-galactoside | | | | | T | | |
| 20 | cyanidin 3-arabinoside | | | | | 399.3 | | |
| 21 | NI | | | | | 2.4[c] | | |
| 22 | cyanidin + rhamnose + pentose | | | | | T | | |
| 23 | pelargonidin 3-arabinoside | | | | | 2.3 | | |
| 24 | cyanidin 3-sambubioside-5-glucoside | | | | | | 82.6 | |
| 25 | cyanidin 3,5-diglucoside | | | | | | | |
| 26 | cyanidin 3-sambubioside | | | | | | 545.9 | 3.39 |
| 27 | pelargonidin 3-sambubioside | | | | | | T | |
| 28 | delphinidin 3-sambubioside | | | | | | | 0.10 |
| 29 | cyanidin 3-sophoroside | | | | | | | 0.11 |
| 30 | cyanidin 3-glucosylrutinoside | | | | | | | 0.49 |
| 31 | cyanidin 3-xylosylrutinoside | | | | | | | 6.93 |
| | total | 10.37 | 10.46 | 2.20 | 0.074 | 1480.0 | 1374.4 | 12.09 |

[a] Trace. [b] Not detected. [c] Calculated as cyanidin 3-glucoside equivalent.

tively identified as cyanidin 3-(6-caffeoyl)-glucoside. Peaks 15 and 17 had the same ionization pattern ($[M]^+$, *m/z* 595; fragment, *m/z* 287) as peak 14, cyanidin 3-(6-coumaroyl)-glucoside, but their exact structures could not be determined due to limited information. Another gooseberry cultivar, Careless, was found to have an anthocyanin profile that differed from the three cultivars above. It contained only two anthocyanins in very low concentrations (**Figure 3C**). Peak 18 had the same mass spectral data as cyanidin 3-glucoside, but its retention time was ~24 min, which was shorter than that of cyanidin 3-glucoside. Thus, this anthocyanin was identified as cyanidin 3-galactoside. Peak 4 was identified as cyanidin 3-rutinoside on the basis of its mass spectral data and retention time. Total concentrations of anthocyanins in Whinham, Lacashine, Dan's Mistake, and Careless are shown in **Table 3**.

*Chokeberry*. Eight anthocyanins were detected in chokeberry (**Figure 3D**), of which the four major anthocyanins were identified as cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, and cyanidin 3-xyloside on the basis of their MS data compared to published data (*20*, *21*, *36*). These four anthocyanins correspond to peaks 18, 3, 20, and 12, respectively. Four minor anthocyanins were detected in chokeberry for the first time. Among them, peak 19 had a molecular ion of 433 ($[M]^+$, *m/z* 433) and a fragment ion of *m/z* 271. The MS data indicated that it was a glucoside or galactoside of pelargonidin, with a retention time of 27.9 min, which is shorter than that of the pelargonidin 3-glucoside standard (~30.9 min). Thus, this anthocyanin was identified as pelargonidin 3-galactoside. MS data of peak 21 indicated that this anthocyanin was an unusual anthocyanidin hexoside, with a molecular ion *m/z* 517 and a fragment ion *m/z* 355. It could not be identified due to limited information. Peak 22 was a cyanidin glycoside, and the difference between molecular weight and the fragment was 278, which can be accounted for by a rhamnose plus a pentose moiety. To our knowledge, there are no published data reporting this structure in chokeberry. The exact structure could not be determined because of limited information. Peak 23 had a molecular ion of 403 ($[M]^+$ *m/z* 403) and a fragment ion of *m/z* 271, which was identified as a pelargonidin 3-pentoside. Considering that its retention time (33.2 min) was shorter than that of cyanidin 3-xyloside (39.5 min), it was identified as pelargonidin 3-arabinoside. The quantification of individual anthocyanins in chokeberry is shown in **Table 3**. Among them, peak 22, the aglycon of which was not identified, was quantified as cyanidin 3-glucoside equivalents.

*Elderberry*. Seven anthocyanins were detected in elderberry (**Figure 4E**). Four of them are well-known anthocyanins (*37*): cyanidin 3-sambubioside-5-glucoside (peak 24), cyanidin 3,5-diglucoside (peak 25), cyanidin 3-sambubioside (peak 26), and cyanidin 3-glucoside (peak 3). Three minor peaks were identified as cyanidin 3-rutinoside (peak 4), pelargonidin 3-glucoside (peak 6), and pelargonidin 3-sambubioside (peak 27), by comparing their MS data and relative retention time with those of anthocyanins discussed above. To our knowledge, all three minor anthocyanins were detected in elderberry for the first time. Remarkably, this is the first time that pelargonidin has been identified in elderberry. The quantification of the individual anthocyanins in elderberry is shown in **Table 3**.

*Red Currant*. One cultivar of red currant, Red Lake, which was analyzed for anthocyanins, contained a total of seven anthocyanins, which were identified (**Figure 4F**). Among them, six corresponded to those that were reported by other investigators (*38*, *39*), namely, peak 29, cyanidin 3-sophoroside; peak 30, cyanidin 3-glucosylrutinoside; peak 26, cyanidin 3-sambubioside; peak 3, cyanidin 3-glucoside; peak 31, cyanidin 3-xylosylrutinoside; and peak 4, cyanidin 3-rutinoside. Peak 28, the retention time of which was shorter than those of all other peaks, had a delphinidin aglycon (fragment *m/z* 303) and a molecular weight of 597. The fragment loss was 304, which could be accounted for by a sambubiose residue. Thus, this anthocyanin was identified as delphinidin 3-sambubioside. This is the first time that the delphinidin derivative has been found in red currant. The concentration of individual anthocyanins in red currant is shown in **Table 3**.

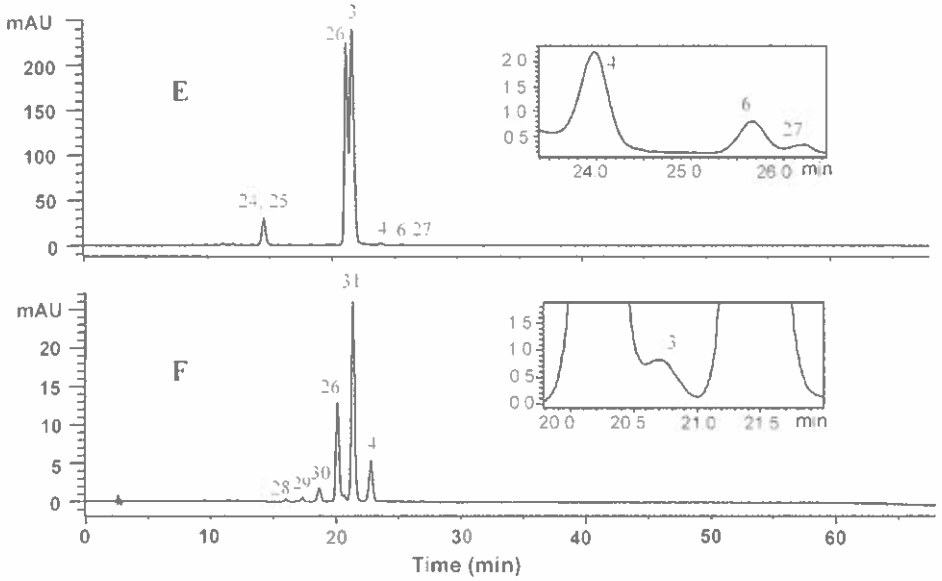



Figure 4. Reverse-phase HPLC chromatograms of anthocyanins detected at 520 nm from (E) elderberry and (F) red currant (cv. Red Lake). Elution gradient 2 was used to separate anthocyanins. Refer to Table 1 for the identification of each numbered peak.

**Characterization and Quantification of Proanthocyanidins.** All cultivars of black currants had similar profiles of PAs (**Figure 5A**). Mass spectrometric data indicated that black currants contained both procyanidins and prodelphinidins. Polymeric PAs (DP > 10) appeared as a major peak in the chromatograms. Previous data (*26*) indicated that the mean degree of polymerization of PAs in black currant was 38.7 with catechin, gallocatechin, and their epimers as subunits (*26*). Total PAs in six cultivars of black currant ranged from 120.6 to 165.8 mg/100 g of FW (**Table 4**). Polymers accounted for >80% of the total PA amount.

Gooseberries and red currant (**Figure 5B**) had profiles of PAs similar to that for black currant but lower concentrations. They contained procyanidins and prodelphinidins, and the predominant PAs were in the polymeric form. Chokeberry contained only procyanidins. The oligomeric procyanidin peaks in chokeberry were well separated and easily distinguishable (**Figure 5C**). The concentration of PAs in chokeberry is among the highest in foods (*9*), which may explain their potent astringent taste. Unlike other berries, elderberry contained no detectable higher oligomers and polymers (**Figure 5D**).

**Variation in Anthocyanin and Proanthocyanidin Profiles.** In black currant, the same profile of the four major anthocyanins occurred in the different cultivars, even though the individual concentrations varied. The detectable minor anthocyanins were different across cultivars. Cyanidin 3-glucoside and cyanidin 3-rutinoside were the major anthocyanins in all three gooseberry cultivars. The other anthocyanins varied greatly from cultivar to cultivar. Cyanidin 3-(6-coumaroyl)-glucoside was one of the major anthocyanins in Whitham but only a minor anthocyanin in Dan's Mistake.

**Antioxidant Capacity and Amounts of Phenolic Compounds.** Results for lipophilic and hydrophilic antioxidant capacities ($ORAC_{FL}$) as well as total phenolics in all berries are presented in **Table 5**. The sum of lipophilic and hydrophilic $ORAC_{FL}$ was regarded as total $ORAC_{FL}$. Comparable antioxidant capacity measurements on other fruits and berries can be found in our recent publication (*6*). Similar to the other berries that we have analyzed, the lipophilic antioxidant capacity is quite low (*6*). However, hydrophilic antioxidant capacities for black

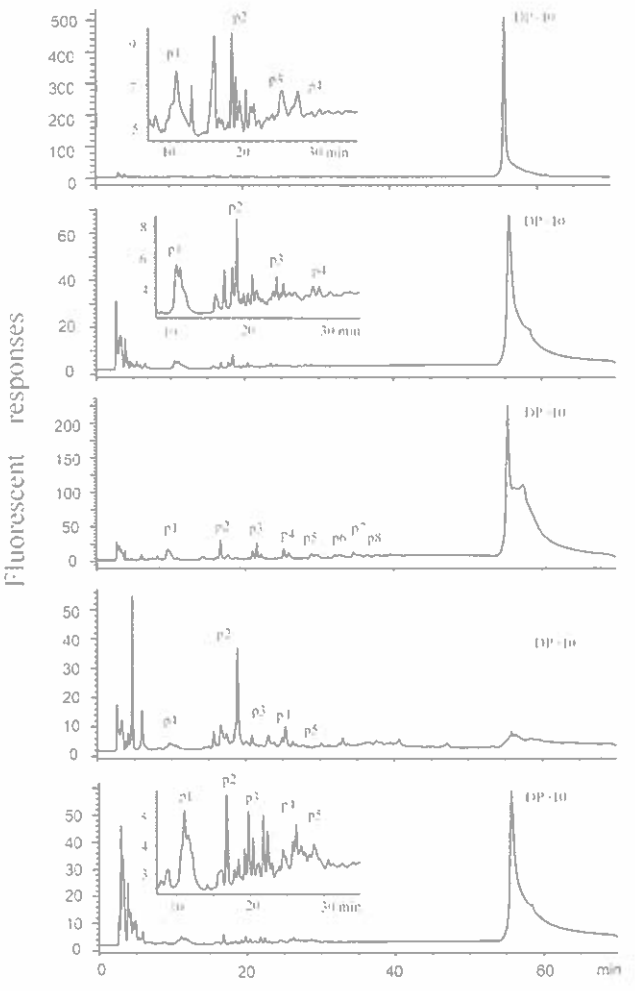



Figure 5. Normal-phase HPLC fluorescence trace of the proanthocyanidins from (A) black currant, (B) gooseberry, (C) chokeberry, (D) elderberry, and (E) red currant. Labels p1–p8 on the peaks indicate the degree of polymerization of proanthocyanidins in the peaks.

Table 4. Proanthocyanidin Concentrations in Different Cultivars of Black Currant and Gooseberry, Chokeberry, Elderberry, and Red Currant (Milligrams per 100 g of FW)[a]

| | monomers | dimers | trimers | 4–6-mers | 7–10-mers | >10-mers | total | type |
|---|---|---|---|---|---|---|---|---|
| black currant | | | | | | | | |
| Ben Alder | 0.81 | 3.34 | 2.43 | 9.75 | 10.86 | 138.6 | 165.8 | pc, pd |
| Ben Nevis | 0.85 | 3.10 | 2.37 | 9.35 | 10.85 | 122.8 | 149.3 | pc, pd |
| Ben Lomond | 0.79 | 3.56 | 2.50 | 9.59 | 10.12 | 100.5 | 127.0 | pc, pd |
| Ben Tirran | 0.69 | 2.62 | 1.91 | 7.89 | 9.37 | 101.9 | 124.4 | pc, pd |
| Titania | 0.78 | 2.97 | 2.15 | 7.36 | 8.69 | 98.7 | 120.6 | pc, pd |
| Ukraine | 0.73 | 2.38 | 1.68 | 6.70 | 8.54 | 110.6 | 130.7 | pc, pd |
| gooseberry | | | | | | | | |
| Whinham | 0.41 | 2.01 | 1.38 | 5.69 | 6.45 | 63.9 | 79.8 | pc, pd |
| Lancashine | 0.65 | 2.80 | 1.82 | 6.92 | 8.55 | 113.3 | 134.0 | pc, pd |
| Dan's Mistake | 0.58 | 1.25 | 1.03 | 4.79 | 5.47 | 77.3 | 90.4 | pc, pd |
| Marigold | 0.60 | 1.52 | 1.12 | 5.16 | 6.41 | 55.8 | 70.6 | pc, pd |
| Careless | 0.36 | 1.00 | 0.73 | 3.64 | 4.49 | 35.4 | 45.6 | pc, pd |
| Leveller | 0.43 | 1.15 | 0.89 | 4.34 | 5.28 | 53.9 | 65.9 | pc, pd |
| chokeberry | 5.17 | 12.48 | 10.29 | 40.32 | 52.87 | 542.6 | 663.7 | pc |
| elderberry | 1.44 | 10.62 | 5.63 | 10.80 | ND | ND | 23.3 | pc |
| red currant | | | | | | | | |
| Red Lake | 1.26 | 1.97 | 1.54 | 6.87 | 7.90 | 41.2 | 60.8 | pc, pd |

[a] Monomers, dimers, and trimers are listed separately. Tetramers through hexamers are pooled together as 4–6-mers. Polymers with DP > 10 are quantified collectively and listed as >10-mers. pc, procyanidins; pd, prodelphinidins.

Table 5. Lipophilic and Hydrophilic $ORAC_{FL}$, Total Phenolics of Black Currant, Gooseberry, Chokeberry, Elderberry, and Red Currant (Based on FW)

| sample | moisture (%) | L-$ORAC_{FL}$[a] ($\mu$mol of TE/g) | H-$ORAC_{FL}$[b] ($\mu$mol of TE/g) | TAC[c] ($\mu$mol of TE/g) | TP[d] (mg of GAE/g) | H-$ORAC_{FL}$/TP[e] |
|---|---|---|---|---|---|---|
| black currant | | | | | | |
| Ben Alder | 80.2 | 0.84 | 100.6 | 101.4 | 13.3 | 7.6 |
| Ben Nevis | 78.5 | 0.75 | 90.6 | 91.3 | 14.1 | 6.4 |
| Ben Lomond | 77.0 | 0.68 | 92.3 | 92.9 | 12.2 | 7.6 |
| Ben Tirran | 78.9 | 0.75 | 86.6 | 87.3 | 13.3 | 6.5 |
| Titania | 78.6 | 1.15 | 49.0 | 50.1 | 8.4 | 5.8 |
| Ukarine | 72.1 | 0.68 | 53.7 | 54.4 | 10.8 | 5.0 |
| gooseberry | | | | | | |
| Whinham | | 0.15 | 39.2 | 39.4 | 5.2 | 7.5 |
| Lancashine | | 0.15 | 41.3 | 41.4 | 6.0 | 6.9 |
| Dan's Mistake | | 0.28 | 37.1 | 37.4 | 6.3 | 5.9 |
| Marigold | | 0.45 | 33.7 | 34.1 | 5.5 | 6.2 |
| Careless | | 0.35 | 20.4 | 20.8 | 3.4 | 6.0 |
| Leveller | | 0.43 | 26.4 | 26.8 | 4.6 | 5.8 |
| chokeberry | 71.8 | 2.42 | 158.2 | 160.6 | 20.1 | 7.9 |
| elderberry | 82.5 | 1.97 | 145.0 | 147.0 | 19.5 | 7.5 |
| red currant | 78.1 | 1.27 | 32.6 | 33.9 | 5.4 | 6.0 |

[a] Lipophilic $ORAC_{FL}$. [b] Hydrophilic $ORAC_{FL}$. [c] Total antioxidant capacity. [d] Total phenolics. [e] Ratio of hydrophilic $ORAC_{FL}$ to total phenolics expressed as $\mu$mol of TE/mg of GAE.

currant, chokeberry, and elderberry are among the highest that we have measured in fresh fruits or berries (*6*).

The concentrations of total phenolics were found to correlate with the antioxidant capacities in berries (*24*, *40*, *41*). However, different types of phenolic compounds may contribute differently to total antioxidant capacity for a given food. The relationships between total $ORAC_{FL}$ and total anthocyanins, total PAs, total phenolics, or total anthocyanins plus total PAs of all samples are presented in **Figure 6**. The best linear relationship was observed between total $ORAC_{FL}$ and total phenolics ($R^2 = 0.96$), followed by total $ORAC_{FL}$ versus total anthocyanins ($R^2 = 0.95$) and total $ORAC_{FL}$ versus total anthocyanin plus total proanthocyanidins ($R^2 = 0.91$). The results are in accordance with our previous data (*24*). However, it is important to remember that these high correlations were obtained only within a certain category of samples. One should be cautious in attempting to extend the conclusion to other diverse groups of samples. Our previous studies have shown that the ratio of total $ORAC_{FL}$ to total phenolics in a wide range of samples varied from 1.7 to 156.4 (*6*), indicating that the relationship between $ORAC_{FL}$ and total phenolics is low across a diverse set of samples. There was a weak correlation ($R^2 = 0.37$) between total $ORAC_{FL}$ and total PAs. Remarkably, as shown in **Figure 6B**, if chokeberry and elderberry were removed from the regression, a much higher correlation between total $ORAC_{FL}$ and total PAs would be observed. These two berries are actually two extreme examples; in chokeberry we found very high concentrations of both anthocyanins and PAs, whereas in elderberry we found very high concentrations of anthocyanins but very low concentrations of PAs. Because the major PAs in these berries are polymers, it appears that on a weight basis polymeric PAs do not contribute as much of the antioxidant capacity as anthocyanins. In addition, PA polymers do not react strongly with the Folin–Ciocalteu reagent, resulting in relatively low total phenolic concentrations in some berries with high PA concentrations.

The berries evaluated in this study represent some interesting and unique differences in phytochemical composition. Some of the extremes in anthocyanin concentrations are represented, with none in some gooseberry cultivars to some of the highest anthocyanin concentrations found in common berries in chokeberry and elderberry. Among black currant cultivars are some berries that have delphinidin aglycon present as the predominant

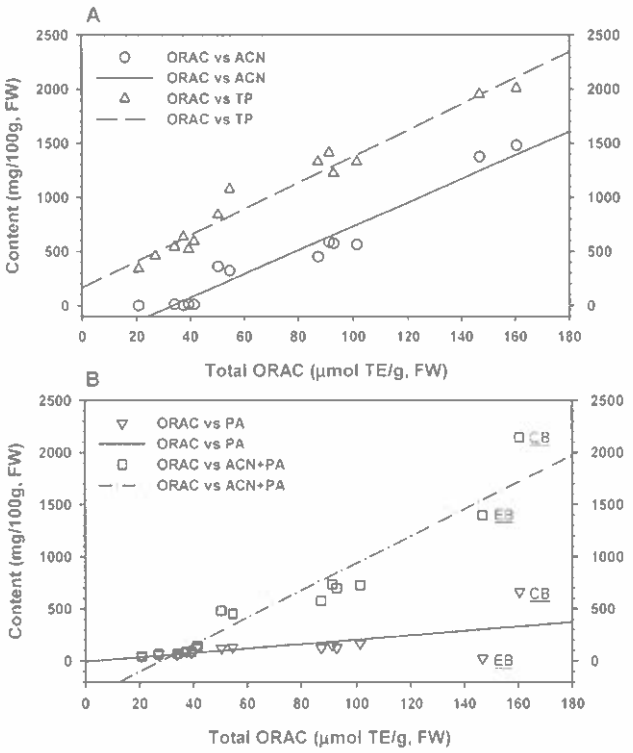



Figure 6. Relationship between total $ORAC_{FL}$ and concentrations of anthocyanins (ACN) or phenolics (TP) (A. $ORAC_{FL}$ vs TP, $y = 7.959x - 10.62$, $R^2 = 0.964$; $ORAC_{FL}$ vs ACN, $y = 0.087x - 35.46$, $R^2 = 0.948$) and relationship between total $ORAC_{FL}$ and concentrations of proanthocyanidins (PA) or anthocyanin plus proanthocyanidins (ACN+PA) (B. $ORAC_{FL}$ vs PA, $y = 0.175x + 43.98$, $R^2 = 0.389$; $ORAC_{FL}$ vs ACN+PA, $y = 0.0709x + 31.074$, $R^2 = 0.911$). Points in (B) labeled as CB or EB refer to chokeberry and elderberry, respectively.

anthocyanin. Elderberry contains an appreciable amount of cyanidin as a diglucoside (sambubioside), which is not common in most berries. All of these differences may have important implications that may become evident from studies of health effects, and as we learn more about the absorption/metabolism of anthocyanins with different aglycons or with anthocyanins containing the complex sugar moieties.

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# Antioxidant Activity in Variously Prepared Elderberry Foods and Supplements

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**Abstract**: Antioxidant capacity of foods and food supplements based on berries and flowers of medicinal plant elderberry (*Sambucus nigra* L.) was assessed. Reducing properties of the samples and extracts were evaluated using amperometric detection at working electrode potential +0.8 V after HPLC separation. Moreover, antiradical activity of selected samples was determined by the means of spectrophotometric DPPH radical scavenging method. Electrochemical activity (EA) of fresh juice pressed from elder fruits amounted to 0.71 g AAE/l with anthocyanins as minor contributors (10.2%). Catechins and phenolic acids were the major active groups. During production of elder berry spread, even more than 90% of the EA compounds found in raw elder berry material can be destroyed. Comparable activity may be found also in the products from elder flowers. Although elder blossom syrups possessed similar EA regardless of the technology used (0.033–0.054 g AAE/kg), their chromatographic patterns were often very different. For example, no flavonols were present in the syrups, if traditional preparation comprising 24-h maceration with citric acid was applied. Analyzing the chromatographic patterns, one can distinguish different base materials and technology, which can be used for the authenticity confirmation. Herbal infusions from elder flowers, which contain more flavonols than are in syrups, were 16–27 times richer in EA than drinks prepared from the syrups after recommended dilution. Only the syrup designed for preventing and treating upper-respiratory viral infections showed the EA (0.09 g AAE/kg) comparable to that of herbal infusion (0.13 g AAE/l).

**Keywords**: elderberry; elder flowers; antioxidants; reducing power; processing

## INTRODUCTION

One of the most important sources of antioxidants among dietary plants is small red fruits. Moreover, other parts of these plants, such as flowers, leaves and bark, used commonly in traditional medicine and healing are often extraordinary rich in antioxidants as well. Black elder (*Sambucus nigra* L.) is an excellent example of such a beneficial plant. Its processing is well established especially in Europe and Mediterranean where numerous food, beverages, and medicinal products are available. Elderberry has been used for generations in traditional herbal medicine as a remedy for colds, sinusitis, and herpes. The berries also have a laxative effect in small doses. Infusions of flowers are known to have diaphoretic, anti-catarrhal, expectorant, diuretic, and anti-inflammatory actions (Wightman 2004; Zakay-Rones *et al.* 2004).

Elderberry medicinal potential comes from its antioxidant potential, a property shared by numerous phytochemicals. Results from various sources have shown that both elderberry fruit and flower are rich in polyphenols. Their antioxidant capacity ranks high when compared to other well known small fruits such as cranberry, mulberry and blueberry, as well as various potent blossom extracts. Using the ORAC method, Wu *et al.* (2004) showed that especially the American elder species (*S. canadensis* L.) had a much higher potential than cranberry and blueberry, two fruits praised for their high antioxidant capacity. In another study (Halvorsen *et al.* 2002), black elder was among the best three small red fruits in

antioxidant potential as measured with the FRAP method. Burdová and Réblová (2008) reported a medium antiradical (against DPPH•) activity in the aqueous extracts of elder flowers, linden flowers, rosehips, and dead-nettle flowers.

The aim of this paper is to assess and compare the antioxidant properties of several common elderberry products on the Czech market, to describe differences in the composition of reducing compounds in the foods and supplements prepared by different methods, and to find the contribution of particular (kinds of) antioxidants to the overall electrochemical activity (EA).

## MATERIAL AND METHODS

*Samples.* Berries from wild plants grown in the countryside of Central Bohemia were picked at optimum fruit maturity, the stalks were removed, and the fruit was frozen and stored at –18°C. The berries were homogenised in a blender for 2 min after defrosting, squeesed and the juice was separated (sample *BJ*). Elderberry-apple fruit spread (*BS*) consisted of apple juice concentrate (51.7%) and elderberries (45.2%; Natudis B.V., NL). Tea (*FT*) was prepared from dried flowers (1.5 g, Megafyt, CZ) and boiling water (250 ml) after 10min infusion. Samples of syrups were: *S1a–c*, traditionally prepared syrups from fresh flowers; the inflorescence with fully opened blossoms was picked and stored at –18°C; 220 g of the flower heads were soaked in cold water (3 l) with citric acid (70 g) for 24 h at 12 ± 1°C; the macerate (sample *FM*) was strained and mixed with sucrose dissolved in water (1.5 kg in 1 l); *S2* (elderberry blossom extract, hibiscus extract, sucrose 55%, Topvet, CZ); *S3* (elder blossom extract, sucrose, citric acid, LL Ltd., CZ); *S4* (elderberry blossom extract, sucrose, lemon juice, citric acid, TBK Hostětín, CZ); *S5* – Yo elder blossom syrup (sucrose, apple concentrate, elder blossom extract, lemon juice, citric acid, aroma, Eckes-Granini, AT). The samples were dissolved or diluted with water – syrups (1:1, v/v), spread (5 g in 10 ml) and juice (1:10, v/v) – and filtered (0.45 mm nylon membrane). Electrochemical activity (EA) of the samples was expressed in g of L-ascorbic acid equivalents (AAE) per kg or l of a sample. All determinations were performed in triplicates at least. The values are expressed together with confidence intervals ($P = 0.05$).

*Antioxidants.* The following compounds were used for the evaluation of the sample extracts: L-ascorbic acid; gallic, 4-hydroxybenzoic, protocatechuic, cinnamic, *p*-coumaric, caffeic, ferulic, and chlorogenic acids; (+)-catechin, (–)-epicatechin, ECG, EGC, EGCG; quercetin, isoquercitrin, and rutin (Fluka, CH, or Aldrich, USA).

*Analyses.* Atlantis $C_{18}$, 150 × 3.9 mm, 3 µm, with a pre-column, gradient elution (pH 6.5/MeCN/5mM NaCl, diode-array (PDA, 996) & electrochemical detectors (ELD, 2465, all Waters, $E_1$ = +0.8 V) were used for HPLC method. The DPPH assay was accomplished after the method described by Brand-Williams *et al.* (1995).

## RESULTS

The most important polyphenols found in elderberry fruit are thought anthocyanins, being responsible for the colour. The HPLC/ELD analysis of fresh juice pressed from black elder fruits revealed only a minor role of the anthocyanins corresponding to 10.2% of the total EA, which amounted to 0.71 g AAE/l (Table 1). The most contributing were cyanidin 3-sambubioside and cyanidin 3-glucoside, which comprised 51% and 40% EA of anthocyanins, respective. The major part of the juice EA was originated from catechins and phenolic acids such as chlorogenic acid; rutin and other flavonols provided 8.1% of the total EA.

Elderberry reaches consumer in a much greater proportion as a processed product than as a fresh fruit. Comparing the beneficial effects of fresh elderberry fruits with that of processed ones, it should be kept in mind that especially anthocyanins stability is affected by numerous factors (Drdák & Daučík 1990). Almost total destruction of anthocyanins as well as chlorogenic acid group occurred during production of the fruit spread containing 45.2% of elderberries, while rutin was more stable and retained on 4.9% of the spread total EA (0.033 g AAE/kg, Table 1). This low AAE value indicated a degradation of most EA compounds (> 90%) during processing provided that the activity of the raw berries was similar to EA of the tested fresh juice.

Elder blossoms are distinguished by their intensive, pleasant odor and serve as a basis for industrially produced soft drinks and as extracts to increase the nutritional value of different foods and diets. Although products such as cough syrups and teas made from elder flower are usually safe taken in moderation (Mills & Bone 2005), after the Czech food regulations (Ministry of Ag-

riculture 1997), elder flowers and berries can be used for the preparation of infusions and teas in mixtures with other plant materials up to 30% (m/m). The consumption of tea infusion of elder flowers is among the most used ways of the elder flower antioxidants' intake. Its EA was found to be equivalent to 21 g AAE/kg of dry flowers (Table 1). The content of rutin found in the infusion was 10.9 ± 0.4 g/kg of dry flowers weight, which is in good agreement with literature (Dawidowicz *et al.* 2006). Rutin was responsible by 63 ± 2 % and the other flavonols by 19 ± 1% for the infusion colour ($Abs_{420nm}$). While the EA in infusions was derived mainly from rutin (27 ± 1%), other flavonols and their glycosides (9.5 ± 0.5%), derivatives of caffeic (chlorogenic) acid and flavan-3-ols, quite different distribution of the active compounds was found within the syrups studied.

Based on dry flowers weight, the acidified 24 h macerate (FM) from fresh flowers resembling traditional preparation of elder blossom syrup revealed only 42% EA comparing to the infusion from dried flowers. Phenolic acids such as protocatechuic acid and derivatives of caffeic acid together with catechins were the major EA compounds. The addition of citric acid adjusting the pH value to 3.1 affected the polyphenoloxidase (PPO) activity as well as subsequent browning. There may be further decrease in PPO activity below pH 4 due to less tight binding of copper in the enzyme, permitting chelators such as citric acid to remove the copper. Lower pH has an effect on the destruction of flavonols and their glycosides during the 24 h maceration and storage, not on the extraction efficiency. Different extraction temperatures (20°C *vs.* 100°C) may influence the yield, but not the composition (data not shown).

EA of the studied syrups depends on the technology used and the content of elder flower and other ingredients. Traditionally prepared syrups from fresh flowers (S1a-c) possessed 0.033–0.045 g AAE/l of syrup and were very pale. Commercially available syrups are prepared mostly with the use of extracts from dry flowers and/or elder blossom aroma. Syrup S2, sold as a food supplement with the declared action against cold and influenza, is more concentrated than syrups intended for a beverage preparation. Its EA (0.090 g AAE/l) was significantly higher than that of the other syrups. L-Ascorbic acid, which was only minor or not detected in the other syrups, was the cause of 57% of EA in S2; rutin participated by 7.5%. Commercially produced S5 with the addition of apple concentrate and elder blossom aroma did not contain detectable amounts of rutin and other flavonols. Close correlation ($r^2$ = 0.98) between EA and colour ($Abs_{420nm}$) was found only for the products prepared from dry flowers, where flavonols were present in significant amounts.

The expected antioxidant activity associated with electrochemical characteristic was compared with a free-radical scavenging capacity. Using DPPH·

Table 1. Electrochemical activity (EA; +0.8 V) of elder flower and berry products with contributions of flavonols and anthocyanins

| Samples | Total EA (g AAE per kg or l) | Flavonols' EA (%) | Anthocyanins' EA (%) |
|---|---|---|---|
| Syrup S1a | 0.045 ± 0.007 | < 0.5 | – |
| S1b | 0.040 ± 0.006 | < 0.5 | – |
| S1c | 0.033 ± 0.007 | < 0.5 | – |
| S2 | 0.090 ± 0.003 | 8.6 ± 0.4 | – |
| S3 | 0.054 ± 0.004 | 10.3 ± 0.3 | – |
| S4 | 0.041 ± 0.004 | 6.5 ± 0.3 | – |
| S5 | 0.045 ± 0.003 | < 0.6 | – |
| Tea FT | 21 ± 2* | 37 ± 2 | – |
| Macerate FM | 8.8 ± 0.8* | < 1 | – |
| Juice BJ | 0.71 ± 0.06 | 8.1 ± 0.3 | 10.2 ± 0.8 |
| Spread BS | 0.033 ± 0.003 | 4.9 ± 0.3 | < 0.5 |

*based on dry flowers' weight

assay, elderberry juice possessed 0.3% and tea infusion, based on dry flowers' weight, 5.2% antiradical activity of L-ascorbic acid. Our results revealed that various forms and preparations comprise often quite different mixtures of active compounds depending on the treatment conditions, while the total EA levels need not vary so much. The data confirmed that both elder flower and fruit can serve as a good source of bioactive polyphenols in human diet and all the studied spectrum of black elder products can be regarded as good candidates for nutritional supplement formulations.

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# *In vitro* antioxidant properties and anthocyanin compositions of elderberry extracts

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Radical scavenging activity
Antioxidant activity

ABSTRACT

In this study, dried elderberry fruits growing wild in Turkey were macerated using different solvents and an infusion was prepared according to traditional methods. All extracts were investigated for their total phenolic content, total monomeric anthocyanins, qualitative–quantitative determination of cyanidin-3-glucoside (by HPLC–UV analysis), anthocyanin compositions (by LC/MS–MS), free radical scavenging activity (DPPH and ABTS) and inhibition of β-carotene/linoleic acid co-oxidation. An extract with 70% ethanol was found to be richer in cyanidin-3-glucoside when compared to the other extracts. The infusion was found to be as rich as the 70% ethanol extract. Ethanol and acetone extracts (both 70%) were found to be more active in the free radical activity and β-carotene bleaching assays. Water extract showed good $ABTS^{\cdot}$ radical scavenging activity when compared with ascorbic acid.

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## 1. Introduction

Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases, cancers and neurodegenerative diseases (Hamid, Aiyelaagbe, Usman, Ameen, & Lawal, 2010; Sharma, Jha, Dubey, & Pessarakli, 2012). Therefore, natural antioxidants and colourants present in foods have attracted interest because of their safety and potential nutritional and therapeutic effects. These natural compounds can also be alternatives to synthetic dyes (Espin, Soler-Rivas, Wichers, & García-Viguera, 2000).

Anthocyanins belong to the most common class of phenolic compounds which occur naturally in fruits and vegetables as glycosides, containing glucose, galactose, rhamnose, xylose or arabinose attached to an aglycon nucleus (Wang & Stoner, 2008). Nowadays, the potential health benefits associated with consumption of anthocyanins are the focus of much research. Intake of anthocyanin is increasing because extracts and juices with high anthocyanin contents from fruits and vegetables such as grape, various berries and red carbage are becoming more and more popular.

The anthocyanin content of berries from *Sambucus*, *Lonicera* and *Viburnum* species have received great attention. Especially juices and extracts from the elderberry, known as *Sambucus nigra* L., have been used in biological studies (Abuja, Murkovic, & Pfannhauser, 1998; Bitsch et al., 2004; Bratu, Guiu, Samarineanu, Gaidargiu, & Porta, 2003; Murkovic, Adam, & Pfannhauser, 2000; Netzel et al., 2005; Roschek, Fink, McMichael, Li, & Alberte, 2009; Wu, Gu, Prior, & McKay, 2004). Elderberries general grow wild in several countries in Europe and are also cultivated on a small scale in some northern European countries (Akbulut et al., 2009). Elderberry has also been used to colour jams, jellies and wines in Europe (Inami, Tamura, Kikuzaki, & Nakatani, 1996). The dried ripe or fresh berries are recommended for the treatment of constipation, as a diuretic and diaphoretic in upper respiratory tract infections, and to alleviate pain. For the treatment of these complaints, juice or tea are consumed. The infusion, prepared from 10 g dried berries standing in cold water for several minutes, is then slowly heated up and boiled briefly. Before filtering, a drawing-time of 5–10 min is recommended (Vlachojannis, Cameron, & Chrubasik, 2009). In Turkey, *S. nigra* occurs sporadically in western and eastern parts, particularly in the northern coastal strip. Elderberry bark, root, stem and fruits have been used particularly by the rural population as medicine and food (Baytop, 1984). But the consumption of elderberry is not very common and only a few studies have been reported (Akbulut, Ercişli, & Tosun, 2009; Yenen & Özgen, 1997). However, none of these studies focus on elderberry anthocyanin composition and identification by liquid chromatography.

The aim of this study was to determine the anthocyanin composition and to evaluate the antioxidant capacity of elderberry

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extracts. Five extracts were prepared with solvents of different polarities and an infusion of elderberry fruits was investigated for its antioxidant activity in *in vitro* test systems. Furthermore, the total phenol and total monomeric anthocyanin were also analysed using spectrophotometric techniques. Anthocyanin contents of the extracts were also characterized by HPLC–UV and LC–MS/MS analyses.

## 2. Materials and methods

### 2.1. Plant material and reagents

Air-dried mature fruits of elderberry were obtained from 'Evçay Company', Yalova, Turkey in 2009. Chromatographic standards were purchased from Sigma Chemical Company. Ultra-pure water was used throughout and was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., MA, USA). All remaining reagents were of the highest purity available and obtained from the Sigma Chemical Company (St. Louis, MO, USA).

### 2.2. Preparation of the extracts

Ground elderberries (5 g) were macerated with 100 ml of water, 70% ethanol, 70% acetone and methanol at room temperature by using a shaker 3 times (48, 48, 24 h) during 5 days in the dark. HCl (0.1%) in methanol extracts were prepared by using an ultrasonic bath at room temperature for 30 min, 3 times. The infusion was prepared according to traditional methods [illegible] and freeze dried. The extracts were evaporated to dryness at 35 °C by a rotary evaporater and stored at −18 °C. Prior to analysis, an aliquot of each extract was dissolved and filtered through a 0.45 μm membrane (Whatman, UK) and used in all the test systems.

### 2.3. Total phenols

Total phenols were estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/100 g extract [illegible]. To *ca.* 6.0 ml $H_2O$, 100 μl of sample was transferred into a 10.0 ml volumetric flask, to which 500 μl undiluted Folin–Ciocalteu reagent was added subsequently. After 1 min, 1.5 ml of a 20% (*w*/*v*) $Na_2CO_3$ solution was added and the volume was made up to 10.0 ml with $H_2O$. After 2 h incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. The data are presented as the average of triplicate analyses.

### 2.4. Total monomeric anthocyanins

Total monomeric anthocyanins were measured according to the 'pH differential method' in triplicate [illegible]. All extracts were dissolved in suitable solvents and used as stock solutions. Appropriate dilution factors for each sample were determined by diluting with 0.025 M potassium chloride buffer (pH 1.0) until the absorbance of the sample at 510 nm was within the linear range of the spectrophotometer. For each sample, two dilutions were prepared, both with 0.025 M potassium chloride buffer (pH 1.0) and with 0.4 M sodium acetate buffer (pH 4.5) diluting each by the previously determined dilution factor. These dilutions were equilibrated for 15 min. The absorbance of each dilution was measured at 510 nm and at 700 nm (to correct for haze), against a blank cell filled with distilled water.

The absorbance of the diluted sample ($A$) was calculated as follows:

$$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}. \quad (1)$$

The monomeric anthocyanin concentration in the original sample was calculated using the following formula:

$$\text{Monomeric anthocyanin pigment (mg/l)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1). \quad (2)$$

Anthocyanin content was calculated as cyanidin-3-glucoside, where MW = 449.2 and $\varepsilon$ = 26,900.

### 2.5. Qualitative–quantitative chromatographic analysis

#### 2.5.1. HPLC–UV analysis

The liquid chromatographic apparatus (Shimadzu LC 10Avp, Ant Ltd. Sti., Istanbul, Turkey) consisted of an in-line degasser, pump and controller coupled to a Shimadzu UV–Vis Spectrophotometer equipped with an automatic injector interfaced to Class VP chromatography manager software (Shimadzu, Japan). Separations were performed on a 250 × 4.6 mm *i.d.*, 5 μm particle size, octadecyl silica gel analytical column (Supelco, PA, USA) operating at 40 °C at a flow rate of 0.4 ml/min. Detection was carried out at 520 nm. Elution was carried out using a binary gradient of formic acid/water (8.5/91.5, *v*/*v*) (solvent A) and tetrahydrofuran/formic acid/acetonitrile/methanol/water (5/8.5/22.5/22.5/41.5, *v*/*v*/*v*/*v*/*v*) (solvent B). The composition of B was increased from 15% to 30% in 10 min, increased to 40% in 10 min and increased to 100% in 5 min, then the composition was decreased to 15% in 5 min. Cyanidin-3-glucoside in all extracts was identified by comparison of its retention time to that of authentic standards under identical analysis conditions and UV spectra. A 10 min equilibrium time was allowed between injections. All standard and sample solutions were injected in triplicate.

#### 2.5.2. HPLC–UV–MS–MS analysis

The identification of some of the anthocyanins present in the elderberry extracts was carried out by means of their molecular weight and their fragments. For this, HPLC with UV detection and tandem mass spectrometry was used (HPLC–UV–MS–MS). The analytical conditions used were those described by [illegible], with some modifications. Samples of each elderberry extract (5 μl) were analysed using an HPLC system equipped with a model LC20AD Shimadzu pump and a model SPD20A Shimadzu UV Detector. The samples were injected by means of a model SIL20A Shimadzu Autosampler. Separations were carried out using an Intersil ODS-3 column (4.6 × 250 mm, 5 μm particle size). The CTO20A Shimadzu Column Oven was kept at 40 °C. The mobile phase was water with 1% formic acid (*v*/*v*) (solvent A) and HPLC grade methanol (solvent B) at a flow rate of 1 ml $min^{-1}$. The linear gradient started with 3% solvent B reaching 5% at 5 min, 8% solvent B at 10 min, 13% solvent B at 15 min, 15% solvent B at 19 min, 40% solvent B at 47 min, 65% solvent B at 64 min, 80% solvent B at 65 min and 98% solvent B, followed by 66 min at isocratic elution until 69 min. At 70 min the gradient reached the initial conditions again. HPLC chromatograms were recorded at 380 and 520 nm. The Shimadzu HPLC–UV system was connected directly to a 3200 Q TRAP mass spectrometer (MS) (AB Sciex, Toronto, Canada). The software used for data acquisition and analysis was Analyst 1.5. The chromatographic conditions were used as described above. For enhanced mass scan (EMS), the MS was operated in positive polarity at a scan rate of 1000 Da/s within the mass range of 200–800 amu. Mass scan (MS) and daughter (MS–MS) spectra were measured from *m*/*z* 100 up to *m*/*z* 800. Collision-induced fragmentation experiments were performed in the ion trap using nitrogen as the collision gas, with the collision energy set at 30–100. The parameters were as follows: Collision Energy Spread (CES) = 15, Declostiring Potential (DP) = 45, Enterance Potential (EP) = 10, Curtain gas (CUR) = 10 and Temperature (TEM) = 600. For the IDA experiment, the criteria were arranged for ions greater than 200,000 *m*/*z* and smaller than

Table 1
Extract yield, total phenols, monomeric anthocyanins, and HPLC qualitative and quantitative data for elderberry.

| Sample | Yield[a] | Spectrophotometric results | | HPLC results | |
|---|---|---|---|---|---|
| | | Total phenols[b] | Total anthocyanins | Cyanidin-3-glucoside[c] | Total anthocyanins[e] |
| (A) | 260 | 8974 ± 37 | 878.5 ± 15 | 100.5 ± 0.72 | 1247 ± 53 |
| (B) | 432 | 7594 ± 3 | 1066.6 ± 55 | 254.3 ± 5.03 | 1326 ± 48 |
| (C) | 404 | 4917 ± 63 | 408.6 ± 85 | 42.4 ± 0.02 | 285 ± 20 |
| (D) | 328 | 8206 ± 167 | 651.1 ± 26 | 175.4 ± 0.63 | 890 ± 50 |
| (E) | 602 | 6399 ± 80 | 600 ± 16 | 68.1 ± 0.07 | 650 ± 15 |
| (F) | 161 | 6715 ± 21 | 734.2 ± 33 | 202.9 ± 1.87 | 951 ± 40 |

(A) Water extract; (B) 70% ethanol extract; (C) methanol extract; (D) 70% acetone extract; (E) acidified methanol; (F) infusion.
[a] Extract yields expressed as milligrams of extract per gram (dry weight) of fruits.
[b] Total phenols expressed as gallic acid equivs. milligrams of gallic acid per 100 gram (dry weight) of extract.
[c] Total monomeric anthocyanins expressed as cyanidin-3-glucoside equivs. milligrams of cyanidin-3-glucoside per 100 gram (dry weight) of extract.
[d] Values (mg/100 g extract) are expressed as means ± standard error.
[e] Total anthocyanins expressed as cyanidin-3-glucoside equivs. milligrams of cyanidin-3-glucoside per 100 g of extract by HPLC method.

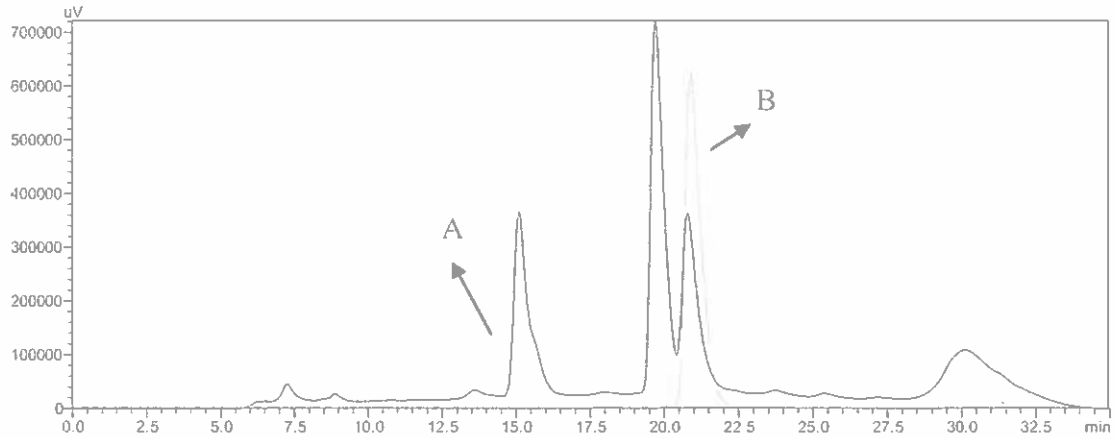



Fig. 1. (A) The HPLC–UV chromatogram of elderberry extract with absorbance at 520 nm; (B) the HPLC–UV chromatogram of cyanidin-3-glucoside standard.

800.000 *m/z*, and excluded former target ions after 3.0 occurrence(s) for 3.000 s.

### 2.6. The antioxidant activity

#### 2.6.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH˙) radical scavenging activity

Serial dilutions were carried out with the stock solutions (10 mg/ml$^{-1}$) of the extracts to obtain the concentrations of 10, 5, $25 \times 10^{-1}$, $125 \times 10^{-2}$, $625 \times 10^{-3}$, $3125 \times 10^{-4}$ mg/ml$^{-1}$. Diluted solutions were mixed with DPPH (equal amounts) and allowed to stand for 30 min for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive control, ascorbic acid. The percentage inhibition was calculated using Eq(3). The $IC_{50}$ value, which is the concentration of the test material that inhibits 50% of the free radical concentration, was calculated as mg/ml$^{-1}$ (Kumarasamy et al., 2007):

$$\text{Percentage inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100. \quad (3)$$

#### 2.6.2. TEAC assay (Trolox Equivalent Antioxidant Capacity)

This assay assesses the capacity of a compound to scavenge the stable ABTS radical in comparison to the antioxidant activity of Trolox, a water-soluble form of vitamin E that is used as a standard. The blue-green ABTS was produced through the reaction of 7 mM ABTS with 2.5 mM sodium persulfate ($Na_2S_2O_8$) (final concentrations) in the dark at room temperature for 12–16 h before use. The concentrated ABTS solution was diluted with ethanol to a final absorbance of 0.7–0.8 at 734 nm. A 10-μl portion of sample (concentrations of 0.6, 0.3 and 0.1 mg/ml$^{-1}$) was added to 990 μl of ABTS solution, and the reduction in absorbance was measured 1 min after addition of Trolox (final concentration 1–20 μM) and up to 40 min after addition of the each extract. The stock solution of Trolox (2.5 mM) was prepared in ethanol. Absorbance was measured on a UV/spectrophotometer (Papandreou et al., 2006).

#### 2.6.3. Determination of inhibition of β-carotene/linoleic acid co-oxidation

Antioxidant activity of the elderberry extracts was determined according to β-carotene bleaching methods (Oomah & Mazza, 1996; Velioglu, Mazza, Gao, & Oomah, 1998). Briefly, 1 ml of β-carotene (0.2 mg/ml$^{-1}$ dissolved in chloroform; Sigma Chemical Co., St. Louis, MO) was added to a flask containing linoleic acid (40 mg) and Tween 80 (400 mg). Chloroform was evaporated under a stream of nitrogen. Distilled water (50 ml) was added and shaken vigorously. A control was prepared without sample or standards with same procedure. Blanks of control and sample were also prepared without β-carotene. Their absorbance were measured on a spectrophotometer at 470 nm. The samples were then subjected to thermal autoxidation by keeping them in a constant temperature water bath at 50 °C for 2 h. The rate of bleaching of β-carotene was monitored by taking the absorbance at 15 min intervals. Antioxidative activity was calculated according to Eq.(4):

$$AA\% = \left[1 - (Ab_{0\text{sample}} - Abs_{120\text{sample}})/(Ab_{0\text{control}} - Abs_{120\text{control}})\right] \times 100. \quad (4)$$

Table 2
HPLC–MS/MS data for elderberry extracts.

| Peak | HPLC Rt (min) | Molecular ion ($MS_1$) $m/z^+$ | Fragment ($MS-MS_2$) $m/z^+$ | Structure | A | B | C | D | E | F |
|---|---|---|---|---|---|---|---|---|---|---|
| X | 26.70 | 611 | 449, 287 | Cyanidin-3,5-diglucoside | + | + | + | + | + | + |
| | | 743 | 581, 449, 287 | Cyanidin-3-sambubioside-5-glucoside | + | + | + | + | + | + |
| Y | 33.03 | 449 | 287 | Cyanidin-3-glucoside | + | + | + | + | + | + |
| | | 581 | 449, 287 | Cyanidin-3-sambubioside | + | + | + | + | | + |
| Z | 52.63 | 633 | 487, 331 | Quercetin-3-rutinoside $Na^+$ adduct | + | + | + | + | | + |

(A): water extract; (B): 70% ethanol extract; (C): methanol extract; (D): 70% acetone extract; (E): Acid methanol extract; (F): infusion.

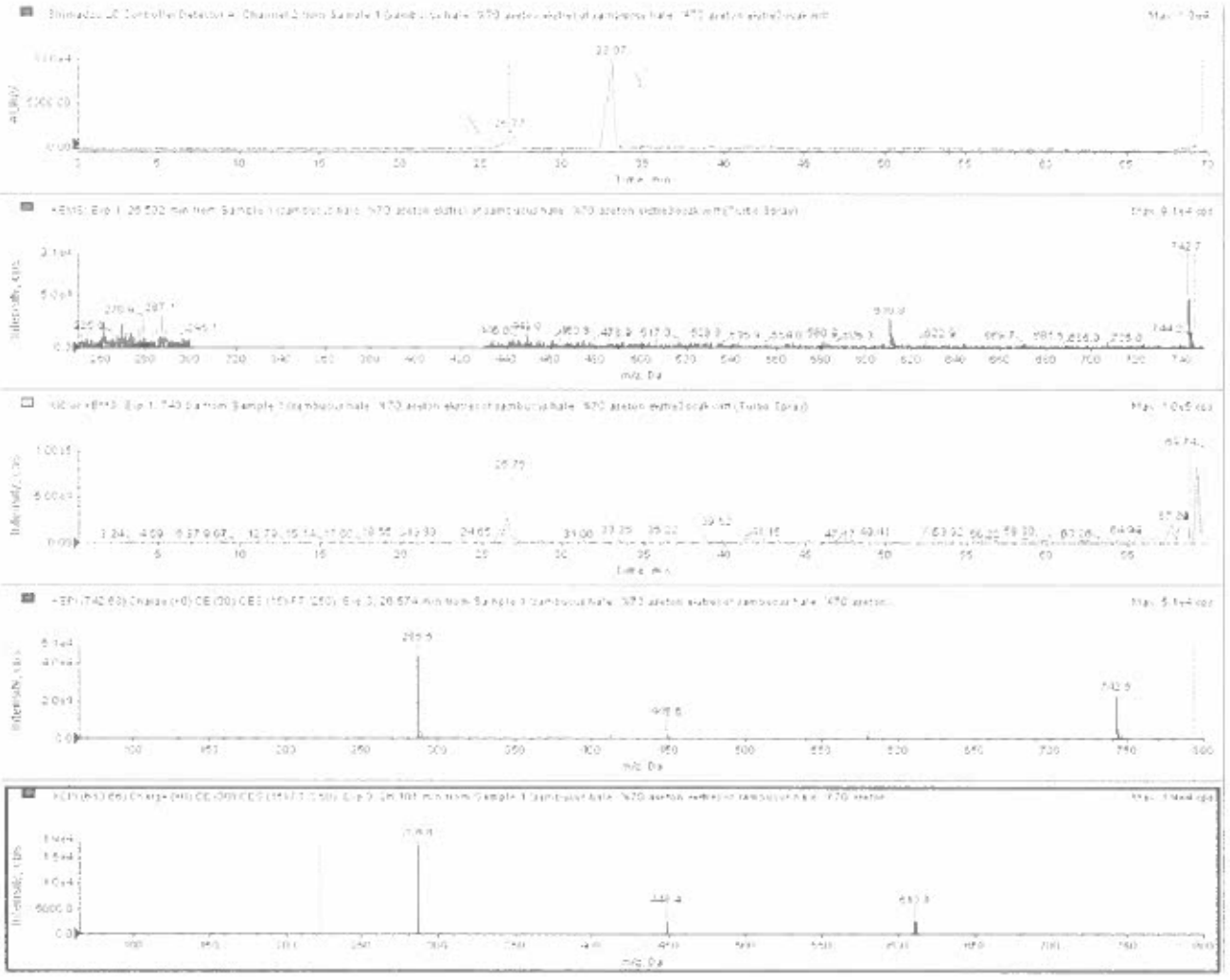


**Fig. 2.** LC chromatogram at 520 nm and EMS–EPI spectra of the X peak at *tR* 26.7 min.

### 2.7. Statistical analysis

Data are presented as mean values ± standard deviation. All the statistical analyses were carried out using SPSS 10.0.1. (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) was performed by ANOVA procedures. Significant differences between means were determined by Tukey's pairwise comparison test at a level of $p < 0.05$. $IC_{50}$ values were estimated using a non-linear regression algorithm.

## 3. Results and discussion

### 3.1. Fraction yields, total phenols, total monomeric anthocyanins and compositional analysis

Ground elderberries were macerated with water, 70% ethanol, 70% acetone, ethanol and methanol at room temperature. Acidified methanol (0.1% HCl Inc.) extracts were prepared using an ultrasonic bath at room temperature for 30 min in triplicate. The

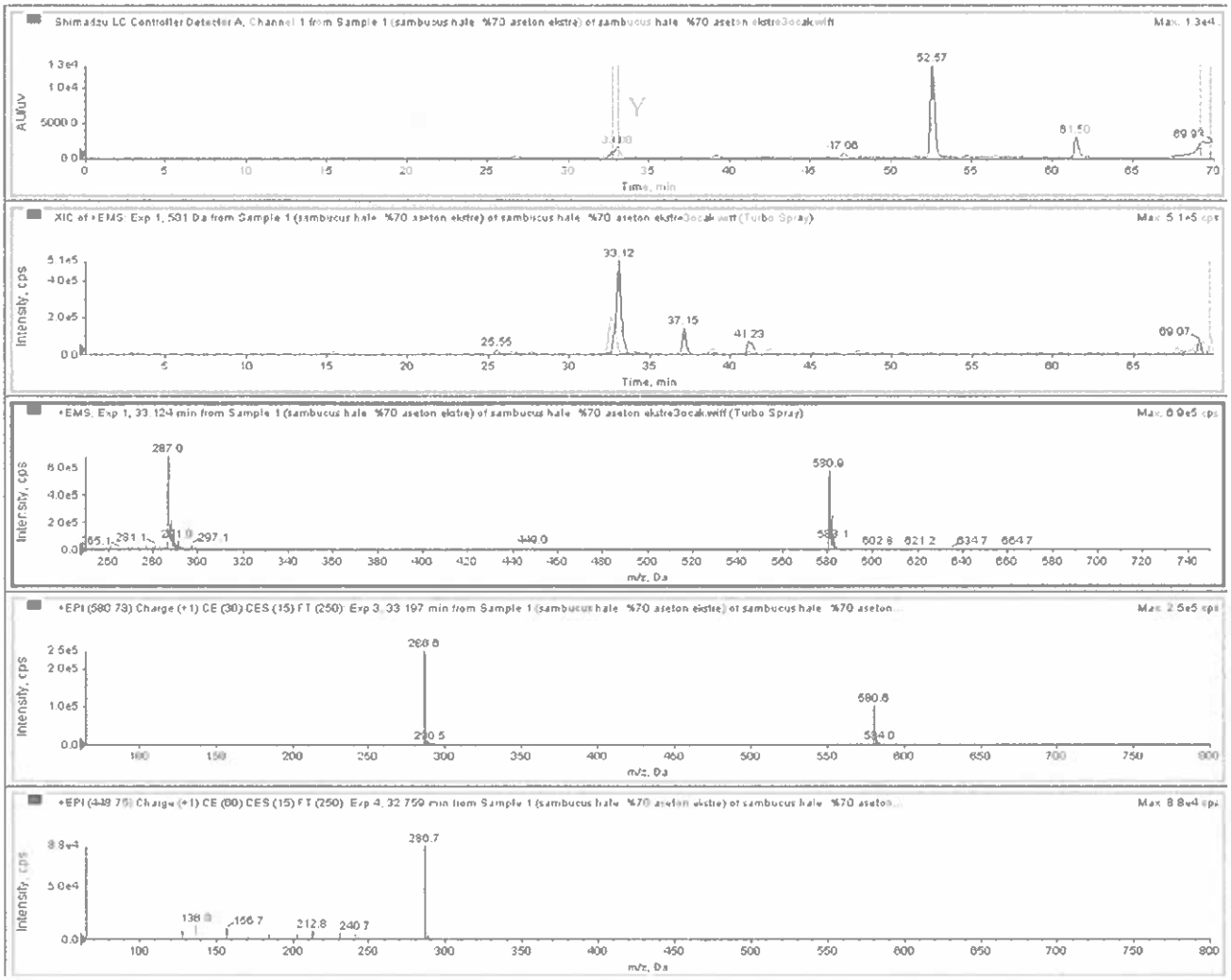



Fig. 3. LC chromatogram at 520 nm and EMS-EPI spectra of the *Y* peak at *tR* 33.08 min.

infusion was prepared according to traditional methods. The results of fractionation, total phenols, total monomeric anthocyanins and the amount of cyanidin-3-glucoside of extracts are presented in Table 1. According to the data presented in Table 1, water, 70% acetone, 70% ethanol, infusion, acidified methanol and methanol extracts contained the highest amount of total phenol content, respectively. The highest yields were obtained also from the acidified methanol, 70% ethanol and methanol extracts.

The results of the qualitative–quantitative analyses of the extracts, carried out using an HPLC apparatus coupled with a UV detector, are presented in Table 1, with a selected chromatogram shown in Fig. 1. Cyanidin-3-glucoside was identified and quantified at 520 nm. Cyanidin-3-glucoside was identified by comparison of the retention time and UV spectra of authentic standard, while quantitative data were calculated from their calibration curves. The 70% ethanol, infusion and 70% acetone extracts were found to be the richest in cyanidin-3-glucoside, as measured by HPLC. In addition, total anthocyanins of each extract obtained from chromatogram at 520 nm were quantified as cyanidin-3-glucoside equivalents (Table 1). When the spectroscopic total anthocyanin analysis data was compared with that from the HPLC method, the total anthocyanins measured by HPLC were higher than those observed in the spectroscopic method, except for the methanol extract. Correlation was determined on the anthocyanin values obtained by the pH differential method and HPLC. The correlation between these two methods was significant ($r^2$ = 0.9302).

All extracts were determined for their total phenolic and anthocyanin contents by spectrophotometric methods. Cyanidin-3-glucoside was also identified and calculated in the extracts by HPLC. Other anthocyanins in the extracts were identified by LC–MS–MS. In order to achieve ionisation of the anthocyanins, HPLC conditions reported by Bermúdez-Soto and Thomas-Barberán (2004) were employed with some modifications. Four different cyanidin glycosides, such as cyanidin-3,5-diglucoside, cyanidin-3-sambubioside-5-glucoside, cyanidin-3-sambubioside and cyanidin-3-glucoside were detected in the 520 nm chromatogram, while quercetin-3-rutinoside $Na^+$ adduct was detected in the 380 nm chromatogram. These compounds were identified by their UV spectra, their molecular weights and their fragmentation patterns. At 520 nm, MS fragmentation ions at *m/z* 287 were attributed to cyanidin derivatives (Table 2). The *X* peak (Fig. 2) was confirmed as cyanidin-3,5-diglucoside (*m/z*$^+$ 611, $MS^2$ fragments at 449, 287) and cyanidin-3-sambubioside-5-glucoside (*m/z*$^+$ 743, $MS^2$ fragments at 581, 449, 287). The *Y* peak (Fig. 3) was characterized as cyanidin-3-sambubioside (*m/z*$^+$ 581, $MS^2$ fragments 449, 287) and cyanidin-3-glucoside (*m/z*$^+$ 449, $MS^2$ fragment 287). The *Z* peak (Fig. 4) obtained from the 380 nm chromatogram was identified as quercetin-3-rutinoside $Na^+$ adduct (*m/z*$^+$ 633, $MS^2$

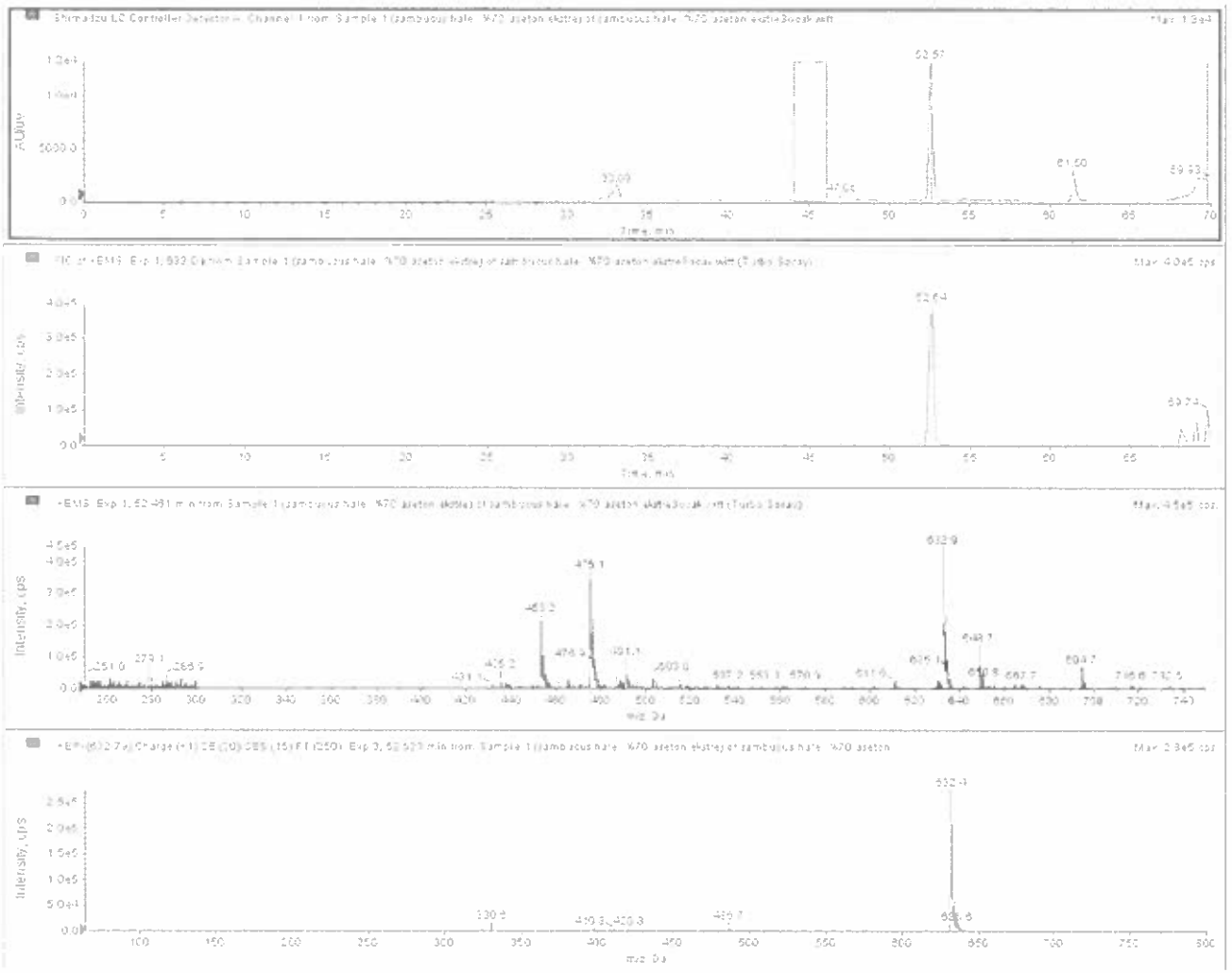



Fig. 4. LC chromatogram at 380 nm and EMS–EPI spectra of the Z peak at *tR* 52.64 min.

fragments at 487, 331). The results are given in [illegible]. Cyanidin-3,5-diglucoside, cyanidin-3-sambubioside-5-glucoside and cyanidin-3-glucoside were identified in all extracts. Cyanidin-3-sambubioside and quercetin-3-rutinoside $Na^+$ adduct were detected in all the extracts except for the acidified methanol extract. These compounds were evaluated according to LC/MS–MS results of [illegible].

### 3.2. DPPH· radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical is a stable radical, with an absorption maximum at 517 nm. When reduced to the hydrazine derivative by an antioxidant *via* electron or hydrogen atom transfer reactions, this absorption maximum decreases (Liu & Luo, 2011). $IC_{50}$ values, defined as the concentration required to scavenge 50% of the available free radicals, were estimated by nonlinear regression for all the extracts. All the extracts showed free radical scavenging activity in this test. According to the results, the 70% acetone extract was the most active free radical scavenger of them all ($IC_{50}$ value 117 μg/ml$^{-1}$). The water extract was the second active free radical scavenger with an $IC_{50}$ value of 123 μg/ml$^{-1}$. However, none of the extracts were as active as the positive control, ascorbic acid ($IC_{50}$ value of 8 μg/ml$^{-1}$). The order of DPPH· radical scavenging ability for the fractions was as follows: 70% acetone > water > 70% ethanol > infusion > acidified methanol > methanol. Also cyanidin-3-glucoside at 1 mg/ml$^{-1}$ concentration showed 80% inhibition, whereas ascorbic acid at the same concentration exhibited 94% inhibition on DPPH· radical. These phenolics have antioxidant activity and they are considered to be responsible for the antioxidant activity.

### 3.3. Inhibition of β-carotene/linoleic acid co-oxidation

Food lipids and cell membranes contain unsaturated fatty acids, linoleic and arachidonic acids, which can be easily oxidised with oxidative agents. Therefore, unsaturated fatty acid–base medium in antioxidant activity tests are important to determine the activities of test samples. The β-carotene–linoleic acid bleaching assay is such a model, widely used to investigate the oxidation of unsaturated fatty acids, especially in the cell wall and food products (Ito-ar Ginger & Rasty, 2008).

The β-carotene/linoleic acid oxidation method evaluates the inhibitory activity of free radicals generated during the peroxidation of linoleic acid. The method is based on spectrophotometric discolouration measurements or oxidation of β-carotene-induced oxidative degradation products of linoleic acid. In the β-carotene/linoleic acid co-oxidation assay, the degree of lipid peroxidation is measured. The inhibition percentages of all extracts of

elderberry are calculated. According to results, some extracts could inhibit the oxidation of linoleic acid. The 70% ethanol extract had the best activity (58% inhibition). The hierarchy of the extracts were 70% ethanol > infusion > 70% acetone > methanol > water > acidified methanol. None of the extracts of elderberry were found to be as active as the positive control BHT. Cyanidin-3-glucoside at 1 mg/ml$^{-1}$ concentration had 43% inhibition on the oxidation of linoleic acid. According to these results, cyanidin-3-glucoside is thought to be responsible for this activity.

### 3.4. TEAC assay (Trolox Equivalent Antioxidant Capacity)

In this assay, the capacity of elderberry extracts to scavenge the ABTS radical ($ABTS^{\cdot+}$) was assessed. This assay is based on the scavenging of the relatively stable blue/green ABTS radical ($ABTS^{\cdot+}$), converting it into a colourless product. The degree of decolourization reflects the amount of $ABTS^{\cdot+}$ that has been scavenged and can be determined spectrophotometrically. The TEAC value is assigned by comparing the scavenging capacity of an antioxidant to that of Trolox (Badarinath et al., 2010). The TEAC values at 30 min were calculated, the Trolox calibration curve was linear ($r^2$ 0.996) and TEAC values of all extracts were calculated according to this curve. The TEAC values of the extracts ranged between 0.89 and 1.97 mmol Trolox equivalents/l. According to our results, the 70% acetone extract (1.96 mM) was as effective as ascorbic acid (1.97 mM) on $ABTS^{\cdot+}$ radical scavenging. Cyanidin-3-glucoside at 1 mg/ml $^{-1}$ conc. had a lower activity than ascorbic acid at the same concentration, whereas it showed better activity than the infusion (1.23 mM), methanol (1.0 mM), acidified methanol (0.89 mM) and 70% ethanol (1.52 mM) extracts. The water extract (1.85 mM) was as active as cyanidin-3-glucoside (1.87 mM).

Several berries are used as natural colourants in different industries and elderberries draw attention because of their anthocyanin-rich content and high antioxidant activity. In elderberry fruits, cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3,5-diglucoside and cyanidin-3-sambubioside-5-glucoside are the main anthocyanins (Inami et al., 1996; Nakajima, Tanaka, Seo, Yamazaki, & Saito, 2004; Wu et al., 2004). The antioxidant and antiradical activities of the juice and extracts of elderberry have been reported. Espin et al. (2000) determined the antiradical activity on DPPH$^{\cdot}$ radical of commercial elderberry juice and other fruit extracts by a spectrophotometric method. According to their results, 3-monoglycoside (97%) and cyanidin 3,5-diglycoside (3%) identified in the juice had a lower DPPH$^{\cdot}$ radical scavenging activity when compared with vitamin E, BHT and BHA. Lugasi and Hovari (2003) reported that elderberry juice had a total polyphenol concentration of 5680 mg/l and this was significantly higher than that of high quality red wines. Hydrogen-donating ability and the reducing power found to be the highest in the juice of elderberry exhibited both antioxidant and DPPH$^{\cdot}$ scavenging properties strongly correlated with the total polyphenol content. In another study, the potent antiradical activity of some berry extracts or commercial juice were determined and their anthocyanin composition was analysed by LC/PDA/ESI–MS. Cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3-sambubioside-5-glucoside and cyanidin-3,5-diglucoside were identified in the commercial juice of elderberry. All extracts were treated by DPPH$^{\cdot}$ and compared with Trolox. According to the results, all the extracts showed lower DPPH$^{\cdot}$ radical scavenging activity than Trolox (Nakajima et al., 2004). Elderberry juice concentrate was found to have a rich phenolic content and high free-radical scavenging activity against both $ABTS^{\cdot+}$ and DPPH$^{\cdot}$ radicals. Cyanidin-3-sambubioside was the most abundant anthocyanin in the elderberry juice concentrate (Bermudez-Soto & Thomas-Barberan, 2004). The alcoholic extracts (80% ethanol in water) from the leaves, berries and flowers of *S. nigra* act as antioxidants, neutralizing the activities of free radicals and inhibiting the co-oxidation reactions of linoleic acid and β-carotene. Cyanidin-3-sambubioside, cyanidin-3-glucoside, rutin, isoquercitrin and astragalin were also identified in elderberry extracts (Dawidowicz, Wianowska, & Baraniak, 2006).

According to our results, the highest total phenolic content was calculated in the water extract, which had a similar TEAC activity when compared to cyanidin-3-glucoside. Furthermore, the water extract was as effective as the 70% acetone extract on DPPH$^{\cdot}$ radical scavenging activity. The infusion of berries was found to possess good antiradical activity correlated with its cyanidin-3-glucoside, total phenol and anthocyanin contents. The methanol extract was found to have the lowest total phenolic, total anthocyanin and cyanidin-3-glucoside levels when compared with the other extracts. Because of this, the methanol extract showed the lowest antiradical and antioxidant activity in all the tested assays.

In conclusion, water and aqueous alcoholic extracts were found to be rich in phenolic and anthocyanin contents. These extracts showed high free radical scavenging activity against both ABTS and DPPH radical suggesting their possible use as antioxidants.

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# Vitamin D

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## Summary


- Vitamin D can be synthesized in the skin upon exposure to sunlight and is then metabolized in the liver and kidney to the metabolically active form called 1α,25-dihydroxyvitamin D. Through binding to the vitamin D receptor (VDR), 1α,25-dihydroxyvitamin D can regulate the expression of hundreds of genes involved in skeletal and other biological functions. *(More information)*
- Vitamin D is essential for maintenance of bone mineralization through the regulation of calcium and phosphorus homeostasis. Vitamin D also exhibits many non-skeletal effects, particularly on the immune, endocrine, and cardiovascular systems. *(More information)*
- Vitamin D is important for normal bone development and maintenance. Severe vitamin D deficiency causes rickets in children and osteomalacia in adults. *(More information)*
- Secondary hyperparathyroidism due to vitamin D insufficiency can increase bone breakdown and precipitate osteoporosis. Randomized clinical trials indicate that supplementation with at least 800 IU/day of vitamin D may reduce the risk of falls and fractures in older individuals. *(More information)*
- Vitamin D can regulate cell differentiation and growth by binding to the vitamin D receptor found in most

- Neurodegenerative diseases
- Adverse pregnancy outcomes
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Sources
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LPI Recommendation

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body cells. Observational studies have reported associations between low sun exposure, poor vitamin D status, and increased risk of developing colorectal and breast cancer. Randomized controlled trials are needed to evaluate whether cancer prevention may benefit from vitamin D supplementation. *(More information)*

- Various observational studies have reported inverse associations between vitamin D status and the susceptibility or severity of autoimmune diseases, including type 1 diabetes mellitus, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus. *(More information)*
- Current evidence from observational studies suggests an inverse relationship between circulating vitamin D concentrations and risk of type 2 diabetes mellitus. It is not yet known whether correcting vitamin D deficiency in individuals with glucose intolerance can decrease the risk of progression to type 2 diabetes mellitus. *(More information)*
- Randomized clinical trials are currently investigating whether vitamin D supplementation can limit cognitive deterioration and disease progression in subjects with neurodegenerative disease. *(More information)*

- Vitamin D insufficiency in pregnant women may be associated with several adverse effects for the mother and newborn. Safety and benefits of vitamin D supplementation during pregnancy both need to be evaluated in clinical trials. *(More information)*
- Observational studies have documented an association between vitamin D deficiency and increased incidence and severity of the coronavirus disease, COVID-19. *(More information)*
- Preliminary studies have shown that vitamin D supplementation may offer promising improvements in the management of atopic dermatitis (eczema) and Crohn's disease. *(More information)*

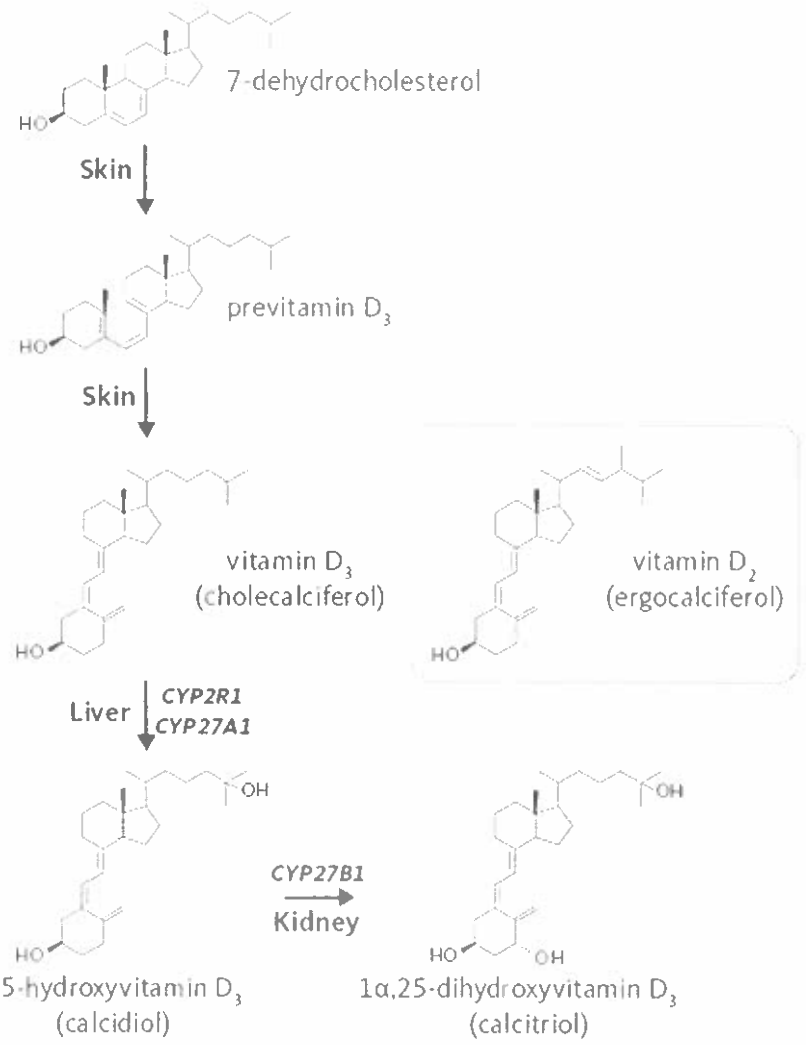
Vitamin D is a fat-soluble vitamin that regulates calcium homeostasis and is vital for bone health (1). While it can also be obtained from dietary sources or supplements, vitamin $D_3$ (cholecalciferol) is synthesized in the human skin from 7-dehydrocholesterol upon exposure to ultraviolet-B (UVB) radiation from sunlight (see the separate article on Vitamin D and Skin Health). Vitamin $D_2$

(ergocalciferol) is a vitamin D analog photosynthesized in plants, mushrooms, and yeasts; vitamin $D_2$ is also sometimes used in vitamin D food fortification (2). When vitamin $D_3$ in skin is inadequate due to insufficient exposure to UVB radiation, oral intake of vitamin D is necessary to meet vitamin D requirements.

## Function

### Vitamin D metabolism

Cholecalciferol and ergocalciferol are biologically inactive precursors of vitamin D and must be converted to biologically active forms in the liver and kidneys (**Figure 1**). Indeed, following dietary intake or synthesis in the epidermis of skin after UVB exposure, both forms of vitamin D enter the circulation and are transported to the liver by the vitamin D-binding protein (and to a lesser extent by albumin). In hepatocytes (liver cells), vitamin D is hydroxylated to form 25-hydroxyvitamin D (calcidiol; calcifediol). Exposure to sunlight or dietary intake of vitamin D increases serum concentrations of 25-hydroxyvitamin D. 25-Hydroxyvitamin D constitutes the major circulating form of vitamin D, and the sum of 25-hydroxyvitamin $D_2$ and 25-hydroxyvitamin $D_3$ concentrations in serum is used as an indicator of vitamin D nutritional status (3). The renal 25-hydroxyvitamin D-1α-hydroxylase enzyme (also known as CYP27B1) eventually catalyzes a second hydroxylation that converts 25-hydroxyvitamin D to 1α,25-dihydroxyvitamin D (calcitriol). The production of 1α,25-dihydroxyvitamin D in the kidneys is regulated by several factors, including serum phosphorus, calcium, parathyroid hormone (PTH), fibroblast growth factor-23 (FGF-23), and 1α,25-dihydroxyvitamin D itself. While the kidney is the main source of 1α-hydroxylase activity, extra-renal production of 1α,25-dihydroxyvitamin D has also been demonstrated in a variety of tissues, including skin, parathyroid gland, breast, colon, prostate, as well as cells of the immune system and bone cells (2). Most of the physiological effects of vitamin D in the body are related to the activity of 1α,25-dihydroxyvitamin D (4). Various forms of vitamin D are listed in **Figure 1**.




Vitamin D synthesis. Previtamin $D_3$ is synthesized in the upper layers of the skin from 7-dehydrocholesterol by the action of ultraviolet light (UVB). A nonenzymatic conversion of previtamin $D_3$ into vitamin $D_3$ (cholecalciferol) then occurs in lower layers of the skin. Vitamin $D_3$ is quickly transported to adipose tissue for storage or liver for activation. In liver cells, several cytochrome P450 (CYP) enzymes can catalyze the 25-hydroxylation of vitamin $D_3$ (or plant-based vitamin $D_2$ or ergocalciferol). The product of this step, 25-hydroxyvitamin $D_3$, is converted into the active form of vitamin $D_3$, 1α,25-dihydroxyvitamin D, in a reaction catalyzed by CYP27B1. This 1α-hydroxylation takes place primarily in the kidney.

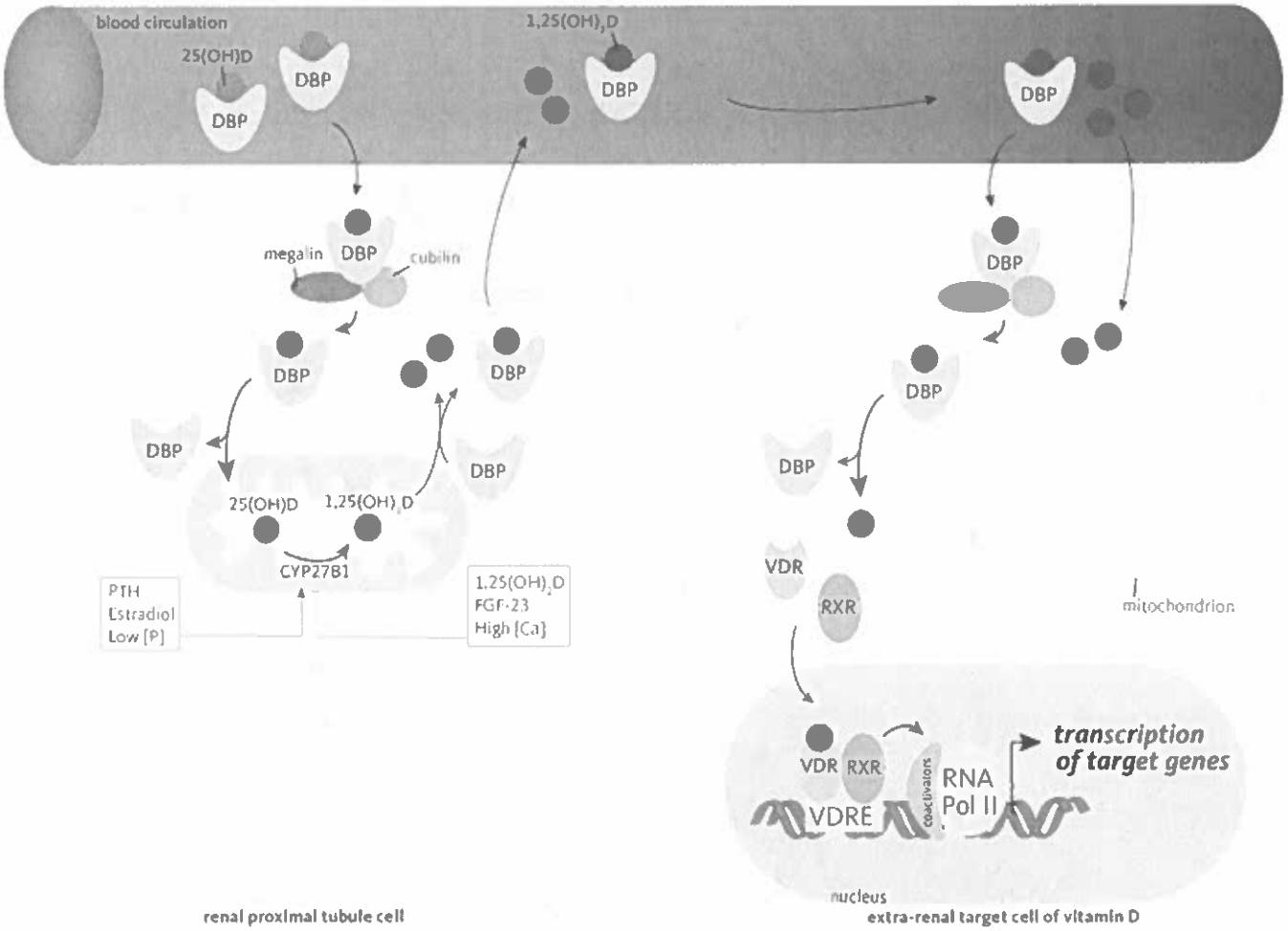
[Figure 1 - Click to Enlarge]

## Mechanisms of action

Most, if not all, actions of vitamin D are mediated through a nuclear transcription factor known as the vitamin D receptor (VDR) (**Figure 2**) (5). Upon entering the nucleus of a cell, 1α,25-dihydroxyvitamin D binds to the VDR and recruits another nuclear receptor known as retinoid X receptor (RXR). In the presence of 1α,25-dihydroxyvitamin D, the VDR/RXR complex binds small

sequences of DNA known as vitamin D response elements (VDREs) and initiates a cascade of molecular interactions that modulate the transcription of specific genes. Thousands of VDREs have been identified throughout the genome, and VDR activation by 1α,25-dihydroxyvitamin D is thought to directly and/or indirectly regulate 100 to 1,250 genes (6).

**Figure 2. Conversion to the Active Form of Vitamin D and VDR-mediated Gene Regulation**



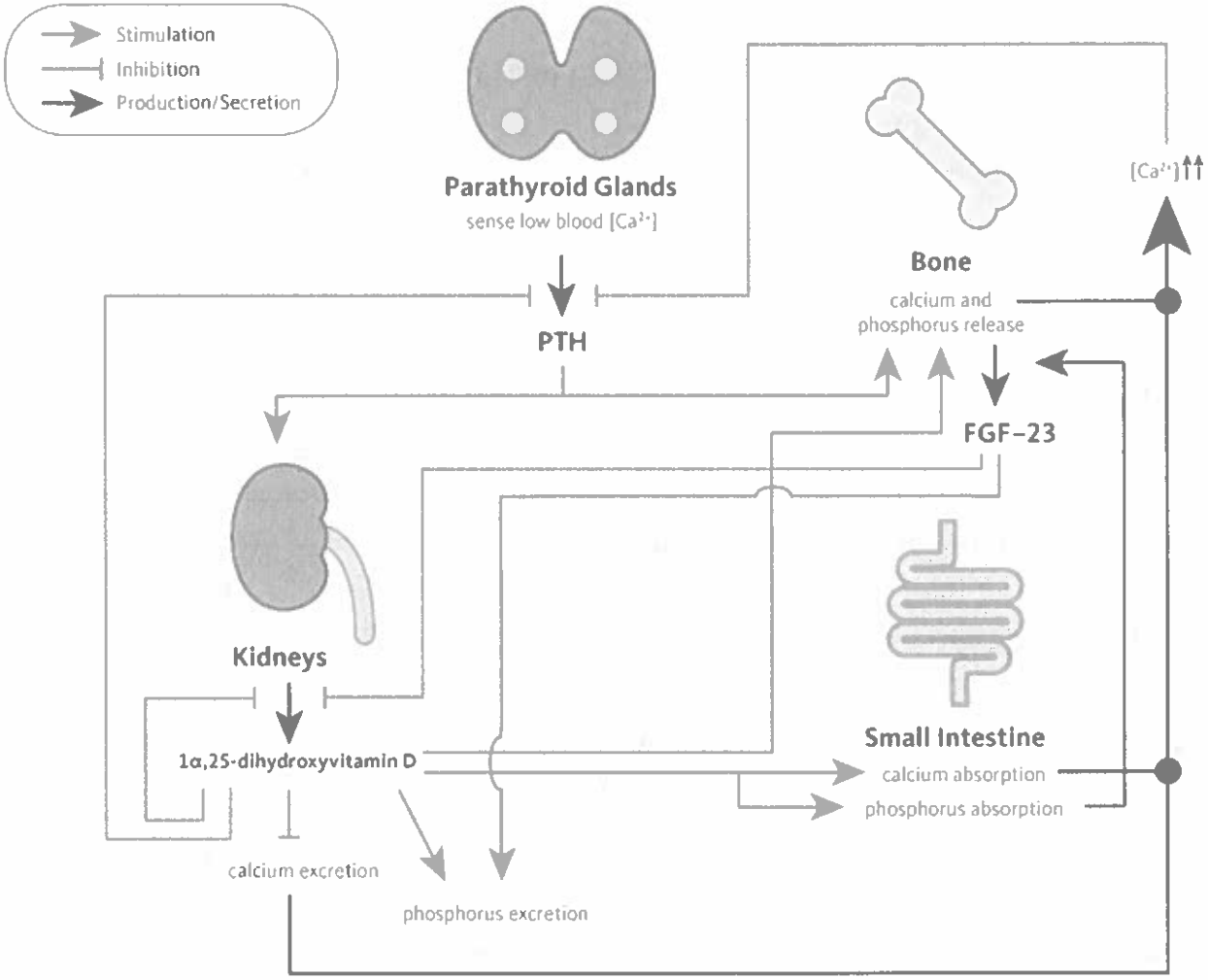

Mechanism of action. 25-Hydroxyvitamin D (25(OH)D) is the main form of vitamin D in the circulation. Most 25(OH)D and 1α,25-dihydroxyvitamin D ($1,25(OH)_2D$) molecules are transported bound to the vitamin D-binding protein and they enter cells via the megalin/tubulin complex. In kidney cells, 1α-hydroxylase (CYP27B1) catalyzes the conversion of 25(OH)D into $1,25(OH)_2D$. Parathyroid hormone (PTH), estradiol, and low phosphorus concentration ([P]) stimulate this reaction, whereas $1,25(OH)_2D$, fibroblast growth factor-23 (FGF-23), and high calcium concentration ([Ca]) inhibit it. $1,25(OH)_2D$ enters the circulation and is transported to extra-renal target tissues where it can regulate gene expression. In the nucleus of target cells, $1,25(OH)_2D$ binds to the vitamin D receptor (VDR), which heterodimerizes with the retinoid X receptor (RXR). VDR-RXR binds to vitamin D response elements (VDRE) in the promoter of vitamin D target genes. *25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1α,25-dihydroxyvitamin D; [Ca], calcium concentration; CYP2R1, 1α-hydroxylase; DBP, vitamin D-binding protein; FGF-23, fibroblast growth factor-23; PTH, parathyroid hormone; [P], phosphorus concentration; RNA Pol II, RNA polymerase II; RXR, retinoid X receptor; VDR, vitamin D nuclear receptor; VDRE, vitamin D response element.*

[Figure 2 - Click to Enlarge]

## Calcium balance

Maintenance of serum calcium concentrations within a narrow range is vital for normal functioning of the nervous system, as well as for bone growth and maintenance of bone density. Vitamin D is essential for the efficient utilization of calcium by the body (1). The parathyroid glands sense serum calcium concentrations and secrete parathyroid hormone (PTH) if calcium concentrations decrease below normal (**Figure 3**). Elevations in PTH stimulate the activity of the 25-hydroxyvitamin $D_3$-1α-hydroxylase enzyme in the kidney, resulting in the increased production of 1α,25-dihydroxyvitamin D. The active vitamin D form, 1α,25-dihydroxyvitamin D, is released into the circulation and transported to target tissues. Within target cells, 1α,25-dihydroxyvitamin binds to and induces the activation of VDR, which leads to changes in gene expression that normalize serum calcium by (1) increasing the intestinal absorption of dietary calcium, (2) increasing the reabsorption of calcium filtered by the kidneys, and (3) mobilizing calcium from bone when there is insufficient dietary calcium to maintain normal serum calcium concentrations (7).

## Figure 3. Regulation of Calcium and Phosphorus Homeostasis




Calcium and phosphorus homeostasis. A slight drop in blood calcium concentration ([$Ca^{2+}$]) results in the secretion of parathyroid hormone (PTH) by the parathyroid glands. PTH simulates the activity of CYP27B1 (25-hydroxyvitamin D-1α-hydroxylase enzyme) that catalyzes the hydroxylation of 25-hydroxyvitamin D into 1α,25-dihydroxyvitamin D. This active form of vitamin D (i) increases the reabsorption of calcium filtered by the kidneys and stimulates phosphorus excretion, (ii) increases the intestinal absorption of both calcium and phosphorus, and (iii) mobilizes calcium (and phosphorus) from bone when dietary calcium intake is insufficient. PTH also stimulates bone resorption that releases calcium and phosphorus. 1α,25-dihydroxyvitamin D inhibits its own production, as well as PTH synthesis, via negative feedback loops. FGF-23 is secreted by bone-forming cells (osteoblasts) in response to an increase in phosphorus intake. FGF-23 inhibits the synthesis of 1α,25-dihydroxyvitamin D and promotes phosphorus excretion in the urine.

*[$Ca^{2+}$], calcium concentration; FGF-23, fibroblast growth factor-23; PTH, parathyroid hormone.*

[Figure 3 - Click to Enlarge]

## Phosphorus balance

The regulations of calcium and phosphorus homeostasis are closely related, and the calciotropic hormones, PTH and 1α,25-dihydroxyvitamin D, can also control serum phosphorus. Specifically, 1α,25-dihydroxyvitamin D increases intestinal phosphorus absorption by stimulating the expression of a sodium-phosphate cotransporter in the small intestine. While PTH increases urinary excretion of phosphorus by reducing reabsorption in the kidney, it is not yet clear whether 1α,25-hydroxyvitamin D can directly regulate renal phosphorus transport. The phosphaturic hormone fibroblast growth factor-23 (FGF-23), secreted by osteoblasts (bone-forming cells), limits the production of 1α,25-hydroxyvitamin D by inhibiting 25-hydroxyvitamin D-1α-hydroxylase (Figure 3) (reviewed in 8).

## Cell differentiation

Cells that are dividing rapidly are said to be proliferating. Differentiation results in the specialization of cells for specific functions. In general, differentiation of cells leads to a decrease in proliferation. While cellular proliferation is essential for growth and wound healing, uncontrolled proliferation of cells with certain mutations may lead to cancer. The active form of vitamin D, 1α,25-dihydroxyvitamin D, inhibits proliferation and stimulates the differentiation of cells through binding to the VDR (1).

## Immunity

Acting through the VDR, 1α,25-dihydroxyvitamin D is a potent immune system modulator. The VDR is expressed by most cells of the immune system, including regulatory T cells and antigen-presenting cells, such as dendritic cells and macrophages (9). Under specific circumstances, monocytes, macrophages, and T cells can express the 25-hydroxyvitamin $D_3$-1α-hydroxylase enzyme and produce 1α,25-dihydroxyvitamin D, which acts locally to regulate the immune response (10, 11). There is considerable scientific evidence that 1α,25-dihydroxyvitamin D has a variety of effects on immune system function, which may enhance innate immunity and inhibit the development of autoimmunity (12). Conversely, vitamin D deficiency may compromise the integrity of the immune system and lead to inappropriate immune responses (see Autoimmune diseases).

## Insulin secretion

The VDR is expressed by insulin-secreting cells of the pancreas, and the results of animal studies suggest that 1α,25-dihydroxyvitamin D plays a role in insulin secretion under conditions of increased insulin demand (13, 14). Cross-sectional and prospective studies suggest that insufficient vitamin D status may have an adverse effect on insulin secretion and glucose tolerance in type 2 diabetes mellitus (noninsulin-dependent diabetes mellitus) (reviewed in 15).

## Blood pressure regulation

The renin-angiotensin system plays an important role in the regulation of blood pressure (16). Renin is an enzyme that catalyzes the cleavage (splitting) of a small peptide (angiotensin I) from a larger protein (angiotensinogen) produced in the liver. Angiotensin-converting enzyme (ACE) catalyzes the cleavage of angiotensin I to form angiotensin II, a peptide that can increase blood

pressure by inducing the constriction of small arteries and by increasing sodium and water retention. The rate of angiotensin II synthesis is dependent on renin (17). Research in mice lacking the gene encoding the VDR indicates that 1α,25-dihydroxyvitamin D decreases the expression of the gene encoding renin through its interaction with the VDR (18). Since inappropriate activation of the renin-angiotensin system can contribute to the development of hypertension, achieving adequate vitamin D status may be important for decreasing the risk of high blood pressure (see Hypertension).

## Deficiency

In vitamin D deficiency, calcium absorption cannot be increased enough to satisfy the body's calcium needs (4). Consequently, PTH production by the parathyroid glands is increased and calcium is mobilized from the skeleton to maintain normal serum calcium concentrations — a condition known as secondary hyperparathyroidism. Although it has long been known that severe vitamin D deficiency has serious consequences for bone health, research suggests that less obvious states of vitamin D deficiency are common and increase the risk of osteoporosis and various other health problems (see Disease Prevention).

### Severe vitamin D deficiency

#### *Rickets*

In infants and children, severe vitamin D deficiency results in the failure of bone to mineralize. The process of mineralization, which involves the production of crystals of calcium phosphate by bone-forming cells, determines the hardness and strength of bones. Vitamin D deficiency severely affects rapidly growing bones. The growth plates of bones continue to enlarge, but in the absence of adequate mineralization, weight-bearing limbs (arms and legs) become bowed. In infants, rickets may result in delayed closure of the fontanels (soft spots) in the skull, and the rib cage may become deformed due to the pulling action of the diaphragm. In severe cases, low serum calcium concentrations (hypocalcemia) may cause seizures. Although fortification of food has led to complacency regarding vitamin D deficiency, nutritional rickets is still being reported throughout the world (19, 20).

#### *Osteomalacia*

Although adult bones are no longer growing, they are in a constant state of turnover, or "remodeling." In adults with severe vitamin D deficiency, the collagenous bone matrix is preserved, but bone mineral is progressively lost, resulting in a softening of bones (osteomalacia), bone pain, and increased risk of osteoporosis (21).

#### *Muscle weakness and pain*

Vitamin D deficiency causes muscle weakness and pain in children and adults. Muscle pain and weakness were prominent symptoms of vitamin D deficiency in a study of Arab and Danish Muslim women living in Denmark (22). In a cross-sectional study of 150 consecutive patients referred to a

clinic in Minnesota for the evaluation of persistent, nonspecific musculoskeletal pain, 93% had serum 25-hydroxyvitamin D concentrations equal to or below 20 ng/mL, with a mean concentration of 12.1 ng/mL, which is indicative of vitamin D insufficiency (23). Loss of muscle strength greatly contributes to increased risk of falling and bone fractures, especially in older people (24). In addition, long-term vitamin D insufficiency may be a contributing factor to osteoporosis in the elderly (see Osteoporosis).

## Risk factors for vitamin D deficiency

Both environmental factors and cultural practices result in variations in vitamin D status:

- Environmental conditions: Geographical locations, including latitude and altitudes, and atmospheric conditions (e.g., air pollution, presence of clouds) greatly influence the intensity of UVB radiation that reaches the ground. Seasonal changes also affect the quality and quantity of UVB rays and thus vitamin D production in skin (25-27).
- Concealed clothing style: In a study of 2,032 Middle Eastern women, who wore a headscarf or covered all skin for religious or cultural reasons, 96% had serum 25-hydroxyvitamin D concentrations less than 20 ng/mL, and 60% had vitamin D concentrations below 12 ng/mL (28). Rickets and osteomalacia are not uncommon in the Middle East and North African regions where children and women cover the majority or all of their skin whenever outside (29).
- Sun safety measures: Sun protection practices, including limiting sun exposure, wearing protective clothing and hats, and applying sunscreens, hinder skin exposure to sunlight and thus result in lower vitamin $D_3$ production and circulating vitamin D metabolites unless there is adequate oral intake. Of note, the application of sunscreen (2 mg/cm$^2$) with a sun protection factor (SPF) of 10 reduces UVB radiation by 90% (30).
- Exclusively breast-fed infants: Infants who are exclusively breast-fed and do not receive vitamin D supplementation are at high risk for vitamin D deficiency, particularly if they have dark skin and/or receive little sun exposure (19). Human milk generally provides 10 to 80 IU of vitamin D per liter (L), which corresponds to 0.2 to 1.5 g/day (8 to 60 IU/day) when using an average daily milk intake of 0.75 L (25 oz) (31). The American Academy of Pediatrics recommends that all breast-fed and partially breast-fed infants be given an oral vitamin D supplement of 400 IU/day (19). Maternal vitamin D supplementation during breast-feeding may contribute to improved vitamin D status of the breast-fed infant, especially in populations with a high prevalence of vitamin D deficiency (32). Older infants and toddlers exclusively fed milk substitutes (e.g., soy-based formulas) and weaning foods that are not vitamin D fortified are at risk for vitamin D deficiency (33).

The efficiency of vitamin D synthesis, absorption, and metabolism also depends on a variety of biological factors:

- Skin pigmentation: People with a dark complexion synthesize less vitamin D on exposure to sunlight than those with light-colored skin (34). A national US survey reported average serum

25-hydroxyvitamin D concentrations of 28.1 ng/mL, 21.6 ng/mL, and 16.9 ng/mL in Caucasian, Mexican American, and African American adults aged ≥20 years old, respectively (25).

- Genetic variations: An international, multicenter, genome-wide association study (GWAS) of 15 cohorts, including ~30,000 participants of European descent — known as the SUNLIGHT [Study of Underlying Genetic Determinants of Vitamin D and Highly Related Traits] consortium — identified common variations (called polymorphisms) in genes involved in cholesterol synthesis, hydroxylation, and vitamin D transport that influence vitamin D status (35). While genetic determinants of low vitamin D status are being identified in populations of European (36, 37) and Asian descent (38, 39), genome-wide association studies are needed in populations of African descent.
- Older age: The elderly have reduced capacity to synthesize vitamin D in skin when exposed to UVB radiation and are more likely to stay indoors or use sunscreen, which prevents vitamin D synthesis. It has been estimated that across Canada, the US, and Europe, the prevalence of vitamin D deficiency ranges between 20%-100% in free-living elderly (40). Moreover, institutionalized adults who are not supplemented with vitamin D are at extremely high risk of vitamin D deficiency (41, 42).
- Chronic kidney disease (CKD): Vitamin D deficiency in patients with impaired renal function is due to a reduced synthesis of 1α,25-dihydroxyvitamin D and an increased loss of 25-hydroxyvitamin D in urine (43).
- Fat malabsorption syndromes: Vitamin D deficiency is common among people with cystic fibrosis and both cholestatic and non-cholestatic liver diseases due to decreased absorption of dietary vitamin D and impaired conversion of vitamin D to 25-hydroxyvitamin D (reviewed in 44).
- Inflammatory bowel disease: People with inflammatory bowel disease like Crohn's disease appear to be at increased risk of vitamin D deficiency, especially those who have had small bowel resections (45).
- Obesity: Obesity (body mass index ≥30 $kg/m^2$) increases the risk of vitamin D deficiency (46). Once vitamin D is synthesized in the skin or ingested, it can be sequestered in body fat stores, making it less bioavailable to people with higher body fat mass. Moreover, vitamin D supplementation trials have shown that obese people reached much lower serum 25-hydroxyvitamin D concentrations compared to normal weight (BMI <25 $kg/m^2$) participants with equivalent oral dosages (47).
- Magnesium deficiency: Recent findings suggest that high magnesium intakes may reduce the risk of vitamin D insufficiency. Magnesium regulates the activity of critical enzymes in vitamin D metabolism, which would explain how magnesium deficiency negatively affects vitamin D status (48).

## Assessing vitamin D nutritional status

Growing awareness that vitamin D insufficiency has serious health consequences beyond rickets and osteomalacia highlights the need for accurate assessment of vitamin D nutritional status. It is currently agreed that the measurement of total serum 25-hydroxyvitamin D concentration (1 ng/mL corresponding to 2.5 nmol/L) is the best indicator to evaluate vitamin D status. However, high-quality evidence is still needed to ensure that the current cutoff values are optimal to define states of insufficiency and deficiency (40). Moreover, only recently, efforts have been made toward the standardization of commercially and laboratory-developed 25-hydroxyvitamin D assays, such that guidelines have been developed using largely unstandardized research data (49). Although laboratory reference values for sufficient vitamin D status were initially based on serum 25-hydroxyvitamin D concentrations from cohorts of healthy individuals, additional studies have suggested that health-based cutoff values aimed at preventing secondary hyperparathyroidism and bone loss should be considerably higher. Indeed, while it is considered that serum 25-hydroxyvitamin D concentrations less than 8 to 10 ng/mL (20 to 25 nmol/L) indicate severe deficiency associated with rickets and osteomalacia, several studies have observed that PTH concentrations (50, 51) and calcium absorption (52) were not optimized with serum 25-hydroxyvitamin D concentrations below 32 ng/mL (80 nmol/L).

Yet, more recent studies have failed to find threshold values of serum 25-hydroxyvitamin D concentrations in relation to PTH suppression and optimal calcium absorption. On the one hand, a cross-sectional analysis of 312,962 clinical samples did not find any evidence of threshold for PTH suppression in the well-fitted curve displaying the inverse association between paired measurements of serum PTH and 25-hydroxyvitamin D, even with 25-hydroxyvitamin D concentrations beyond 70 ng/mL (175 nmol/L) (53). This contradicted an analysis of the US National Health and Nutrition Examination Survey (NHANES 2003-2006) that estimated maximum PTH suppression for 25-hydroxyvitamin D concentrations of 40 ng/mL (100 nmol/L) and above (54). In addition, both studies identified evidence of mild hyperparathyroidism (serum PTH >65 pg/mL) in individuals with serum 25-hydroxyvitamin D concentrations well beyond 20 ng/mL (50 nmol/L), questioning the use of serum PTH as a sensible indicator of vitamin D insufficiency (53, 54). On the other hand, a randomized, placebo-controlled trial in postmenopausal women with vitamin D insufficiency (serum 25-hydroxyvitamin D <20 ng/mL) supplemented with daily vitamin $D_3$ doses from 400 to 4,800 IU found little change (6%) in calcium absorption over a normal 25-hydroxyvitamin D concentration range of 20 to 66 ng/mL (55).

The current cutoffs proposed by the Institute of Medicine (IOM) are as follows: deficiency as serum 25-hydroxyvitamin D values ≤12 ng/mL (≤30 nmol/L), insufficiency as serum 25-hydroxyvitamin D values of 12 to 19 ng/mL (30 to 49 nmol/L), and sufficiency as serum 25-hydroxyvitamin D values of 20 to 50 ng/mL (50 to 125 nmol/L) (56). The dietary reference intakes (EAR, RDA) set by the IOM are based on achieving circulating 25-hydroxyvitamin D concentrations (20 to 50 ng/mL) that are adequate to maintain bone health and optimal calcium absorption (57).

Yet, considering the potential role of circulating concentrations lower than 30 ng/mL in the burden of many chronic diseases (6), the US Endocrine Society has suggested to define cutoff values as follows: deficiency as serum 25-hydroxyvitamin D values ≤20 ng/mL (≤50 nmol/L), insufficiency as serum 25-hydroxyvitamin D values of 21 to 29 ng/mL (51 to 74 nmol/L), and sufficiency as serum 25-hydroxyvitamin D values of 30 to 100 ng/mL (75 to 250 nmol/L) (40). This alternate target range is supported by some observational studies, but it is not based on data from randomized controlled trials (see Disease Prevention) (47). With these latter cutoff values, studies from across the world have estimated that hypovitaminosis D is widespread and that children and adults of all ages are equally at risk of insufficiency and deficiency (58). Data from supplementation studies indicate that vitamin D intakes of at least 800 to 1,000 IU/day are required by adults living in temperate latitudes to achieve serum 25-hydroxyvitamin D concentrations of at least 30 ng/mL (75 nmol/L) (40).

Finally, total serum 25-hydroxyvitamin D concentrations may not always adequately reflect vitamin D bioavailability (59), and additional evidence is needed to improve the determination of vitamin D status in different ethnic populations.

## The Recommended Dietary Allowance (RDA)

In 2010, the Food and Nutrition Board (FNB) of the IOM set a Recommended Dietary Allowance (RDA) based on the amount of vitamin D needed for bone health. While the RDA was increased from the adequate intake (AI) set in 1997, the optimal levels of recommended intakes and serum 25-hydroxyvitamin D to minimize hyperthyroidism and maximize bone health in the general population remain controversial (40). The RDA for vitamin D is listed in **Table 1** by life stage and gender.

Table 1. Recommended Dietary Allowance (RDA) for Vitamin D

| Life Stage | Age | Males | | Females | |
|---|---|---|---|---|---|
| | | µg/day | IU/day | µg/day | IU/day |
| Infants (AI) | 0-6 months | 10 | 400 | 10 | 400 |
| Infants (AI) | 6-12 months | 10 | 400 | 10 | 400 |
| Children | 1-3 years | 15 | 600 | 15 | 600 |
| Children | 4-8 years | 15 | 600 | 15 | 600 |
| Children | 9-13 years | 15 | 600 | 15 | 600 |
| Adolescents | 14-18 years | 15 | 600 | 15 | 600 |
| Adults | 19-70 years | 15 | 600 | 15 | 600 |

| | | | | | |
|---|---|---|---|---|---|
| Adults | 71 years and older | 20 | 800 | 20 | 800 |
| Pregnancy | all ages | - | - | 15 | 600 |
| Breast-feeding | all ages | - | - | 15 | 600 |

# Disease Prevention

## Mortality

In a nine-year follow-up analysis of the Third US National Health and Nutrition Examination Survey (NHANES III) that included 15,099 participants (of which 77% were Caucasians), serum concentrations of 25-hydroxyvitamin D — standardized as per the methodology developed by the Vitamin D Standardization Program [VDSP] — were examined in relation to mortality. The analysis suggested an increase in all-cause mortality with decreasing serum 25-hydroxyvitamin D concentrations <16 ng/mL (60). In contrast, the risk of all-cause mortality varied little for baseline serum 25-hydroxyvitamin D concentrations in the range of 16 to 40 ng/mL (60). Similar results were obtained in a meta-analysis of eight prospective cohort studies that considered the relationship between standardized 25-hydroxyvitamin D concentrations and mortality during a median follow-up period of 10.5 years. The risk of death was found to be 19% higher with 25-hydroxyvitamin D concentrations between 12 and 15.99 ng/mL and 56% higher with concentrations <12 ng/mL compared to the risk associated with concentrations between 30 and 39.99 ng/mL (61). A meta-analysis of 73 prospective cohort studies, including >800,000 participants, found that the lowest versus highest tertile of serum 25-hydroxyvitamin D concentrations was associated with greater risks of all-cause mortality (+35%), mortality due to cardiovascular disease (+35%), and mortality due to cancer (+14%) (62). Yet, a Mendelian randomization analysis — which limits bias due to confounding and reverse causation (63) — of data from three large Danish cohorts of 95,766 adults found a significant association of genetically low plasma 25-hydroxyvitamin D concentrations with all-cause and cancer-related mortality, but not with cardiovascular disease-related mortality (64). Finally, two meta-analyses of randomized controlled trials have suggested a modest reduction in all-cause mortality in older people supplemented with vitamin D and calcium, but not vitamin D alone (62, 65). Additional placebo-controlled trials need to examine further whether supplementation with vitamin D alone or in combination with calcium might help prevent premature death in replete individuals.

## Osteoporosis

### *Vitamin D status, osteoporosis, and risk of fracture*

Although the causes of osteoporosis are multifactorial, vitamin D insufficiency can be an important etiological factor in older adults. Osteoporosis affects one-third of women aged 60 to 70 years and two-thirds of women aged 80 years and above (66). A multinational (18 different countries with latitudes ranging from 64 degrees north to 38 degrees south) survey of more than 2,600 postmenopausal women with osteoporosis revealed that 31% of subjects had 25-hydroxyvitamin D

concentrations <20 ng/mL (50 nmol/L) (67). In addition, a case-control study that included 111 hip fracture patients and 73 controls (median age, 83 years) found that lower serum concentrations of both 25-hydroxyvitamin D and vitamin $K_1$ in patients compared to controls were associated with an increased risk of hip fracture (68). Without sufficient vitamin D from sun exposure or dietary intake, intestinal calcium absorption can be significantly reduced. This increases PTH secretion by the parathyroid glands; sustained PTH elevation may result in increased bone resorption, which in turn may increase the risk of osteoporotic fracture (69).

## *Vitamin D supplementation and bone mineral density*

The progressive loss of bone mineral density (BMD) leading to osteopenia (pre-osteoporosis) and osteoporosis is commonly observed in older adults, especially the elderly. The results of a meta-analysis of 23 randomized controlled trials with more than 4,000 participants (mean age, 59 years) showed little evidence for an effect of vitamin D supplementation on BMD at any of the five skeletal sites examined, including lumbar spine, femoral neck, trochanter, forearm, and total body. A significant increase in BMD was reported only at the femoral neck (70). It was, however, suggested that individuals in this age group would have adequate calcium intake and thus normal bone metabolism, explaining the lack of an effect of vitamin D in strengthening bone mass (71). Conversely, in older individuals, vitamin D supplementation is essential to correct and maintain adequate concentrations of serum 25-hydroxyvitamin D and to prevent secondary hyperparathyroidism and BMD loss (72).

## *Vitamin D supplementation and risk of fracture*

A prospective cohort study that followed more than 72,000 postmenopausal women in the US for 18 years found that those who consumed at least 600 IU/day of vitamin D from diet and supplements had a 37% lower risk of osteoporotic hip fracture than women who consumed less than 140 IU/day of vitamin D (73). However, daily supplementation with 400 IU of vitamin $D_3$, in combination with 1,000 mg calcium, did not significantly reduce risk of hip fracture compared to a placebo in 36,282 postmenopausal women from the Women's Health Initiative trial (74), suggesting that there might be a threshold of vitamin D intake that is necessary to observe reductions in fracture risk. Results of a genetic analysis of data from this trial also suggested that beneficial effects of vitamin D and calcium supplementation on fracture risk might be limited to women with the lowest genetic risk of low BMD (75). Yet, this trial has been questioned for reasons that include poor adherence and the fact that participants were allowed to take additional vitamin D and calcium supplements that might have confounded the results. In addition, use of hormone replacement therapy was not considered in the study of the effect of vitamin D and calcium on skeletal health in postmenopausal women despite being a major confounding factor in this population (57, 76).

Another trial, the Randomised Evaluation of Calcium Or vitamin D (RECORD) study, reported that oral supplemental vitamin $D_3$ (800 IU/day) alone, or in combination with calcium (1,000 mg/day), did not prevent the occurrence of osteoporotic fractures in elderly adults who had already

experienced a low-trauma, osteoporotic fracture (77). In this latter study as well, a number of limitations, including poor adherence and/or the fact that vitamin D supplementation did not raise serum 25-hydroxyvitamin D concentrations to a level that would protect against fractures, might explain the lack of an effect (78). Despite high adherence to treatment, the incidence of non-vertebral fracture was similar in postmenopausal women supplemented with vitamin $D_3$ (initial dose of 200,000 IU followed by 100,000/month) or placebo for over three years in the Vitamin D Assessment (ViDA) trial (79).

Nevertheless, the US Preventive Services Task Force that conducted the meta-analysis of 11 randomized, placebo-controlled trials, including 52,915 older people (of whom 69% were postmenopausal women), found that the supplementation of vitamin D (300-1,000 IU/day) and calcium (500-1,200 mg/day) for up to seven years resulted in a 12% reduction in the risk of any new fracture (80). Another meta-analysis of 11 randomized, double-blind, placebo-controlled trials on the effect of vitamin D supplementation in 31,022 individuals (91% women) 65 years and older indicated that those with the highest vitamin D intake (792-2,000 IU/day) had a 30% lower risk of hip fracture and a 14% lower risk of any non-spine fracture (81). Finally, a third meta-analysis of trials that examined the effect of combined vitamin D and calcium in preventing fractures in older men and postmenopausal women also concluded that the risk of new fractures, including hip fractures, was significantly reduced in those supplemented compared to controls (82). Interestingly, the three meta-analyses have found that the prevention of fractures by supplemental vitamin D and calcium was limited to institutionalized, older people. Indeed, the risk of fracture was not significantly reduced by vitamin D in community-dwelling seniors (80-82).

### *Vitamin D supplementation and postural balance, muscle strength, and risk of fall*

A meta-analysis of seven observational studies in 840 fallers and 1,330 non-fallers found significantly lower serum 25-hydroxyvitamin D concentrations in fallers than in non-fallers (83). Moreover, another meta-analysis of four cohorts from three observational studies reported a modest yet significant inverse association between vitamin D status and the risk of fall (83). Several randomized controlled trials have examined the impact of vitamin D supplementation on muscle strength, postural balance, or risk of fall in older subjects. A meta-analysis of these trials found limited evidence of an effect of vitamin D supplementation on muscle strength and mobility, based on only one type of test for each outcome. Nevertheless, in a recent randomized, double-blind, placebo-controlled study in 160 postmenopausal women (ages, 50-65 years) with suboptimal vitamin D status (mean serum 25-hydroxyvitamin D concentration <20 ng/mL), supplementation with 1,000 IU/day of vitamin $D_3$ significantly improved vitamin D status, as well as upper and lower limb muscle strength and postural balance parameters (84, 85). The risks of fall and recurrent falls were found to be two- to three-fold greater in women in the control group than in those supplemented with vitamin $D_3$ (85). In contrast, another 12-month randomized controlled study in 200 older adults (of which 58% had a baseline serum 25-hydroxyvitamin D concentration <20 ng/mL) showed no benefits regarding lower extremity function or odds of falling in those

supplemented with 2,000 IU/day (+/- 10 µg of calcidiol) compared to those who received 800 IU/day (86). The recently published post-hoc analysis of the ViDA trial found no differences in odds of falling and number of falls reported by 5,108 community-dwelling participants (ages, 50-84 years) regardless of whether they were randomized to receive supplemental vitamin D (100,000 IU/month, i.e. ~3,350 IU/day) or a placebo for a mean 3.4 years (79). Most ViDA participants had serum 25-hydroxyvitamin D concentrations ≥20 ng/mL, which might at least partly explain the lack of an effect of vitamin D on falls (87).

Overall, the current evidence suggests that vitamin $D_3$ supplements of 800-1,000 IU/day may be helpful in reducing falls and fracture rates in older adults. In order for vitamin D supplementation to be effective in preserving bone health, adequate dietary calcium (1,000 to 1,200 mg/day) should be consumed (see the article on Calcium) (88).

## Cancer

Ecologic studies first suggested an association between Northern latitudes, vitamin D deficiency, and cancer incidence (89). Since the 1980s, several prospective cohort studies have examined the association of vitamin D intake or status and various types of cancer. A 2013 systematic review and meta-analysis of 16 prospective studies, including 137,567 subjects, reported an 11% reduction in total cancer incidence and a 17% reduction in cancer mortality with each 20 ng/mL (50 nmol/L) increase in circulating 25-hydroxyvitamin D concentrations. Yet, a sex-based subgroup analysis of eight studies found an inverse association between circulating vitamin D and cancer mortality in women, but not in men (90). In addition, increasing evidence suggests that a few variations in the gene coding for the vitamin D receptor (VDR) might influence individual vitamin D status and subsequently modify the susceptibility to site-specific cancers (91) and influence cancer survival (92). Finally, many malignant tumors have been found to express the VDR, including breast, lung, skin (melanoma), colon, and bone (93), suggesting that they might be susceptible to the effects of vitamin D. Numerous experimental studies have demonstrated that biologically active forms of vitamin D, such as 1α,25-dihydroxyvitamin D and its analogs, upon binding to the VDR, can control cell fate by inhibiting proliferation and/or inducing cell differentiation or death (apoptosis) of a number of cancerous cell types (94).

### *Colorectal cancer*

The geographic distribution of colon cancer mortality resembles the historical geographic distribution of rickets (95), providing circumstantial evidence that decreased sunlight exposure and diminished vitamin D nutritional status may be related to an increased risk of colon cancer. Evidence from observational studies has largely supported this hypothesis. A recent meta-analysis of four prospective cohort studies, four cross-sectional studies, and seven case-control studies found an inverse relationship between circulating vitamin D and incidence of colorectal adenoma — a benign tumor that may transform to become malignant (96). The analysis identified a 32% risk reduction between top versus bottom quantiles of serum 25-hydroxyvitamin D concentrations (96). Additionally, there is strong evidence from meta-analyses of prospective

cohort studies to suggest that higher vitamin D intakes and serum 25-hydroxyvitamin D concentrations are associated with reductions in colorectal cancer risk (97-99). The most recent meta-analysis of four prospective cohort, 17 nested case-control, and three case-control studies found a 38% reduced risk of colorectal cancer with high versus low quantiles of circulating 25-hydroxyvitamin D concentrations (100). A dose-response analysis estimated that serum 25-hydroxyvitamin D concentrations of ~20 to 30 ng/mL (compared to ≤12 ng/mL) were associated with a 17% lower risk of colorectal cancer, and the risk was even lower (-35%) with a serum concentration of 55 ng/mL (100). An earlier dose-response analysis based on five nested case-control studies had estimated that serum 25-hydroxyvitamin D concentrations ≥33 ng/mL (compared to ≤12 ng/mL) were associated with a 50% lower risk of colorectal cancer (101).

However, in a seven-year, randomized, double-blind, placebo-controlled trial in 36,282 postmenopausal women participating in the Women's Health Initiative study, a combination of supplemental vitamin $D_3$ (400 IU/day) and calcium (1,000 mg/day) did not lower incidence of colorectal cancer (102). Another randomized controlled trial of vitamin $D_3$ supplementation (1,000 IU/day), with or without calcium supplementation (1,200 mg/day), found no reduction in the risk of colorectal adenoma recurrence over a three-to-five year period, compared to placebo, after initial adenoma removal in participants (103). Whether these daily vitamin D doses are too low to detect any effect on cancer incidence is not clear (101, 104). Additional randomized clinical trials are needed to assess whether vitamin D supplementation could help prevent colorectal cancer. Moreover, it is uncertain whether genetic variations (polymorphisms) in the sequence of genes involved in vitamin D metabolism and function can influence the relationship between vitamin D status and risk of colorectal adenoma or colorectal cancer (105-107).

Finally, growing evidence suggests that adequate vitamin D status may be linked to better survival of colorectal cancer patients. A meta-analysis of five prospective studies found a 35% reduced risk of colorectal cancer-specific mortality in cancer patients with higher serum 25-hydroxyvitamin D concentrations. A dose-response analysis estimated that every 8 ng/mL increase in 25-hydroxyvitamin D concentration was associated with a 10% decrease in colorectal cancer mortality (108).

### *Breast cancer*

Although ecologic evidence suggests that breast cancer mortality rises with increasing latitudes and decreasing sunlight exposure (109), the most current observational data provide little support for an association between vitamin D nutritional status and risk of breast cancer. An early prospective study of women who participated in the First US National Health and Nutrition Examination Survey (NHANES I) found that Caucasian women with adequate sunlight exposure and dietary vitamin D intake had a significantly reduced risk of breast cancer 20 years later (110). Nonetheless, when this study was included in a meta-analysis with nine more recent prospective studies, there was no significant difference in the risk of developing breast cancer between the highest and lowest levels of vitamin D intakes (111). Moreover, whether an association exists between circulating vitamin D

concentrations and risk of breast cancer is unclear. One meta-analysis of 14 observational studies (9,110 cases and 16,244 controls) reported an overall risk reduction of 16% when the highest quantile of serum 25-hydroxyvitamin D concentrations was compared to the lowest. This inverse association was statistically significant in postmenopausal women but not in premenopausal women (112). Yet, another meta-analysis that included a similar set of 14 prospective studies (two studies were different) found no overall association (111). Additionally, a meta-analysis of studies conducted in patients in the early stage of breast cancer identified associations between inadequate vitamin D status and increased risks of recurrence and death (113). Evidence from randomized controlled trials is currently too limited to conclude whether vitamin D supplementation may reduce breast cancer incidence (reviewed in 114).

Nonetheless, three meta-analyses have found an inverse association between circulating vitamin D concentrations and breast cancer-related mortality (111, 115, 116). In one meta-analysis of one retrospective and five prospective cohort studies, the highest versus lowest categories of serum vitamin D concentrations was associated with a 33% reduction in mortality; a dose-response analysis found a 12% reduction per 8 ng/mL increase in serum vitamin D (115).

Finally, current evidence does not suggest that specific genetic variations in the gene coding for the VDR may influence the risk of breast cancer (117, 118).

### *Other types of cancer*

Evidence associating vitamin D status with other types of cancer is currently limited. While incidence of prostate cancer appears to be inversely associated with the availability of sunlight, prospective cohort studies have not generally found significant relationships between serum 25-hydroxyvitamin D concentrations and subsequent risk of developing prostate cancer (119, 120). In fact, some studies have suggested an increased risk of prostate cancer with higher circulating vitamin D concentrations. For example, a nested case-control study of men (622 cases and 1,451 controls) from Scandinavia found a U-shaped relationship between serum 25-hydroxyvitamin D concentrations and prostate cancer risk. In that study, serum 25-hydroxyvitamin D concentrations of 7.6 ng/mL or lower, or 32 ng/mL or higher, were associated with increased prostate cancer risk (121). A meta-analysis of 17 nested case-control studies, three prospective cohort studies, and one retrospective cohort study found a 17% increased risk of prostate cancer in individuals in the highest versus lowest categories of blood 25-hydroxyvitamin D concentrations (122). Potential confounding factors that might explain the detection of a slight increase in prostate cancer cases in men with high circulating vitamin D concentrations have been highlighted in a recent publication (123).

Finally, recent meta-analyses of observational studies found an inverse relationship between vitamin D status and risk of lung cancer (124, 125) and bladder cancer (126, 127). Yet, in the few and often heterogeneous studies published to date, serum 25-hydroxyvitamin D concentrations were not associated with other cancer types, including non-Hodgkin's lymphoma (128), ovarian cancer (129), gastric cancer (130), or skin cancers (131).

## Autoimmune diseases

Insulin-dependent diabetes mellitus (type 1 diabetes mellitus), multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) are examples of autoimmune diseases. Autoimmune diseases occur when the body mounts an immune response against its own tissue, rather than a foreign pathogen. In type 1 diabetes mellitus, insulin-producing β-cells of the pancreas are the target of an inappropriate immune response. In MS, the targets are the myelin-producing cells of the central nervous system, and in RA, the targets are the collagen-producing cells of the joints (132). SLE is characterized by the presence of a large spectrum of autoantibodies resulting in potential damage to multiple tissues (133). Autoimmune responses are mediated by immune cells called T cells. The biologically active form of vitamin D, 1α,25-dihydroxyvitamin D, has been found to modulate T cell responses, such that the autoimmune responses are diminished. Ecologic studies have found that the prevalence of autoimmune diseases (particularly for MS; 134) increases as latitude increases, suggesting that lower exposure to UVB radiation and associated decreases in skin vitamin D synthesis may play a role in the pathology of these diseases. Results of several prospective cohort studies also suggest that adequate vitamin D status at different ages (including *in utero*, early childhood, and during adolescence) could possibly decrease the risk of autoimmune diseases.

### *Type 1 diabetes mellitus*

Lower levels of circulating vitamin D have been reported in patients newly diagnosed with type 1 diabetes mellitus compared to age- and sex-matched non-diabetic subjects (135, 136). A greater prevalence of vitamin D insufficiency and deficiency has also been observed in prediabetic children who developed multiple islet autoantibodies (antibodies against insulin-secreting pancreatic cells) compared to autoantibody-negative children. However, a prospective study that followed the cohort of prediabetic children found that their vitamin D status, defined as either insufficient, deficient, or sufficient, was not associated with rate of progression to type 1 diabetes after 5 or 10 years of follow up (137). An earlier prospective cohort study of children born in Finland during the year 1966 and followed for 30 years found that children supplemented with vitamin D during the first year of life had an 88% lower risk of developing type 1 diabetes compared to those receiving no supplementation. Moreover, children suspected of having had rickets (severe vitamin D deficiency) during the first year of life showed a significantly higher risk of developing type 1 diabetes (138). Thus, vitamin D supplementation appears protective against type 1 diabetes onset, and suboptimal vitamin D status in infancy may have long-term effects on immune responses later in life.

There are also limited data suggesting that maternal vitamin D insufficiency during pregnancy may influence the risk of type 1 diabetes in offspring. In a recent case-control study, the risk of childhood onset of type 1 diabetes was more than two-fold greater in children whose mothers had serum 25-hydroxyvitamin D concentrations <21.6 ng/mL (54 nmol/L) during the last trimester of pregnancy compared to children born from women with serum 25-hydroxyvitamin D >35.6 ng/mL (89 nmol/L) (139). Other case-control studies have found that vitamin D supplementation during

pregnancy was associated with a lower risk of their children developing diabetes-related autoantibodies (140, 141). However, a larger study conducted in mothers of children at increased genetic risk for diabetes reported no association between the appearance of islet autoantibodies and/or diabetes onset in offspring in the first year of life and maternal vitamin D intake during pregnancy (142). Another case-control study failed to observe a relationship between serum 25-dihydroxyvitamin D during early pregnancy and type 1 diabetes diagnosis in offspring (143). Large prospective studies are needed to establish whether maternal vitamin D status during pregnancy can influence the risk of type 1 diabetes in offspring.

Finally, the relationship of polymorphisms in vitamin D metabolism-related genes and type 1 diabetes is currently under investigation. It has been proposed that specific polymorphisms in genes, such as *CYP27B1* (coding for 25-hydroxyvitamin $D_3$-1α-hydroxylase) and *VDR*, may be functionally relevant to the action of vitamin D and may thus affect disease susceptibility. In a study conducted in 8,517 children and adolescents with type 1 diabetes and 7,320 control subjects, polymorphisms in genes involved in cholesterol synthesis and vitamin D hydroxylation were linked to circulating vitamin D concentrations and diabetic status (26).

## *Multiple sclerosis*

Low levels of sun exposure and vitamin D deficiency appear to be associated with the development of multiple sclerosis (MS). Poor vitamin D status may compromise the function of specific immune cells critical in the regulation of various immune responses and help trigger autoimmunity in MS (144). Genetic determinants of low vitamin D status have been recently linked to an increased susceptibility to adult-onset MS in a Mendelian randomization analysis of data from the Multiple Sclerosis Genetics Consortium (145). This echoed the results of several observational studies that suggested an association between vitamin D sufficiency and decreased MS risk. A retrospective study of levels of ambient UV radiation and cases of MS conducted in Australia revealed that MS incidence in offspring was inversely correlated to maternal exposure to UV during early pregnancy (146). Sun exposure was also used as a surrogate marker for vitamin D exposure in a recent case-control study that included 1,660 MS patients and 3,050 controls. The authors found that infrequent outdoor activities and the use of sunscreen during early childhood and adolescence were associated with an increased risk of developing MS later in life (147). In a cross-sectional study, sun exposure and intake of cod liver oil (rich in vitamin D) during childhood were linked to later symptom onset among veterans with relapsing MS (148). Additionally, a case-control study in US military personnel, including 257 cases of diagnosed MS, found that Caucasian subjects in the highest quintile of serum 25-hydroxyvitamin D (>39.6 ng/mL) had a 62% lower risk of developing MS compared to the lowest quintile (<25.3 ng/mL) (149). Further, in two large cohorts of over 187,000 US women followed for at least 10 years, vitamin D supplement use (≥400 IU/day) was associated with a 41% reduction in the risk of developing MS (150). Another prospective, uncontrolled study monitored incidence of relapse in relation to vitamin D status in 156 patients with relapsing-remitting MS before and after they were given supplemental vitamin D (100,000 IU/month; 6-42 months, median of 31 months), in addition to first-line immunomodulatory therapy

(151). Each 4 ng/mL increase in serum 25-hydroxyvitamin D concentration was associated with a 14.9% decrease in incidence of relapse (151). In a multicenter study conducted in patients newly diagnosed with a clinically isolated syndrome (CIS) and treated with interferon (IFN)-β, vitamin D status was predictive of MS disease activity and progression. Higher serum 25-hydroxyvitamin D concentrations (≥20 ng/mL or ≥50 nmol/L) in the first year following CIS diagnosis predicted a longer time to MS diagnosis, lower number of new lesions, and lower changes in lesion and brain volume during the subsequent four years of follow-up (152). However, a retrospective study suggested that vitamin D status in patients with relapsing-remitting MS had no predictive value regarding the time to conversion to secondary progressive MS, which is characterized by a worsening of disability (153).

Clinical trials have failed to demonstrate any benefit of vitamin D supplementation, alone or in combination with IFN-β treatment, with respect to relapse rates and disability-related symptoms in MS patients (154, 155). In other trials, supplemental vitamin $D_3$ also failed to demonstrate immunomodulary activities (156-159). In a recent randomized, placebo-controlled trial in 53 IFN-β-treated patients with relapsing-remitting MS, supplementation with vitamin $D_3$ (7,000 IU/day for four weeks, followed by 14,000 IU/day until week 48) showed little effect on the proportion of some regulatory T and B lymphocytes over the 48-week study period. Vitamin $D_3$ only appeared to help maintain the proportion of anti-inflammatory $CD4^+$ T cells — which decreased in patients given placebo — but failed to enhance their reactivity when stimulated with 1.25-dihydroxyvitamin D *in vitro* (157). In another trial, supplementation with vitamin $D_3$ (10,400 IU/day for three months) to patients with relapsing-remitting MS was found to reduce the proportion of pro-inflammatory IL-17-producing $CD4^+$ T cells, which are thought to play a central role in MS development (160).

### *Rheumatoid arthritis*

Vitamin D deficiency may also be implicated in the etiology and/or progression of rheumatoid arthritis (RA), although evidence is mainly from animal studies. The absence of vitamin D receptors (VDR) in genetically modified mice has been linked to higher levels of inflammation and increased susceptibility to autoimmunity (161). When transgenic mice that spontaneously develop inflammatory arthritis are also deficient in VDR, they develop a more aggressive form of chronic arthritis (162). Also, specific polymorphisms in the *VDR* gene have been linked to an increased susceptibility to RA in certain populations, although how these genetic variants influence vitamin D functionality is not fully understood (163-165). The current data, however, point to a role for vitamin D in modulating the inflammatory process that underlies many chronic diseases, including RA. Several cross-sectional studies in individuals with moderate-to-high levels of inflammation have reported either no association or an inverse association between circulating 25-dihydroxyvitamin D and markers of inflammation. Nonetheless, there is a lack of intervention trials to show whether vitamin D supplementation could limit inflammation and reduce disease risk (including RA) in subjects with high inflammation levels (166).

At this time, it remains unclear whether the prevalence of vitamin D deficiency is linked to RA incidence. In a large cohort study of nearly 30,000 postmenopausal US women, subjects with the highest total vitamin D intakes (≥467.7 IU/day) had a 33% lower risk of developing RA after 11 years of follow-up than those with the lowest intakes (<221.4 IU/day) (167). Yet, more recent analyses of two large cohorts of nearly 200,000 US women followed for several decades found no association between reported vitamin D dietary intakes (using food frequency questionnaires) during adolescence or adulthood and incidence of RA later in life (168, 169). Moreover, several studies that explored the relationship between circulating vitamin D and disease activity in RA patients have reported mixed results (reviewed in 170). Yet, two recent meta-analyses of observational studies found an inverse relationship between vitamin D status and disease activity in RA patients, assessed using the Disease Activity Score 28 (DAS28) (171, 172). Finally, there is a dearth of studies exploring the effect of vitamin D supplementation on disease activity in arthritis subjects. A small randomized, double-blind, placebo-controlled study in 22 RA patients failed to demonstrate improvements in disease activity and inflammation level in subjects supplemented with calcium (1,500 mg/day) and high doses of vitamin $D_2$ (ergocalciferol; averaging over 4,500 IU/day) for a year compared to placebo (173). Another three-month, randomized controlled trial in 41 women with early RA found no additional benefits of supplemental vitamin $D_3$ (one bolus dose of 300,000 IU) to standard care (methotrexate and glucocorticoids) regarding T-helper lymphocyte enumeration, cytokine production, or clinical parameters including disease activity (174). Supplemental vitamin D also failed to reduce disease recurrence rate in RA patients enrolled in two small randomized controlled trials (175, 176). Since these studies have several limitations, including small sample size, additional research is warranted.

## *Systemic lupus erythematosus*

More prevalent and severe in non-Caucasian populations (Hispanics, African descendants, and Asians) (177), systemic lupus erythematosus (SLE) is an autoimmune disease with heterogeneous clinical manifestations. The disease can potentially affect most tissues and organs, including skin (skin rash and photosensitivity), kidneys (nephritis), and joints (arthritis). There is evidence of a role for vitamin D in the prevention of SLE in animal models (178). Interestingly, a recent meta-analysis of 11 case-control studies found that specific *VDR* polymorphisms were linked to SLE in Asians particularly (179). However, the functional relevance of such genetic variants is not known (180). Analyses of two large prospective cohort studies of nearly 200,000 US women failed to show an association between dietary vitamin D intake (measured by food frequency questionnaire) during adolescence or adulthood and incidence of SLE later in life (168, 169).

Yet, a suboptimal vitamin D status is commonly observed in subjects with SLE, and this is partly explained by the lack of sunlight exposure, which tends to aggravate disease symptoms (181, 182). Serum concentrations of 25-hydroxyvitamin D were inversely correlated with measures of disease activity in a cohort of 378 patients with SLE (183). The correction of vitamin D insufficiency with high levels of vitamin $D_3$ (100,000 IU/week for one month followed by 100,000 IU/month for six months) in 20 subjects with SLE was linked to a reduction in signs of immune imbalance and in levels of

autoantibodies typically detected in SLE, suggesting a therapeutic value for vitamin D in disease treatment (184). Another prospective study conducted in 52 vitamin D-deficient patients with cutaneous lupus erythematosus (a type of lupus with skin disorders only) reported a reduction in disease severity in the group supplemented with vitamin $D_3$ (1,400 IU/day initially, followed by 800 IU/day) and calcium for one year compared to untreated patients (185). Supplementation with vitamin $D_3$ (200 IU/day for one year) was also able to reduce the level of inflammatory cytokines in a randomized, placebo-controlled study conducted in 267 patients with SLE (186). In another randomized, placebo-controlled trial, supplementation with vitamin $D_3$ (50,000 IU/week for six months) improved SLE disease activity index (SLEDAI) and European Consensus Lupus Activity Measurement (ECLAM) scores, as well as some measures of fatigue in young adults with juvenile-onset SLE (187). However, in two other recent studies, supplementation with vitamin $D_3$ (weekly/monthly bolus doses equivalent to ~800 to 7,000 IU/day for 6 to 24 months) improved vitamin D status in SLE patients but failed to show any benefit regarding disease activity (188, 189). While oral vitamin D administration to SLE patients is well tolerated, its efficacy remains questionable and deserves further investigation in clinical trials.

### *Summary*

Thus, evidence from human epidemiological studies suggests that while it cannot yet be concluded that vitamin D supplementation is beneficial in prevention or treatment of autoimmune disease, it is reasonable to assume that correcting vitamin D insufficiency and maintaining sufficient levels could possibly help decrease disease risk (190).

## Cardiovascular disease

### *Hypertension (high blood pressure)*

Hypertension is a well-known risk factor for cardiovascular disease (CVD) (191). The results of observational and clinical studies suggest a role for vitamin D in lowering blood pressure, which may be partly explained by the fact that 1α,25-dihydroxyvitamin D inhibits renin synthesis (see Function). Thus, vitamin D deficiency and subsequent upregulation of the renin-angiotensin system may contribute to high blood pressure and CVD risk. It has also been suggested that elevated PTH concentrations may increase the risk of hypertension and CVD (6). Yet, in a recent prospective cohort study of 3,002 individuals (mean age, 59 years at baseline), the incidence of hypertension, which affected 41% of participants during the nine year follow-up period, was not higher in those with serum 25-hydroxyvitamin D concentrations lower than 20 ng/mL and was only marginally associated with elevated PTH concentrations (192). Nevertheless, a meta-analysis of seven prospective studies, including a total of 48,633 participants with nearly 5,000 incident hypertension cases, found a 30% lower risk of hypertension in those in the top versus bottom tertiles of serum 25-hydroxyvitamin D concentrations. The dose-response analysis estimated that every 10 ng/mL increase in serum 25-hydroxyvitamin D concentration was associated with a 12% lower risk of hypertension (193). Another meta-analysis of four prospective and 14 cross-sectional studies also reported an inverse relationship between circulating 25-hydroxyvitamin D and hypertension (194).

## *Endothelial dysfunction*

Vascular endothelium dysfunction, which contributes to an increased risk of cardiovascular disease (CVD), is common in patients with chronic kidney disease (CKD) (195). In CKD patients, abnormal endothelial function is associated with low values of flow-mediated dilation (FMD) of the brachial artery, a surrogate marker of vascular health. In a recent study conducted in subjects with mild-to-moderate CKD, serum 25-hydroxyvitamin D concentrations were positively associated with FMD values, suggesting a link between suboptimal vitamin D status and endothelial dysfunction (196). In a preliminary intervention study, 26 patients with moderate CKD and vitamin D insufficiency (mean value, 17.2 ng/mL) were supplemented twice with 300,000 IU of vitamin $D_3$ (at weeks 1 and 8) and followed for a total of 16 weeks. Vitamin D supplementation nearly doubled serum 25-hydroxyvitamin D concentrations and decreased PTH concentrations by 68.5%; improved vitamin D status was accompanied by increased FMD values and reduced levels of endothelial dysfunction markers (197). A recent meta-analysis of 12 small randomized controlled trials in participants at high risk for CVD found a significant increase in FMD with vitamin D supplementation (daily doses, 2,500-5,000 IU; weekly dose, 50,000 IU; monthly dose, 60,000 IU; single-bolus doses, 100,000-200,000 IU) for eight weeks to six months (198).

## *Cardiovascular events in observational studies and clinical trials*

To date, the many epidemiological studies investigating the relationship between vitamin D and outcomes of CVD have provided mixed results (reviewed in 199). Recent Mendelian randomization studies found no association between genetically low serum 25-hydroxyvitamin D concentrations and risks of coronary heart disease, ischemic heart disease, or myocardial infarction (200, 201), suggesting that associations reported in observational studies may be due to confounding or reverse causation. In the RECORD trial in 5,292 older people (see Osteoporosis), supplementation with 800 IU/day of vitamin $D_3$ (± calcium) reduced the risk of first cardiac failure but had no effect on the risk of myocardial infarction and stroke compared to supplementation with calcium alone or placebo (202). Data on the effect of vitamin D supplementation on cardiovascular events were collected from 21 randomized controlled studies (including the RECORD trial) in 13,033 participants (≥60 years old) and combined in a meta-analysis (202). No effect of vitamin D (including vitamin D analogs) was found for major cardiovascular events, including heart failure, myocardial infarction, and stroke over follow-up periods of 1 to 6.2 years (202). However, caution is advised when interpreting these results since the trials were initially designed to evaluate the effect of vitamin D on bone health, and cardiovascular outcomes were not primary endpoints. Several randomized controlled trials exploring the effect of vitamin D supplementation on CVD risk are currently underway (203), including two large trials, the Vitamin D and Omega-3 Trial (VITAL) in the US (204) and the D-Health trial in Australia (205). The results of one randomized controlled trial, the Vitamin D Assessment (ViDA) trial in New Zealand, were recently published. The total number of CVD events and time to first CVD event during follow-up did not differ between those supplemented with vitamin $D_3$ (initial dose of 200,000 IU for the first month followed by monthly doses of 100,000 IU) and those given a placebo for a median 3.3 years (206).

## Type 2 diabetes mellitus

People with metabolic syndrome are at increased risk for type 2 diabetes mellitus (noninsulin-dependent diabetes mellitus) and cardiovascular disease (CVD). Metabolic syndrome refers to several metabolic disorders, including dyslipidemia, hypertension, insulin resistance, and obesity. A recent study found that the prevalence of type 2 diabetes was associated with low levels of serum 25-hydroxyvitamin D (<30 ng/mL) in 1,801 patients with metabolic syndrome. During an eight-year follow-up period, lower risks of all-cause mortality (72% lower risk) and CVD-specific mortality (64% lower risk) were reported in individuals with serum 25-hydroxyvitamin D concentrations over 30 ng/mL (75 nmol/L) when compared to those with concentrations below 10 ng/mL (25 nmol/L) (207).

In healthy people, vitamin D sufficiency is positively correlated with insulin sensitivity and adequate pancreatic β-cell function. Conversely, vitamin D deficiency might affect glucose homeostasis and cause impaired glucose tolerance and insulin resistance (208). In a cross-sectional study conducted in 12,719 adults, of whom 4,057 had prediabetes (i.e., an increased risk of developing type 2 diabetes), the prevalence of prediabetes was associated with lower concentrations of serum 25-hydroxyvitamin D (≤32.4 ng/mL). Subjects with the lowest concentrations of serum 25-hydroxyvitamin D (≤17.7 ng/mL) were more likely to be current smokers, obese, and have hypertension (209). Vitamin D insufficiency in high-risk individuals may accelerate the progression to overt diabetes. In a prospective study of 2,378 middle-aged men and women followed for 8 to 10 years, the risk for progression to type 2 diabetes from prediabetes was 62% lower in women and 60% lower in men in the highest compared to the lowest quartile of circulating vitamin D (>28.4 ng/mL vs. <18.5 ng/mL). A dose-response analysis measured an average 23% reduction in the risk of progression to type 2 diabetes for every 4 ng/mL (10 nmol/L) increment in serum 25-hydroxyvitamin D concentration (210). A recent review and meta-analysis of 18 prospective cohort studies, including over 210,000 participants followed for a median period of 10 years, found that individuals in the top third of vitamin D levels (reported as either circulating vitamin D or dietary intakes) had lower risks of developing type 2 diabetes (19% lower risk) and metabolic syndrome (14% lower risk) compared to those in the bottom third (211). In another meta-analysis of nine prospective studies, including 28,258 older people (mean age, 67.7 years), lower versus higher circulating vitamin D concentrations at baseline were found to be associated with a 17% higher risk of developing type 2 diabetes over a median follow-up period of 7.3 years (212). Currently, limited evidence suggests that vitamin D supplementation may improve insulin sensitivity in individuals with glucose intolerance or manifest type 2 diabetes (213-216). There is a need for well-designed clinical trials to examine whether maintaining adequate vitamin D levels can prevent adverse metabolic outcomes in healthy and at-risk individuals.

## Neurodegenerative diseases

### *Cognitive impairment and Alzheimer's disease*

Alzheimer's disease (AD) is the most common form of dementia, characterized by the presence of extra-neuronal β-amyloid plaques and intra-neuronal Tau protein aggregates (known as neurofibrillary tangles) in the brain. Mechanistic models currently investigated in animal research suggest that vitamin D deficiency or disorders of vitamin D metabolism and/or the disruption of the vitamin D-VDR pathway in the cerebral regions of the cortex and hippocampus may be involved in the degeneration of neurons and loss of cognitive functions (217). Experimental evidence supporting a role for vitamin D in calcium channel regulation, neuroprotection, and immunomodulation in the central nervous system also implies that low vitamin D status may precede or contribute to cognitive dysfunction with age (218).

A number of observational studies have examined cognitive decline and degenerative brain disease in the elderly in relation to dietary intake of vitamin D and serum 25-hydroxyvitamin D concentration. In a large French cohort study on osteoporosis and hip fractures in postmenopausal women, impairments in global cognitive performance, assessed with the Pfeiffer Short Portable Mental State Questionnaire (SPMSQ), were associated with lower dietary intakes of vitamin D (<1,400 IU/week vs. ≥1,400 IU/week) in 5,596 elderly women (mean age, 80.5 years) (219). A seven-year follow-up study of a subgroup of 498 women indicated that the risk of Alzheimer's disease (but not other types of dementia) was 77% lower in those in the highest versus the lowest quintiles of vitamin D dietary intakes at baseline (220). Some, but not all, observational studies have found an association between low serum 25-hydroxyvitamin D concentrations and mild cognitive impairment in older adults (219, 221, 222). The cross-sectional and longitudinal analysis of two prospective studies, which included 1,604 men (223) and 6,257 women (224) aged 65 and over, reported a 60% greater odds of cognitive impairment at baseline and a 58% increased risk of cognitive decline during a four-year follow-up period in women, but not in men, with vitamin D deficiency (circulating 25-hydroxyvitamin D <10 ng/mL vs. ≥30 ng/mL). In the nested case-control, multiethnic, Singapore Kidney Eye Study, which included 2,273 individuals (mean age, 70.4 years), serum 25-hydroxyvitamin D concentrations were inversely correlated with cognitive deficits affecting retrograde episodic memory, semantic memory, and orientation in time, as assessed by the Abbreviated Mental Test (AMT) (225).

Yet, systematic reviews and meta-analyses of observational studies have given mixed results regarding the association of vitamin D status with cognitive performance and AD (226-230). Moreover, the recent analysis of data from 1,182 men followed for 18 years in the Uppsala Longitudinal Study of Adult Men (ULSAM) failed to find associations of genetic determinants of vitamin D synthesis, vitamin D intakes, and plasma 25-hydroxyvitamin D concentrations with risks of cognitive impairments, AD, vascular dementia, or all-cause dementia (231). In contrast, another Mendelian randomization study associated genetic determinants of low vitamin D status to higher risk of AD in the International Genomics of Alzheimer's Project dataset (17,008 AD cases and 37,154 healthy cases) (232).

Nevertheless, the prevalence of vitamin D insufficiency/deficiency ranges between 70% and 90% in older adults, and correcting low concentrations of serum 25-hydroxyvitamin D may help improve cognitive processes, in particular executive functions (233). In a small, non-randomized controlled study in an outpatient clinic, global cognitive function was assessed at baseline and after 16 months in 20 patients supplemented with 800 IU/day (or 100,000 IU/month) of vitamin D and in 24 control subjects. The supplementation of outpatients with vitamin D resulted in the correction of low vitamin D status (average serum 25-hydroxyvitamin D concentration was 16.8 ng/mL at baseline and 30 ng/mL at 16 months) and was associated with a significantly improved scoring in cognition tests compared to the non-supplemented group (234). In a small randomized, placebo-controlled clinical trial in 32 mild-to-moderate AD patients receiving nasal insulin, high doses of vitamin $D_2$ supplementation for eight weeks (up to 36,000 IU/day) did not significantly improve cognitive performance compared to low doses (1,000 IU/day) (235). More research is needed to investigate a causal relationship between vitamin D repletion and potential long-term cognitive benefits in older adults. Further, it is of great importance to evaluate whether correcting vitamin D deficiency in cognitively impaired subjects can improve the impact of anti-dementia therapy (236).

### *Parkinson's disease*

Parkinson's disease (PD) has been associated with a high prevalence of vitamin D insufficiency among patients, especially those with greater mobility problems (237). A case-control study of 296 outpatients with a mean age of 65 years indicated that 23% of PD subjects had serum 25-hydroxyvitamin D concentrations lower than 20 ng/mL compared to 16% and 10% of AD and healthy individuals, respectively (238). In a prospective cohort study conducted among 3,173 men and women aged 50-79 years and free of PD at baseline, individuals in the highest quartile of serum 25-hydroxyvitamin D (≥20 ng/mL for women and ≥22.8 ng/mL for men) had a 67% lower risk of PD compared to those in the lowest quartile (≤10 ng/mL for women and ≤11.2 ng/mL for men) (239). Meta-analyses pooling data from observational studies all showed that vitamin D inadequacy was more likely reported in subjects with PD than in healthy controls (240-242).

In a randomized, double-blind, placebo-controlled study, 112 PD patients (mean age, 72 years) on standard PD treatment were supplemented with 1,200 IU/day of vitamin D or a placebo for 12 months. Vitamin D supplementation nearly doubled serum 25-hydroxyvitamin D concentration (from mean of 22.5 ng/mL to 41.7 ng/mL) in supplemented subjects and limited the progression of PD, as indicated by a greater proportion of patients who showed no worsening (as assessed by the Hoehn and Yahr stage and the United Parkinson Disease Rating Scale part II) in the supplemented group compared to the placebo group (243). It is not known whether vitamin D insufficiency has a role in the pathogenesis of the disease, but the repletion of vitamin D may provide health benefits that go beyond the prevention and/or the treatment of PD. For example, vitamin D deficiency may contribute to the increased risk of osteoporosis and bone fracture in individuals with neurologic disorders, including PD and multiple sclerosis (244-246). Interestingly, sunlight exposure was found

to be associated with improved vitamin D status, higher bone mineral density of the second metacarpal bone, and lower incidence of hip fracture in a prospective study conducted in 324 elderly people with PD (247).

## Adverse pregnancy outcomes

A systematic review and meta-analysis of 31 observational studies on maternal vitamin D status and pregnancy outcomes indicated that vitamin D insufficiency may be associated with gestational diabetes mellitus, preeclampsia, and bacterial vaginosis in pregnant women. Low maternal serum vitamin D during pregnancy was also linked to an increased risk for small-for-gestational age infants and low-birth-weight infants, but not for Cesarean section (248). However, the number of intervention trials is currently too limited to draw conclusions as to whether vitamin D supplementation during pregnancy might reduce the incidence of the above-mentioned adverse outcomes (249).

### *Gestational diabetes mellitus*

Abnormal hyperglycemia due to pancreatic β-cell dysfunction characterizes the onset of gestational diabetes mellitus (GDM) in pregnant women without known type 2 diabetes mellitus. This condition is associated with serious adverse maternal outcomes, including preeclampsia, high risk of Cesarean delivery, and life-long increased risk of developing metabolic syndrome and type 2 diabetes mellitus. GDM may also contribute to increased risks of fetal macrosomia (excessive birth weight), neonatal hypoglycemia, infant respiratory distress, and increased life-long risk for obesity, glucose intolerance, type 2 diabetes mellitus, and cardiovascular disease in the offspring (reviewed in 250).

A recent prospective study conducted in 655 pregnant women found that the mean serum 25-hydroxyvitamin D concentration during the first trimester of pregnancy was significantly lower in 54 women who developed incident GDM compared to the rest of the cohort (23 ng/mL vs. 25.4 ng/mL). After multiple adjustments for confounding factors of vitamin D status and GDM risk (including overweight/obesity and prior history of type 2 diabetes and GDM), the study found each 7.5 ng/mL decrease in serum 25-hydroxyvitamin D concentration during early pregnancy was associated with a 48% higher risk of developing GDM (251). Low serum 25-hydroxyvitamin D concentrations (<29.4 ng/mL) during the second trimester of pregnancy were also associated with GDM incidence in a nested case-control study of 118 women with GDM and 219 matched control subjects (252). Five meta-analyses (248, 253-256), including observational studies of moderate-to-high quality, also reported that maternal serum vitamin D concentrations during pregnancy were inversely related to the risk of developing GDM despite evidence of bias amongst studies, such as the use of different methods for serum 25-hydroxyvitamin D measurement, measures done in different trimesters, and different criteria to assess GDM (reviewed in 257).

Further, evidence for the role of vitamin D in glucose regulation during pregnancy was reported in a small randomized, double-blind, placebo-controlled trial in 54 pregnant women diagnosed with GDM. The supplementation with 50,000 IU of vitamin $D_3$ twice during a six-week period (at day 1 and day 21) resulted in significantly lower fasting plasma glucose and serum insulin concentration, reduced insulin resistance, and improved insulin sensitivity compared to placebo (258). This suggests that vitamin D deficiency may adversely affect glucose tolerance during pregnancy and contribute to the onset of GDM. Yet, the potential benefits of vitamin D supplementation in the prevention of glucose intolerance and GDM during pregnancy have not been assessed. A multicentered, randomized controlled trial (DALI) is ongoing in Europe to evaluate the effects of vitamin D and lifestyle interventions (healthy eating and physical activity) on the metabolic status of pregnant women at risk of GDM (inclusion criteria: pre-pregnancy BMI ≥29 kg/m$^2$) (259). Preliminary findings suggest that healthy eating and physical activity can help lower gestational weight gain, when compared to standard-of-care; however, these lifestyle changes are unlikely to reduce the risk of GDM among obese pregnant women (260). The results regarding the effect of vitamin D supplementation in the DALI study are yet to be published.

### *Health outcomes in offspring*

During pregnancy, increased intestinal calcium absorption and mobilization of calcium from the skeleton allows accretion of calcium within the fetal skeleton. Yet, observational studies that examined the relationship between maternal vitamin D status and measures of fetal bone growth have not provided consistent results (261, 262). In addition, recent data from the Maternal Vitamin D Osteoporosis Study (MAVIDOS) suggested no difference in whole-body bone mineral content (BMC) of newborns from mothers randomized to daily supplementation with either vitamin $D_3$ (1,000 IU) or placebo from <17 weeks' gestation until delivery (263). Further, the risk of fracture in Danish children ages 10 to 18 years was similar regardless of whether their mothers were exposed to extra vitamin D from fortification during pregnancy (264). While maternal vitamin D supplementation during pregnancy effectively prevents the neonate's risk of vitamin D deficiency at birth (265), there is little evidence that neonatal vitamin D status influences the risk of fracture later during childhood (266).

A few observational studies have given rather weak evidence in support of a relationship between maternal vitamin D sufficiency during pregnancy and incidence of respiratory conditions and allergies in children (267). A randomized controlled trial found that the supplementation of 108 pregnant women in the third trimester (at week 27 of gestation until delivery) with either 800 IU/day or a bolus dose of 200,000 IU of vitamin $D_3$ did not decrease the risk of wheezing, allergic rhinitis, food allergy diagnosis, lower respiratory tract infections, or eczema in offspring at three years of age compared to placebo (N=50) (268). A more recent double-blind, randomized controlled trial found that vitamin $D_3$ supplementation of 295 Danish pregnant women, from weeks 22 to 26 of gestation until delivery, with 2,800 IU/day (70 µg/day) — compared to 400 IU/day (10 µg/day) of vitamin $D_3$ (i.e., the current recommendation in Denmark) (N=286) — reduced the risk of troublesome episodes of lung symptoms by 17% in offspring during the first three years of life

(269). However, no differences were reported regarding the risk of persistent wheeze, asthma, allergic sensitization, respiratory tract infections, or eczema between treatment and control groups (269). In a similar randomized, double-blind, controlled trial — the Vitamin D Antenatal Asthma Reduction Trial — conducted in 777 US pregnant women with a history of asthma, allergic rhinitis, or eczema (or whose partner had such an history), supplementation with 2,400 IU/day (60 µg/day) or 400 IU/day (10 µg/day) did not result in differences in the risk of developing asthma or recurrent wheezing in their children at age three years (270). Despite the lack of significance reported in individual studies, the pooled analysis of the three trials found a 19% reduction in the risk of recurrent wheeze in children whose mothers received high-dose versus low-dose vitamin D supplementation during pregnancy (271). In a cohort of 378 mother-child pairs, high serum 25-hydroxyvitamin D concentrations measured in the 34$^{th}$ week of pregnancy were associated with an increase in food allergy of the child during the first two years of life, warranting careful evaluation of the safety of vitamin D supplementation during pregnancy (272).

Since vitamin D insufficiency has been linked to autoimmunity (see Autoimmune diseases), it has also been proposed that poor maternal vitamin D status during pregnancy may contribute to an increased risk of autoimmune diabetes (insulin-dependent type 1 diabetes mellitus) in the offspring. Yet, the results of a study of 3,723 children at high genetic risk for type 1 diabetes and followed for a mean 4.3 year-period found that maternal intake of vitamin D (from food and/or supplements) during the third trimester of pregnancy (assessed through food frequency questionnaires) was not associated with advanced β-cell autoimmunity or clinical diabetes (142). In a nested case-control study, there was no difference in mean serum concentration of 25-hydroxyvitamin D during the first trimester of pregnancy between 343 mothers of children with type 1 diabetes and 343 control mothers (143). A follow-up study suggested that specific maternal *VDR* polymorphisms, rather than vitamin D status, may be linked to an increased susceptibility to developing type 1 diabetes in children (273). Another nested case-control study (119 mothers of children with type 1 diabetes and 129 control mothers) found an inverse association between maternal vitamin D-binding protein — but not 25-hydroxyvitamin D — concentration during the third trimester of pregnancy and the risk of type 1 diabetes in children (274). At present, there is no established causality between maternal vitamin D status during pregnancy and risk of autoimmune disease in offspring.

## Acute respiratory infections

More than 200 viruses are responsible for causing familiar infections of the upper respiratory tract (URT), known as the common cold, resulting in symptoms of nasal congestion and discharge, cough, sore throat, and sneezing (275). The analysis of cross-sectional data from 18,883 participants (ages 12 years and older) of the Third US National Health and Nutrition Examination Survey (NHANES III) reported an inverse relationship between serum 25-hydroxyvitamin D concentrations and recent (self-reported) URT infection (URTI). Compared to levels of circulating vitamin D of 30 ng/mL or above, the risk of URTI was 24% higher in individuals with concentrations between 10 and 29 ng/mL and 36% higher in those with levels below 10 ng/mL (276). A subgroup

analysis indicated that low concentrations of serum 25-hydroxyvitamin D in subjects with asthma and chronic obstructive pulmonary disease (COPD) were linked to a greater susceptibility to URTI when compared to people without pulmonary disease.

In a randomized, double-blind, placebo-controlled trial conducted in 322 healthy adults (ages ≥18 years), supplementation with monthly doses of vitamin $D_3$ (200,000 IU for the first two months and 100,000 IU for the following 16 months) significantly raised the mean serum 25-hydroxyvitamin D concentration (from 29 ng/mL to 48 ng/mL) in the intervention group but did not decrease the occurrence of URTI compared to placebo (277). Moreover, in a larger, multicenter, four-arm clinical trial in 2,259 subjects (aged 45-75 years) with a history of colorectal adenoma, daily vitamin $D_3$ supplementation of 1,000 IU did not reduce the number or the duration of URTI episodes during winter or the rest of the year, even among participants with the lowest serum 25-hydroxyvitamin D concentrations at baseline (278). In addition, the post-hoc analysis of data from a randomized, placebo-controlled trial in 644 individuals (ages 60-84 years) found that monthly supplementation with 30,000 IU or 60,000 IU of vitamin $D_3$ for a maximum period of one year did not significantly decrease the rate of antibiotic prescriptions for bacterial airway infections. The stratified analysis, however, found that doses of 60,000 IU/month reduced the risk of using antibiotics in participants ≥70 years old by 47% (279). In addition, compared to placebo, the supplementation of pregnant women with vitamin $D_3$ (with 2,000 IU/day) for three months until birth followed by the supplementation of their infants (800 IU/day) from birth to six months of age significantly reduced the number of acute respiratory infections after the intervention period in children 6 to 18 months old (280). Interestingly, despite significant heterogeneity among trials, the pooled analysis of these data with that of 21 additional trials suggested an overall 12% reduction in incidence of URTI with vitamin $D_3$ given as bolus doses (every week, month, or every three months), daily doses, or a combination of bolus and daily doses (281). Subgroup analyses revealed a 42% reduction in URTI risk with vitamin $D_3$ supplementation in subjects with baseline serum 25-hydroxyvitamin D concentrations <10 ng/mL, while there was no protective effect of vitamin $D_3$ in those with concentrations ≥10 ng/mL. Moreover, large bolus doses (≥30,000 IU) were found to be ineffective compared to daily or weekly doses such that, in subgroup analyses that excluded bolus doses, daily and weekly vitamin $D_3$ regimens appeared to be protective against URTI regardless of baseline vitamin D status. Finally, the effect of vitamin $D_3$ supplementation on URTI did not appear to vary with age, BMI, the presence of asthma or COPD, and flu vaccination status (281).

The findings of a large clinical trial in New Zealand, the Vitamin D Assessment (ViDA) Study, confirmed the ineffectiveness of large bolus doses of vitamin D in reducing the risk of acute respiratory infections (282). In this double-blind, placebo-controlled trial in 5,110 older adults (ages 50-84 at baseline), supplementation with an initial dose of 200,000 IU of vitamin $D_3$ followed by 100,000 IU/month of vitamin $D_3$ for a median of 3.3 years had no effect on the incidence of (self-reported) acute respiratory infections (HR, 1.01; 95% CI, 0.94-1.07). Upon data stratification by vitamin D status, a similar null result (HR, 1.08; 95% CI, 0.95-1.23) was seen in study participants with blood vitamin D concentrations ≤20 ng/mL (≤50 nmol/L; 282).

The recently completed VITAL (Vitamin D and Omega-3 Trial) may provide additional evidence of an effect of daily dosing (2,000 IU/day of vitamin D) on risk of airway infections in older adults, although the prevention of infectious disease is a secondary outcome in the trial (204, 283, 284).

In a trial in preschool-age children, vitamin D supplementation with 2,000 IU/day for at least four months did not decrease the incidence (285) or severity (286) of URTI in winter compared to supplementation with 400 IU/day for at least four months. Supplementation with 2,000 IU/day led to significantly higher mean serum 25-hydroxyvitamin D concentrations compared to the lower dose (48.7 ng/mL vs. 36.8 ng/mL), but the 400 IU dose may have been sufficient to prevent URTI in these young children (285). In a placebo-controlled trial in 1,300 healthy children and adolescents in rural Vietnam (baseline 25-hydroxyvitamin D concentrations of 26 ng/mL for both the control and intervention group), supplementation with 14,000 IU/week of vitamin D for eight months significantly reduced the incidence of non-influenza viral respiratory infections by 24%, but not the incidence of influenza A or B infection (287).

## Coronavirus disease, COVID-19

The coronavirus disease, COVID-19, is caused by infection with the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). This virus originated in Wuhan, China in late 2019 and rapidly spread throughout the world causing a global pandemic. Flu-like symptoms, including cough, fever, fatigue, and difficulty breathing, as well as several other diverse symptoms characterize COVID-19 (see the websites of the CDC and the WHO). The effects of the disease vary widely, with the most severe resulting in pneumonia, respiratory distress, and death. Some individuals infected with SARS-CoV-2, however, are asymptomatic but can transmit virus to others (288). Moreover, SARS-CoV-2 infected individuals can spread disease before they experience symptoms (i.e., presymptomatic transmission) (289), placing importance on public health measures like hand washing, face mask wearing, social distancing, and testing and contact tracing to curtail the pandemic.

A number of observational studies have examined the correlation of vitamin D status and COVID-19 incidence, with most — but not all (290-292) — finding vitamin D deficiency or insufficiency associated with an increased risk of SARS-CoV-2 infection. In a retrospective cohort study of more than 191,000 US residents administered a SARS-CoV-2 test during a three-month period in spring 2020, SARS-CoV-2 positivity was strongly associated with a lower serum 25-hydroxyvitamin D concentration, measured at a single point in the 12 months prior to the viral test (293). In this study, each 1 ng/mL increment of serum 25-hydroxyvitamin D was linked to a 1.6% lower risk of SARS-CoV-2 positivity, with the risk being the lowest at serum concentrations of 55 ng/mL and above (293). In a retrospective, population-based study among 7,807 people in Israel, including 782 SARS-CoV-2 positive cases, plasma 25-hydroxyvitamin D concentrations below 30 ng/mL were associated with a 50% higher risk of SARS-CoV-2 infection compared to higher vitamin D concentrations (294). The vitamin D status assessment in this study was done prior to the viral testing, but details on the time frame are lacking. The temporal relationship of the vitamin D status

measure and COVID diagnostic test is also a concern in an analysis of a large dataset from the UK Biobank, which found vitamin D status was not associated with SARS-CoV-2 infection: the study utilized vitamin D status assessments done 10 to 14 years prior to the COVID-19 diagnostic test (291). Consistent with findings of most available studies, a small retrospective cohort study (295) and five case-control studies (296-300) reported an inverse association of vitamin D status and SARS-CoV-2 infection. A 2021 meta-analysis of 10 observational studies, including 361,934 participants, reported an association between vitamin D insufficiency or deficiency and increased risk of COVID-19 (OR, 1.43; 95% CI: 1.00-2.05), although heterogeneity across studies was high (301). Further, in an analysis of data from the UK Biobank, regular use of vitamin D supplements was found to be associated with a 34% lower risk of SARS-CoV-2 infection compared nonusers of vitamin D supplements (292).

Additionally, most of the available observational research suggests an association between low vitamin D status and COVID-19 severity, with studies finding vitamin D deficiency linked to an increased risk of disease severity, as measured by need for hospitalization, intensive care unit admission (302), stage of pneumonia (in men but not in women; 303), need for non-invasive ventilation (304), need for invasive mechanical ventilation (305), or a combination of these and other indicators (297). Another study found that insufficient vitamin D status, defined as plasma 25-hydroxyvitamin D concentrations below 30 ng/mL, was associated with an increased likelihood of COVID-related hospitalization, but the association failed to reach statistical significance ($p=0.061$) except in a subanalysis of individuals over 50 years of age (OR, 2.71; 95% CI: 1.55-4.78; $p<0.001$) (294). In contrast to most of the evidence to date, one case-control study found vitamin D deficiency was not linked to COVID-19 severity (298). Further, a few studies have looked at the relationship of vitamin D status and COVID-19 mortality, with three finding vitamin D deficiency to be associated with an increased risk of death (303, 305, 306) and two finding no association (304, 307), although one of the studies finding no association used vitamin D status measurements more than 10 years prior to the COVID-19 diagnostic testing (307).

Since there are several known risk factors for severe illness from COVID-19, including advanced age, obesity, and preexisting type 2 diabetes or cardiovascular disease (308), it is important for observational studies to control for potential confounders. Two quasi-experimental studies reported that bolus supplementation with vitamin $D_3$ (either 50,000 IU per month or 80,000-100,000 IU every two to three months) prior to or during SARS-CoV-2 infection was associated with a less severe illness and improved survival in frail elderly COVID-19 patients (309, 310). A number of randomized controlled trials of vitamin D supplementation in COVID-19 prevention and treatment are currently underway; the results of these trials will inform on the causality of the association. Nevertheless, the currently available data indicate that improving vitamin D status through supplementation represents a modifiable risk factor for COVID-19.

## Disease Treatment

### Atopic dermatitis

Atopic dermatitis or eczema is particularly prevalent in industrialized countries, affecting 10%-20% of children and 1%-3% of adults. Atopic dermatitis is a chronic inflammatory skin disorder characterized by dry and pruritus (itchy) areas of the skin in affected subjects. Local skin inflammation and immune dysfunction can damage the epidermal barrier and increase the susceptibility to skin infections and atopic reactions in affected individuals. The disease is often associated with other atopic diseases, including food allergies, asthma, and allergic rhinitis (311).

While the etiology of the disease is not fully elucidated, it has been suggested that vitamin D deficiency might contribute to the onset and/or the severity of the disease (312). Recently, using large-scale datasets from Caucasian people of European descent, including the UK Biobank resource (313) and the SUNLIGHT (35), the GABRIEL asthma (314), and EAGLE eczema (315) consortia, a Mendelian randomization study found no association between genetically low serum 25-hydroxyvitamin D concentrations and risks of atopic dermatitis, asthma, or high serum immunoglobulin (Ig)-E concentrations (316).

A number of randomized controlled studies have examined whether vitamin D might be an effective adjunct tool in disease management, possibly through regulating local inflammatory reactions and stimulating antimicrobial activities in the skin. Moreover, the beneficial effect of phototherapy observed in specific cases of atopic dermatitis may be partly mediated by the action of vitamin D (311). In a small randomized, double-blind, placebo-controlled study in 45 patients with atopic dermatitis and low vitamin D status (70% of subjects had serum 25-hydroxyvitamin D concentrations <20 ng/mL), daily administration of 1,600 IU of oral vitamin $D_3$, alone or together with 600 IU/day of vitamin E, for a period of 60 days significantly reduced the extent and intensity of eczema, as assessed by the SCORAD (SCORing Atopic Dermatitis) score (317). Vitamin $D_3$ (1,600 IU/day for 60 days) also improved vitamin D status and reduced disease severity in 53 patients with atopic dermatitis in another small randomized trial (318). More recently, vitamin $D_3$ (1,000 IU/day for one month) improved the severity of winter-related atopic dermatitis in Mongolian children, as shown by changes in Eczema Area and Severity Index (EASI) and Investigator's Global Assessment (IGA) scores (319). A meta-analysis of four small trials (including those cited above) confirmed that supplemental vitamin D can lead to measurable clinical improvements in affected individuals (320). Larger trials are needed to strengthen these preliminary findings and determine the most appropriate and effective supplementation regimen. Of note, topical treatment of psoriasis with vitamin D analogs has been approved by the US Food and Drug Administration (FDA) and may be effective in the management of other skin disorders (321).

## Inflammatory bowel diseases

Several ill-defined environmental and genetic factors are thought to contribute to the development of the inappropriate immune response to the intestinal microbiota that causes ulcerative colitis (UC) and Crohn's disease (CD). While specific *VDR* polymorphisms may be linked to an increased susceptibility to developing UC and CD (322), higher vitamin D intakes and predicted circulating levels were found to be associated with a reduced incidence of UC and CD in a large cohort of

72,719 women (323). A meta-analysis of six observational studies found an inverse association between vitamin D status and severity of CD (324). Three studies have investigated whether vitamin $D_3$ could benefit patients with CD, possibly through reducing intestinal inflammation. In one multicenter, double-blind, placebo-controlled study, the relapse rate in CD patients in remission after one year of treatment was significantly lower in those supplemented daily with 1,200 IU of vitamin $D_3$ and 1,200 mg of calcium compared to those who received calcium alone (13% vs. 29%) (325). In a second pilot study, incremental daily doses of vitamin $D_3$, from 1,000 IU up to 5,000 IU, were administrated over a 24-week period to 18 CD patients in order to achieve circulating 25-hydroxyvitamin D concentrations >40 ng/mL. Although half of the patients failed to achieve 40 ng/mL, the mean 25-hydroxyvitamin D concentration was raised to 45 ng/mL (from a baseline mean of 16 ng/mL), and the overall improvement in vitamin D status was associated with a significant decrease in disease severity as assessed by Crohn's Disease Activity (CDAI) scores (326). In a three-month, randomized, double-blind, placebo-controlled trial in 27 CD patients in remission, daily supplementation with vitamin $D_3$ (2,000 IU) improved vitamin D status but had no significant effect on intestinal permeability ('leaky gut') or measures of inflammation and disease activity (327). Yet, the study suggested that achieving serum 25-hydroxyvitamin D concentrations ≥30 ng/mL might help reduce intestinal inflammation and improve patients' quality of life. Additional studies are needed to confirm the therapeutic efficacy of vitamin D in inflammatory bowel diseases.

## Cardiovascular disease

The prospective analysis of 41,504 electronic medical records in the Intermountain Heart Collaborative study found that only one-third of patients had adequate serum 25-hydroxyvitamin D concentrations (>30 ng/mL); vitamin D insufficiency (serum 25-hydroxyvitamin D concentrations ≤30 ng/mL) was associated with increased prevalence and incidence of many cardiovascular conditions, including hypertension, coronary artery disease, heart failure, and stroke (328). Suboptimal vitamin D status has also been linked to arterial stiffness and vascular endothelial dysfunction — strong determinants of incident hypertension and adverse cardiovascular outcomes (329).

### *Hypertension*

Several intervention studies have evaluated the effect of vitamin D supplementation on blood pressure. An early controlled clinical trial in 18 men and women with untreated mild hypertension living in the Netherlands found that exposure to UVB radiation three times weekly for six weeks during the winter increased serum 25-hydroxyvitamin D concentrations by 162%, lowered PTH concentrations by 15%, and decreased 24-hour ambulatory systolic and diastolic blood pressure measurements by an average of 6 mm Hg (330). A recent meta-analysis of 16 randomized controlled trials involving 1,879 participants, either healthy or with pre-existing cardiometabolic conditions (including hypertension), found no significant reduction in systolic and diastolic blood pressures with vitamin D supplementation (800-8,571 IU/day for five weeks to one year). However, a subgroup analysis of six trials found a significant reduction of 1.31 mm Hg in diastolic blood pressure in individuals with preexisting conditions. While improvements in blood pressure may be

expected in cases of vitamin D insufficiency/deficiency, the authors noted that suboptimal vitamin D status in participants was not exclusively observed in those with cardiometabolic conditions (331). In contrast, in two recent intervention studies — the Styrian vitamin D hypertension trial (332) and the Vitamin D therapy in individuals at high risk of hypertension trial [DAYLIGHT] (333) — subjects with (pre)hypertension supplemented with vitamin $D_3$ (400 to 4,000 IU/day for two to six months) showed no evidence of blood pressure lowering, regardless of their baseline vitamin D status (insufficient or adequate, according to the IOM's current cutoffs).

Conditions that decrease vitamin D synthesis in the skin, such as having dark-colored skin, living in temperate latitudes, and aging, are associated with increased prevalence of hypertension (334), suggesting that vitamin D may reduce blood pressure levels in selected groups of individuals. In the above-mentioned meta-analysis, one four-arm, double-blind, placebo-controlled clinical trial was conducted in 283 African Americans randomized to receive daily vitamin $D_3$ supplements of 1,000 IU, 2,000 IU, or 4,000 IU for a period of three months. Systolic blood pressure was decreased by 0.66 mm Hg with 1,000 IU/day, 3.4 mm Hg with 2,000 IU/day, and by 4 mm Hg with 4,000 IU/day while it increased by 1.7 mm Hg in the placebo group when compared to baseline. A significant reduction of 0.2 mm Hg in systolic blood pressure was detected per 1 ng/mL incremental increase in 25-hydroxyvitamin D concentration. However, there was no statistical difference on three-month change in blood pressure between vitamin $D_3$ and placebo (335). Another randomized, placebo-controlled study in 150 elderly participants (mean age, 77 years) showed that supplementation with 100,000 IU of vitamin $D_3$ every three months for one year did not significantly lower blood pressure compared to placebo (336).

Further research is needed to determine whether vitamin D supplementation is helpful in the prevention or management of hypertension.

### *Congestive heart failure*

Congestive heart failure (also called cardiac insufficiency) is characterized by increased heart rate and subsequent hypertrophy of the left heart ventricle. Cardiac insufficiency is associated with a reduced left ventricular ejection fraction (LVEF), assessed by echocardiography. Inhibitors of angiotensin-converting enzyme (ACE) (see Blood pressure regulation) are currently used as first-line therapy for patients with heart failure. In a cross-sectional study in healthy patients who underwent coronary angiography, serum 25-hydroxyvitamin D concentrations <30 ng/mL were associated with poorer coronary flow rates (337). Suboptimal vitamin D status has also been linked to poorer prognosis in patients with heart failure (338). Over the past several years, a number of intervention studies have examined the effect of vitamin D supplementation in those with cardiac insufficiency. In a 12-week randomized, double-blind, placebo-controlled study, daily supplementation with 1,200 IU of vitamin $D_3$ in children with chronic congestive heart failure led to a significant increase in itamin D status accompanied by an improved heart muscle performance (increased LVEF), as well as by lower levels of PTH and pro-inflammatory cytokines (339). In another randomized, double-blind, placebo-controlled trial in 64 elderly patients with heart failure, participants receiving 800

mg/day of calcium and 50,000 IU/week of vitamin $D_3$ did not perform significantly better at physical performance tasks (used as proxy to assess aerobic capacity and skeletal muscle strength) compared to those supplemented with calcium only (340). A recent meta-analysis of seven small randomized placebo-controlled trials in 573 subjects with heart failure showed that vitamin D supplementation (from 1,000 IU/day to 50,000 IU/week) for six weeks to nine months could reduce serum concentrations of PTH, tumor necrosis factor-α (TNF-α), and C-reactive protein (CRP). Yet, there were no significant differences in LVEF, circulating interleukin-10 (IL-10) concentration, and renin concentration between patients treated with vitamin D and those given a placebo (341). Finally, in the EVITA (Effect of vitamin D on all-cause mortality) trial in patients with end-stage heart failure and inadequate vitamin D status (baseline serum 25-hydroxyvitamin D values, 8.6-19.7 ng/mL), supplementation with 4,000 IU/day for three years did not reduce the risk of mortality compared to placebo (342).

## Sources

### Sunlight

Solar ultraviolet-B radiation (UVB; wavelengths of 290 to 315 nanometers) stimulates the production of vitamin $D_3$ in the epidermis of the skin (343). Sunlight exposure can provide most people with their entire vitamin D requirement. Children and young adults who spend a short time outside two or three times a week will generally synthesize all the vitamin D they need to prevent deficiency. One study reported that serum vitamin D concentrations following exposure to one minimal erythemal dose of simulated sunlight (the amount required to cause a slight pinkness of the skin) to the whole body was equivalent to ingesting approximately 10,000 to 25,000 IU of vitamin D (344). People with dark-colored skin synthesize markedly less vitamin D on exposure to sunlight than those with lighter complexion (34). Additionally, older adults have diminished capacity to synthesize vitamin D from sunlight exposure and frequently use sunscreen or protective clothing in order to prevent skin cancer and sun damage. The application of sunscreen with an SPF factor of 10 reduces production of vitamin D by 90% (30). In latitudes around 40 degrees north or 40 degrees south (Boston is 42 degrees north), there is insufficient UVB radiation available for vitamin D synthesis from November to early March. Ten degrees farther north or south (Edmonton, Canada), the "vitamin D winter" extends from mid-October to mid-March. It has been estimated that up to 15 minutes of daily sun exposure on the hands, arms, and face around 12 pm throughout the year at 25 degrees latitude (Miami, FL) and during the spring, summer, and fall at 42 degrees (Boston, MA) latitude may provide a light-skinned individual with 1,000 IU of vitamin D (345).

### Food sources

Vitamin D is found naturally in only a few foods, such as some fatty fish (mackerel, salmon, sardines), fish liver oils, eggs from hens that have been fed vitamin D, and mushrooms exposed to sunlight or UV light. In the US, milk and infant formula are fortified with vitamin D so that they contain 400 IU (10 µg) per quart. However, other dairy products, such as cheese and yogurt, are not

always fortified with vitamin D. Some cereal, bread, and fruit juices may also be fortified with vitamin D. Accurate estimates of average dietary intakes of vitamin D are difficult because of the high variability of the vitamin D content of fortified foods (346). The vitamin D content (sum of vitamin $D_2$ and vitamin $D_3$) of some vitamin D-rich foods is listed in **Table 2** in both international units (IU) and micrograms (µg). For more information on the nutrient content of specific foods, search USDA's FoodData Central. The 25-hydroxyvitamin D metabolite is also present at low levels is certain foods, including meats, dairy products, and eggs (347, 348).

Table 2. Some Food Sources of Vitamin D

| Food | Serving | Vitamin D (IU) | Vitamin D (µg) |
|---|---|---|---|
| Pink salmon, canned | 3 ounces | 465 | 11.6 |
| Mackerel, canned (boneless) | 3 ounces | 248 | 6.2 |
| Sardines, canned | 3 ounces | 164 | 4.1 |
| Milk, low-fat (1%), fortified with vitamin D | 8 fluid ounces | 108 | 2.7 |
| Orange juice, fortified with vitamin D | 8 fluid ounces | 100 | 2.5 |
| Cereal, fortified | 1 serving (usually 1 cup) | 40-50 | 1.0-1.3 |
| Egg yolk | 1 large | 37 | 0.9 |

## Supplements

Most vitamin D supplements available without a prescription contain cholecalciferol (vitamin $D_3$). Multivitamin supplements generally provide 400 IU-1,000 IU (10 µg-25 µg) of vitamin $D_2$ or vitamin $D_3$. Single-ingredient vitamin D supplements may provide 400 to 50,000 IU of vitamin $D_3$, but 400 IU is the most commonly available dose (66). A number of calcium supplements may also provide vitamin D. A meta-analysis of randomized controlled trials suggested that bolus doses of vitamin $D_2$ (ergocalciferol) may not always be as effective as vitamin $D_3$ in raising serum 25-hydroxyvitamin D concentrations, yet no difference in efficacy was found with daily supplementation with vitamin $D_2$ or vitamin $D_3$ (349). Nonetheless, a 25-week, randomized, double-blind, placebo-controlled trial found daily supplementation with 1,000 IU of vitamin $D_3$ initiated at the end of summer to be more efficacious than vitamin $D_2$ in maintaining summertime concentrations of 25-hydroxyvitamin D during fall and winter months (350).

There is growing interest in using the hydroxylated form of cholecalciferol, 25-hydroxyvitamin $D_3$ (calcidiol; calcifediol), as a supplement. This vitamin D metabolite is synthesized by the liver of humans and animals from cholecalciferol produced in skin or consumed in the diet, and is present in some foods (e.g., meats, milk, eggs) at low levels (347, 348). In general, clinical studies have found calcifediol is two to five times more potent at increasing blood concentrations of 25-hydroxyvitamin D compared to supplementation with equivalent doses of cholecalciferol (351-359). Supplementation with calcifediol may thus represent a means to improve vitamin D status rapidly and consistently with lower doses than cholecalciferol and may benefit those individuals with conditions that decrease intestinal absorption of cholecalciferol (359). However, the 25-hydroxyvitamin $D_3$ form is presently not available as an over-the-counter supplement in the United States.

## Safety

### Toxicity

Vitamin D toxicity (hypervitaminosis D) has not been observed to result from sun exposure. The reason is that excessive sunlight exposure generates a number of biologically inert photoproducts from 7-dehydrocholesterol and cholecalciferol (3). Vitamin D toxicity induces abnormally high serum calcium concentration (hypercalcemia), which could result in bone loss, kidney stones, and calcification of organs like the heart and kidneys if untreated over a long period of time. Hypercalcemia has been observed following daily doses of greater than 50,000 IU of vitamin D (360). Overall, research suggests that vitamin D toxicity is very unlikely in healthy people at intake levels lower than 10,000 IU/day (361-363). However, the Food and Nutrition Board of the IOM conservatively set the tolerable upper intake level (UL) at 4,000 IU/day (100 µg/day) for all adults (**Table 3**). Certain medical conditions can increase the risk of hypercalcemia in response to vitamin D, including primary hyperparathyroidism, sarcoidosis, tuberculosis, and lymphoma (361). People with these conditions may develop hypercalcemia in response to any increase in vitamin D nutrition and should consult a qualified health care provider regarding any increase in vitamin D intake.

Table 3. Tolerable Upper Intake Level (UL) for Vitamin D

| Age Group | µg/day | IU/day |
|---|---|---|
| Infants 0-6 months | 25 | 1,000 |
| Infants 6-12 months | 37.5 | 1,500 |
| Children 1-3 years | 62.5 | 2,500 |
| Children 4-8 years | 75 | 3,000 |
| Children 9-13 years | 100 | 4,000 |

| | | |
|---|---|---|
| Adolescents 14-18 years | 100 | 4,000 |
| Adults 19 years and older | 100 | 4,000 |

## Drug interactions

The following medications should not be taken at the same time as vitamin D because they can decrease the intestinal absorption of vitamin D: cholestyramine (Questran), colestipol (Colestid), orlistat (Xenical), and mineral oil (364, 365). The following medications increase the metabolism of vitamin D and may decrease serum 25-hydroxyvitamin D concentrations: phenytoin (Dilantin), fosphenytoin (Cerebyx), phenobarbital (Luminal), carbamazepine (Tegretol), and rifampin (Rimactane) (6). Cimetidine, a $H_2$ blocker that suppresses stomach acid secretion, inhibits the hydroxylation of vitamin D in the liver (366). Treating acid reflux, gastroesophageal reflux disease (GERD), or ulcers with proton-pump inhibitors (omeprazole, lansoprazole) might interfere with calcium absorption and increase the risk of fracture such that patients are advised to take calcium and vitamin D supplements (367). The oral antifungal medication, ketoconazole, inhibits the 25-hydroxyvitamin $D_3$-1α-hydroxylase enzyme and has been found to reduce serum 1α,25-hydroxyvitamin D concentrations in healthy men (368). The Endocrine Society also recommends monitoring vitamin D status of patients on glucocorticoids and HIV treatment drugs because these medications increase the catabolism of 25-hydroxyvitamin D (40). The use of some cytostatic agents (cell growth inhibitors) may also increase the degradation of 25-hydroxyvitamin D and α,25-hydroxyvitamin D in cancer patients under chemotherapy (6). The induction of hypercalcemia by toxic levels of vitamin D may precipitate cardiac arrhythmia in patients on digoxin (Lanoxin) (366). Hypercalcemia may also reduce the effectiveness of verapamil (Calan) and diltiazem (Cardizem) in atrial fibrillation (366).

## Linus Pauling Institute Recommendation

The Linus Pauling Institute recommends that generally healthy adults take 2,000 IU (50 µg) of supplemental vitamin D daily. Most multivitamins contain 400 IU (10 µg) of vitamin D, and single-ingredient vitamin D supplements are available for additional supplementation. Sun exposure, diet, skin color, and body mass index (BMI) have variable, substantial impact on body vitamin D levels. To adjust for individual differences and ensure adequate body vitamin D status, the Linus Pauling Institute recommends aiming for a serum 25-hydroxyvitamin D concentration of at least 30 ng/mL (75 nmol/L). Observational studies suggest that serum 25-hydroxyvitamin D concentrations between 30 ng/mL and 60 ng/mL are associated with lower risks of adverse health outcomes, including cancers and autoimmune diseases.

The American Academy of Pediatrics currently suggests that all infants, children, and adolescents receive 400 IU of supplemental vitamin D daily (19). Consistent with the recommendations of the Endocrine Society (40), the Linus Pauling Institute recommends daily intakes of 400 to 1,000 IU (10

to 25 µg) of vitamin D in infants and 600 to 1,000 IU (15 to 25 µg) of vitamin D in children and adolescents. Given the average vitamin D content of breast milk, infant formula, and the diets of children and adolescents, supplementation may be necessary to meet these recommendations.

### Older adults (>50 years)

Daily supplementation with 2,000 IU (50 µg) of vitamin D is especially important for older adults because aging is associated with a reduced capacity to synthesize vitamin D in the skin upon sun exposure.

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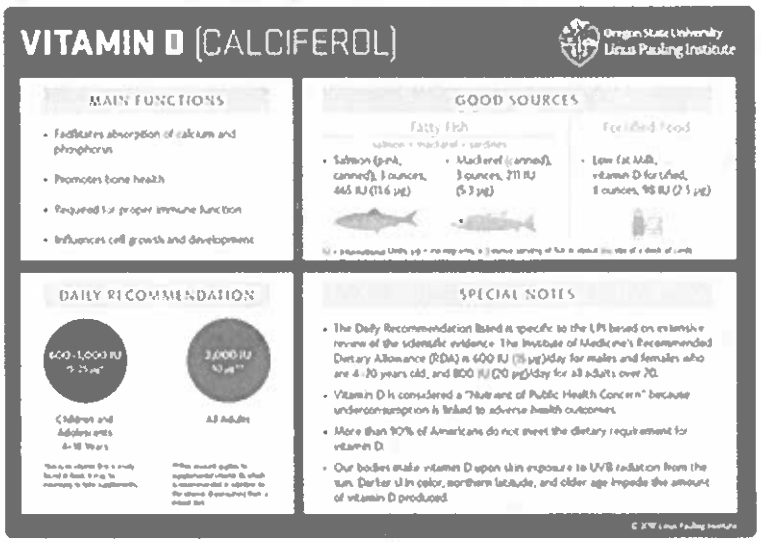
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# Selenium

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## Summary


- Selenium exerts various biological functions mainly as part of the amino acid, selenocysteine, which is found in at least 25 selenocysteine-containing proteins (selenoproteins). *(More information)*
- Five glutathione peroxidases, three thioredoxin reductases, three iodothyronine deiodinases, and one methionine sulfoxide reductase B1 are among the best characterized selenoproteins with known functions. *(More information)*
- Impaired antioxidant protection in selenium-deficient individuals may affect physiological responses to stress. Keshan cardiomyopathy and Kashin-Beck osteoarthropathy are diseases occurring specifically in areas of selenium deficiency in Asia. *(More information)*
- The current recommended dietary allowance (RDA) set by the US Institute of Medicine is 55 µg/day for adolescents and adults of all ages. *(More information)*
- Early observational studies have found either null or inverse associations between selenium exposure and risk of site-specific cancers. However, current evidence from intervention trials in selenium-replete participants does not support a protective effect of selenium supplementation against cancer. *(More information)*
- Preliminary evidence from randomized controlled trials suggests that selenium supplementation may prevent

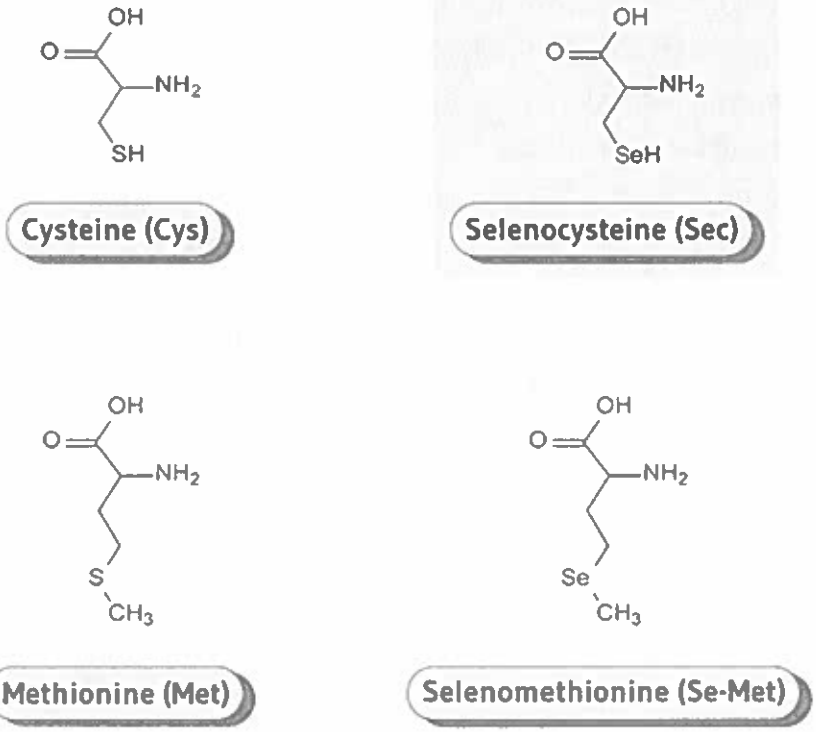
› Drug interactions
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viral load progression and increase immune cell count in HIV-positive patients. *(More information)*

- The levels and chemical forms of selenium in plant-based food vary according to the composition and selenium content of soil in which the plants are grown. Selenium-rich food sources include Brazil nuts, grains, seafood, organ meats, poultry, and dairy products. *(More information)*
- The tolerable upper intake level (UL) for selenium is 400 µg/day for adolescents and adults and includes both selenium obtained from food, which averages about 100 µg/day for adults in the US, and selenium from supplements. *(More information)*
- Because some evidence suggests that high serum selenium concentrations may have adverse effects on glycemic control, individuals with high selenium status and/or those at risk for type 2 diabetes mellitus should avoid taking selenium supplements. *(More information)*

Selenium is a trace element that is essential in small amounts, but like all essential elements, selenium can be toxic at high levels. Unlike plants, most animals — including humans — require selenium for the appropriate functioning of a number of selenium-dependent enzymes known as selenoproteins. During protein synthesis (translation), the amino acid selenocysteine is incorporated into elongating proteins at very specific locations in the amino acid sequence in order to form functional selenoproteins. Although higher plants do not appear to require selenium for survival, they can incorporate it non-specifically into sulfur-containing molecules when the mineral is present in the soil (1). Of note, in animals, the amino acid selenomethionine can be nonspecifically incorporated into proteins in place of methionine (2). However, only selenocysteine-containing proteins are regarded as selenoproteins (**Figure 1**).

Figure 1. Cysteine, Methionine, and Selenium-containing Analogs




Twenty-five human genes code for selenocysteine (Sec)-containing proteins known as selenoproteins. The incorporation of the amino acid Sec into proteins during translation is controlled by a regulatory element (named SECIS) in the 3'-untranslated region of mRNAs coding for selenoproteins. During translation, the recoding of the codon UGA as a signal to insert a Sec (rather than as a "stop codon") into elongating proteins requires the recognition of the SECIS sequence and the presence of a specific Sec-tRNA. Selenomethionine (Se-Met)-containing proteins are not selenoproteins per se. While the incorporation of Sec into selenoproteins is regulated and essential for proper folding and specific selenoprotein functions, the substitution of methionine (Met) with Se-Met is a nonspecific mechanism that does not result in structural and/or functional changes in proteins.

# Function

## Selenoproteins

Twenty-five genes coding for selenoproteins have been identified in humans (3). The insertion of selenocysteine into selenoproteins during translation is directed by the presence of a selenocysteine-insertion sequence (SECIS) within selenoprotein mRNAs. Briefly, the recognition of SECIS by the translational machinery results in the recruitment of specific translational factors that decode in-frame UGA codons by inserting selenocysteine into elongating selenoproteins (4).

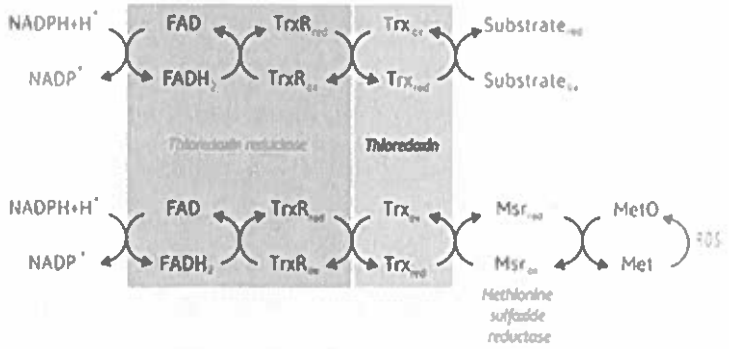
Research is gradually uncovering the metabolic functions of all human selenoproteins, including splicing variants (3). Some of the selenoproteins with an identified function include:

### *Glutathione peroxidases*

Five selenium-containing glutathione peroxidases (GPx1-4 and GPx6) have been identified: GPx1 (cytosolic GPx), GPx2 (epithelial cell-specific GPx expressed in intestinal lining and lungs), GPx3 (highly expressed in thyroid gland and kidneys), GPx4 (phospholipid-hydroperoxide GPx; PHGPx), and GPx6 (expressed in the olfactory epithelium) (4). GPx isoenzymes are all antioxidant enzymes that reduce potentially damaging reactive oxygen species (ROS), such as hydrogen peroxide and lipid hydroperoxides, to harmless products like water and alcohols by coupling their reduction with the oxidation of glutathione (Figure 2). Spermatogenesis and male fertility are highly dependent on GPx4 and selenoprotein P (SEPP1; see below). In the testes, GPx4 reduces phospholipid hydroperoxides, hence protecting immature spermatozoa cells against oxidative stress. GPx4 is also a major structural protein of the capsule embedding mature sperm mitochondrial helix involved in sperm motility. SEPP1 is essential for selenium supply to the testes, and animal models lacking the *SEPP1* gene are infertile due to poor selenium tissue bioavailability, defective GPx4 synthesis, and impaired sperm maturation (5).

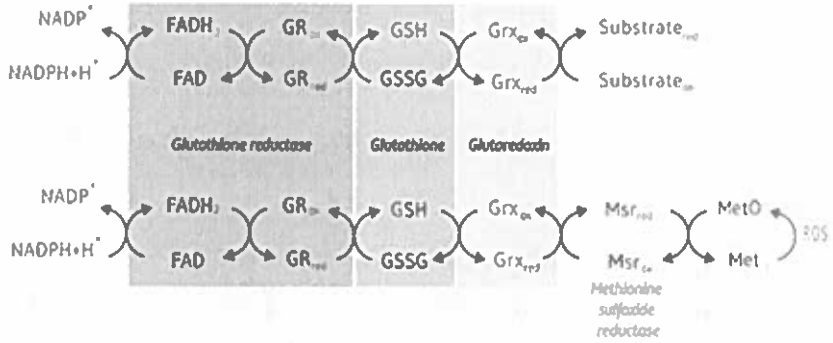
Figure 2. Selenoproteins in Thiol-based Antioxidant Systems

a) Thioredoxin antioxidant system

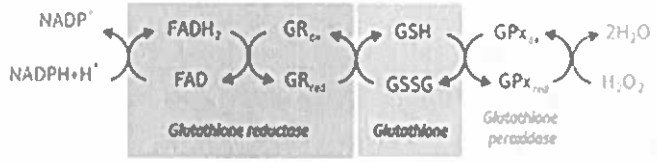



b) Glutathione antioxidant system

Glutaredoxin antioxidant system




Glutathione peroxidase system




Selenoproteins (in red) are involved in two major thiol/disulfide-based antioxidant systems: (a) the thioredoxin (Trx) and (b) the glutathione (GSH) antioxidant systems. (a) Substrate reduction by the Trx system results from the transfer of electrons from NADPH to thioredoxin (Trx) via the selenoprotein thioredoxin reductase (TrxR). The family of methionine sulfoxide reductases (Msrs), which includes the selenoenzyme MsrB1 and catalyzes the reduction of methionine sulfoxide (MetO) to methionine (Met), can be readily reduced by both Trx and glutaredoxin (Grx) systems. (b) In the Grx system, electrons are transferred from NADPH to Grx via GSH reductase and GSH. Among the many substrates of the GSH-based redox system is the family of glutathione peroxidases (some of them are selenoenzymes) that can catalyze the conversion of hydrogen peroxide ($H_2O_2$) to water. *FAD, flavin adenine dinucleotide; GPx, GSH peroxidase; GR, GSH reductase; GSH, glutathione; GSSG, oxidized GSH; Grx, glutaredoxin; $H_2O_2$, hydrogen peroxide; Met, methionine; MetO, methionine sulfoxide; Msr, methionine sulfoxide reductase; NADP, phosphorylated nicotinamide adenine dinucleotide; ox, oxidized form; red, reduced form; ROS, reactive oxygen species; TrxR, thioredoxin reductase; Trx, thioredoxin.*

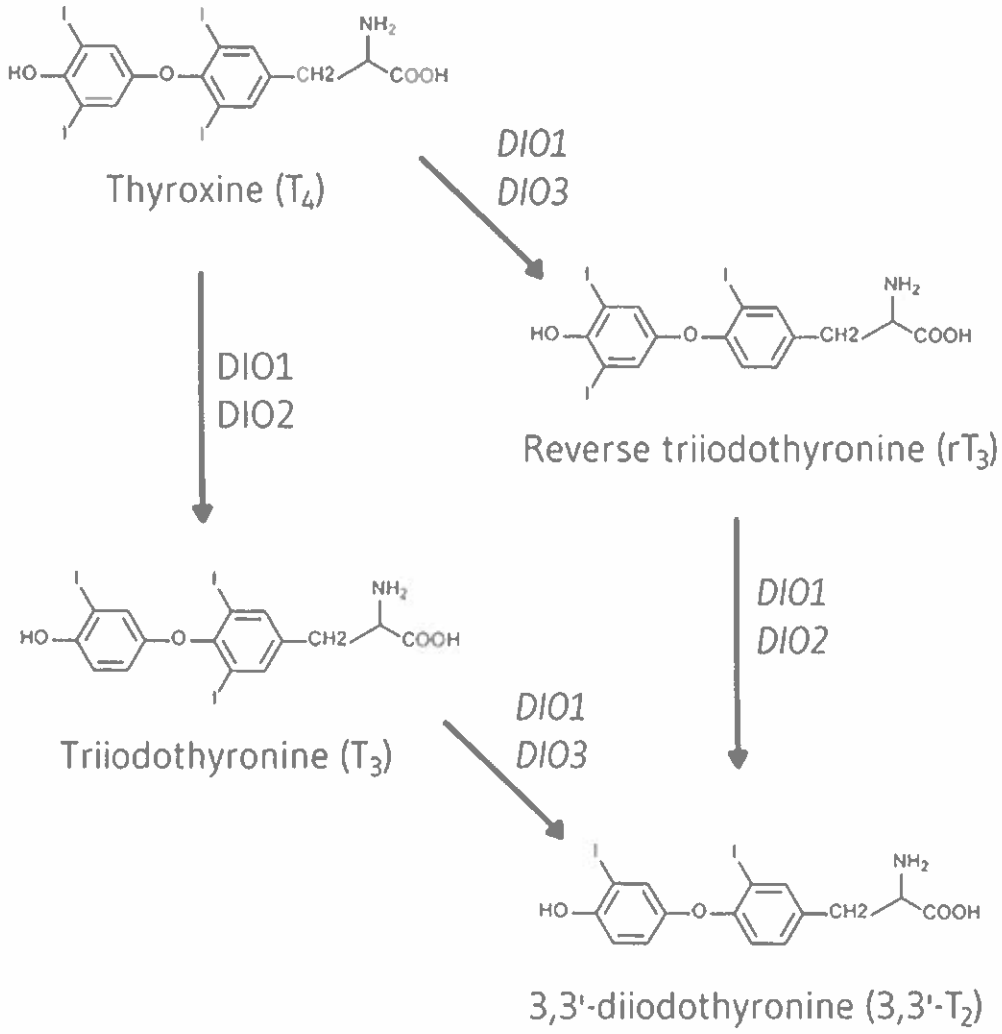
## *Thioredoxin reductases*

In mammals, three selenocysteine-containing thioredoxin reductase (TrxR) isoenzymes have been identified in the thioredoxin system: cytosolic TrxR1, mitochondrial TrxR3, and testes-specific thioredoxin glutathione reductase TGR. TrxRs are homodimeric enzymes, and each monomer contains FAD- and NADPH-binding domains and a selenocysteine-containing catalytic site. TrxRs catalyze the reduction of a wide range of substrates, including thioredoxin and protein disulfide isomerase (PDI) (see **Figure 2** above). TrxRs also serve as electron donors for the regeneration of small antioxidants, possibly recycling ascorbic acid (vitamin C), α-lipoic acid, α-tocopherol (vitamin E), and coenzyme $Q_{10}$ from their oxidized forms (6). The maintenance of thioredoxin in a reduced form by TrxRs is important for regulating cell growth and survival. The protein thioredoxin, together with TrxR1 (or TrxR3), NADPH, and FAD, constitute the thioredoxin antioxidant system involved in the reduction of antioxidant enzymes (e.g., peroxiredoxins, methionine sulfoxide reductases, and ribonucleotide reductase) and of many oxidation/reduction (redox)-sensitive signaling proteins (7). TrxR1 is one of the most investigated selenoproteins and regarded as one of the major antioxidant enzymes and redox regulators in mammalian cells.

### *Iodothyronine deiodinases (thyroid hormone deiodinases)*

The thyroid gland releases very small amounts of biologically active thyroid hormone (triiodothyronine or $T_3$) and larger amounts of an inactive form of thyroid hormone ($T_3$ precursor: thyroxine or $T_4$) into the circulation. Most of the biologically active $T_3$ in the circulation and inside cells is generated by the removal of one iodine atom from $T_4$ in a reaction catalyzed by selenium-dependent iodothyronine deiodinase enzymes. Two different selenium-dependent iodothyronine deiodinases (DIOs type 1 and 2) can deiodinate $T_4$, thus increasing circulating $T_3$, while a third iodothyronine deiodinase (DIO type 3) can convert both $T_3$ and $T_4$ to inactive metabolites (**Figure 3**) (8). Of note, inactivation of the genes encoding DIOs in rodent models has revealed a role for DIO type 1 in iodine homeostasis and the importance of DIOs type 2 and 3 in the maturation of auditory and visual systems during fetal development (8). Thus the importance of selenium in normal development, growth, and metabolism is not limited to its role in the regulation of thyroid gland function.

Figure 3. Deiodination of Thyroid Hormones




Iodothyronine deiodinases (DIOs) are selenoenzymes that catalyze the deiodination (removal of iodine) from iodothyronines. Specifically, DIO1 and DIO2 catalyze the deiodination of thyroxine ($T_4$) that generates the biologically active triiodothyronine ($T_3$). DIO3 inactivates $T_3$ and $T_4$ by removing iodine atoms from the inner ring.

## *Selenoprotein P*

Selenoprotein P (SEPP1) is predominantly produced by the liver, a major storage site for selenium, and secreted in the plasma. The full-length glycoprotein contains a selenium-rich domain with nine selenocysteine residues, as well as a thioredoxin-like catalytic site with one selenocysteine residue. SEPP1 constitutes the major form of selenium transport to peripheral tissues (9). SEPP1 also functions as an antioxidant that protects cells from oxidative damage by enabling full activity of thioredoxin reductases and glutathione peroxidases through adequate supply of selenium to extrahepatic tissues (see Glutathione peroxidases). SEPP1 appears to be especially critical for selenium homeostasis in the brain and testes where apolipoprotein E receptor 2 (apoER2)

facilitates the uptake of SEPP1. Megalin is another SEPP1-specific lipoprotein receptor that helps limit urinary selenium loss through SEPP1 re-uptake by the kidneys (10). Moreover, SEPP1 has been recently implicated in the regulation of glucose metabolism and insulin sensitivity (11).

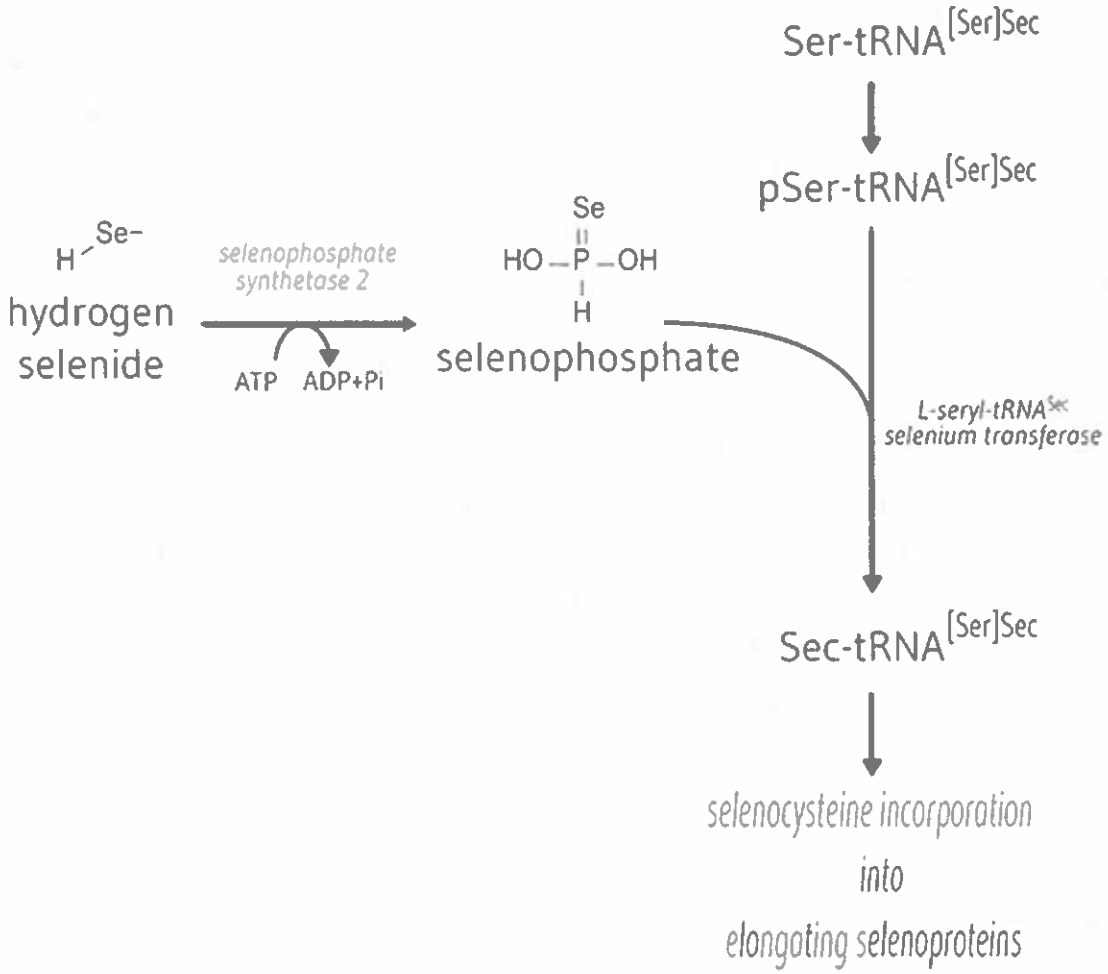
### *Selenoprotein W*

Selenoprotein W (SEPW or SelW) exists in different isoforms (homologues) and is highly conserved across species. In humans, SEPW is expressed in numerous tissues, with highest levels found in skeletal muscle and heart (12). SEPW contains a selenocysteine residue and a cysteine residue that binds to a glutathione molecule, suggesting a role in redox regulation (13). The expression of SEPW is correlated with selenium status and appears to be sensitive to low-selenium supply (14, 15). SEPW expression in the brain has been found to confer protection against oxidative stress-induced neuronal cell death (16). SEPW also appears to be a negative regulator for 14-3-3 proteins. Indeed, 14-3-3 inhibition by SEPW in breast cancer cells was found to increase cell proliferation and cell survival through increasing resistance to genotoxic stress (17). In skeletal muscle cells, SEPW was shown to reduce the binding of 14-3-3 to TAZ, allowing TAZ translocation to the nucleus and subsequent activation of muscle cell differentiation genes (18). Finally, SEPW was found to prevent the degradation of the epidermal growth factor receptor (EGFR) in breast and prostate epithelial cells in culture. EGFR is constitutively activated in many tumors, and evidence of a role for SEPW in EGFR activation and signaling may help shed light on the relationship between selenium status and cancer risk (19).

### *Selenophosphate synthetase 2*

There is no free pool of the amino acid selenocysteine in cells such that selenocysteine synthesis takes place on a specialized tRNA during the translation of selenoprotein mRNAs. The reaction is catalyzed by pyridoxal 5′-phosphate (PLP)-dependent L-seryl-tRNA$^{Sec}$ selenium transferase and uses selenophosphate (monoselenium phosphate) as the selenium donor (**Figure 4**) (20). Selenophosphate synthetase 2 is a selenoenzyme that catalyzes the ATP-dependent synthesis of selenophosphate from hydrogen selenide (**Figure 4**) (3).

Figure 4. Synthesis of Selenocysteine




Selenocysteine (Sec) synthesis takes place on a special tRNA initially aminoacetylated (charged) with the amino acid serine (Ser). Ser-tRNA$^{[Ser]Sec}$ is then phosphorylated to produce pSer-tRNA$^{[Ser]Sec}$. Pyridoxal 5'-phosphate (PLP)-dependent L-seryl-tRNA$^{Sec}$ selenium transferase catalyzes the conversion of Ser-tRNA$^{[Ser]Sec}$ into Sec-tRNA$^{[Ser]Sec}$ using selenophosphate as selenium donor. Selenophosphate is synthesized from hydrogen selenide in a reaction catalyzed by the selenoenzyme selenophosphate synthetase 2. Selenocysteine is incorporated into elongating proteins during translation. *Se, selenium; Ser-tRNA, L-seryl-tRNA; Sec-tRNA, L-selenocysteinyl-tRNA.*

## *Methionine-R-sulfoxide reductase B1 (formerly selenoprotein R)*

The methionine sulfoxide reduction system is involved in the protection against oxidative stress and is especially critical for the regeneration of proteins damaged by reactive oxygen species (ROS). Indeed, ROS can oxidize methionine residues (methionine sulfoxides) within proteins and potentially impair their activities. In humans, two stereospecific families of methionine sulfoxide reductases (MsrA and MsrB) are encoded by a single *MSRA* gene and three *MSRB* genes (*MSRB1-3*). MsrA catalyzes the reduction of the S-form of methionine sulfoxide; the R-form of methionine sulfoxide is reduced by MsrB1, 2, or 3. Only MsrB1 has been characterized as a selenoprotein with

one selenocysteine residue in its catalytic site. MsrB1 appears to be involved in the redox regulation of certain proteins. In macrophages, reorganization of the actin cytoskeleton necessary for chemotaxis and phagocytosis requires MsrB1-dependent reduction of methionine-R-sulfoxide residues within actin (21). Studies using *MSR* gene inactivation in mice have also shown that methionine sulfoxide reduction is implicated in the regulation of the methionine cycle (reviewed in 22). Both the thioredoxin (Trx) and GSH-dependent glutaredoxin (Grx) antioxidant systems have been found to reduce methionine sulfoxide reductases *in vitro* and/or *in vivo* (see **Figure 2** above) (22).

### *15 kDa selenoprotein*

15 kDa selenoprotein (selenoprotein 15; SEP15) is highly expressed in several tissues, including prostate, kidney, testes, liver, and brain (23). Although its function is not known, SEP15 was found to interact with the endoplasmic reticulum UDP-glucose:glycoprotein glucosyltransferase (UGGT), an enzyme involved in the quality control of glycoprotein folding (24, 25). Because SEP15 has a thioredoxin-like catalytic site, SEP15 is thought to either regulate UGGT activity or the redox state of UGGT substrates (26). Mice lacking a functional SEP15 were found to develop nuclear cataract (lens opacification) at a very early age suggesting that SEP15 may be critical to the quality control system of protein folding in the lens (27). SEP15 may also be implicated in anticancer mechanisms (reviewed in 28).

### *Selenoprotein S*

The mammalian selenoprotein S (known as SEPS1, SelS, or VCP-interacting membrane selenoprotein [VIMP]) is an endoplasmic reticulum (ER) membrane protein. SEPS1 is involved in the cellular response to ER stress (ER-associated degradation; ERAD) activated by the detection of misfolded proteins. SEPS1 contributes to the removal and transfer (retrotranslocation) of misfolded proteins from the ER lumen to the cytosol where proteins are tagged with ubiquitin before being degraded. A polymorphism or variation in the sequence within an ER-response element located in the *SEPS1* promoter was found to result in reduced *SEPS1* promoter activity and gene expression (29). The polymorphism corresponding to the substitution of a guanine (G) by an adenine (A) at nucleotide -105 (-105G>A) has been associated with increased plasma levels of pro-inflammatory cytokines. In addition, a case-control study recently reported that the A allele was more prevalent in individuals affected by Hashimoto thyroiditis (HT) — a T-cell-mediated autoimmune disease resulting in the destruction of thyroid cells — than in healthy controls (30). Other associations between *SEPS1* polymorphism (including -105G>A) and susceptibility to various conditions, such as preeclampsia, coronary artery disease, or gastrointestinal cancers, strongly suggest a role for this selenoprotein in the regulation of inflammatory and immune responses (31-34).

Other less well-characterized selenoproteins, which are also localized in the ER lumen and/or membrane, include selenoproteins K, M, N, and T (35).

## Nutrient interactions

### *Antioxidant nutrients*

The importance of selenium to biological systems, and specifically to the cellular redox (pro-oxidant/antioxidant) balance, is derived from its presence as selenocysteine in the catalytic site of selenoproteins (see Function). Other minerals that are critical components of antioxidant enzymes include copper (as superoxide dismutase), zinc (as superoxide dismutase), and iron (as catalase). Selenium acts in synergy with the antioxidant vitamins, vitamin C (ascorbic acid) and vitamin E (α-tocopherol), by regenerating them from their oxidized forms and promoting maximal antioxidant protection (36-38).

### *Iodine*

While iodine is an essential component of thyroid hormones, the selenium-containing iodothyronine deiodinases (DIOs) are enzymes required for the conversion of thyroxine ($T_4$) to the biologically active thyroid hormone, triiodothyronine ($T_3$) (see Function). DIO1 activity may also be involved in regulating iodine homeostasis (39). The selenoenzymes, glutathione peroxidases, also play a critical role in thyroid function because they catalyze the degradation of peroxides generated during thyroid hormone synthesis (8). The epidemiology of coexisting iodine and selenium deficiencies in central Africa, but not in China, has been linked to the prevalence of myxedematous cretinism, a severe form of congenital hypothyroidism accompanied by mental and physical retardation. Selenium deficiency may be only one of several undetermined factors that might exacerbate the detrimental effects of iodine deficiency (40). Interestingly, selenium deficiency in rodents was found to have little impact on DIO activities as it appears that selenium is being supplied in priority for adequate synthesis of DIOs at the expense of other selenoenzymes (8).

## Deficiency

Insufficient selenium intake may negatively affect the activity of several selenium-responsive enzymes, including glutathione peroxidases (GPx1 and GPx3), iodothyronine deiodinases, selenoprotein W, and methionine-R-sulfoxide reductase B1 (MsrB1). Even when severe, isolated selenium deficiency does not usually result in obvious clinical illness. Yet, compared to subjects with adequate selenium status, selenium-deficient individuals might be more susceptible to additional physiological stresses (41). Prolonged selenium deficiency may likely contribute to Keshan and Kashin-Beck diseases (see below).

## Individuals at increased risk of selenium deficiency

Selenium deficiency has been reported in chronically ill patients who were receiving total parenteral nutrition (TPN) without added selenium for prolonged periods of time. Muscular weakness, muscle wasting, and cardiomyopathy (inflammation and damage to the heart muscle) have been observed in these patients. Nowadays, TPN solutions are routinely supplemented with selenium. The risk of selenium deficiency may be increased following bariatric surgery or in severe gastrointestinal conditions, such as Crohn's disease. Some specialized medical diets like those used

to treat certain metabolic disorders, including phenylketonuria, homocystinuria, and maple syrup urine disease, need to be supplemented with selenium to ensure optimum selenium status in patients (42).

## Keshan disease

Keshan disease is a fatal form of dilated cardiomyopathy that was first described in young women and children in a selenium-deficient region in China. The acute form of the disease is characterized by the sudden onset of cardiac insufficiency, while the chronic form results in moderate-to-severe heart enlargement with varying degrees of cardiac insufficiency (43). The incidence of Keshan disease is closely associated with very low dietary intakes of selenium and poor selenium nutritional status. Selenium supplementation (in the form of sodium selenite; $Na_2SeO_3$) was found to protect people from developing Keshan disease but could not reverse heart muscle damage once it had occurred (43). A recent case-control study reported that selenium-responsive glutathione peroxidase 1 (GPx1) activity was significantly lower in Keshan patients compared to healthy individuals. Interestingly, a specific *GPX1* polymorphism resulting in a proline-to-leucine transition at position 198 (Pro198Leu) is associated with a reduction in GPx1 activity and found to be more prevalent in Keshan patients. This *GPX1* polymorphism might confer a greater susceptibility to Keshan disease in carriers with low selenium nutritional status (44).

While selenium deficiency is a major etiological factor of Keshan disease, the seasonal and annual variation in disease occurrence suggested that other factors, especially an infectious agent, might be involved in addition to selenium deficiency (45). Coxsackie virus B3 is one virus type that has been isolated from Keshan patients, and animal studies have shown that this virus was capable of causing an inflammation of the heart (myocarditis) in selenium-deficient mice. Studies in mice also indicated that oxidative stress induced by selenium deficiency could result in changes in the viral genome, such as to convert a relatively harmless strain of coxsackie virus B3 into a myocarditis-causing strain (43). Although not proven in Keshan disease, it is possible that selenium deficiency may increase the virulence of viruses with the potential to invade and damage the heart muscle (46).

## Kashin-Beck disease

Kashin-Beck disease (KBD) is another endemic condition that affects an estimated 2.5 million people in Tibet, northern and central China, North Korea, and southeastern Siberia (47). KBD is characterized by the degeneration of articular cartilage between joints (osteoarthritis) that can result in joint deformities and dwarfism in the most severe forms of the disease. The disease affects children as young as two years old. As with Keshan disease, KBD is prevalent in selenium-deficient provinces and thus generally affects people with very low selenium intakes (47). Recent studies have suggested that increased susceptibility to KBD in selenium-deficient populations might result from a reduced antioxidant protection associated with polymorphisms in *GPX* genes

(48, 49). Yet, the etiology appears to be multifactorial, as a number of other causative factors have been suggested for KBD, including fungal toxins in grain, iodine deficiency, and contaminated drinking water (43).

Recent meta-analyses of a few small trials and prospective cohort studies have indicated that improving selenium nutritional status in children living in endemic areas may help reduce KBD incidence (50). Also, there is limited evidence to suggest that selenium supplementation could be useful in the treatment of patients with KBD. A meta-analysis of 10 randomized controlled trials reported a significant increase in the repairing rate of bone lesions in KBD children supplemented with sodium selenite for at least one year (51). Larger trials of higher quality are needed to assess whether selenium supplementation could result in disease remission.

## The Recommended Dietary Allowance (RDA)

The dietary reference intakes (DRIs) for selenium were last revised in 2000 by the Food and Nutrition Board (FNB) of the US Institute of Medicine. The most recent RDA is based on the estimated average requirement (EAR) needed to maximize antioxidant enzyme glutathione peroxidase (GPx) activity in plasma (52).

Table 1. Recommended Dietary Allowance (RDA) for Selenium

| Life Stage | Age | Males (µg/day) | Females (µg/day) |
|---|---|---|---|
| Infants | 0-6 months | 15 (AI) | 15 (AI) |
| Infants | 7-12 months | 20 (AI) | 20 (AI) |
| Children | 1-3 years | 20 | 20 |
| Children | 4-8 years | 30 | 30 |
| Children | 9-13 years | 40 | 40 |
| Adolescents | 14-18 years | 55 | 55 |
| Adults | 19 years and older | 55 | 55 |
| Pregnancy | all ages | - | 60 |
| Breast-feeding | all ages | - | 70 |

Of note, the third National Health and Nutrition Examination Survey (NHANES III) reported that over 99% of the US participants had serum selenium concentrations consistent with selenium requirements being met (52), suggesting selenium supplementation is not needed for Americans.

## Disease Prevention

## Cancer

### *Animal studies*

There has been considerable research on the effect of selenium supplementation on the incidence of cancer in animals. More than two-thirds of over 100 published studies in 20 different animal models of spontaneous, viral, and chemically induced cancers found that selenium supplementation (to at least adequate intake levels) significantly reduces tumor incidence, especially in comparison to selenium-deficient diets (53). Evidence of cancer-inhibiting effects of selenium has provided a strong rationale for investigating potential associations between selenium intake and cancer risk in humans.

### *Observational studies*

Most of the early epidemiological evidence from case-control and nested case-control studies suggested either null or inverse associations between selenium exposure and risk of site-specific cancers (54). Markers of selenium exposure include toenail and blood selenium content, as well as plasma glutathione peroxidase (GPx) activity. However, it is not clear whether they adequately reflect selenium exposure from dietary and supplemental sources (see Sources) or selenium distribution in tissues and organs that may be affected by cancer. In the Danish Prospective Diet, Cancer, and Health prospective study that followed over 57,000 men and women for 14 years, the risk of rectal cancer was found to be 42% higher in current smokers compared to nonsmokers. No difference between smokers and nonsmokers regarding supplemental and dietary intakes of antioxidant micronutrients, including selenium, was found to contribute to the association of smoking and rectal cancer (55). Yet, because studies have consistently reported lower blood selenium concentrations and GPx activities in smokers compared to nonsmokers (reviewed in 56), estimation of selenium intakes might not be a reliable marker of selenium exposure in this population. Also, the chemical forms of selenium found in food are varied (see Sources) and may have very different biological and toxicological effects (57, 58).

A recent Cochrane review included 55 completed observational studies — mostly with a nested case-control design — published over three decades (54). A meta-analysis of 16 of these observational studies, including over 144,000 individuals, reported that higher versus lower selenium status was associated with a 31% lower risk of cancer at any site and a 40% lower risk of cancer-related mortality. A significantly lower risk was reported for bladder cancer (5 studies) and prostate cancer (17 studies); however, higher selenium status was not inversely associated with risks of breast cancer (8 studies), lung cancer (12 studies), colorectal cancer (5 studies), and gastric cancer (5 studies) (54). Another meta-analysis of 16 observational studies reported an inverse relationship between breast cancer and serum selenium concentrations (59). Gender differences in cancer susceptibility have been reported in some studies, although consistent evidence of different effects in men and women appears to be lacking.

Single nucleotide variations (polymorphisms) in the sequence of genes can modify gene expression level and the stability and activity of the synthesized proteins. For example, a proline-to-leucine transition caused by a specific polymorphism in the *GPX1* gene (rs1050450 C>T) is associated with reduced GPx1 enzymatic activity. The description of several polymorphisms in genes encoding selenoproteins has led to evaluation of possible associations with selenium status and cancer incidence. Notably, certain polymorphisms in genes coding for selenoproteins have been associated with increased risks of gastric and colorectal cancers (reviewed in 60). Additionally, a number of studies have investigated the effect of selenoprotein polymorphisms on the relationship between selenium status and prostate cancer risk. A nested case-control study within the EPIC-Heidelberg cohort has combined genotyping for several selenoprotein variants with markers of selenium status (61). Briefly, the study showed that a *GPX1* gene polymorphism (rs1050450 C>T) affected the association between selenium concentrations and prostate cancer risk. Specifically, selenium concentrations were found to be inversely associated with prostate cancer risk only among carriers of the *GPX1 T* allele. Additional variants in selenoprotein genes may mitigate the effect of selenium status on the risk of prostate cancer (62, 63). In another nested case-control study within the Physicians' Health Study (PHS), individuals in the highest versus lowest quartile of selenium concentrations were found to have a reduced risk of prostate cancer-related mortality except if carrying a specific variant in 15kDa selenoprotein gene (*SEP15* rs561104 G>A) (64). More research is needed to further unravel the mechanisms underlying the influence of gene-diet interactions on the risk of developing cancer.

## *Intervention trials*

**Community-based studies**: A very early intervention study of selenium supplementation was undertaken in China among a general population of 130,471 individuals living in a high-risk area for viral hepatitis B infection and liver cancer. The trial provided table salt enriched with sodium selenite to the population of one township (20,847 people) using four other townships as controls. During an eight-year follow-up period, the average incidence of liver cancer was reduced by 35% in the selenium-supplemented population, while no reduction was found in the control populations. In a clinical trial in the same region, 226 individuals with evidence of chronic hepatitis B infection were supplemented daily with either 200 µg of selenium in the form of a selenium-enriched yeast tablet or a placebo yeast tablet. During the 4-year follow-up period, 7 out of 113 individuals on the placebo developed primary liver cancer, while none of the 113 subjects supplemented with selenium developed liver cancer (65).

**Randomized controlled trials**: The double-blind, placebo-controlled Nutritional Prevention of Cancer (NPC) study in 1,312 older adults with a history of nonmelanoma skin cancer found that supplementation with 200 µg/day of selenium-enriched yeast (selenized yeast) for an average of 7.4 years resulted in a 52% decrease in prostate cancer incidence in men (reviewed in 66). The protective effect of selenium supplementation was greatest in men with lower baseline plasma selenium and prostate-specific antigen (PSA) levels. A reduced incidence in lung, colorectal, and total cancer was also associated with supplementation with 200 µg/day (67) but not with 400

µg/day of selenium-enriched yeast (68). Besides, selenium supplementation increased the risk of one type of skin cancer (squamous cell carcinoma) by 25%. A larger randomized, placebo-controlled intervention trial (the SELECT study) in more than 35,000 middle-aged and selenium-replete men, randomized to receive selenium (in the form of selenomethionine, 200 µg/day) and/or vitamin E supplementation, was halted because of concerns regarding an increased risk of type 2 diabetes with selenium and increased risk of prostate cancer with vitamin E (69, 70). In addition, the supplementation of selenium, alone or together with vitamin E, did not show any benefits regarding the risk of prostate, lung, or colorectal cancers after 5.5 years of follow-up (71, 72). Outcomes from other smaller trials (reviewed in 66) also suggested either a lack of an effect or the possibility of an increased risk of cancer. The lack of a beneficial effect of selenium supplementation was supported in a recent meta-analysis of randomized controlled trials (54).

## Cardiovascular disease

Low activity levels of the selenoenzymes, glutathione peroxidases (GPx), have been reported in oxidative stress-related diseases, including cardiovascular disease (CVD) (73). Theoretically, the maintenance of an optimal selenium status has the potential to protect against oxidative stress (including lipid peroxidation) and could eventually prevent chronic inflammation and cardiovascular disorders. Yet, analyses of cross-sectional data from 13,887 US adults included in the Third National Health and Nutrition Examination Survey (NHANES III, 1988-1994) failed to show any significant associations between serum selenium concentrations and mortality from CVD, coronary artery/heart disease (CAD), or stroke (74). In addition, while individuals with renal insufficiency are at higher risk of developing CAD compared to those with normal kidney function, that risk was not found to be greater with low rather than normal selenium concentrations in serum (≤98 ng/mL vs. >98 ng/mL) (75).

A cross-sectional study based on NHANES 2003-2004 data from 2,638 participants ages 40 years and over found that the risk of high blood pressure (hypertension), a major contributing factor for CVD, was 73% higher in individuals in the upper versus lowest quintile of serum selenium concentrations (≥150 ng/mL vs. <122 ng/mL) (76). Yet, a recent systematic review of the literature failed to find enough evidence to support any relationship between serum selenium concentrations and hypertension (77). Besides, a few observational studies have also reported associations between normal-to-high selenium status and elevated serum lipid levels in selenium-replete populations, speculating that selenium might interfere with lipid metabolism and adversely affect cardiovascular health (78, 79). At present, randomized controlled trials have not provided consistent results regarding the effect of selenium supplementation on lipid levels nor have they demonstrated any additional cardiovascular benefits of selenium in individuals with suboptimal or optimal selenium intakes (80).

# Disease Treatment

## Immune dysfunction

Selenium deficiency has been associated with impaired immunity and chronic inflammation (81). A considerable amount of research conducted in cell culture and animal models indicates that selenium plays essential roles in regulating the migration, proliferation, differentiation, activation, and optimal function of immune cells, thus influencing innate immunity, B-cell dependent antibody production, and T-cell immunity (reviewed in 82). Recent evidence on the role of selenium and selenoproteins in the production of lipid mediators (called eicosanoids) involved in inflammatory responses suggests that selenium supplementation might mitigate dysfunctional inflammatory responses that contribute to the pathogenesis of many chronic health conditions (83). At present, randomized controlled trials are needed to evaluate the potential benefits of selenium supplementation in inflammatory disorders, such as asthma (84) and inflammatory bowel disease (85).

## Infectious diseases

### *HIV/AIDS*

In areas of widespread malnutrition, deficiencies in micronutrients (including selenium) are common in individuals infected by the human immunodeficiency virus (HIV) that causes acquired immunodeficiency syndrome (AIDS). Before antiretroviral therapy (ART) became the standard for HIV treatment, observational studies had consistently reported associations between low serum selenium concentrations and HIV infection in well-nourished subjects (86). Poor selenium status has been linked to increased risks of dilated cardiomyopathy and mortality in HIV-infected children and adults, as well as mother-to-child HIV transmission and perinatal mortality (reviewed in 87. Early laboratory studies have suggested that HIV might disrupt normal antioxidant defenses in infected T-cells by reducing the levels of selenoproteins, i.e., thioredoxine reductases and glutathione peroxidases (87). Interestingly, a recent cross-sectional study found that HIV-seropositive individuals receiving ART for more than two years had undetectable plasma viral loads, higher CD4 lymphocyte T-cell counts, and adequate serum selenium concentrations compared to ART-naïve subjects (88). Because the antioxidant activity of selenoproteins may interfere with viral replication in HIV-infected immune cells (89,90), it has been suggested that selenium supplementation might serve as a potential adjunct to ART for HIV patients.

A few trials of selenium supplementation in HIV-infected individuals have been conducted. A randomized, double-blind, placebo-controlled trial in 186 HIV-positive adults initially found that selenium supplementation at 200 µg/day for two years significantly decreased the rate of hospital admissions (91). Another randomized, double-blind, placebo-controlled trial in 174 HIV-positive individuals reported that 200 µg/day of selenium supplementation (in the form of selenium-enriched yeast) for nine months increased serum selenium concentrations, improved CD4 lymphocyte T-cell count, and prevented any progression of the HIV viral load (92). In a third double-blind trial in Tanzania, 913 pregnant women between 12 and 27 weeks' gestation were randomized to receive 200 µg/day of selenium (as selenomethionine) or a placebo until six months after birth. Selenium supplementation had no effect on maternal CD4, CD8, and CD3 T-cell counts and on HIV

viral load, but it significantly decreased the risk of acute or persistent diarrhea (93, 94). In addition, the risk of death between six weeks and six months postpartum was significantly reduced in infants of mothers supplemented with selenium compared to placebo (94).

A recent four-armed trial in Botswana randomized 878 HIV-positive adults at an early stage of the infection to receive either a placebo treatment, multivitamins (vitamins B, C, and E), 200 µg/day of selenium, or both multivitamins and selenium for 24 months (95). Unlike selenium alone, supplementation with multivitamins (with or without selenium) reduced the risk of immune decline by significantly increasing the time before ART initiation became necessary (i.e., when CD4 T-cell count fell below 251 cells/mcL) compared to placebo. In the study, a combined outcome of (1) CD4 T-cell count falling below 251 cells/mcL; (2) occurrence of AIDS-defining conditions; and (3) AIDS-related death — whichever happened first — was used to evaluate disease progression in the different arms of treatment. Compared to placebo, there was a longer period of time from randomization to the date of the composite outcome in individuals supplemented with multivitamins plus selenium, but not in those who received multivitamins or selenium alone (95). More research is needed to replicate these preliminary results, especially in settings and communities where malnutrition hastens the progression of HIV infection and access to antiretroviral therapy may be limited.

### *Sepsis*

The systemic inflammatory response syndrome (SIRS) results from a systemic inflammatory response that can be due to an infection (sepsis) (96). Severe sepsis and septic shock — defined as persistent sepsis-induced low blood pressure — are associated with elevated mortality rates in critically ill patients (96, 97). Because systemic inflammatory responses involve excessive oxidative stress, it has been suggested that providing antioxidant nutrients like selenium may improve the outcome of critically ill patients in intensive care units. Two recent meta-analyses of randomized controlled trials found that intravenous selenium supplementation (as sodium selenite) in critically ill patients with SIRS, sepsis, or septic shock resulted in significantly reducing the risk of mortality by 17% to 27% (98, 99). More trials are needed to identify the appropriate schedule of selenium administration (in terms of dose, route, and treatment duration) and to assess additional outcomes (e.g., infectious complications and length of hospital stay).

## Autoimmune thyroid diseases

Hashimoto thyroiditis (HT; chronic autoimmune thyroiditis) is an autoimmune disease characterized by T-cell infiltration in the thyroid gland and circulating autoantibodies (predominantly raised against thyroid peroxidase), causing prolonged inflammation, tissue damage, and hypothyroidism (8). While the function of the thyroid gland of healthy individuals is usually protected from variations in selenium supply, selenium deficiency and genetic polymorphisms affecting the activity of selenoproteins might be potential contributing factors to autoimmune thyroid diseases. A recent systematic review (100) identified four randomized controlled trials that evaluated the effect of selenium supplementation as an adjunct treatment to

$T_4$ replacement therapy (levothyroxine) in HT patients (101-104). While three out of four studies suggested a reduction in levels of circulating autoantibodies, none of them provided information on whether selenium may improve mood- and health-related symptoms to allow for a decreased dosage of levothyroxine. Another randomized controlled trial found that selenium supplementation improved the well being of patients affected by another autoimmune thyroid disease leading to hyperthyroidism (Graves disease) (105). The results of two ongoing, randomized, placebo-controlled trials — the CATALYST in HT patients and the GRASS trial in patients with Graves disease — may provide insight into an effect of selenium on thyroid-specific quality-of-life criteria and inform clinical decision making (106, 107).

## Sources

### Food sources

The richest food sources of selenium are organ meats and seafood, followed by muscle meats. Drinking water is not considered to be a significant source of selenium in North America. However, in areas, where high levels of selenium in soil contribute to the selenium content of the water, higher levels of selenium may be found in wells used for drinking water (108). In general, there is wide variation in the selenium content of plants and grains, especially because some plants, including garlic, Brazil nuts, and multiple *Brassica* species, tend to accumulate selenium ('selenium accumulators'), while other assimilate selenium to a lesser extent ('non-accumulators'). The assimilation of selenium by plants also depends on soil selenium content. Brazil nuts grown in areas of Brazil with selenium-rich soil may provide more than 100 µg of selenium in one nut, while those grown in selenium-poor soil provide 10 times less (109). In the US, grains are a good source of selenium, but fruit and vegetables tend to be relatively poor in selenium.

Various chemical forms (species) of selenium are found in selenium accumulators, including selenate (inorganic selenium), selenomethionine, selenocysteine, selenium-methyl-selenocysteine, and γ-glutamyl-selenium-methyl-selenocysteine. Although the two latter compounds are predominant in plants of the *Allium* and *Brassicaceae* families (which include garlic, onion, and broccoli), wheat, other grains (including Brazil nuts), and soy are rich in selenomethionine and contain smaller amounts of selenocysteine and selenate. Less is known about selenium species and distribution in dietary sources of animal origin. Animal nutrition and growth conditions certainly contribute to the selenium species formed and their relative quantities, and it is assumed that the metabolic pathway of dietary selenium in animals is similar to that in humans. Selenocysteine is predominantly formed in animals fed inorganic selenium, while selenomethionine is derived from dietary sources of plant origin (reviewed in 110).

In the US, the national survey NHANES III reported mean dietary intakes ranging between 100.5 µg/day and 158.5 µg/day in adults ages 19-50 years (52). **Table 2** lists some good food sources of selenium and their selenium content in micrograms (µg). For more information on the selenium content of specific foods, search USDA's FoodData Central.

Table 2. Some Common Food Sources of Selenium

| Food | Serving | Selenium (µg) |
|---|---|---|
| Brazil nuts (from selenium-rich soil) | 1 ounce (6 kernels) | 543.5* |
| Tuna (yellowfin, cooked, dry heat) | 3 ounces | 92.0 |
| Oysters (Pacific, raw) | 3 ounces | 65.4 |
| Clams (mixed, cooked, steamed) | 3 ounces | 54.4 |
| Halibut (Atlantic and Pacific, cooked, dry heat) | 3 ounces | 47.1 |
| Shrimp (cooked, steamed) | 3 ounces | 42.1 |
| Salmon (Chinook, cooked, dry heat) | 3 ounces | 39.8 |
| Noodles (egg, cooked, enriched) | 1 cup | 38.2 |
| Crab (queen, cooked, steamed) | 3 ounces | 37.7 |
| Pork (lean, tenderloin, cooked, roasted) | 3 ounces | 32.5 |
| Beef (lean, plate steak, cooked, grilled) | 3 ounces | 30.6 |
| Chicken (light-meat, cooked, roasted) | 3 ounces | 25.8 |
| Rice (brown, long-grain, cooked) | 1 cup | 19.1 |
| Sunflowers seed kernels (dried) | ¼ cup | 18.6 |
| Whole-wheat bread | 2 slices | 16.4 |
| Milk (fat free or skim) | 8 fluid ounces (1 cup) | 7.6 |

*Above the tolerable upper intake level (UL) of 400 µg/day.

## Supplements

Selenium supplements are available in several forms. Sodium selenite and sodium selenate are inorganic forms of selenium. Sodium selenate is almost completely absorbed, but a significant amount is excreted in the urine before it can be incorporated into proteins. Sodium selenite is only about 50% absorbed but is better retained than selenate once it is absorbed. Selenomethionine is about 90% absorbed (52); however, only about 34% may then actually be converted to free selenomethionine (111). Selenomethionine and selenium-enriched yeast, which mainly supply selenomethionine, are also available as supplements. The consumer should be aware that some forms of selenium-enriched yeast on the market contain yeast plus mainly inorganic forms of selenium.

Although both inorganic and organic forms of selenium can be metabolized to selenocysteine by the body and incorporated into selenoenzymes, they may not equally contribute to the maintenance of an adequate selenium status. In intervention trials, supplementation with an organic form of selenium (selenomethionine) more effectively increased blood selenium concentrations compared to supplementation with inorganic forms (i.e., sodium selenite and sodium selenate) (110). Yet, inorganic forms may increase plasma glutathione peroxidase (GPx) activity more effectively than organic forms (reviewed in 112). It has also been suggested that the incorporation of selenomethionine in place of methionine into tissue proteins may ensure that selenium is available upon protein turnover (110).

## Selenium-enriched foods

Selenium-enriched foods have been of interest to scientists, especially because of the suggestion that some of the chemical forms of selenium produced by plants might be more potent modifiers of cancer risk than those currently available in supplements. Although there is currently no evidence of long-term health benefits associated with the consumption of selenium-enriched foods, results from animal studies and intervention trials suggest that protein-based sources of selenium are more effective at increasing GPx activity than selenium-enriched yeast and selenomethionine (112). Food fortification may also represent a cost-effective strategy to improve selenium nutritional status in populations at risk of inadequacy (113).

# Safety

## Toxicity

Although selenium is required for health, high doses of selenium can be toxic. Acute and fatal toxicities have occurred with accidental or suicidal ingestion of gram quantities of selenium. Clinically significant selenium toxicity was reported in 13 individuals after taking supplements that contained 27.3 mg (27,300 µg) per tablet due to a manufacturing error. Chronic selenium toxicity (selenosis) may occur with smaller doses of selenium over long periods of time. The most frequently reported symptoms of selenosis are hair and nail brittleness and loss. Other symptoms may include gastrointestinal disturbances, skin rashes, a garlic breath odor, fatigue, irritability, and neurologic disorders. In an area of China with a high prevalence of selenosis, toxic effects occurred with increasing frequency when blood selenium concentrations reached a level corresponding to an intake of 850 µg/day.

The Food and Nutrition Board (FNB) of the US Institute of Medicine set the tolerable upper intake level (UL) for selenium at 400 µg/day in adults based on the prevention of hair and nail brittleness and loss and early signs of chronic selenium toxicity (52). The UL of 400 µg/day for adults includes both selenium obtained from food and selenium from supplements (**Table 3**).

Table 3. Tolerable Upper Intake Level (UL) for Selenium

| Age Group | UL (µg/day) |
|---|---|
| Infants 0-6 months | 45 |
| Infants 6-12 months | 60 |
| Children 1-3 years | 90 |
| Children 4-8 years | 150 |
| Children 9-13 years | 280 |
| Adolescents 14-18 years | 400 |
| Adults 19 years and older | 400 |

## Do selenium supplements increase the risk for type 2 diabetes mellitus?

A few studies have examined the relationship between selenium status and type 2 diabetes mellitus. In the cross-sectional analysis of NHANES III (1988-1994) data from 8,876 adult participants, the highest versus lowest quintile of serum selenium concentrations (≥137 ng/mL vs. <111 ng/mL) was associated with an increased risk of type 2 diabetes (114). Data analyses from 917 participants (≥40 years of age) of NHANES 2003-2004 also indicated an increased prevalence of type 2 diabetes in the highest versus lowest quartile of serum selenium concentrations (≥147 ngm/L vs. <124 ng/mL). Individuals in the highest versus lowest quartile of serum selenium concentrations also had higher levels of plasma glucose and glycated hemoglobin, suggestive of poor glycemic control (115). The randomized, double-blind, placebo-controlled study in 1,312 participants in the Nutritional Prevention of Cancer (NPC) trial found that selenium supplementation (200 µg/day; mean follow-up of 7.7 years) significantly increased the risk for type 2 diabetes in participants in the highest tertile of baseline plasma selenium concentrations (116). In addition, in the Selenium and Vitamin E Cancer Prevention Trial (SELECT), more cases of type 2 diabetes were found in the selenium group (200 µg/day; median follow-up of 5.5 years) than in the placebo group, but this was only a trend and not statistically significant (72).

At present, the mechanisms behind these observations are not well understood. An increase in insulin sensitivity has been reported in individuals with congenital (inborn) deficiency of most selenoproteins (117). Results from several animal studies also indicated that selenium supplementation and selenoproteins may interfere with insulin action and glucose homeostasis (reviewed in 118). On the other hand, recent studies have found that impaired glucose metabolism in patients with type 2 diabetes may affect SEPP1 expression and selenium homeostasis (11, 119, 120). While more research is needed to fully understand the interplay between carbohydrate metabolism and selenium homeostasis, the use of selenium supplements is considered unlikely to increase the risk of type 2 diabetes in healthy individuals but should be discouraged in those with high selenium status and/or at increased risk for developing type 2 diabetes (118).

## Drug interactions

At present, few interactions between selenium and medications have been reported (121). For example, the anticonvulsant drug valproic acid and the chemotherapeutic agent cisplatin may lower circulating selenium concentrations in treated subjects (122, 123). Also, supplemental sodium selenite was found to reduce the toxicity of the antibiotic nitrofurantoin and the herbicide paraquat in animal studies (124).

### *Antioxidant supplements and statins*

A three-year randomized controlled trial in 160 patients with documented coronary artery disease (CAD) and low high-density-lipoprotein (HDL) levels found that a combination of simvastatin (Zocor) and niacin increased HDL2 subfraction levels, inhibited the progression of coronary artery stenosis (narrowing), and decreased the frequency of cardiovascular events (125). Surprisingly, when an antioxidant combination (1,000 mg of vitamin C, 800 IU of vitamin E (*d*-α-tocopherol), 100 µg of selenium, and 25 mg of β-carotene daily) was taken with the simvastatin-niacin combination, the protective effects were diminished. However, the individual contribution of selenium cannot be established, and other studies have reported that antioxidant vitamins alone could interfere with the action of HDL-raising drugs, including statins (126).

## Linus Pauling Institute Recommendation

The average American diet is estimated to provide about 100 µg/day of selenium, an amount that is well above the current RDA (55 µg/day) and appears sufficient to optimize plasma and cellular glutathione peroxidase (GPx) activity. A recent 10-week randomized controlled study in healthy British adults (ages, 50-64 years) estimated that about 105 µg/day of total selenium intake was required to maximize the plasma concentrations of selenoprotein P (SEPP1), another useful biomarker of selenium status (127). However, a similar trial in an American cohort with higher baseline plasma selenium concentrations found no effect of selenium supplementation on SEPP1 concentrations (128). While the amount of selenium in multivitamin/mineral (MVM) supplements varies considerably, MVM supplements rarely provide more than the Daily Value (DV) of 70 µg. Eating a varied diet and taking a daily MVM supplement should provide sufficient selenium for most people in the US and help improve selenium status in populations with lower selenium intakes outside the US.

### Men

At present, the effect of selenium supplementation on cancer risk is not clear enough to support a general recommendation for an extra selenium supplement, especially in men with serum selenium concentrations consistent with adequate selenium intakes. The SELECT trial found that 200 µg/day of selenium did not reduce the risk of prostate cancer (72), refuting the results of the NPC trial (see Cancer). Another recent multicenter, randomized, double-blind, placebo-controlled trial (the Negative Biopsy Trial) in 699 men at high risk of prostate cancer found no effect of either 200 µg/day or 400 µg/day of selenium on prostate cancer risk during a mean follow-up of 36

months (129). In addition, because current evidence suggests a U-shaped relationship between selenium status and prostate cancer risk (130), men should avoid taking supplemental selenium that would exceed 200 µg/day.

### Women

At present, there is no clinical evidence showing that selenium supplementation above recommended levels decreases the risk of breast cancer although some, but not all, observational studies have found an inverse relationship between selenium status and breast cancer in women (59).

### Older adults (>50 years)

Aging has not been associated with significant changes in the requirement for selenium. A five-year, randomized, double-blind, placebo-controlled trial in healthy Danish older people (ages at inclusion, 60-74 years) found that selenium supplementation (100-300 µg/day) had little-to-no impact on circulating levels of antioxidant enzymes, including GPx (131).

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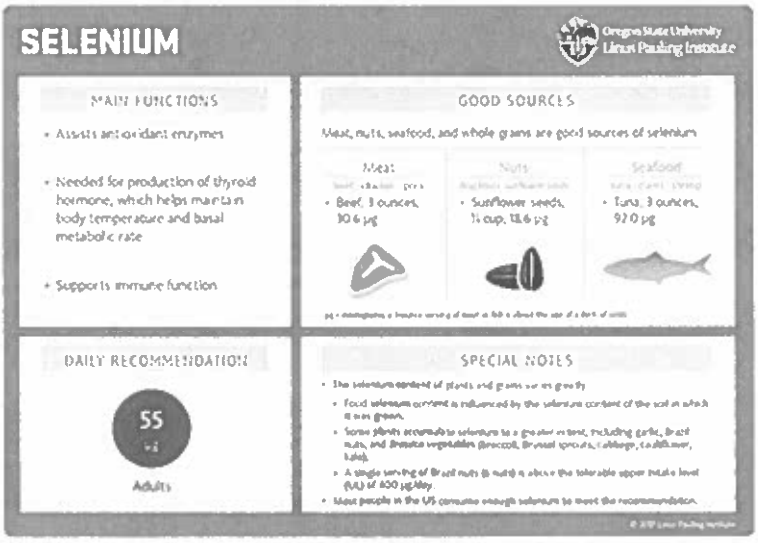
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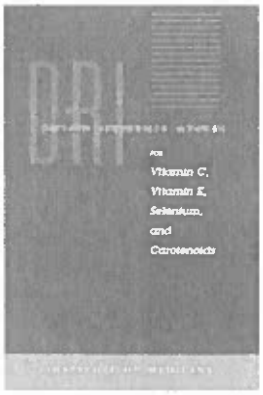
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# Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids (2000)

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# 2

# Vitamin C, Vitamin E, Selenium, and β-Carotene and Other Carotenoids: Overview, Antioxidant Definition, and Relationship to Chronic Disease

## OVERVIEW

This report focuses on vitamin C, vitamin E, selenium, and β-carotene and other carotenoids (α-carotene, β-cryptoxanthin, lutein, lycopene, and zeaxanthin). These compounds have frequently been called dietary antioxidants since in some cases they counteract oxidative damage to biomolecules (Halliwell, 1996), and the possibility exists that increased intakes of these compounds may protect against chronic disease. Although the term dietary antioxidants is a convenient description, these compounds are multifunctional, and some of the actions observed in vivo may not represent an antioxidant function, even though the compounds have been classified as antioxidant nutrients (Sies and Stahl, 1995).

Therefore, in this report the above compounds were evaluated with respect to their role in human nutrition, without limiting the investigation to antioxidant properties. Information was reviewed regarding the minimum amount of these nutrients required to prevent deficiency diseases, as well as the amounts that might impact on chronic diseases, regardless of whether the effect was an antioxidant effect or not. Resolution of any impact of these compounds on chronic disease will require evaluation of the many human intervention trials that are still under way (Table 2-1).

Four main tasks were assigned to the Dietary Reference Intakes Panel on Dietary Antioxidants and Related Compounds. The first task was to develop a definition of a dietary antioxidant; the second



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**TABLE 2-1** Intervention Trials: Antioxidants and Chronic Diseases

| Study | Country | Study Type[a] | Study Population |
|---|---|---|---|
| Skin Cancer Prevention Study (Greenberg et al., 1990) | U.S.[c] | Secondary prevention; randomized, double-blind, placebo-controlled intervention | 1,805 men and women with recent nonmelanoma skin cancer, aged 40–89 y |
| Linxian Cancer Prevention Study (Blot et al., 1993) | China | Primary prevention; randomized, double-blind, placebo-controlled intervention | 29,584 poorly nourished men and women, aged 40–69 y |
| α-Tocopherol, β-Carotene Cancer Prevention Study (ATBC Cancer Prevention Group, 1994) | Finland | Primary prevention; randomized, double-blind, placebo-controlled intervention | 29,133 male cigarette smokers, aged 50–69 y |
| Polyp Prevention Study (Greenberg et al., 1994) | U.S. | Secondary prevention; randomized, double-blind, placebo-controlled intervention | 864 men and women with recent nonmelanoma skin cancer |
| The β-Carotene and Retinol Efficacy Trial (Omenn et al., 1996) | U.S. | Primary prevention; randomized, double-blind, placebo-controlled intervention | 14,254 heavy smokers and 4,060 asbestos workers, aged 45–69 y |
| Cambridge Heart Antioxidant Study (Stephens et al., 1996) | U.K.[c] | Secondary prevention; randomized, double-blind, placebo-controlled intervention | 2,002 patients with coronary atherosclerosis, mean age 62 y |

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| | Duration of Treatment (yr) | Daily Dose | Primary Disease Outcome | Results[b] |
|---|---|---|---|---|
| | 5 | 50 mg β-carotene | Skin cancer | No effect on occurrence of new nonmelanoma skin cancers |
| | 5.25 | 15 mg β-carotene, 30 mg α-tocopherol, 50 mg selenium | Cancer | 9% reduction in total mortality; 13% decrease in cancer mortality; 21% decrease in stomach cancer deaths; 10% decrease in cerebrovascular mortality (nonsignificant) |
| | 6 | 50 mg α-tocopherol and/or 20 mg β-carotene | Lung cancer | 50% increase in hemorrhagic stroke deaths among vitamin E group; 11% increase in ischemic heart disease deaths among β-carotene group; 18% increase in lung cancer among β-carotene group; no effect of vitamin E on lung cancer |
| | 3 | 25 mg β-carotene, 1,000 mg vitamin C, 100 mg α-tocopherol | Colorectal cancer | No reduced incidence of adenomas |
| | 4 | 30 mg β-carotene, 25,000 IU retinol (as retinyl palmitate) | Lung cancer | 28% increase in lung cancer; 26% increase in CVD[c] (nonsignificant); 17% increase in total mortality among treatment group |
| | 1.1 | 100 or 800 IU (268 or 537 mg) α-tocopherol | CVD death or nonfatal MI[d] | 77% decrease in risk of subsequent nonfatal MI; no benefit on cardiovascular mortality |

*continued*

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**TABLE 2-1** Continued

| Study | Country | Study Type[a] | Study Population |
|---|---|---|---|
| Physicians' Health Study (Hennekens et al., 1996) | U.S. | Primary prevention: randomized, double-blind, placebo-controlled intervention | 22,071 male physicians, aged 40–84 y |
| Nutritional Prevention of Cancer Study (Clark et al., 1996, 1998) | U.S. | Secondary prevention: randomized, double-blind, placebo-controlled intervention | 1,312 men and women with history of basal or squamous cell carcinoma; aged 18–80 y |
| GISSI-Prevention Trial (GISSI-Prevenzione Investigators; 1999) | Italy | Secondary prevention: randomized, double-blind, placebo-controlled intervention | 11,324 patients with recent MI |
| Women's Health Study (Lee et al., 1999) | U.S. | Primary prevention; randomized, double-blind, placebo-controlled intervention | 39,876 healthy women, aged ≥45 y |
| Heart Outcomes Prevention Evaluation Study (HOPE Study Investigators, 2000) | Canada | Secondary prevention; randomized, double-blind, placebo-controlled intervention | 9,541 high-risk men and women, aged ≥55 y |
| *Studies In Progress* | | | |
| MRC/BHF Heart Protection Study (MRC/BHF, 1999) | U.K. | Secondary prevention trial | 20,536 high-risk men and women, aged 40–80 y |

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| Duration of Treatment (y) | Daily Dose | Primary Disease Outcome | Results[b] |
|---|---|---|---|
| 12 | 50 mg β-carotene (alternate days) | Cancer, CVD | No effects on CVD or cancer including among smokers |
| 13 | 200 µg selenium | Skin cancer, prostate cancer | No effect on incidence of skin cancer; 63% reduction in prostate cancer incidence; reduction in total cancer mortality and total cancer incidence |
| 3–5 | 300 mg α-tocopherol and/or 1 g ω-3 PUFA[a] | Total mortality | No benefit from vitamin E; 15% decrease in risk of death, nonfatal MI, and stroke from ω-3 PUFA |
| 21 | 50 mg β-carotene (alternate days) | MI, stroke, or CVD death | No effect on incidence of cancer, CVD, or total mortality |
| 1–6 | 100 IU (268 mg) α-tocopherol, ACE[b] inhibitor | MI, stroke, or CVD death | No benefit from vitamin E |
| 2–3 | 20 mg β-carotene, 600 mg α-tocopherol, 250 mg vitamin C | Total mortality | No results yet |

*continued*

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**TABLE 2-1** Continued

| Study | Country | Study Type[a] | Study Population |
|---|---|---|---|
| Women's Antioxidant Cardiovascular Study (Manson et al., 1995) | U.S. | Secondary prevention; randomized, double-blind, placebo-controlled intervention | 8,000 women with prior CVD event or ≥3 coronary risk factors, aged ≥40 y |
| Women's Health Study (Buring and Hennekens, 1992) | U.S. | Primary prevention; randomized, double-blind, placebo-controlled intervention | 39,876 healthy women, aged ≥45 y |
| Physician's Health Study II (Hennekens, 1998) | U.S. | Primary prevention; randomized, double-blind, placebo-controlled intervention | 15,000 healthy male physicians, aged ≥55 y |
| SUVIMAX (Hercberg et al., 1998) | France | Primary prevention; randomized, double-blind, placebo-controlled intervention | 12,735 men and women, aged 35–60 y |

[a] A primary prevention trial is one in which the study participants have no history of the disease outcome being investigated. Participants in a secondary prevention trial have had a prior occurrence of the outcome being investigated.

[b] Unless noted otherwise, results are statistically significant.

[c] U.S. = United States.

[d] CVD = cardiovascular disease.

task was to select, in addition to vitamin C, vitamin E, and β-carotene, other food components which might prove to be antioxidants and play a role in health; the third task was to assess the role of these compounds in health; and the fourth task was to develop Dietary Reference Intakes (DRIs) for the selected nutrients. The panel was asked to evaluate vitamin C, vitamin E, and β-carotene and other antioxidants. Since other dietary carotenoids share many,

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| Duration of Treatment (y) | Daily Dose | Primary Disease Outcome | Results[b] |
|---|---|---|---|
| 1 | 50 mg β-carotene, 600 IU α-tocopherol, 500 mg vitamin C (alternate days) | CVD | No results yet |
| NR[c] | 600 IU α-tocopherol, 100 mg aspirin (alternate days) | MI, stroke, or CVD death | No results yet |
| NR | 50 mg β-carotene, 100 IU α-tocopherol (alternate days), 500 mg vitamin C, multivitamin (Centrum silver) | CVD, cancer, eye diseases | No results yet |
| 8 | 6 mg β-carotene, 30 mg α-tocopherol, 120 mg vitamin C, 100 μg selenium, 20 mg zinc | Cancer, ischemic heart disease | No results yet |

[a] U.K. = United Kingdom.
[b] MI = myocardial infarction.
[c] PUFA = polyunsaturated fatty acid.
[d] ACE = angiotension converting enzyme.
[e] NR = not reported.

although not all, of the properties of β-carotene, additional carotenoids (α-carotene, β-cryptoxanthin, lutein, lycopene, and zeaxanthin) were added as related nutrients that would be investigated. In addition, because of the important role that selenium plays as a cofactor for oxidant defense enzymes, it was also included as a related nutrient.

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## DEFINITION AND CRITERIA FOR A DIETARY ANTIOXIDANT

### *Definition of a Dietary Antioxidant*

The definition was based on several criteria: (1) the substance is found in human diets; (2) the content of the substance has been measured in foods commonly consumed; and (3) in humans, the substance decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species in vivo. Thus, the definition of a dietary antioxidant is as follows:

> *A dietary antioxidant is a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans.*

This stringent definition, with supporting information, was slightly modified from the proposed definition published earlier (IOM, 1998). The earlier report stated that the nutritional recommendations that will be presented in the final report for some of these dietary components may not be determined by or related to their possible action as antioxidants. The primary indicators for developing the Dietary Reference Intakes (DRIs) are based on specific actions and effects of the compounds that may or may not be attributed to their antioxidant activity. In particular, criteria chosen upon which to base EARs and thus RDAs for any of the compounds investigated are not related specifically to ameliorating chronic diseases because a scientific justification for such a requirement was not found.

The European Commission Concerted Action on Functional Food also adopted stringent criteria regarding the relationship between free-radical events and human disease, and whether antioxidants are capable of modulating these events and thus reducing the risk of disease (Diplock et al., 1998). The summary report concluded that, "there is at present insufficient evidence available on which to base a firm conclusion that antioxidants are capable of reducing risk of disease" (Diplock, 1998). Recommendations for the nutrients in this report are similar. However, it should be pointed out that a large number of human intervention studies are under way using the compounds discussed in this report, and the results of these studies may give rise to new conclusions.

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### *Are Vitamin C, Vitamin E, Selenium, and β-Carotene and Other Carotenoids Dietary Antioxidants?*

*Vitamin C* functions physiologically as a water-soluble antioxidant by virtue of its very strong reducing power (high redox potential) and facile regeneration via ubiquitous reductants such as glutathione, nicotinamide adenine dinucleotide, and nicotinamide adenine dinucleotide phosphate. The primary method used to estimate the requirement relates to the vitamin C intake needed to maintain near-maximal neutrophil ascorbate concentration with minimal urinary excretion. Because smokers suffer increased oxidative stress and metabolic turnover of vitamin C, their requirement is increased. Although there is ample evidence that vitamin C administration can result in decreases in markers of oxidative stress, a Recommended Dietary Allowance (RDA) derived from a direct antioxidant function of vitamin C could not be calculated because of the lack of a quantitative relationship between this antioxidant function and a health-related endpoint. Since vitamin C reduces markers of oxidative stress, it meets the definition of a dietary antioxidant.

*Vitamin E* functions as a chain-breaking antioxidant that prevents the propagation of lipid peroxidation. To estimate the requirement, data were examined on the intake of vitamin E that would prevent hydrogen peroxide-induced lysis of erythrocytes. Under these circumstances, vitamin E is acting as an ex vivo antioxidant, maintaining a normal physiological function in humans. Although it is not yet possible to relate vitamin E intake to a lowering of chronic disease risk, it still meets the definition of a dietary antioxidant.

*Selenium* functions through selenoproteins, several of which are oxidant defense enzymes. The criterion used to estimate the requirement for selenium relates to the intake needed to maximize the activity of the plasma selenoprotein glutathione peroxidase, an oxidant defense enzyme. It is not clear if the diseases associated with selenium deficiencies, Keshan disease or Kashin-Beck disease, are due to oxidative stress. The selenium in several selenoproteins has a biochemical role in oxidant defense, thus maintaining normal physiological function, and as such plays a role as a dietary antioxidant.

*β-Carotene and other carotenoids* function as sources of vitamin A and, due to this provitamin A activity, can prevent vitamin A deficiency. Because no other specific nutrient functions have been identified at this time, no requirements have been established for any of the carotenoids. β-Carotene and the other carotenoids display in vitro antioxidant activity, but the evidence that they act as in vivo

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antioxidants in humans is still controversial. Therefore, β-carotene does not meet the definition of a dietary antioxidant. Similar conclusions have been drawn for α-carotene, β-cryptoxanthin, lycopene, lutein, and zeaxanthin.

## OXIDATIVE STRESS, ANTIOXIDANTS, AND CHRONIC DISEASE

### *Oxidative Stress*

There have been many proposals as to how oxidative stress can be defined. Because all cells are exposed to oxidants, generated either endogenously from metabolism or exogenously from a variety of environmental insults, the problem arises as to what constitutes an oxidative stress. Furthermore, different cells can be exposed to the same level of oxidants, but depending on the level of antioxidants or protective mechanisms available to the cell, they may or may not experience an oxidative stress. Sies (1985) defined oxidative stress as "a disturbance in the prooxidant-antioxidant balance in favor of the former." Others have amplified this definition to include "short- and/or long-term disturbance of the prooxidant-antioxidant balance resulting in adverse effects that are due either to impaired antioxidation or to favored prooxidation" (Biesalski et al., 1997). Here, oxidative stress is defined as an *imbalance* between the production of various reactive species and the ability of the organism's natural protective mechanisms to cope with these reactive compounds and prevent adverse effects.

The primary reactive species include reactive oxygen species (ROS) and reactive nitrogen species (RNS). These in turn react in the body and generate radical intermediates of lipids, proteins, and nucleic acids that ultimately form the chemical end products of oxidative stress. The physiological consequences of these end products have been hypothesized to be the causes of many chronic diseases as well as the natural aging process (Ames, 1998; Halliwell, 1997). The protective mechanisms include protective enzymes, antioxidant or quenching compounds produced by the organism, and similar compounds made available in the diet. The evidence that chronic disease results from an imbalance between formation and removal of reactive species is discussed below.

The primary defensive compounds are antioxidants that can interact with and quench reactive radical species, and enzymes that can inactivate these species or their products. All of the compounds in this report—vitamin C, vitamin E, selenium in the form of selenoproteins, and β-carotene and other carotenoids—are in vitro

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antioxidants, and some evidence exists that they have in vivo antioxidant actions. The protective enzymes include, among others, superoxide dismutase, catalase, and selenium-containing glutathione peroxidases and thioredoxin reductase. Other antioxidant mechanisms include stimulation of the expression of antioxidant or repair enzymes, as well as chelation of transition metals. A recent review of the relationship between antioxidant supplementation and oxidative damage concludes that, with the exception of vitamin E and possibly vitamin C reducing markers of lipid peroxidation, the evidence is insufficient that antioxidant supplementation leads to a material reduction in oxidative damage in humans (McCall and Frei, 1999).

### *Biomarkers of Oxidative Stress*

There have been numerous tests described to evaluate the level of markers of oxidative stress in living systems. Many of these markers represent oxidative breakdown products of normal tissue components and metabolites. Products derived from oxidized lipids include age pigments (lipofuscin), aldehydes, alkanes, and prostanoids such as the $F_2$-isoprostanes derived from unsaturated fatty acids. Oxidation of proteins produces protein carbonyls and amino acid derivatives such as methionine sulfoxide and nitrotyrosine, derived from the reaction of peroxynitrite ($ONOO^-$) with tyrosine. Purine and pyrimidine metabolites that are derived from oxidized nucleic acids can be detected in tissues and in urine. Since virtually all tissues are exposed to ROS and RNS, there will always be a baseline production of these biomarker molecules. What is important in evaluating the extent of oxidative stress is the *change* in the level of the biomarker compared to a baseline or steady-state level.

It is quite clear that dietary change can alter the levels of some of the biomarkers described above, and this phenomenon has been reported when the dietary intake of fruits and vegetables has been increased. Examples of such studies include reductions in biomarkers of deoxyribonucleic acid (DNA) damage (Pool-Zobel et al., 1997) or of lipid peroxidation (Miller et al., 1998), following consumption of diets rich in fruits and vegetables. Many of these studies have been reviewed recently (Halliwell, 1999).

A major problem with the use of biomarkers such as those described above is not knowing whether they reflect processes in the initiation of a disease state or whether they are products of a disease state. The validity of biomarkers, either intermediate or final, will not be settled until they are evaluated as part of intervention trials.

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A causal relationship between the formation of a biomarker of oxidant stress and a chronic disease has not yet been validated.

Some attempts have been made to determine a more global measure of oxidative stress, such as TRAP (total radical-trapping antioxidant capability) (Wayner et al., 1985), ORAC (oxygen radical absorbance capacity) (Cao et al., 1993), TEAC (trolox equivalent antioxidant capacity) (Miller et al., 1993), and FOX (ferrous oxidation/xylenol orange) (Jiang et al., 1992) assays. However, because these markers do not measure the same oxidants or antioxidant defenses (Cao and Prior, 1998), it is difficult to validate them as useful markers of oxidant stress.

### *Evidence of Oxidative Stress and Chronic Disease*

Aerobic metabolism produces energy, waste products such as carbon dioxide, and a small, but steady stream of radical by-products that are capable of reacting with all of the body's constituents to form oxidative damage products (Ames et al., 1993; Chance et al., 1979). Calculations have been made of the number of radical species formed per cell per day, but it is not at all clear how these numbers relate to diseases. Since the entire population is exposed to oxidative stresses and only a small fraction develops a chronic disease, it is clear that at this time, it is not understood how to evaluate the role of oxidative stress in the development of chronic disease. The potential role of oxidative stress in six chronic disease areas and aging is described briefly below.

#### *Cancer*

DNA is subject to damage, and either an exogenous or an endogenous mutagen can produce damage at a faster rate than the normal protective process of enzymatic repair (Ames et al., 1995). Under these circumstances, elevated levels of excision products will appear in the urine. The observation that 8-oxy-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is a major urinary product of both oxidative damage to DNA and damage by ionizing radiation that produces ROS, has served as the basis for the hypothesis that oxidative DNA damage is carcinogenic (Ames et al., 1995). The presence of 8-oxodG in DNA would lead to a guanine-to-thymine transversion that would result in a DNA mutation and, depending on its location, an altered gene product. 8-OxodG is also produced by ionizing radiation, and since this process can be carcinogenic, it has been assumed that any condition that produces 8-oxodG will also

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be carcinogenic. However, a causal relationship between oxidative stress to DNA and cancer in humans has not yet been established (Poulsen et al., 1998).

A great deal of evidence based on epidemiological studies indicates that consuming diets rich in fruits and vegetables is associated with both a decrease in oxidative damage to DNA (Halliwell, 1998) and a lower risk of a number of common cancers. There are several mechanisms that could account for these observations, in addition to antioxidant components that scavenge radical intermediates. Other mechanisms include the modification of carcinogen activation by the inhibition of phase 1 enzymes, modification of carcinogen detoxification by phase 2 enzymes, and suppression of the abnormal proliferation associated with preneoplastic lesions (Wargovich, 1997).

### *Cardiovascular Disease*

Of all the chronic diseases in which excess oxidative stress has been implicated, cardiovascular disease has the strongest supporting evidence. A coherent pathogenetic mechanism has been developed to account for the earliest stage in atherogenesis, namely, the development of the fatty streak lesion. Hypercholesterolemia and, particularly, increased concentrations of low-density lipoproteins (LDLs) cause the accumulation of cholesterol-loaded "foam cells" beneath the endothelial lining of major arteries, which in turn develop into a fatty streak. This lesion is clinically benign but is the precursor of later lesions (the fibrous plaque and the complex lesion) that ultimately give rise to clinical manifestations (angina pectoris and myocardial infarction) (Steinberg and Witztum, 1990).

An elevated LDL cholesterol level sufficiently increases the risk of cardiovascular disease. Brown and Goldstein (1986) demonstrated that the molecular defect in individuals with familial hypercholesterolemia was the absence of functional LDL receptors. The histological lesions in these patients cannot be differentiated from that of lesions in individuals who have normal LDL receptors. The implication therefore is that normal LDL taken up by way of the normal LDL receptor cannot be the basis for the formation of foam cells. In other words, the LDL must first be modified somehow and the modified form must be taken up into the monocytes or macrophages via one or more alternative receptors ultimately and develop into foam cells. One such modification, and the most extensively studied, is LDL oxidation, and several macrophage receptors have been shown to take up oxidatively modified LDL (oxLDL) and

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thus develop foam cells (Navab et al., 1995; Steinberg, 1997; Steinberg and Witztum, 1990; Steinberg et al., 1989; Steinbrecher, 1997).

In addition to its ability to cause foam cell formation, oxLDL can contribute to atherogenesis by virtue of a number of properties that differ from those of normal LDL (reviewed in Steinberg, 1997). The key question is whether these potentially proatherogenic properties of oxLDL are of sufficient importance that inhibition of the generation of oxLDL by antioxidants will have a significant impact on the rate of progression of atherosclerotic lesions.

The finding that a relationship existed between LDL levels and the incidence of cardiovascular disease was followed by the observation that oxLDL was associated with the development of atherosclerotic lesions in experimental animals. This association led to the hypothesis that oxLDL is the causative agent in the development of cardiovascular disease (Steinberg et al., 1989). This hypothesis has served as the basis for a number of human intervention trials, testing whether antioxidant agents capable of decreasing the extent of oxidation of LDL and thus decreasing oxLDL concentration might prove useful in decreasing the incidence of cardiovascular disease.

Several biomarkers of oxidative stress have been used to evaluate the extent of oxLDL formation and/or the extent of oxidative damage to lipids in general. OxLDL formation has been assessed after treating individuals with dietary antioxidants and evaluating the presence of oxLDL. In addition there have been reports of a potent effect of supplementary antioxidants in lowering the extent of ex vivo oxLDL formation. The results are not consistent, and as such, it is still not possible to conclude that dietary antioxidants prevent the formation of oxLDL in vivo. In addition, this modulation of LDL oxidation still must be validated as a marker of risk for cardiovascular disease (Zock and Katan, 1998).

The appearance of $F_2$-isoprostanes in urine has been suggested by a number of investigators as a reliable index of in vivo free radical generation and oxidative lipid formation. There is very strong evidence from animal studies that $F_2$-isoprostanes increase in plasma and urine as a result of oxidative stress, and in humans, these products are elevated in smokers (Morrow et al., 1995). Evidence is gradually accumulating that supplementary antioxidants, and vitamin E in particular, can both affect the level of $F_2$-isoprostanes in animal models of atherosclerosis and decrease the extent of arterial wall lesions (Pratico et al., 1998). Similar results have been obtained in humans (Hodis et al., 1995). Although some reviews point out that the protective role of antioxidants in animal models of atherosclerosis is only partially confirmed in human studies (Faggiotto et al.,

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1998), others are much more supportive of a protective role for vitamin E in coronary heart disease (Pryor, 2000).

### *Cataracts*

There have been a number of observational epidemiological studies of the risk of developing cataracts in humans. Many of these studies indicate that the risk of cataracts may be inversely proportional to the serum level of antioxidants (Knekt et al., 1992; Taylor et al., 1995) or may be reduced by supplement use (Jacques et al., 1997). These studies, however, have been considered to be inconclusive (Christen et al., 1996a; Leske et al., 1998). In the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, 5 to 8 years of daily supplementation with either 50 mg of vitamin E or 20 mg of β-carotene or both resulted in no difference in the prevalence of cataracts in the men in this study (Teikari et al., 1997).

### *Age-Related Macular Degeneration*

This irreversible disease, which is the major form of blindness in the elderly in the United States, Canada, and Europe, has been related to antioxidants found in the diet. This is because the pigment in the macular region of the normal retina consists of the two xanthophyll carotenoids, lutein and zeaxanthin (Bone et al., 1988; Handelman et al., 1988). This observation, coupled with the epidemiological observations of an inverse relationship between the risk of age-related macular degeneration (AMD) and the ingestion of fruits and vegetables (Goldberg et al., 1988), led a number of groups to propose that the basis of this chronic disease was a nutritional deficiency of green, leafy vegetables and yellow and orange fruits and vegetables that were rich in lutein and zeaxanthin (EDCOG, 1992; Seddon et al., 1991; Snodderly, 1995). Another association was the observation that smokers, who have lower plasma levels of carotenoids, also have a lower macular pigment (lutein and zeaxanthin) density (Hammond et al., 1996) and an increased risk of developing AMD (Christen et al., 1996b). However, all of these reports are associative in nature and do not demonstrate a causal relationship between deficiencies of lutein and zeaxanthin and development of AMD.

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### *Central Neurodegenerative Diseases*

There is increasing evidence that a number of common neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis may include adverse responses to oxidative stress. Small intervention trials in patients with these diagnoses have reported some improvement with either vitamin E (Muller, 1994; Sano et al., 1997) or vitamin C (Morris et al., 1998; Riviere et al., 1998), but it is still too early to draw any conclusions as to the usefulness of these compounds in these diseases, or to their ability to delay onset of the disease.

### *Diabetes Mellitus*

Cardiovascular complications are the major causes of death in diabetes. The incidence of coronary heart disease in type II diabetes is significantly higher than that in the general population (Kannel and McGee, 1979). In addition, individuals with diabetes experience microvascular complications (retinopathy, neuropathy, and nephropathy) secondary to their hyperglycemia. Thus, oxidative processes also may play an important role in the development or progression of diabetes mellitus.

In vitro oxidation of LDL from patients with diabetes mellitus proceeds at an accelerated rate, which suggests that they are more susceptible to the atherogenic process (Chisolm et al., 1992; Nishigaki et al., 1981; Reaven et al., 1995; Tsai et al., 1994). The several ways in which oxLDL is potentially more atherogenic than native LDL have been discussed above (see section "Cardiovascular Disease").

Diabetics tend to have smaller, denser LDL (associated with hypertriglyceridemia) and these LDLs are more susceptible to oxidative modification ex vivo (Feingold et al., 1992). The TRAP of plasma from patients with insulin-dependent diabetes is decreased (Tsai et al. 1994), which may account in part for the greater susceptibility of their LDL to oxidation.

Microvascular complications are believed to be the ultimate consequences of nonenzymatic glycosylation and the progressive accumulation of advanced glycosylation end products (AGEs). There is evidence that the formation of these complex carbohydrate-protein and carbohydrate-lipid complexes is accompanied and accelerated by oxidative processes, which may lead to diabetic complications (Brownlee et al., 1988; Dyer et al., 1993; Hunt et al., 1988; McCance et al., 1993; Mullarkey et al., 1990). The glycosylation process is associated with increased formation of free radicals, and the possi-

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bility that treatment with antioxidants might slow the development of AGEs is under investigation.

The issue of whether modifications observed in plasma and tissue proteins in patients with diabetes are due to an oxidative stress or a stress from reactive carbonyls has been discussed by Baynes and Thorpe (1999). A number of studies have tried altering the pathobiology of diabetes by treating patients with either single antioxidant-type compounds or combinations of compounds with antioxidant properties. The results have been inconclusive, which may reflect the fact that the underlying pathology is not caused exclusively by an oxidative stress, but by an inability to metabolize and inactivate reactive carbonyls appropriately. Under these circumstances, the oxidative damage may be exacerbated, resulting in an increase in many of the markers associated with oxidative stress but not caused directly by oxidative stress (Baynes and Thorpe, 1999).

### *Aging*

Aging is not in itself a chronic disease, but rather is characterized by the active or passive presence of a chronic disease (cardiovascular disease, cancer, cataracts, Alzheimer's disease, etc.). It is not clear if an accumulation of chronic insults and weakened defenses renders the aging individual more susceptible to various diseases. Do antioxidants play a role in preventing aging or prolonging life? There is no direct evidence in humans for such an effect, although vitamin E supplementation appears to improve some immune responses in the elderly (Meydani et al., 1997). There have been suggestions that supplementing older animals with antioxidants may improve various physiological functions (Hagen et al., 1999), but the only experimental intervention that has resulted in prolongation of the life span of the animals has been the drastic reduction of food consumption (Pariza and Boutwell, 1987). Whether such a protocol would delay aging in humans has not yet been studied. Whether dietary antioxidants can lead to healthier aging remains to be proven.

## CONCLUSIONS

There is little doubt that an imbalance in the production of free radicals and other reactive species and the natural protective systems available to organisms can lead to the production of oxidized products of lipids, nucleic acids, and proteins. These oxidation products, or biomarkers of this imbalance, may be related to early

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events in certain chronic diseases. However, they have not yet been adequately validated as markers of the onset, progression, or regression of any chronic diseases. Although vitamin C, vitamin E, and selenium have been shown to decrease the concentrations of some of the biomarkers associated with oxidative stress, the relationship between such observations and chronic disease remain to be elucidated. As a consequence, it has not been possible to establish that dietary antioxidants or other nutrients that can alter the levels of these biomarkers are themselves causally related to the development or prevention of chronic diseases.

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# Vitamin C

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Español

## Summary


- Vitamin C, also known as L-ascorbic acid, is a water-soluble vitamin. Unlike most mammals and other animals, humans do not have the ability to synthesize vitamin C and must obtain it from the diet. *(More information)*
- Vitamin C is an essential cofactor in numerous enzymatic reactions, e.g., in the biosynthesis of collagen, carnitine, and neuropeptides, and in the regulation of gene expression. It is also a potent antioxidant. *(More information)*
- Prospective cohort studies indicate that higher vitamin C status, assessed by measuring circulating vitamin C, is associated with lower risks of hypertension, coronary heart disease, and stroke. *(More information)*
- There is some evidence to suggest that vitamin C may be a useful adjunct to conventional medical practice to reduce myocardial injury and arrhythmia following a cardiac procedure or surgery in patients with cardiovascular disease. *(More information)*
- There are insufficient data to suggest a link between vitamin C status and the risk of developing a given type of cancer. Most observational studies examining vitamin C intake in relation to cancer incidence have found no association. Randomized controlled trials have reported no effect of vitamin C supplementation on cancer risk. *(More information)*

- Sources
  - Food sources
  - Supplements
- Safety
  - Toxicity
  - Kidney stones
  - Drug interactions
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- Current evidence of the efficacy of intravenous vitamin C in cancer patients is limited to observational studies, uncontrolled interventions, and case reports. There is a need for large, longer-duration phase II clinical trials that test the efficacy of intravenous vitamin C in cancer progression and overall survival. *(More information)*
- Overall, regular use of vitamin C supplements shortens the duration of the common cold but does not reduce the risk of becoming ill. Taking supplements once cold symptoms have already begun has no proven benefits. *(More information)*
- Vitamin C supplements are available in many forms, but there is little scientific evidence that any one form is better absorbed or more effective than another. *(More information)*
- There is no scientific evidence that large amounts of vitamin C (up to 10 grams [g]/day in adults) exert any adverse or toxic effects. An upper intake level of 2 g/day is recommended in order to prevent some adults from experiencing diarrhea and gastrointestinal disturbances. *(More information)*
- Supplemental vitamin C increases urinary oxalate concentrations, but whether an increase in urinary oxalate elevates the risk for kidney stones is not yet known. Those predisposed for kidney stone formation may consider avoiding high-dose (≥1 g/day) vitamin C supplementation. *(More information)*

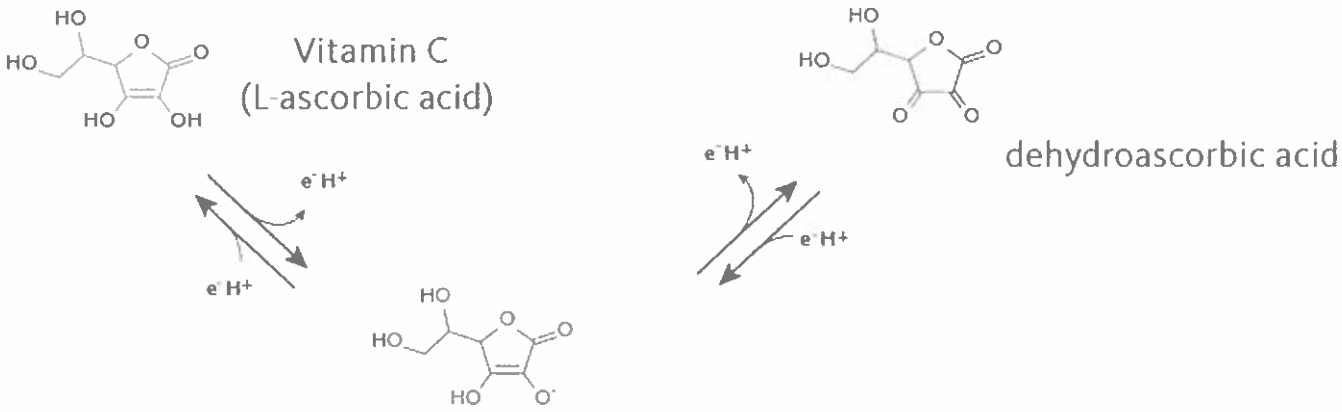
## Function

Vitamin C (L-ascorbic acid) is a potent reducing agent, meaning that it readily donates electrons to recipient molecules (**Figure 1**). Related to this oxidation-reduction (redox) potential, two major functions of vitamin C are as an antioxidant and as an enzyme cofactor (1).

Vitamin C is the primary water-soluble, non-enzymatic antioxidant in plasma and tissues. Even in small amounts, vitamin C can protect indispensable molecules in the body, such as proteins, lipids (fats), carbohydrates, and nucleic acids (DNA and RNA), from damage by free radicals and reactive oxygen species (ROS) that are generated during normal metabolism, by active immune cells, and through exposure to toxins and pollutants (e.g., certain chemotherapy drugs and cigarette smoke). Vitamin C also participates in redox recycling of other important antioxidants; for example, vitamin C is known to regenerate vitamin E from its oxidized form (see the article on Vitamin E).

The role of vitamin C as a cofactor is also related to its redox potential. By maintaining enzyme-bound metals in their reduced forms, vitamin C assists mixed-function oxidases in the synthesis of several critical biomolecules (1). These enzymes are either monooxygenases or dioxygenases (see **Table 1**). Symptoms of vitamin C deficiency, such as poor wound healing and lethargy, likely result from the impairment of these vitamin C-dependent enzymatic reactions leading to the insufficient synthesis of collagen, carnitine, and catecholamines (see Deficiency). Moreover, several dioxygenases involved in the regulation of gene expression and the maintenance of genome integrity require vitamin C as a cofactor. Indeed, research has recently uncovered the crucial role played by enzymes, such as the TET dioxygenases and Jumonji domain-containing histone demethylases, in the fate of cells and tissues (see **Table 1**). These enzymes contribute to the epigenetic regulation of gene expression by catalyzing reactions involved in the demethylation of DNA and histones.

**Figure 1. Vitamin C**




Vitamin C (L-ascorbic acid) is an electron donor. Vitamin C can sequentially donate two electrons. Vitamin C can donate electrons to reactive free radicals, which then become reduced. The loss of one electron results in vitamin C being oxidized to the ascorbate free radical, which is relatively unreactive compared to other free radicals. The ascorbate free radical can be reduced to vitamin C by gaining one electron or be further oxidized to dehydroascorbic acid by losing another electron. Dehydroascorbic acid is only stable for a few minutes and is then either irreversibly hydrolyzed to form 2,3-diketogulonic acid or reduced to semidehydroascorbic acid and vitamin C (not shown here). However, the efficacy of the *in vivo* reduction reactions that yield vitamin C from dehydroascorbic acid and semidehydroascorbic acid appears to be low as vitamin C deficiency occurs in 30 days when vitamin C is removed from the diet of healthy people. Thus, most of the vitamin C is likely oxidized to dehydroascorbic acid, which is irreversibly metabolized.

[Figure 1 - Click to Enlarge]

Table 1. Enzymes Requiring Vitamin C as a Cofactor in Mammals (1, 2)

| Enzymes* | Functions |
|---|---|
| **Monooxygenases** | |
| Dopamine β-monooxygenase | Norepinephrine (Noradrenaline) biosynthesis |
| Peptidyl-glycine α-amidating monooxygenase | Amidation of peptide hormones |
| **Dioxygenases** | |
| 3 Prolyl 4-hydroxylase isoenzymes | Collagen hydroxylation |
| 3 Prolyl 3-hydroxylase isoenzymes | Collagen hydroxylation |
| 3 Lysyl hydroxylase isoenzymes | Collagen hydroxylation |
| 4 Hypoxia-inducible factor (HIF) isoenzymes | HIF hydroxylation |
| Trimethyllysine hydroxylase | Carnitine biosynthesis |
| γ-Butyrobetaine hydroxylase | Carnitine biosynthesis |
| 4-Hydroxyphenylpyruvate dioxygenase | Tyrosine metabolism |
| Ten-eleven translocation (TET) family of dioxygenases | Demethylation of DNA |
| Jumonji domain-containing histone demethylases | Demethylation of histones |

*Monooxygenases catalyze the hydroxylation of one substrate, whereas dioxygenases catalyze a reaction that couples the hydroxylation of a specific substrate with the conversion (decarboxylation) of α-ketoglutarate into succinate.

The capacity of vitamin C to influence the methylation status of DNA and histones in mammalian cells supports a role for the vitamin in health and disease beyond what was previously understood, in particular by safeguarding genome integrity (3, 4).

## *Role in immunity*

Vitamin C affects several components of the human immune system *in vitro*; for example, vitamin C has been shown to stimulate both the production (5-9) and function (10, 11) of leukocytes (white blood cells), especially neutrophils, lymphocytes, and phagocytes. Specific measures of functions stimulated by vitamin C include cellular motility (10), chemotaxis (10, 11), and phagocytosis (11). Neutrophils, mononuclear phagocytes, and lymphocytes accumulate vitamin C to high concentrations, which can protect these cell types from oxidative damage (12-14). In response to invading microorganisms, phagocytic leukocytes release non-specific toxins, such as superoxide

radicals, hypochlorous acid ("bleach"), and peroxynitrite; these reactive oxygen species kill pathogens and, in the process, can damage the leukocytes themselves (15). Vitamin C, through its antioxidant functions, has been shown to protect leukocytes from self-inflicted oxidative damage (14). Phagocytic leukocytes also produce and release cytokines, including interferons, which have antiviral activity (16). Vitamin C has been shown to increase interferon production *in vitro* (17). Additional studies have reported that vitamin C enhances the chemotactic and microbial killing capacities of neutrophils and stimulates the proliferation and differentiation of B- and T-lymphocytes (reviewed in 18).

It is widely thought by the general public that vitamin C boosts immune function, yet human studies published to date are conflicting. Disparate results are likely due to study design issues, often linked to a lack of understanding of vitamin C pharmacokinetics and requirements (19, 20).

Finally, vitamin C increases the bioavailability of iron from foods by enhancing intestinal absorption of non-heme iron (see the article on Iron) (21).

## Bioavailability

Depletion-repletion pharmacokinetic experiments demonstrated that plasma vitamin C concentration is tightly controlled by three primary mechanisms: intestinal absorption, tissue transport, and renal reabsorption (22). In response to increasing oral doses of vitamin C, plasma vitamin C concentration rises steeply at intakes between 30 and 100 mg/day. Plasma concentrations of ascorbate reach steady-state at concentrations between 60 and 80 micromoles/L (µmol/L). This is typically observed at doses between 200 to 400 mg/day in healthy young adults, with some degree of individual variation (23, 24).

One hundred percent absorption efficiency is observed when ingesting vitamin C at doses up to 200 mg at a time. Higher doses (>500 mg) result in fractionally less vitamin C being absorbed as the dose increases. Once plasma vitamin C concentrations reach saturation, additional vitamin C is largely excreted in the urine. Notably, intravenous administration of vitamin C bypasses absorptive control in the intestine such that very high concentrations of vitamin C can be achieved in the plasma; within a few hours, renal excretion restores vitamin C to baseline plasma concentrations (see Cancer Treatment) (25).

While plasma vitamin C concentration reflects recent dietary intake, leukocyte (white blood cell) vitamin C is thought to be an indicator of body stores. However, leukocyte vitamin C concentration does not accurately reflect vitamin C in several tissues and may specifically underestimate vitamin C uptake into skeletal muscle (26). Yet, plasma concentrations of vitamin C ≥50 µmol/L are sufficient to saturate muscle tissue vitamin C.

There is also some limited evidence suggesting that individuals who carry certain polymorphisms in genes involved in vitamin C transport and detoxification mechanisms may have lower plasma vitamin C concentrations even with high vitamin C intakes (see also Vascular complications of

diabetes mellitus) (reviewed in 27).

Due to the pharmacokinetics and tight regulation of plasma vitamin C, supplementation with vitamin C will have variable effects in vitamin C-replete (plasma concentrations near saturation) versus sub-optimal (plasma concentrations <50 µmol/L), marginally deficient (plasma concentrations <23 µmol/L), or severely deficient (plasma concentrations <11 µmol/L) individuals (28). Scientific studies investigating vitamin C efficacy to prevent or treat disease need to assess baseline vitamin C status before embarking on an intervention or statistical analysis (22, 29-31).

For a more detailed discussion on the bioavailability of different forms of vitamin C, see the article, The Bioavailability of Different Forms of Vitamin C.

## Deficiency

Severe vitamin C deficiency has been known for many centuries as the potentially fatal disease, scurvy. By the late 1700s, the British navy was aware that scurvy could be cured by eating oranges or lemons, even though vitamin C would not be isolated until the early 1930s. Symptoms of scurvy include subcutaneous bleeding, poor wound closure, and bruising easily, hair and tooth loss, and joint pain and swelling. Such symptoms appear to be related to the weakening of blood vessels, connective tissue, and bone, which all contain collagen. Early symptoms of scurvy like fatigue may result from diminished levels of carnitine, which is needed to derive energy from fat, or from decreased synthesis of the catecholamine norepinephrine (see Function). Scurvy is rare in developed countries because it can be prevented by as little as 10 mg of vitamin C daily (32). However, cases have occurred in children and the elderly on very restricted diets (33, 34).

## The Recommended Dietary Allowance (RDA)

The recommended dietary allowance (RDA) for vitamin C is based on the amount of vitamin C intake necessary to maintain neutrophil vitamin C concentration with minimal urinary excretion of vitamin C and is proposed to provide sufficient antioxidant protection (**Table 2**) (35). The recommended intake for smokers is 35 mg/day higher than for nonsmokers, because smokers are under increased oxidative stress from the toxins in cigarette smoke and generally have lower blood concentrations of vitamin C (36).

Table 2. Recommended Dietary Allowance (RDA) for Vitamin C

| Life Stage | Age | Males (mg/day) | Females (mg/day) |
|---|---|---|---|
| Infants | 0-6 months | 40 (AI) | 40 (AI) |
| Infants | 7-12 months | 50 (AI) | 50 (AI) |
| Children | 1-3 years | 15 | 15 |
| Children | 4-8 years | 25 | 25 |

| | | | |
|---|---|---|---|
| Children | 9-13 years | 45 | 45 |
| Adolescents | 14-18 years | 75 | 65 |
| Adults | 19 years and older | 90 | 75 |
| Smokers | 19 years and older | 125 | 110 |
| Pregnancy | 18 years and younger | - | 80 |
| Pregnancy | 19 years and older | - | 85 |
| Breast-feeding | 18 years and younger | - | 115 |
| Breast-feeding | 19 years and older | - | 120 |

## Disease Prevention

The amount of vitamin C required to help prevent chronic disease is higher than the amount required for prevention of scurvy. Information regarding vitamin C and the prevention of chronic disease is based on both observational prospective cohort studies and randomized controlled trials (29, 37). Prospective cohort studies can examine the incidence of a specific disease in relation to vitamin C intake or body status in a cohort of participants who are followed over time. In contrast, trials are intervention studies that can establish a causal relationship between an exposure and an outcome, e.g., by evaluating the effect of vitamin C supplementation on the incidence of chronic disease in participants randomly assigned to receive either vitamin C or placebo for a given length of time.

### Cardiovascular disease

#### *Endothelial dysfunction*

Endothelial dysfunction is considered to be an early step in the development of atherosclerosis. Alterations in the structure and function of the vascular endothelium that lines the inner surface of all blood vessels are associated with the loss of normal nitric oxide-mediated endothelium-dependent vasodilation. Endothelial dysfunction results in widespread vasoconstriction and coagulation abnormalities. The measurement of brachial artery flow-mediated dilation (FMD) is often used as a functional marker of endothelial function; FMD values are inversely correlated with the risk of future cardiovascular events (38). A 2014 meta-analysis of 44 randomized controlled trials in subjects with or without chronic diseases summarized the effect of supplemental vitamin C on endothelial function by measuring FMD (19 studies), assessing forearm blood flow (20 studies), or by pulse wave analysis (5 trials) (39). Short-term supplementation with vitamin C was found to reduce endothelial dysfunction in subjects with heart failure, atherosclerosis, or diabetes mellitus, but it had no effect in those with hypertension. Vitamin C also limited endothelial dysfunction that was experimentally induced in healthy volunteers (39). Improved endothelial function was observed with daily vitamin C doses above 500 mg (39).

## *Hypertension*

Hypertension is a major risk factor for cardiovascular disease, including coronary heart disease, stroke, and atrial fibrillation. An analysis that combined data from three, large, independent prospective cohorts, (1) Nurses' Health Study 1 (NHS1; 88,540 women, median age 49 years); (2) Nurses' Health Study 2 (NHS2; 97,315 women, median age 36 years); and (3) Health Professionals Follow-up Study (HPFS; 37,375 men, median age 52 years), found no association between the level of vitamin C intake and risk of developing hypertension (40). On the other hand, when plasma vitamin C concentration was measured, cross-sectional studies have consistently indicated an inverse relationship between plasma vitamin C concentration and blood pressure in both men and women (41-43). A 15-year follow-up of about 2,500 participants in the Coronary Artery Risk Development in Young Adults (CARDIA) study found that higher plasma vitamin C and a higher diet quality score were independently associated with a reduced risk of developing hypertension (44). Interestingly, there was no relationship between diet score and risk of hypertension in those with the lowest plasma vitamin C, and plasma vitamin C was positively associated with risk of hypertension in those with low diet scores (44).

A meta-analysis of 29 small randomized controlled trials of short durations (median duration, 8 weeks) in 1,407 participants (10 to 120 subjects per trial; including both normotensive and hypertensive subjects) found that daily supplementation with 60 to 4,000 mg of vitamin C (median dose, 500 mg) reduced systolic blood pressure by 3.84 mm Hg and diastolic blood pressure by 1.48 mm Hg (45). Good quality long-term trials are needed to examine whether the anti-hypertensive effect of vitamin C is sustained over time and eventually results in a reduced risk of cardiovascular events.

It is important for individuals with significantly elevated blood pressure not to rely on vitamin C supplementation alone to reduce their hypertension. They should instead seek or continue treatment with anti-hypertensive medication and make dietary and lifestyle changes in consultation with their health care provider.

## *Cardiovascular disease risk*

Coronary heart disease (CHD) is characterized by the buildup of plaque inside the arteries that supply blood to the heart (atherosclerosis). Over years of buildup and accumulated damage to the coronary arteries, CHD may culminate in a myocardial infarction or heart attack. Many prospective cohort studies have examined the relationship between vitamin C intake from diet and supplements and CHD risk, the results of which have been pooled and analyzed in two separate analyses (46, 47). In 2004, a pooled analysis of nine prospective cohort studies found that supplemental vitamin C intake (≥400 mg/day for a mean of 10 years), but not dietary vitamin C intake, was inversely associated with CHD risk (46). Conversely, a 2008 meta-analysis of 14 cohort studies concluded that dietary, but not supplemental, vitamin C intake was inversely related to CHD risk (47). The most recent large prospective cohort study found an inverse association between

dietary vitamin C intake and CHD mortality in Japanese women, but not in men (48). In spite of the variable association depending on source, these analyses indicate an overall inverse association between higher vitamin C intakes and CHD risk.

Limitations inherent to dietary assessment methodology, such as recall bias, measurement error, and residual confounding, may account for some of the inconsistent associations between vitamin C intake and CHD risk. In order to overcome such limitations, some prospective studies measured plasma or serum concentrations of vitamin C as a more reliable index of vitamin C intake and biomarker of body vitamin C status.

The European Investigation into Cancer and Nutrition (EPIC)-Norfolk prospective cohort study investigated the relationship between vitamin C status and incident heart failure in healthy adults (9,187 men and 11,112 women, aged 58.1+/-9.2 years) (49). After a mean follow-up of 12.8 years, plasma vitamin C was inversely associated with incident cases of heart failure. Specifically, plasma vitamin C ranged from approximately 23 to 70 µmol/L in men and 33 to 82 µmol/L in women; across this range, every 20 µmol/L increase in plasma vitamin C was associated with a 9% reduction in risk of heart failure. Although a primary source of dietary vitamin C, consumption of fruit and vegetables — assessed by food frequency questionnaire — was not found to be associated with a lower risk of congestive heart failure (49). This highlights the fact that limitations associated with dietary assessment methods such as food frequency questionnaires may be overcome by using biomarkers of nutrient intake (50, 51).

A 2017 review of eight published randomized controlled trials found inconsistent results from seven trials reporting on the effect of vitamin C supplementation on serum cholesterol and triglycerides, established risk factors for cardiovascular disease (52). Only one large trial in more than 14,000 older men participating in the Physicians' Health Study II (PHS II) reported on cardiovascular outcomes. PHS II found that vitamin C supplementation (500 mg/day) for an average of eight years had no significant effect on major cardiovascular events, total myocardial infarction, or cardiovascular mortality (53). Notably, this study had several limitations (54), including no measurement of vitamin C status and the recruitment of a well-nourished study population.

There is a need for better quality studies to examine the effect of vitamin C on cardiovascular endpoints in participants with elevated risk of cardiovascular disease.

### *Stroke*

A cerebrovascular event, or stroke, can be classified as hemorrhagic or ischemic. Hemorrhagic stroke occurs when a weakened blood vessel ruptures and bleeds into the surrounding brain tissue. Ischemic stroke occurs when an obstruction within a blood vessel blocks blood flow to the brain. Most (~80%) cerebrovascular events in high-income countries are ischemic in nature and associated with atherosclerosis as an underlying condition (55, 56).

With respect to vitamin C and cerebrovascular disease, a prospective cohort study that followed more than 2,000 residents of a rural Japanese community for 20 years found that the risk of stroke in those with the highest serum concentrations of vitamin C was 29% lower than in those with the lowest serum concentrations of vitamin C (57). Similarly, the EPIC-Norfolk study, a 10-year prospective cohort study in 20,649 adults, found that individuals with plasma vitamin C concentrations in the top quartile (25%) had a 42% lower risk of stroke compared to those in the lowest quartile (≥66 µmol/L vs. <41 µmol/L) (58). In both the Japanese (57) and EPIC-Norfolk (58) populations, blood vitamin C concentrations were highly correlated with fruit and vegetable intake. Therefore, as in many studies of vitamin C intake and chronic disease risk, it is difficult to separate the effects of vitamin C from the effects of other components of fruit and vegetables. For example, potassium — found at high levels in bananas, potatoes, and other fruit and vegetables — is known to be important in blood pressure regulation, and elevated blood pressure is a major risk factor for stroke (see the article on Potassium). A 2013 meta-analysis of 17 prospective cohort studies reported a 19% lower risk of stroke with the highest versus lowest dietary vitamin C intakes and a 38% lower risk with the highest versus lowest circulating vitamin C concentrations (59).

A randomized, double-blind, placebo-controlled trial in more than 14,000 older men participating in the Physicians' Health Study II (PHS II) found that vitamin C supplementation (500 mg/day) for an average of eight years had no significant effect on the incidence of or mortality from any type of stroke (53). Other trials also failed to show any evidence of an effect of vitamin C on the risk of stroke. A meta-analysis of 10 trials that examined antioxidant vitamins, of which five included vitamin C, found no association between any antioxidant vitamin (vitamin C, vitamin E, or β-carotene), administered alone or in combination, and risk of stroke (60).

## Cancer

Overall, observational prospective cohort studies have reported no or modest inverse associations between vitamin C intake and the risk of developing a given type of cancer (37, 61-63). Additional detail is provided below for those cancer subtypes with substantial scientific information obtained from prospective cohort studies. Randomized, double-blind, placebo-controlled trials that have tested the effect of vitamin C supplementation (alone or in combination with other antioxidant nutrients) on cancer incidence or mortality have shown no effect (64).

### *Breast cancer*

Two large prospective studies found dietary vitamin C intake to be inversely associated with breast cancer incidence in certain subgroups. In the Nurses' Health Study, premenopausal women with a family history of breast cancer who consumed an average of 205 mg/day of vitamin C from food had a 63% lower risk of breast cancer than those who consumed an average of 70 mg/day (65). In the Swedish Mammography Cohort, overweight women who consumed an average of 110 mg/day

of vitamin C had a 39% lower risk of breast cancer compared to overweight women who consumed an average of 31 mg/day (66). More recent prospective cohort studies have reported no association between dietary and/or supplemental vitamin C intake and breast cancer (67-69).

## *Stomach cancer*

A number of observational studies have found increased dietary vitamin C intake to be associated with decreased risk of gastric (stomach) cancer, and laboratory experiments indicate that vitamin C inhibits the formation of carcinogenic N-nitroso compounds in the stomach (70-72). A nested case-control study in the EPIC study found a 45% lower risk of gastric cancer incidence in individuals in the highest (≥51 µmol/L) versus lowest (<29 µmol/L) quartile of plasma vitamin C concentration; no association was observed between dietary vitamin C intake and gastric cancer (73).

Infection with the bacteria, *Helicobacter pylori* (*H. pylori*), is known to increase the risk of stomach cancer and is associated with lower vitamin C content of stomach secretions (74, 75). Although two intervention studies failed to show a reduction in stomach cancer incidence with vitamin C supplementation (35), some research suggests that vitamin C supplementation may be a useful addition to standard *H. pylori* eradication therapy in reducing the risk of gastric cancer (76). Because vitamin C can inactivate urease (an enzyme that facilitates *H. pylori* survival and colonization of the gastric mucosa at low pH) *in vitro*, vitamin C may be most effective as a prophylactic agent in those without achlorhydria (77, 78).

## *Colon cancer*

By pooling data from 13 prospective cohort studies comprising 676,141 participants, it was determined that dietary intake of vitamin C was not associated with colon cancer, while total intake of vitamin C (i.e., from food and supplements) was associated with a 19% reduced risk of colon cancer (79). Each of the cohort studies used self-administered food frequency questionnaires at baseline to assess vitamin C intake. Although the analysis adjusted for several lifestyle and known risk factors, the authors noted that other healthy behaviors and/or folate intake may have confounded the association.

## *Non-Hodgkin lymphoma*

A population-based, prospective study, the Iowa Women's Health Study, collected baseline data on diet and supplement use in 35,159 women (aged 55-69 years) and evaluated the risk of developing non-Hodgkin lymphoma (NHL) over 19 years of follow-up (80). Overall, an inverse association between fruit and vegetable intake and risk of NHL was observed. Additionally, dietary, but not supplemental, intake of vitamin C and other antioxidant nutrients (carotenoids, proanthocyanidins, and manganese) was inversely associated with NHL risk. Another large, multi-center, prospective study — the Women's Health Initiative — that followed 154,363 postmenopausal women for 11 years found that dietary and supplemental vitamin C intake at baseline was inversely associated with diffuse B-cell lymphoma, a subtype of NHL (81).

### *Other site-specific cancer types*

The Physicians' Health Study II was a randomized, placebo-controlled trial that examined the effect of vitamin E (400 IU/day), vitamin C (500 mg/day), and a multivitamin supplement on the risk of cancer in 14,641 middle-aged male physicians over 10.3 years (7.6 years of active treatment plus 2.8 years post-treatment follow-up) (82). Supplementation with vitamin C had no effect on the overall risk of cancer or on the risk of prostate, bladder, or pancreatic cancer; there was a marginal reduction in colorectal cancer incidence with vitamin C compared to placebo (82).

## Type 2 diabetes mellitus

In the National Institutes of Health (NIH)-American Association of Retired Persons (AARP) Diet and Health study that included 232,007 participants, the use of vitamin C supplements for at least seven times a week was associated with a 9% lower risk of developing type 2 diabetes mellitus compared to non-supplement use (83). In a cohort of 21,831 adults followed for 12 years in the EPIC-Norfolk study, high plasma vitamin C was found to be strongly associated with a reduced risk of diabetes (84). Additionally, several cross-sectional studies reported inverse associations between circulating vitamin C concentrations and markers of insulin resistance or glucose intolerance, such as glycated hemoglobin (HbA1c) concentration (50, 85, 86). Yet, short-term randomized controlled studies have found no effect of vitamin C supplementation on fasting glucose, fasting insulin, and HbA1c concentrations in healthy individuals (87). It is not known whether supplemental vitamin C could improve markers of glycemic control in subjects at risk of diabetes.

## Adverse pregnancy outcomes

A 2015 meta-analysis of 29 randomized controlled trials found that administration of vitamin C during pregnancy, alone or in combination with a few other supplements, failed to reduce the risks of stillbirth, perinatal death, intrauterine growth restriction, preterm birth, premature rupture of membranes, and preeclampsia (88). Nonetheless, vitamin C supplementation led to a 36% lower risk of placental abruption and to a significant increase in gestational age at birth (88). Another meta-analysis of 40 randomized controlled trials in 276,820 women found no effect of vitamin C, alone or combined with vitamin E or multivitamins, when supplemented during pregnancy (starting prior to 20 weeks' gestation), on the risks of overall fetal loss, miscarriage, stillbirth, and congenital malformation (89).

Cigarette smoking during pregnancy causes intrauterine growth restriction and preterm birth, among other pregnancy complications (90, 91), and is the primary cause of childhood respiratory illness (92). For some still unclear reasons, smoking has been associated with a lower risk of preeclampsia during pregnancy (93). A secondary analysis of a multicenter, randomized, double-blind, placebo-controlled trial in nearly 10,000 pregnant women found no reduction in the risk of preeclampsia with supplemental vitamin C (1,000 mg/day) and vitamin E (400 IU/day), regardless of women's smoking status during pregnancy. However, antioxidant supplementation resulted in reduced risks of placental abruption and preterm birth in women who smoked during pregnancy but not in non-smokers (94). Another pilot multicenter trial found better lung function during the

first week of life and lower risk of wheezing through one year of age in infants whose smoking mothers were randomized to receive vitamin C (500 mg/day) rather than a placebo during pregnancy (95). The Vitamin C to Decrease the Effects of Smoking in Pregnancy on Infant Lung Function [VCSIP] study is an ongoing trial designed to confirm these preliminary observations using more accurate measurements of pulmonary function in a larger sample of women randomized to receive supplemental vitamin C or placebo (96).

## Alzheimer's disease

In the US, Alzheimer's disease (AD) is the most common form of dementia, affecting 5.5 million individuals 65 years and over (97). Oxidative stress, neuroinflammation, β-amyloid plaque deposition, Tau protein-forming tangles, and neuronal cell death in the brain of subjects affected by AD have been associated with cognitive decline and memory loss. Lower vitamin C concentrations in the cerebrospinal fluid (CSF) and brain extracellular matrix of a mouse model of AD were found to increase oxidative stress and accelerate amyloid deposition and disease progression (98). In another AD mouse model that was lacking the ability to synthesize vitamin C, supplementation with a high versus low dose of vitamin C reduced amyloid deposition in the cortex and hippocampus and limited blood-brain barrier impairments and mitochondrial dysfunction (99).

The majority of large, population-based studies examining the relationship of vitamin C intake or supplementation with AD incidence have reported null results (100). In contrast, observational studies reported lower plasma vitamin C concentrations in AD patients compared to cognitively healthy subjects (101) and found better cognitive function or lower risk of cognitive impairment with higher plasma vitamin C (100).

Few studies have measured vitamin C concentration in the CSF, which more closely reflects the vitamin C status of the brain. Vitamin C is concentrated in the brain through a combination of active transport into brain tissue and retention via the blood-brain barrier (100). Although CSF vitamin C is maintained at concentrations several-fold higher than plasma vitamin C, the precise function of vitamin C in cognitive function and AD etiology is not yet fully understood (102). In a small, longitudinal biomarker study in 32 individuals with probable AD, a higher CSF-to-plasma vitamin C ratio at baseline was associated with a slower rate of cognitive decline at one year of follow-up (103). Impaired blood-brain barrier integrity may affect the brain's ability to retain vitamin C and thus to maintain a high CSF-to-plasma vitamin C ratio. The significance of the CSF-to-plasma vitamin C ratio in AD progression requires further study.

The effect of vitamin C supplementation, in combination with other antioxidants, on CSF biomarkers and cognitive function has been examined in only a few trials involving AD patients. In a small (n=23), open-label trial, combined supplementation with vitamin C (1,000 mg/day) and vitamin E (400 IU/day) to AD patients taking a cholinesterase inhibitor significantly increased antioxidant levels and decreased lipoprotein oxidation in CSF after one year, but had no effect on the clinical course of AD compared to controls (104). A similar finding was obtained in a double-blind, randomized controlled trial in which combined supplementation with vitamin C (500

mg/day), vitamin E (800 IU/day), and α-lipoic acid (900 mg/day) for 16 weeks reduced lipoprotein oxidation in CSF but elicited no clinical benefit in individuals with mild-to-moderate AD (n=78) (105). In this latter trial, a greater decline in the Mini Mental State Examination (MMSE) score was observed in the supplemented group, however, the significance of this observation remains unclear. A third placebo-controlled trial in mildly cognitively impaired older adults (ages, 60-75 years) found that one-year supplementation with vitamin C (400 mg/day) and vitamin E (300 mg/day) improved antioxidant blood capacity but had no effect on MMSE scores (106).

At this time, avoidance of vitamin C deficiency or insufficiency, rather than supplementation in replete individuals, seems prudent for the promotion of healthy brain aging (101).

## Cataracts

The lens of the eye focuses light, producing a clear, sharp image on the retina, a layer of tissue on the inside back wall of the eyeball. Age-related changes to the lens (thickening, loss of flexibility) and oxidative damage contribute to the formation of cataract, i.e., cloudiness or opacity in the lens that interferes with the clear focusing of images on the retina.

In humans, vitamin C concentration is about 15 to 20 times higher in the aqueous humor — fluid that fills the anterior and posterior chambers of the eye — than in plasma, suggesting that the vitamin may be playing an important role in the eye (107). Decreased vitamin C concentrations in the lens of the eye have been associated with increased severity of cataracts (108). A meta-analysis of observational studies found that a reduced risk of age-related cataract with higher dietary vitamin C intakes in case-control studies and with higher circulating vitamin C concentrations in cross-sectional studies. However, no such associations were found in pooled analyses of prospective cohort studies (109). In fact, two prospective cohort studies in Swedish men (110) and women (111) reported that high-dose single nutrient supplements of vitamin C were associated with an increased risk of cataract, especially in those on corticosteroid therapy.

A 2012 review of nine randomized controlled trials found no substantial effect of β-carotene, vitamin C, and vitamin E, administered individually or in combination over 2.1 to 12 years, on the risk of cataracts or cataract surgery (112). Although trials do not currently support the use of high-dose supplementation with vitamin C in cataract prevention, there is a consistent inverse association observed between high daily intake of fruit and/or vegetables (>5 servings/day) and risk of cataract (113).

## Gout

Gout, a condition that afflicts more than 4% of US adults (114), is characterized by abnormally high blood concentrations of uric acid (urate) (115). Urate crystals may form in joints, resulting in inflammation and pain, as well as in the kidneys and urinary tract, resulting in kidney stones. The tendency to exhibit elevated blood uric acid concentrations and develop gout is often inherited; however, dietary and lifestyle modification may be helpful in both the prevention and treatment of gout (116). In an observational study that included 1,387 men, higher intakes of vitamin C were

associated with lower serum concentrations of uric acid (117). In a cross-sectional study conducted in 4,576 African Americans, the odds of having hyperuricemia was associated with dietary intakes high in fructose, low in vitamin C, or with high fructose-to-vitamin C ratios (118). A prospective study that followed a cohort of 46,994 men for 20 years found that total daily vitamin C intake was inversely associated with incidence of gout, with higher intakes being associated with greater risk reductions (119). The results of this study also indicated that supplemental vitamin C may be helpful in the prevention of gout (119).

A 2011 meta-analysis of 13 randomized controlled trials in healthy individuals with elevated serum uric acid revealed that vitamin C supplementation (a median dose of 500 mg/day for a median duration of 30 days) modestly reduced serum uric acid concentrations by 0.35 mg/dL compared to placebo (120). Such a reduction falls within the range of assay variability and is unlikely to be clinically significant (121). An eight-week, open-label, controlled trial randomized 40 subjects with gout to receive either allopurinol (standard-of-care), vitamin C, or both treatments (122). The effect of vitamin C, alone or with allopurinol, decreasing serum uric acid was modest and much less than that of allopurinol alone. The trial did not examine the effect of vitamin C on other outcomes associated with gout (122).

Although observational studies suggested that supplemental vitamin C may be helpful to prevent incident and recurrent gout, this has not been demonstrated by intervention studies undertaken thus far. In addition, there is currently little evidence to support a role for vitamin C in the management of patients with gout (123).

## Mortality

Two large prospective cohort studies assessed the relationship between dietary and supplemental vitamin C intakes and mortality. In the Vitamins and Lifestyle Study, 55,543 men and women (ages 50-76 years) were questioned at baseline on their use of dietary supplements during the previous 10 years (124). After five years of follow-up, vitamin C supplement use was associated with a small decreased risk of total mortality, although no association was found with cardiovascular disease- or cancer-specific mortality. In the second prospective cohort study, the Diet, Cancer and Health Study, 55,543 Danish adults (ages 50-64 years) were questioned at baseline about their lifestyle, diet, and supplement use during the previous 12 months (125). No association between dietary or supplemental intake of vitamin C and mortality was found after approximately 14 years of follow-up. In contrast, a 2014 meta-analysis of 10 prospective cohort studies in 17,696 women with breast cancer found a lower risk of total and breast cancer-specific mortality with higher supplemental and dietary vitamin C intakes (126). A 2012 meta-analysis of 29 trials found no effect of oral vitamin C, given alone or in combination with other antioxidants, on all-cause mortality (127).

In parallel to these dietary assessment studies, a strong inverse association between plasma vitamin C and mortality from all-causes, cardiovascular disease, and ischemic heart disease (and cancer in men only) was observed in the EPIC-Norfolk multicenter, prospective cohort study (128). After approximately four years of follow-up in 19,496 men and women (ages 45-79 years), a dose-

response relationship was observed such that each 20 µmol/L increase in plasma vitamin C was associated with an estimated 20% risk reduction in all-cause mortality. Similarly, higher serum vitamin C concentrations were associated with decreased risks of cancer-specific and all-cause mortality in 16,008 adults from the US National Health and Nutrition Examination Survey (NHANES) III (1994-1998) (129).

## Disease Treatment

### Cardiovascular disease

#### *Complications of cardiac procedures and surgeries*

**Periprocedural myocardial injury:** Coronary angioplasty (also called percutaneous transluminal coronary angioplasty) is a nonsurgical procedure for treating obstructive coronary heart disease (CHD), including unstable angina pectoris, acute myocardial infarction, and multivessel CHD. Angioplasty involves temporarily inserting and inflating a tiny balloon into the clogged artery to help restore the blood flow to the heart. Periprocedural myocardial injury that occurs in up to one-third of patients undergoing otherwise uncomplicated angioplasty increases the risk of morbidity and mortality at follow-up.

One randomized, placebo-controlled trial has examined the effect of intravenous vitamin C administered to patients with stable angina undergoing elective coronary angioplasty (130). Administration of a 1-gram (g) vitamin C infusion one hour prior to the angioplasty reduced the concentrations of oxidative stress markers and improved microcirculatory perfusion compared to placebo (130). Another trial randomized 532 patients to receive a 3-g vitamin C infusion or a placebo (saline solution) within six hours prior to coronary angioplasty (131). Vitamin C treatment substantially reduced the incidence of periprocedural myocardial injury, as assessed by a reduction in the concentrations of two markers of myocardial injury, namely creatine kinase and troponin-I (131). A recent randomized controlled trial assessed the effect of vitamin C and vitamin E administration on reperfusion damage in patients who experienced acute myocardial infarction and underwent coronary angioplasty (see below) (132).

**Myocardial reperfusion injury:** Reperfusion injury refers to tissue damage occurring at the time of blood flow restoration (reperfusion) following transient ischemia. The heart muscle may become oxygen-deprived (ischemic) as the result of myocardial infarction or with aortic clamping during coronary artery bypass graft (CABG) surgery. Increased generation of reactive oxygen species (ROS) when the heart muscle's oxygen supply is restored might be an important contributor to myocardial damage occurring at reperfusion (133). Myocardial reperfusion injury leads to complications, such as reperfusion arrhythmias (see Atrial fibrillation) and myocardial stunning.

Vitamin C is depleted during and following cardiac surgery (134) and this might be due to the direct quenching of ROS, the regeneration of other antioxidants, and/or a massive synthesis of catecholamines (dopamine, epinephrine, norepinephrine) (135). Two randomized controlled trials conducted in the 1990s reported a reduction in reperfusion-induced oxidative stress and

myocardial injury with intravenous (136) or oral (137) vitamin C administration prior to CABG surgery (reviewed in 135). A more recent randomized, double-blind, placebo-controlled trial has been designed to examine the effect of vitamin C and vitamin E administration on ischemia-reperfusion damage in 99 patients with acute myocardial infarction undergoing coronary angioplasty (132). Vitamin C infusion (sodium ascorbate: 3.20 mmol/min for 1 hour then 0.96 mmol/min for 2 hours) prior to reperfusion followed by oral supplementation with vitamin C (1 g/day) and vitamin E (400 IU/day) for 84 days effectively prevented a reduction in antioxidant capacity at reperfusion and for the next six to eight hours. The protocol also limited microvascular dysfunction (i.e., improved microcirculatory perfusion) and improved left ventricular ejection fraction at discharge (on day 84) (138, 139). However, no difference in the infarct size between antioxidant vitamin treatment and placebo was seen (138).

**Atrial fibrillation:** Atrial fibrillation is the most common type of cardiac arrhythmia. It is also a common post-cardiac surgery complication, leading to an increased risk of cardiovascular morbidity (e.g., heart failure, stroke) and mortality. Three meta-analyses of prospective cohort studies and randomized controlled trials have reported an overall reduction in the risk of post-operative atrial fibrillation following administration of primarily oral vitamin C (140-142). In most trials, participants received 2 g of vitamin C prior to undergoing CABG or valve replacement surgery and 1 to 2 g/day for five days post-surgery. Although only a minority of trials delivered vitamin C intravenously, this administration route appeared to be more effective at reducing the risk of atrial fibrillation — presumably due to higher plasma concentrations achieved (140). Of note, a subgroup analysis in one of the meta-analyses showed a reduction of post-operative atrial fibrillation with vitamin C in non US-based trials (10 trials) but no effect of vitamin C in US-based trials (5 trials) (140).

### *Cerebral ischemia-reperfusion injury*

A small randomized controlled trial performed in 60 ischemic stroke patients showed that intravenous vitamin C administration (500 mg/day for 10 days, initiated day 1 post-stroke) had no effect on serum markers of oxidative stress or neurological outcomes compared to placebo (143).

### *Vascular complications of diabetes mellitus*

Cardiovascular disease (CVD) is the leading cause of death in individuals with diabetes mellitus. The role of increased oxidative stress in the occurrence of vascular complications in subjects with diabetes has led to hypothesis that higher intakes of antioxidant nutrients could help lower the risk of CVD in diabetic subjects (144). A 2018 meta-analysis of randomized controlled trials investigating the effect of antioxidant vitamin supplementation in patients with type 2 diabetes found that most improvement in markers of oxidative stress and blood glucose control could be attributed to vitamin E (145). Another meta-analysis of trials found no effect of vitamins E and C, alone or in combination, on measures of β-cell function and insulin resistance (146). Yet, most studies were small and of short duration and thus did not assess the consequence of long-term use of antioxidant vitamins on the risk of vascular complications in diabetic patients. One 12-month

randomized placebo-controlled trial in 456 participants with type 2 diabetes treated with metformin examined the effect of vitamin C (500 mg/day) or acetylsalicylic acid (aspirin; 100 mg/day) on risk factors for diabetes-related complications such as CVD (147). Both vitamin C and aspirin reduced fasting blood glucose and HbA1c concentrations and improved blood lipid profile in metformin-treated patients. Compared to placebo, both treatments were found to be more likely to limit risk factors contributing to diabetes-related complications, as well as to lower the risk of future cardiovascular events over a 10-year period (estimated using the Framingham risk score) (147).

Of note, it is possible that genetic differences among diabetic patients influence the effect of vitamin C supplementation on cardiovascular risk. In particular, a specific allele of the haptoglobin gene (Hp), namely Hp2, appears to be associated with an increased risk of diabetic vascular complications. Carriers of two copies of the Hp2 allele (Hp2-2) express a Hp protein that has a lower capacity to bind and remove pro-oxidant, free hemoglobin (Hb) from plasma, compared to Hp proteins coded by the Hp1-1 and Hp1-2 genotypes. When the results of the Women's Antioxidant Vitamin Estrogen (WAVE) trial were reanalyzed based on Hp genotype, antioxidant therapy (1,000 mg/day of vitamin C + 800 IU/day of vitamin E) was associated with improvement of coronary atherosclerosis in diabetic women with Hp1-1 genotype but worsening of coronary atherosclerosis in those carrying the Hp2-2 genotype (148). Results from another study by the same investigators suggested that vitamin C could not prevent the oxidation of high-density lipoprotein (HDL)-cholesterol by glycated Hb-Hp2-2 complexes *in vitro* nor restore impaired HDL function in diabetic mice carrying the Hp2-2 genotype (149).

## Sepsis

Sepsis and septic shock — defined as persistent sepsis-induced low blood pressure — are associated with elevated mortality rates in critically ill patients (150, 151). Because systemic inflammatory responses involve excessive oxidative stress, it has been suggested that providing antioxidant nutrients like vitamin C may improve the outcome of critically ill patients in intensive care units. In addition, hypovitaminosis C is common in critically ill patients, especially in those with septic shock, and persists despite enteral/parenteral nutritional therapy providing recommended amounts of vitamin C (152). Vitamin C requirements are likely to be increased in this population due to the hypermetabolic response driven by the systemic inflammatory reaction (152, 153). Intravenous administration of 50 mg or 200 mg of vitamin C per kg per day for 96 hours to patients with sepsis admitted in intensive care unit was found to correct vitamin C deficiency. Vitamin C also prevented the rise of Sequential Organ Failure Assessment (SOFA) and Acute Physiologic Assessment and Chronic Health Evaluation (APACHE) II scores — used to assess severity of illness and risk of mortality — observed in placebo-treated patients (154). Vitamin C infusion also lowered the concentration of markers of inflammation and endothelial injury in patients compared to placebo (154). In another randomized, double-blind, controlled trial in 28 critically ill patients with septic shock, infusion of 25 mg of vitamin C per kg every six hours for 72 hours significantly limited the requirement to vasopressor norepinephrine — decreasing both the dose and duration of

treatment — and dramatically improved the 28-day survival rate (155). Similar results have been reported in septic patients given intravenous vitamin C (1.5 g/6 h), hydrocortisone (50 mg/6 h), and thiamin (200 mg/12 h) until hospital discharge. Compared to standard-of-care, this intervention cocktail more than halved the mean duration of vasopressor use (18.3 h versus 54.9 h) and reduced the odds of mortality by nearly 90% (156). Although intravenous vitamin C administration appears to be safe and well tolerated, there is a non-negligible risk of oxalate nephropathy (a rare cause of kidney failure) in these critically ill patients (157).

See also the section on Sepsis in the Symposium on Vitamin C — Part of the LPI's 9th International Conference on Diet and Optimum Health.

## Cancer

### *Route of administration*

Studies in the 1970s and 1980s conducted by Linus Pauling, Ewan Cameron, and colleagues suggested that large doses of vitamin C (10 g/day infused intravenously for 10 days followed by at least 10 g/day orally indefinitely) were helpful in increasing the survival time and improving the quality of life of terminal cancer patients (158). Controversy surrounding the efficacy of vitamin C in cancer treatment ensued, leading to the recognition that the route of vitamin C administration is critical (22, 159). Compared to orally administered vitamin C, intravenous vitamin C can result in 30 to 70-fold higher plasma vitamin C concentrations (25). Higher plasma concentrations achieved via intravenous vitamin C administration are comparable to those that are toxic to cancer cells in culture. The anticancer mechanism of intravenous vitamin C action is under investigation. It may involve the production of high levels of hydrogen peroxide, selectively toxic to cancer cells (22, 160-162), or the deactivation of hypoxia inducible factor, a prosurvival transcription factor that protects cancer cells from various forms of stress (159, 163, 164). Vitamin C likely also plays a role in the maintenance of genome integrity and in the protection against cellular transformation through regulating DNA and histone demethylating enzymes (see Function) (165).

### *Safety*

Current evidence from controlled clinical trials indicates that intravenous vitamin C is generally safe and well tolerated in cancer patients. Of note, because intravenous administration of 80 g of vitamin C precipitated hemolytic anemia in two subjects with glucose-6-phosphate dehydrogenase deficiency, patients due to receive high-dose vitamin C infusion are systematically screened for this genetic disorder (166). Four phase I clinical trials in patients with advanced cancer found that intravenous administration of vitamin C at doses up to 1.5 g/kg of body weight (equivalent to about 100 g/day for an average weight [70 kg] person) and 70 to 80 g/m$^2$ was well tolerated and safe in pre-screened patients (167-170). A few observational studies in cancer patients undergoing chemotherapy and/or radiotherapy reported that complementary intravenous vitamin C treatment was associated with a reduction in treatment-associated side effects and an improved quality of life

(171). A phase I study in nine patients with metastatic pancreatic cancer showed that millimolar concentrations of plasma vitamin C could be reached safely when administered in conjunction with the cancer chemotherapy drugs, gemcitabine and erlotinib (168).

### *Sensitivity to vitamin C*

Retrospective *in vitro* colony formation assays revealed that patient leukemic cells displayed variable sensitivity to vitamin C treatment: leukemic cells from seven out of the nine patients who experienced a significant clinical benefit were sensitive to vitamin C *in vitro* (i.e., "responders"); the leukemic cells from the remaining six patients were not sensitive to vitamin C (i.e., "non-responders"). Thus, *in vitro* vitamin C sensitivity assays may provide predictive value for the clinical response to intravenous vitamin C treatment. The mechanisms underlying differential sensitivity to vitamin C are under investigation. *In vitro* experiments performed using 11 different cancer cell lines demonstrated that sensitivity to vitamin C correlated with the expression of catalase, an enzyme involved in the decomposition of hydrogen peroxide (172). Approximately one-half of the cell lines tested were resistant to vitamin C cytotoxicity, a response associated with high levels of catalase activity.

Sensitivity to vitamin C may also be determined by the expression of sodium-dependent vitamin C transporter-2 (SVCT-2), which transports vitamin C into cells (173). Higher SVCT-2 levels were associated with enhanced sensitivity to vitamin C in nine different breast cancer cell lines. Moreover, SVCT-2 was significantly expressed in 20 breast cancer tissue samples, but weakly expressed in normal tissues. Finally, mutations in genes coding for vitamin C-dependent TET demethylases, mutations that are common in cancer cells, may also contribute to resistance to vitamin C treatment (165).

### *Efficacy*

Current evidence of the efficacy of intravenous vitamin C in cancer patients is limited to observational studies, uncontrolled interventions, and case reports (174, 175). There is a need for larger, longer-duration phase II clinical trials that test the efficacy of intravenous vitamin C in disease progression and overall survival (176).

See also the section on Cancer in the Symposium on Vitamin C — Part of the LPI's 9th International Conference on Diet and Optimum Health.

## Common cold

The work of Linus Pauling stimulated public interest in the use of doses greater than 1 g/day of vitamin C to prevent the common cold (177). In the past 40 years, numerous placebo-controlled trials have examined the effect of vitamin C supplementation on the prevention and treatment of colds. A 2013 meta-analysis of 53 placebo-controlled trials evaluated the effect of vitamin C supplementation on the incidence, duration, or severity of the common cold when taken as a continuous daily supplement (43 trials) or as therapy upon onset of cold symptoms (10 trials) (178).

Regarding the incidence of colds, a difference was observed between two groups of participants. Regular supplementation with vitamin C (0.25 to 2 g/day) did not reduce the incidence of colds in the general population (23 trials); however, in participants undergoing heavy physical stress (e.g., marathon runners, skiers, or soldiers in subarctic conditions), vitamin C supplementation halved the incidence of colds (5 trials). A benefit of regular vitamin C supplementation was also seen in the duration of colds, with a greater benefit in children than in adults: The pooled effect of vitamin C supplementation was a 14% reduction in cold duration in children and an 8% reduction in adults. Finally, no significant effect of vitamin C supplementation (1-8 g/day) was observed in therapeutic trials in which vitamin C was administered after cold symptoms occurred.

In addition, a 2013 systematic review by the same investigators identified only two small randomized, double-blind, placebo-controlled trials that examined the effect of vitamin C on the incidence of respiratory infection-induced asthma (179). One trial found that vitamin C supplementation (1 g/day) for 14 weeks reduced the risk of asthma attacks precipitated by respiratory infection. The other trial randomized subjects diagnosed with infection-related asthma to receive 5 g/day of vitamin C or a placebo for one week; a lower proportion of participants was found to present with bronchial hypersensitivity to histamine — which characterizes chronic asthma — in the vitamin C group compared to the control group (reviewed in 179). These observations need to be confirmed in larger, well-designed trials.

## Asthma

A 2013 systematic review identified 11 randomized controlled studies that evaluated the effect of vitamin C on asthma (eight trials) or exercise-induced bronchoconstriction (three trials) (180). Exercise-induced bronchoconstriction is a transient narrowing of the airways that occurs after exercise and is indicated by a ≥10% decline in Forced Expiratory Volume in 1 second ($FEV_1$). In the three trials that included a total of 40 participants with exercise-induced bronchoconstriction, vitamin C administration before exercise (a 0.5-g dose on two subsequent days in one trial, a single dose of 2 g in the second trial, and 1.5 g daily for two weeks in the third trial) significantly reduced the exercise-induced decline in $FEV_1$. Among the five out of eight trials in asthmatic subjects that reported on $FEV_1$ outcomes, none found a difference between vitamin C supplementation and placebo (180).

## Lead toxicity

Although the use of lead paint and leaded gasoline has been discontinued in the US, lead toxicity continues to be a significant health problem, especially in children living in urban areas. Abnormal growth and development have been observed in infants of women exposed to lead during pregnancy, while children who are chronically exposed to lead are more likely to develop learning disabilities, behavioral problems, and to have a low IQ. In adults, lead toxicity may result in kidney damage, high blood pressure, and anemia.

Several cross-sectional studies have reported an inverse association between vitamin C status and blood lead concentration. For instance, in a study of 747 older men, blood lead concentration was significantly higher in those who reported total dietary vitamin C intakes averaging less than 109 mg/day compared to those with higher vitamin C intakes (181). A much larger study of 19,578 people, including 4,214 children from 6 to 16 years of age, found higher serum vitamin C concentrations to be associated with significantly lower blood lead concentrations (182). A US national survey of more than 10,000 adults found that blood lead concentrations were inversely related to serum vitamin C concentrations (183).

Cigarette smoking or second-hand exposure to cigarette smoke contributes to increased blood lead concentration and a state of chronic low-level lead exposure. An intervention trial in 75 adult male smokers found that supplementation with 1,000 mg/day of vitamin C resulted in significantly lower blood lead concentration over a four-week treatment period compared to placebo (184). A lower dose of 200 mg/day did not significantly affect blood lead concentration, although serum vitamin C concentrations were not different from those in the group who took 1,000 mg/day.

The mechanism(s) by which vitamin C reduces blood lead concentration is not known, yet it has been proposed that vitamin C could inhibit intestinal absorption (184) or enhance urinary excretion of lead (185).

## Sources

Unlike plants and most animals, humans have lost the ability to synthesize vitamin C endogenously and therefore have an essential dietary requirement for this vitamin (see The Recommended Dietary Allowance). Results from 7,277 participants in the US National Health and Nutrition Examination Survey (NHANES) 2003-2004 indicated that an estimated 7.1% of individuals ages ≥6 years were deficient in vitamin C — based on serum vitamin C concentrations <11.4 µmol/L (36). The national study identified smokers and those of lower socioeconomic status to both be at higher risk for vitamin C deficiency (36).

### Food sources

As shown in Table 3, different fruit and vegetables vary in their vitamin C content, but five servings (2½ cup-equivalents) of a variety of fruit and vegetables should average out to about 150 to 200 mg of vitamin C, especially if vitamin C-rich fruits are consumed. If you wish to check foods for their vitamin C content, search USDA's FoodData Central.

Table 3. Some Food Sources of Vitamin C

| Food | Serving | Vitamin C (mg) |
|---|---|---|
| Kiwifruit, Zespri SunGold | 1 fruit (81 g) | 131 |
| Grapefruit juice, pink, raw | ¾ cup (6 ounces) | 94 |

| | | |
|---|---|---|
| Orange juice, raw | ¾ cup (6 ounces) | 93 |
| Strawberries | 1 cup, whole | 85 |
| Grapefruit juice, white, raw | ¾ cup (6 ounces) | 70 |
| Kiwifruit | 1 fruit (74 g) | 69 |
| Orange | 1 medium | 65 |
| Sweet red pepper, raw | ½ cup, chopped | 59 |
| Broccoli, cooked | ½ cup | 51 |
| Grapefruit, raw | ½ medium | 44 |
| Brussel sprouts, cooked | ½ cup | 37 |
| Potato, white, flesh and skin | 1 medium, baked | 22 |
| Tomato, red, ripe, raw | 1 medium | 17 |
| Banana, raw | 1 medium | 10 |
| Apple, raw | 1 medium | 8 |
| Spinach, raw | 1 cup | 8 |

## Supplements

Vitamin C (L-ascorbic acid) is available in many forms, but there is little scientific evidence that any one form is better absorbed or more effective than another. Most experimental and clinical research uses ascorbic acid or its sodium salt, called sodium ascorbate. Natural and synthetic L-ascorbic acid are chemically identical and there are no known differences regarding biological activities or bioavailability (186).

### *Mineral ascorbates*

Mineral salts of vitamin C are considered less acidic than vitamin C and therefore are considered "buffered." Some people find them less irritating to the gastrointestinal tract than ascorbic acid. Sodium ascorbate and calcium ascorbate are the most common forms, although a number of other mineral ascorbates are available. Sodium ascorbate provides 111 mg of sodium (889 mg of ascorbic acid) per 1,000 mg of sodium ascorbate, and calcium ascorbate generally provides 90 to 110 mg of calcium (890-910 mg of ascorbic acid) per 1,000 mg of calcium ascorbate.

### *Vitamin C with flavonoids*

Flavonoids are a class of water-soluble plant pigments that are often found in vitamin C-rich fruit and vegetables, especially citrus fruit and berries (see the article on Flavonoids). There is little evidence that the flavonoids in most commercial preparations increase the bioavailability or

efficacy of vitamin C (187). Some, yet not all, studies in animal models such as vitamin C-deficient guinea pigs or genetically scorbutic rats found an increased uptake of vitamin C in peripheral circulation and specific organs in the presence of flavonoids. However, studies conducted in humans found no differences in bioavailability of vitamin C from flavonoid-rich whole fruit or fruit juice and synthetic vitamin C (reviewed in 186).

### *Vitamin C and metabolites*

One supplement, Ester-C®, contains mainly calcium ascorbate and includes small amounts of the vitamin C metabolites, dehydroascorbic acid (oxidized ascorbic acid), calcium threonate, and trace amounts of xylonate and lyxonate. Although these metabolites are purported to increase the bioavailability of vitamin C, the only published study in humans addressing this issue found no difference between Ester-C® and commercially available vitamin C tablets with respect to the absorption and urinary excretion of vitamin C (187). Ester-C® should not be confused with ascorbyl palmitate, which is also marketed as "vitamin C ester" (see below).

### *Ascorbyl palmitate*

Ascorbyl palmitate is a vitamin C ester (i.e., ascorbic acid linked to a fatty acid). In this case, vitamin C is esterified to the saturated fatty acid, palmitic acid, resulting in a fat-soluble form of vitamin C. Ascorbyl palmitate has been added to a number of skin creams due to interest in its antioxidant properties, as well as its importance in collagen synthesis (see the separate article, Vitamin C and Skin Health) (188). Although ascorbyl palmitate is also available as an oral supplement, most of it is likely hydrolyzed to ascorbic acid and palmitic acid in the digestive tract before it is absorbed (189). Ascorbyl palmitate is marketed as "vitamin C ester," which should not be confused with Ester-C® (see above).

### *Other formulations of vitamin C*

One small placebo-controlled, cross-over trial in 11 men showed that the oral administration of 4 g of vitamin C resulted in a greater vitamin C concentration in plasma over a four-hour period when vitamin C was encapsulated in liposomes compared to unencapsulated vitamin C (190). Although liposomal encapsulation could increase vitamin C bioavailability, plasma vitamin C concentrations were much lower than those achieved with intravenous vitamin C administration (190).

For a more detailed review of scientific research on the bioavailability of different forms of vitamin C, see The Bioavailability of Different Forms of Vitamin C.

## Safety

### Toxicity

A number of possible adverse health effects of very large doses of vitamin C have been identified, mainly based on *in vitro* experiments or isolated case reports, and include genetic mutations, birth defects, cancer, atherosclerosis, kidney stones, "rebound scurvy," increased oxidative stress, excess

iron absorption, vitamin $B_{12}$ deficiency, and erosion of dental enamel. However, none of these alleged adverse health effects have been confirmed in subsequent studies, and there is no reliable scientific evidence that doses of vitamin C up to 10 g/day in adults are toxic or detrimental to health. The concern of kidney stone formation with vitamin C supplementation is discussed below.

With the latest RDA published in 2000, a tolerable upper intake level (UL) for vitamin C was set for the first time (**Table 4**). A UL of 2 g (2,000 mg) daily was recommended in order to prevent generally healthy adults from experiencing diarrhea and gastrointestinal disturbances (35). Such symptoms are not generally serious, especially if they resolve with temporary discontinuation of vitamin C supplementation.

Table 4. Tolerable Upper Intake Level (UL) for Vitamin C

| Age Group | UL (mg/day) |
|---|---|
| Infants 0-12 months | Not possible to establish* |
| Children 1-3 years | 400 |
| Children 4-8 years | 650 |
| Children 9-13 years | 1,200 |
| Adolescents 14-18 years | 1,800 |
| Adults 19 years and older | 2,000 |

*Source of intake should be from foods or formula only.

## Kidney stones

Because oxalate is a metabolite of vitamin C, there is some concern that high vitamin C intake could increase the risk of calcium oxalate kidney stones. Some (24, 191, 192), but not all (193-195), studies have reported that supplemental vitamin C increases urinary oxalate concentrations. Whether any increase in oxalate levels would translate to an elevation in risk for kidney stones has been examined in several epidemiological studies. Two large prospective cohort studies, one following 45,251 men for six years and the other following 85,557 women for 14 years, reported that consumption of ≥1,500 mg of vitamin C daily did not increase the risk of kidney stone formation compared to those consuming <250 mg daily (196, 197). On the other hand, two other large prospective studies reported that a high intake of vitamin C was associated with an increased risk of kidney stone formation in men (198, 199). Specifically, the Health Professionals Follow-Up Study collected data on dietary and supplemental vitamin C intake every four years in 45,619 male health professionals (ages 40-75 years) (198). After 14 years of follow-up, it was found that men who consumed ≥1,000 mg/day of vitamin C had a 41% higher risk of kidney stones compared to men consuming <90 mg of vitamin C daily. In the Cohort of Swedish Men study, self-reported use

of single-nutrient vitamin C supplements (taken seven or more times per week) at baseline was associated with a two-fold higher risk of incident kidney stones among 48,840 men (ages 45-79 years) followed for 11 years (199). Despite conflicting results, it may be prudent for individuals predisposed to oxalate kidney stone formation to avoid high-dose vitamin C supplementation.

## Drug interactions

Overall, evidence suggesting specific drugs can lower blood vitamin C concentrations in humans is limited. Dihydropyridine calcium channel blockers (e.g., nicardipine, nifedipine) can inhibit vitamin C uptake by intestinal cells *in vitro*. However, a reduction in blood vitamin C concentrations with these drugs has not been reported in humans (200). Aspirin can impair vitamin C status if taken frequently (201).

Conversely, there are case reports suggesting that supplemental vitamin C may lower blood concentrations of some medications, such as fluphenazine (the antipsychotic drug, Prolixin) and indinavir (the antiretroviral drug, Crixivan) (200). There is some evidence, though controversial, that vitamin C interacts with anticoagulant medications like warfarin (Coumadin). Large doses of vitamin C may block the action of warfarin and thus lower its effectiveness. Individuals on anticoagulants should limit their vitamin C intake to <1 g/day and have their prothrombin time monitored by the clinician following their anticoagulant therapy (200). In addition, vitamin C may bind aluminum in the gut and increase the absorption of aluminum-containing compounds (e.g., aluminum-containing antacids, aluminum-containing phosphate binders). People with impaired kidney function may be at risk for aluminum toxicity when supplemental vitamin C is taken at the same time as these compounds (200, 201). Finally, supplemental vitamin C may increase blood estrogen concentrations in women using oral contraceptives or hormone replacement therapy (200).

The potential effect of antioxidants during chemotherapy is not well understood, yet only likely to be an issue if a specific chemotherapeutic agent acts through an oxidative mechanism, which is uncommon (171). It is not clear whether vitamin C given parenterally could diminish or increase the efficacy of chemotherapy drugs — in particular, akylating agents (e.g., cyclophosphamide, busulfan), antitumor antibiotics (e.g., doxorubicin, bleomycin), and arsenic trioxide. Patients are advised to discuss with their oncologist before using vitamin C supplements (200, 201).

Because high doses of vitamin C have also been found to interfere with the interpretation of certain laboratory tests (e.g., serum bilirubin, serum creatinine, and the stool guaiac assay for occult blood), it is important to inform one's health care provider of any recent supplement use.

### *Antioxidant supplements and HMG-CoA reductase inhibitors (statins)*

A three-year randomized controlled trial in 160 patients with documented coronary heart disease and low blood HDL concentrations found that a combination of simvastatin (Zocor) and niacin increased HDL concentration, inhibited the progression of coronary artery stenosis (narrowing), and decreased the frequency of cardiovascular events, such as myocardial infarction and stroke (202). Surprisingly, when an antioxidant combination (1,000 mg vitamin C, 800 IU vitamin E,

100 mg selenium, and 25 mg β-carotene daily) was taken with the simvastatin-niacin combination, the protective effects were diminished. Since the antioxidants were taken together in this trial, the individual contribution of vitamin C cannot be determined. In contrast, a much larger trial in more than 20,000 men and women with coronary heart disease or diabetes mellitus found that simvastatin and an antioxidant combination (600 mg vitamin E, 250 mg vitamin C, and 20 mg β-carotene daily) did not diminish the cardioprotective effects of simvastatin therapy over a five-year period (203). These contradictory findings indicate that further research is needed on potential interactions between antioxidant supplements and cholesterol-lowering drugs, such as HMG-CoA reductase inhibitors (statins).

## Does vitamin C promote oxidative damage under physiological conditions?

Vitamin C is known to function as a highly effective antioxidant in living organisms. However, in test tube experiments, vitamin C can interact with some free metal ions and lead to the generation of potentially damaging free radicals. Although free metal ions are not generally found under physiological conditions, the idea that high doses of vitamin C might be able to promote oxidative damage *in vivo* has received a great deal of attention. Widespread publicity has been given to a few studies suggesting a pro-oxidant effect of vitamin C (204, 205), but these studies turned out to be either flawed or of no physiological relevance. A comprehensive review of the literature found no credible scientific evidence that supplemental vitamin C promotes oxidative damage under physiological conditions or in humans (206).

## Linus Pauling Institute Recommendation

Combined evidence from metabolic, pharmacokinetic, and observational studies, and from randomized controlled trials supports consuming sufficient vitamin C to achieve plasma concentrations of at least 60 µmol/L. While most generally healthy young adults can achieve these plasma concentrations with daily vitamin C intake of at least 200 mg/day, some individuals may have a lower vitamin C absorptive capacity than what is currently documented. Thus, the Linus Pauling Institute recommends a vitamin C intake of 400 mg daily for adults to ensure replete tissue concentrations (29) — an amount substantially higher than the RDA yet with minimal risk of side effects.

This recommendation can be met through food if the diet includes at least several servings of vitamin C-rich fruit and vegetables (e.g., citrus fruit, kiwifruit, peppers; see Food sources) as part of the daily recommended fruit and vegetable intake (see article on Fruit and Vegetables). Most multivitamin supplements provide at least 60 mg of vitamin C.

## Older adults (>50 years)

Whether older adults have higher requirements for vitamin C is not yet known with certainty, yet some older populations have been found to have vitamin C intakes considerably below the RDA of 75 and 90 mg/day for women and men, respectively (207). A vitamin C intake of at least 400 mg

daily may be particularly important for older adults who are at higher risk for age-related chronic diseases. Pharmacokinetic studies in older adults have not yet been conducted, but there is some evidence suggesting that the efficiency of one of the molecular mechanisms for the cellular uptake of vitamin C declines with age (208). Because maximizing blood concentrations of vitamin C may be important in protecting against oxidative damage to cells and biological molecules, a vitamin C intake of at least 400 mg daily might benefit older adults who are at higher risk for chronic diseases caused, in part, by oxidative damage, such as heart disease, stroke, certain cancers, and cataract.

For more information on the difference between Dr Linus Pauling's recommendation and the Linus Pauling Institute's recommendation for vitamin C intake, select the highlighted text.

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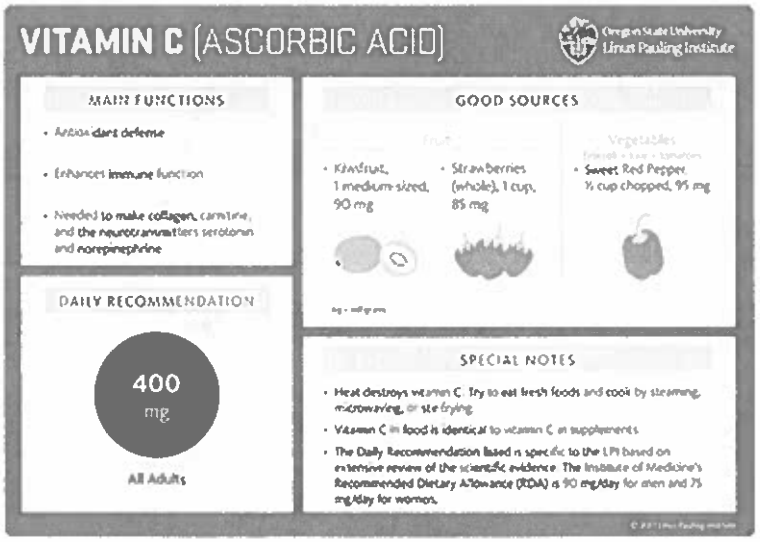
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# Vitamin C and E supplementation hampers cellular adaptation to endurance training in humans: a double-blind, randomised, controlled trial

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**Key points**

- Recent studies have indicated that antioxidant supplementation may blunt adaptations to exercise, such as mitochondrial biogenesis induced by endurance training. However, studies in humans are sparse and results are conflicting.
- Isolated vitamin C and E supplements are widely used, and unravelling the interference of these vitamins in cellular and physiological adaptations to exercise is of interest to those who exercise for health purposes and to athletes.
- Our results show that vitamin C and E supplements blunted the endurance training-induced increase of mitochondrial proteins (COX4), which is important for improving muscular endurance.
- Training-induced increases in $\dot{V}_{O_2max}$ and running performance were not detectably affected by the supplementation.
- The present study contributes to understanding of how antioxidants may interfere with adaptations to exercise in humans, and the results indicate that high dosages of vitamins C and E should be used with caution.

**Abstract** In this double-blind, randomised, controlled trial, we investigated the effects of vitamin C and E supplementation on endurance training adaptations in humans. Fifty-four young men and women were randomly allocated to receive either 1000 mg of vitamin C and 235 mg of vitamin E or a placebo daily for 11 weeks. During supplementation, the participants completed an endurance training programme consisting of three to four sessions per week (primarily of running), divided into high-intensity interval sessions [4–6 × 4–6 min; >90% of maximal heart rate ($HR_{max}$)] and steady state continuous sessions (30–60 min; 70–90% of $HR_{max}$). Maximal oxygen uptake ($\dot{V}_{O_2max}$), submaximal running and a 20 m shuttle run test were assessed and blood samples and muscle biopsies were collected, before and after the intervention. Participants in the vitamin C and E group increased their $\dot{V}_{O_2max}$ (mean ± S.D.: 8 ± 5%) and performance in the 20 m shuttle test (10 ± 11%) to the same degree as those in the placebo group (mean ± S.D.: 8 ± 5% and 14 ± 17%, respectively). However, the mitochondrial marker cytochrome c oxidase subunit IV (COX4) and cytosolic peroxisome proliferator-activated receptor-$\gamma$ coactivator 1 $\alpha$ (PGC-1$\alpha$) increased in the m. vastus lateralis in the placebo group by 59 ± 97% and 19 ± 51%,

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respectively, but not in the vitamin C and E group (COX4: −13 ± 54%; PGC-1α: −13 ± 29%; $P \leq 0.03$, between groups). Furthermore, mRNA levels of CDC42 and mitogen-activated protein kinase 1 (MAPK1) in the trained muscle were lower in the vitamin C and E group than in the placebo group ($P \leq 0.05$). Daily vitamin C and E supplementation attenuated increases in markers of mitochondrial biogenesis following endurance training. However, no clear interactions were detected for improvements in $\dot{V}_{O_2max}$ and running performance. Consequently, vitamin C and E supplementation hampered cellular adaptations in the exercised muscles, and although this did not translate to the performance tests applied in this study, we advocate caution when considering antioxidant supplementation combined with endurance exercise.

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## Introduction

Aerobic endurance exercise is highly recommended by health authorities for its rewarding effects on health (Garber *et al.* 2011) and, in many sports, high muscular aerobic energy capacity and $\dot{V}_{O_2max}$ are prerequisites for elite performance (Saltin & Astrand, 1967). Strategies for obtaining optimal endurance training effects include not only certain training methods, such as interval training (Gibala, 2007), but also nutritional measures (Hawley *et al.* 2011). Supplements containing antioxidants and vitamins are widely used for the purpose of improving health and athletic achievement (Petroczi *et al.* 2007; Kennedy *et al.* 2013). Isolated vitamin C and E supplements are among the most commonly used, despite tentative evidence for the purported effects of these vitamins on health, sport performance and recovery from muscle damage (Padayatty *et al.* 2003; Nikolaidis *et al.* 2012).

Contrary to common beliefs, studies have recently demonstrated that antioxidant supplementation may interfere with exercise-induced cell signalling in skeletal muscle fibres (Ristow & Zarse, 2010; Hawley *et al.* 2011). In turn, such changes in cell signalling may potentially blunt or block adaptations to training (Peternelj & Coombes, 2011; Gliemann *et al.* 2013; Morales-Alamo & Calbet, 2014). For example, Gomez-Cabrera *et al.* (2008) investigated whether high dosages of vitamin C affected adaptation to endurance exercise training in both an animal and a human model (1000 mg day$^{-1}$ in the human study; male participants). Interestingly, endurance performance increased to a greater extent in animals treated with the placebo compared with animals treated with vitamin C. Furthermore, markers for mitochondrial biogenesis [i.e. peroxisome proliferator-activated receptor-γ co-activator 1-α (PGC-1α)] increased only in animals treated with the placebo. In the human experiment, changes in $\dot{V}_{O_2max}$ did not differ significantly between the supplement and placebo groups. Unfortunately, these authors did not test endurance capacity or collect muscle biopsies from participants to verify the results of the animal study. In another study with untrained and trained male participants, Ristow *et al.* (2009) demonstrated that 4 weeks of vitamin C (1000 mg day$^{-1}$) and E (400 IU day$^{-1}$) supplementation blunted training-induced increases in the mRNA expression of genes associated with mitochondrial biogenesis and endogenous antioxidant systems in skeletal muscle (e.g. PGC-1α and glutathione peroxidase). Furthermore, Braakhuis *et al.* (2014) observed that supplementation with 1000 mg day$^{-1}$ of vitamin C for 3 weeks slowed female runners during training, although no differences were found in a 5 km time trial or in an incremental treadmill test after the intervention period.

Contrary to these studies, Yfanti *et al.* (2010, 2011, 2012) found no negative effects of vitamin C (500 mg day$^{-1}$) and E (400 IU day$^{-1}$) supplementation in male participants who trained five times per week for 12 weeks on a cycle ergometer. The antioxidant supplementation did not influence changes in $\dot{V}_{O_2max}$ and maximal power output (cycling), or activity of the enzymes citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) in skeletal muscle. Similarly, Roberts *et al.* (2011) reported no effects of vitamin C (1000 mg day$^{-1}$) supplementation on adaptations to high-intensity running training in male participants. $\dot{V}_{O_2max}$ and endurance performance (10 km time trial and YoYo tests) improved equally in supplemented and placebo groups. The conflicting results from these human studies are reflected in the findings of recent animal studies (Braakhuis, 2012; Gomez-Cabrera *et al.* 2012; Nikolaidis *et al.* 2012).

Accordingly, it seems clear that antioxidant supplementation potentially inhibits favourable cellular responses to endurance training. By contrast, the discrepancy between studies invites further investigation. Therefore, we studied the influence of vitamin C and E supplementation on adaptations to aerobic endurance training, hypothesising that high dosages of vitamin C and E, ingested shortly before and after exercise, would blunt physiological adaptations to 11 weeks of endurance

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training. The hypothesis was tested in a study using a double-blind, randomised, controlled trial design, in which both training and nutrition were tightly controlled. We combined performance tests with physiological measurements ($\dot{V}_{O_2max}$) and biochemical/molecular analyses of blood and muscle.

## Methods

### Participants

Fifty-four young, healthy men and women participated in the experiment (Table 1 and Fig. 1). Forty of the volunteers were defined as recreationally endurance-trained individuals because they had been endurance training between one and four times per week for 6 months prior to the study. The endurance training consisted mainly of running and cycling. Fourteen volunteers were defined as untrained because they had not trained regularly (maximum of one session per week) during the previous 6 months. Sixty-eight volunteers were recruited to the study, but 14 participants (seven from each group) dropped out of the study during the training intervention. Five participants were injured during training (ankle sprains, Achilles pains) and nine dropped out for reasons unrelated to the study.

The volunteers were instructed not to take any form of supplements or medication (except contraceptives). Individuals who did use multi-vitamin supplements were asked to stop taking them at least 2 weeks before the beginning of the study.

The study was approved by the Regional Ethics Committee for Medical and Health Research of South-East Norway and performed in accordance with the Helsinki Declaration. All participants signed a written consent form.

### Experimental design

After pre-tests and assessments (e.g. $\dot{V}_{O_2max}$ and muscle biopsies), participants were randomly allocated to a vitamin C and E supplement group or a placebo group. The randomisation was stratified by gender and $\dot{V}_{O_2max}$. All participants started to take supplements or placebo as they started on the endurance training programme. All tests were replicated after 11 weeks of training. The experiment was a double-blind, randomised, controlled trial.

Blood samples and muscle biopsies collected before the intervention period were preceded with 3 days of rest and were scheduled to occur again 3 days after the last exercise session. However, for practical reasons, a few participants provided samples 2 or 4 days after the last exercise session. There was no group bias in the sampling time-points.

### Supplementation and nutrition

The C and E vitamin and placebo pills were produced under Good Manufacturing Practice (GMP) requirements at Petefa AB (Västra Frölunda, Sweden). Each vitamin pill contained 250 mg of ascorbic acid and 58.5 mg DL-α-tocopherol acetate. The placebo pills had the same shape and appearance as the vitamin pills.

The pills were analysed by a commercial company (Vitas AS, Oslo, Norway) 2 years after production; no sign of degradation of the vitamins was found (per pill: mean ± S.D. vitamin C: 255 ± 7 mg; mean ± S.D. vitamin E: 62 ± 2 mg). The experiments were conducted within this time period. No traces of the vitamins were found in the placebo pills.

Participants consumed two pills (500 mg vitamin C and 117 mg vitamin E) 1–3 h before every training session and two pills within 1 h after training. On non-training days, participants ingested two pills in the morning and two pills in the evening. Thus, the daily dosage was 1000 mg of vitamin C and 235 mg of vitamin E. Supplement intake was confirmed in a training diary.

Participants were asked to drink no more than two glasses of juice and four cups of coffee or tea per day. Juices especially rich in antioxidants, such as grape juice, were to be avoided.

We aimed to keep the participants in energy balance and encouraged them to continue their normal diets. Participants completed a weighed food registration dietary assessment over 4 days (Black *et al.* 1991) at the start and end of the intervention period. Participants used a digital food scale with precision to 1 g (Vera 67002; Soehnle-Waagen GmbH & Co., Murrhardt, Germany). The dietary registrations were analysed with a nutrient analysis programme (Mat på data 4.1; Norwegian Nutrition Society, Oslo, Norway).

### Body composition

A bioimpedance apparatus (Inbody 720; Biospace Co., Ltd, Seoul, South Korea) was used to assess body composition before and after the training intervention. The apparatus has been validated (compared with Dual-energy X-ray absorptiometry, DXA) for estimating fat mass and lean mass in men and women (Anderson *et al.* 2012).

### Endurance training

The training programme was divided into three periods (Table 2). In period 1, participants exercised three times per week in two continuous sessions (30 min and 60 min) and one interval session (4 × 4 min). In period 2, one extra interval session was added to give a total of four sessions per week. In periods 2 and 3, the number of runs per interval session was increased, although exercise intensity was similar throughout the training period. The exception was that less experienced runners (untrained participants)

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used three to six sessions to gradually increase the intensity. Intensity was high in every session, except during the 60 min run (moderate intensity). Running was the main form of exercise, but one running session per week could be substituted by cycling, cross-country skiing or a similar whole-body activity.

Training intensity was controlled using the Borgs scale (rating of perceived exertion) and heart rate monitors (RS400/RS800CX; Polar Electro Oy, Kempele, Finland). Heart rate monitors were worn in every session and training data were collected and controlled by the investigators. Moreover, each participant was instructed to complete a training diary in which he or she logged mean heart rate, running distance and perceived effort (not reported).

### $\dot{V}_{O_2max}$ and submaximal workloads

All participants took part in a familiarisation session for $\dot{V}_{O_2max}$ measurements using a mixing chamber (Oxycon Pro; Erich Jaeger GmbH, Hoechberg, Germany) on a treadmill (ELG 90/200 Sport; Woodway GmbH, Weil am Rhein, Germany). The pre-test for $\dot{V}_{O_2max}$ started with 7 min at two submaximal running speeds (5.3% inclination), corresponding to 60% and 85% of the $\dot{V}_{O_2peak}$ reached during the familiarisation session. $\dot{V}_{O_2}$, respiratory exchange ratio (RER), heart rate (measured using the RS400; Polar Electro Oy) and ratings of perceived exertion (Borgs scale) were measured during the last 2 min at each speed. Capillary blood from a finger-stick was sampled within 1 min after each workload and blood lactate concentration was measured (YSI 1500 Sport Lactate Analyzer; YSI, Inc., Yellow Springs, OH, USA). The same submaximal running velocities were used in both the pre- and post-tests.

After a 10 min rest, the participants performed the $\dot{V}_{O_2max}$ test. Running speed (5.3% inclination) was increased by 1 km h$^{-1}$ in three 1 min stages, before 0.5 km h$^{-1}$ increases per minute until exhaustion (total duration: 4–8 min). Lactate was measured as detailed above.

**20 m shuttle run test (Beep test).** The 20 m shuttle run test is a multi-stage shuttle run test that measures aerobic fitness and has demonstrated good reliability (Leger *et al.* 1988). Participants ran a distance of 20 m between two lines and placed one foot on the line each time a beep sounded (from a CD player); the interval between beeps decreased over time. The test had 21 levels. It started at a speed of 8 km h$^{-1}$ and increased by 0.5 km h$^{-1}$ per minute. Participants ran until exhaustion, which was defined as failure to complete the distance within the time limit after one warning. Untrained participants completed a familiarisation session before this test.

**Muscle tissue sampling and pre-analytic handling.** Muscle biopsies from the mid-portion of the right m. vastus lateralis were collected before and after the training intervention. The post-training insertion was located proximally to the site of the pre-training biopsy (approximately 3 cm). The procedure was conducted under local anaesthesia (xylocain adrenalin, 10 mg ml$^{-1}$ + 5 $\mu$g ml$^{-1}$; AstraZeneca PLC, London, UK). Approximately 200 mg (2–3 × 50–150 mg) of muscle tissue was obtained using a modified Bergström technique. Tissue intended for homogenisation and protein measurements was quickly washed in physiological saline, and fat, connective tissue and blood were removed before the sample was weighed and quickly frozen in isopentane cooled on dry ice. Tissue intended for mRNA analyses was placed in RNAlater (Ambion; Life Technologies, Inc., Carlsbad, CA, USA). Samples for immunohistochemistry were mounted in Tissue-Tek (cat. no. 4583; Sakura Finetek USA, Inc., Torrance, CA, USA) and quickly frozen in isopentane cooled on liquid nitrogen. All muscle samples were stored at −80°C for later analyses.

**Protein immunoblot.** About 50 mg of muscle tissue was homogenised and fractionated into cytosol, membrane, nuclear and cytoskeletal fractions, using a commercial fractionation kit according to the manufacturer's procedures (ProteoExtract Subcellular Proteo Extraction Kit, no. 539790, Calbiochem; EMD Biosciences GmbH, Schwalbach, Germany). Protein concentrations were assessed with a commercial kit (BioRad DC protein micro plate assay, nos 0113, 0114, 0115; Bio-Rad Laboratories, Inc., Hercules, CA, USA), a filter photometer (Expert 96; ASYS Hitech Cambridge, UK), and the software provided (Kim Version 5.45.0.1; Daniel Kittrich).

Cytosol, membrane and nuclear fractions were analysed by the western blotting technique. Equal amounts of protein were loaded per well (9–30 $\mu$g) and separated on 4–12% SDS-PAGE gels under denatured conditions for 35–45 min at 200 volts in cold MES running buffer (NuPAGE MES SDS running buffer; Invitrogen, Inc., Carlsbad, CA, USA). Proteins were thereafter transferred onto a PDVF-membrane (Immuno-blot, cat. no. 162-0177; Bio-Rad Laboratories, Inc.), at 30 volts for 90 min in cold transfer buffer (NuPAGE transfer buffer, cat. no. NP0006-1; Life Technologies, Inc.). Membranes were blocked at room temperature for 2 h in a 5% fat-free skimmed milk and 0.05% TBS-T solution [TBS, cat. no. 170-6435 (Bio-Rad Laboratories, Inc.); Tween 20, cat. no. 437082Q (VWR International, Radnor, PA, USA); skimmed milk, cat. no. 1.15363 (Merck KGaA, Darmstadt, Germany)]. Blocked membranes were incubated with antibodies against heat shock protein 60 (HSP60) (mouse anti-HSP60, cat. no. ADI-SPA-807, diluted 1 : 4000; Enzo Life Sciences, Inc., Farmingdale, NY, USA), HSP70 (mouse anti-HSP70, cat. no. ADI-SPA-810, diluted 1 : 4000; Enzo Life Sciences Inc.), and COX 4 (mouse anti-COX4, cat.

**Table 1. Characteristics of participants in the vitamin C and E supplementation group and the placebo group**

| | Vitamin C and E group ($n$ = 27: 14 women, 13 men) | Placebo group ($n$ = 27: 14 women, 13 men) |
|---|---|---|
| Age (years) | 25 ± 5 | 24 ± 6 |
| Height (m) | 1.74 ± 0.10 | 1.76 ± 0.10 |
| Body mass (kg) | 74 ± 14 | 70 ± 12 |
| $\dot{V}_{O_2max}$ (ml·min$^{-1}$·kg$^{-1}$) | 53 ± 9 | 53 ± 8 |

Data are mean ± S.D.

no. Ab14744, diluted 1 : 1000; Abcam Plc, Cambridge, UK) overnight at 4°C, followed by incubation with secondary antibody (goat anti-mouse, cat. no. 31430, diluted 1 : 30000; Thermo Fisher Scientific, Inc., Hanover Park, IL, USA) at room temperature for 1 h. All antibodies were diluted in a 1% fat-free skimmed milk and 0.05% TBS-T solution. Membranes with the PGC-1$\alpha$ molecular weight were blocked at room temperature for 2 h in a 1% BSA solution (BSA 10% in PBS; deionized $H_2O$; cat. no. 37525; Thermo Fisher Scientific, Inc.). Blocked membranes were incubated with primary antibodies against PGC-1$\alpha$ [rabbit-anti-PGC-1$\alpha$, C-Terminal (777–7979), cat. no. 516557, diluted 1 : 2000; Calbiochem, EMD Millipore Corp., Billerica, MA, USA) overnight at 4°C, followed by incubation with secondary antibody (goat anti-rabbit IgG, cat. no. 7074, diluted 1 : 1000; Cell Signaling Technology, Inc., Beverly, MA, USA) at room temperature for 1 h. Both primary and secondary antibodies were diluted in 1% BSA and deionized $H_2O$ solution. Between stages, membranes were washed in 0.05% TBS-T solution. Bands were visualised using an HRP-detection system (Super Signal West Dura Extended Duration Substrate, cat. no. 34076; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Chemiluminescence was measured using a CCD image sensor (Image Station 2000R or Image Station 4000R; Eastman Kodak, Inc., Rochester, NY, USA), and band intensities were calculated with Carestream molecular imaging software (Carestream Health, Inc., Rochester, NY, USA). All samples were run as duplicates and mean values were used for statistical analyses.

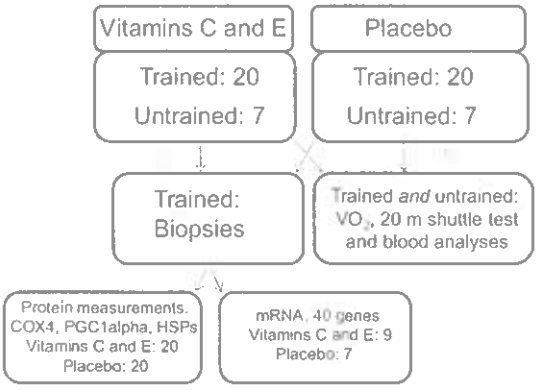



**Figure 1. Numbers of endurance-trained and untrained participants in each group**
Numbers of participants in tests and analyses applied. PGC-1$\alpha$, peroxisome proliferator-activated receptor-$\gamma$ coactivator 1 $\alpha$; HSPs, heat shock proteins.

**Immunohistochemistry.** Cross-sections 8 $\mu$m thick were cut using a microtome at −20°C (CM3050; Leica Microsystems GmbH, Wetzlar, Germany) and mounted on microscope slides (Superfrost Plus; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The sections were then air-dried and stored at −80°C. The muscle sections were blocked for 30 min with 1% BSA (cat. no. A4503; Sigma-Aldrich Corp., St Louis, MO, USA) and 0.05% PBS-T solution (cat. no. 524650; Calbiochem, EMD Biosciences, Inc., San Diego, CA, USA). They were then incubated with antibodies against myosin heavy chain type 2 (1 : 1000; SC71; gift from Professor S. Schiaffino), CD31 (capillaries; 1 : 200; clone JC70A, M0823; Dako A/S, Glostrup, Denmark) and dystrophin (1 : 1000; cat. no. ab15277; Abcam Plc) overnight at 4°C followed by incubation with appropriate secondary antibodies (Alexa Fluor, cat. no. A11005 or A11001; Invitrogen, Inc.). Between stages, sections were washed for 3 × 5 min in 0.05% PBS-T solution. Muscle sections were finally covered with a coverslip and glued with ProLong Gold Antifade Reagent with DAPI (cat. no. P36935; Invitrogen Molecular Probes, Eugene, OR, USA) and left to dry overnight at room temperature. Muscle sections were visualised using a high-resolution camera (DP72; Olympus Corp., Tokyo, Japan) mounted on a microscope (BX61; Olympus Corp.) with a fluorescence light source (X-Cite 120PCQ; EXFO Photonic Solutions Inc., Mississauga, Ontario, Canada). Fibre type distribution, fibre cross-sectional area and capillaries were identified using TEMA software (CheckVision, Hadsund, Denmark). All staining counts were manually approved or corrected independently by two investigators. Capillarisation was expressed as capillaries around each fibre (CAF) and CAF related to fibre area (CAFA), for type 1 and type 2 (2A and 2X) fibres.

**Gene expression analyses.** Total RNA was isolated using an RNeasy Fibrous Tissue Mini Kit (cat. no. 74704; Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. RNA quantity and quality were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and Agilent Bioanalyser combined with an Agilent RNA 6000 Nano Kit (Agilent Technologies, Inc., Palo Alto, CA, USA). A High-Capacity cDNA Reverse Transcription Kit (cat. no. 4368814; Applied Biosystems, Inc., Foster City, CA, USA) was used for cDNA synthesis. Quantitative RT–PCR was performed in a 7900HT

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Table 2. Outline of the endurance training programme

| Weeks | Period | Day 1 | Day 2 | Day 3 | Day 4 |
|---|---|---|---|---|---|
| 1–3 | 1 | Continuous: 30 min: 82–87% of $HR_{max}$; Borg: 15–17 | Interval: 4 × 4 min: >90% of $HR_{max}$; Borg: 16–18 | Continuous: 45–60 min: 72–82% of $HR_{max}$; Borg: 13–16 | |
| 4–8 | 2 | Continuous: 30 min: 82–87% of $HR_{max}$; Borg: 15–17(18) | Interval: 5 × 4 min: >90% of $HR_{max}$; Borg: 16–18 | Continuous: 60 min: 72–82% of $HR_{max}$; Borg: 13–16 | Interval: 4 × 6 min: >90% of $HR_{max}$; Borg: 16–18 |
| 9–11 | 3 | Continuous: 30 min: 82–87% of $HR_{max}$; Borg: 15–17(18) | Interval: 6 × 4 min: >90% of $HR_{max}$; Borg: 16–18 | Continuous: 60 min: 72–82% of $HR_{max}$; Borg: 13–16 | Interval: 5 × 6 min: >90% of $HR_{max}$; Borg: 16–18 |

$HR_{max}$, maximal heart rate; Borg, Borg scale of perceived exertion (6–20).

Fast Real-Time PCR System (Applied Biosystems, Inc.) using 140 ng cDNA in a custom-made Taq-Man Low Density Array (Applied Biosystems, Inc.). Primers for the following genes were included in the array (abbreviated name; Applied Biosystems assay ID): *CRYAB* (Hs00157107_m1); *CAT* (Hs00156308_m1); *CDC42* (Hs00741586_m1); *CS* (Hs00830726_s1); *COL4A1* (Hs01007469_m1); *COX4I1* (Hs00971639_m1); *CYCS* (Hs01588973_m1); *ESRRA* (Hs00607062_g1); *FOXO1* (Hs01054576_m1); *SLC2A4* (Hs00168966_m1); *GPX1* (Hs00829989_g1); *HIF1A* (Hs00936368_m1); *HMOX1* (Hs00157965_m1); *HSPB2* (Hs00155436_m1); *HSPD1* (Hs01036747_m1); *HSPA1A:HSPA1B* (Hs00359147_s1); *HSF1* (Hs00232134_m1); *IGF2* (Hs00171254_m1); *IL6* (Hs99999032_m1); *LAMA4* (Hs00158588_m1); *MAPK1* (Hs01046830_m1); *MAPK3* (Hs00385075_m1); *NFKB1* (Hs00231653_m1); *NFKB2* (Hs00174517_m1); *NID2* (Hs00201233_m1); *NOX1* (Hs00246589_m1); *CYBB* (Hs00166163_m1); *NOX3* (Hs00210462_m1); *NOX4* (Hs01558199_m1); *NOX5* (Hs00225846_m1); *NQO1* (Hs00168547_m1); *NFE2L1* (Hs00231457_m1); *NFE2L2* (Hs00232352_m1); *NRF1* (Hs00602161_m1); *PPARGC1B* (Hs00991676_m1); *PPARGC1A* (Hs01016724_m1); *PPARA* (Hs00947539_m1); *PPARG* (Hs01115512_m1); *RELA* (Hs00153294_m1); *SOD1* (Hs00916476_m1); *SOD2* (Hs00167309_m1); *TXN* (Hs00828652_m1), and *VEGFA* (Hs00900055_m1). Endogenous controls included in the assay were: 18S, *GAPDH* (Hs99999905_m1); *GUSB* (Hs99999908_m1); *HPRT1* (Hs99999909_m1), and *TBP* (Hs99999910_m1). RQ Manager Version 1.2 (Applied Biosystems, Inc.) and Microsoft Excel 2010 were used for data analysis. Expression levels were quantified using the cycle threshold (Ct) normalised against the average of the endogenous controls *GUSB* and *HPRT1*. ΔCt represents the Ct value of the target gene minus (average) Ct value of the endogenous control and is used to calculate $2^{-\Delta Ct}$. A target gene was determined as 'not expressed' when the average Ct was ≥35.

**Blood sampling and handling.** Venous blood was collected in the morning after 12 h of fasting. Heparin- and EDTA-coated tubes were immediately centrifuged at 1500 *g* for 10 min at 4°C. Care was taken to keep the collected plasma cooled (on ice) between steps, and to freeze the treated samples rapidly in dry ice. Heparin plasma destined for vitamin C analysis was immediately mixed in equal volumes with metaphosphoric acid before freezing; the further procedure is described by Karlsen *et al.* (2005). Vitamin E was analysed in EDTA plasma, as described by Bastani *et al.* (2012). Plasma (heparin) 8-iso $PGF_{2\alpha}$ analyses have previously been described by Bastani *et al.* (2009). All samples were stored at −80°C until analysis.

**Statistics.** The numbers of participants included in the different tests and analyses are given in Fig. 1. All data were tested for Gaussian distribution with the D'Agostino–Pearson omnibus normality test. A two-way ANOVA was used to evaluate the effect of training (time) and vitamin C and E supplementation (absolute values, pre and post). A Holm–Sidak multiple comparisons test was applied for *post hoc* analyses. Between-groups differences in relative changes (%) from before to after the intervention period (pre–post changes) were assessed with an unpaired Student's *t* test or the Mann–Whitney test (depending on distribution). Relative changes within each group were assessed with a paired Student's *t* test or Wilcoxon signed rank test (depending on distribution). For mRNA data, Mann–Whitney *U* tests were used to compare changes between groups, and Wilcoxon signed rank tests were used for within-group analyses. Data are given as the mean ± S.D. in the text and tables. The figures display maximum and minimum values, 25th and 75th quartiles and medians (boxplots) as some of the biochemical variables were not normally distributed. Outliers were defined by Tukey's rule. Effect size was calculated as the differences between the group means divided by the combined S.D. Graphpad Prism Version 6.00 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses.

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## Results

The participants reported 97 ± 5% adherence to the supplements. A survey conducted after the training period confirmed that group affiliation had remained concealed from participants. The vitamin C and E supplementation raised plasma levels of both vitamin C [before: 81 ± 24 $\mu$M; after: 114 ± 30 $\mu$M ($P < 0.001$)] and vitamin E [$\alpha$-tocopherol; before: 27 ± 7 $\mu$M; after: 35 ± 11 $\mu$M ($P = 0.009$)] (Fig. 2). No changes were found in the placebo group [vitamin C: before: 80.9 ± 17.2 $\mu$M; after: 81.1 ± 19.9 $\mu$M ($P = 0.70$); vitamin E: before 25.9 ± 6.6 $\mu$M; after: 26.6 ± 4.2 $\mu$M ($P = 0.66$)].

In contrast to the vitamin C and E supplementation group (before: 87.1 ± 49 pg ml$^{-1}$; after: 85.5 ± 43 pg ml$^{-1}$), 8-iso PGF-2$\alpha$ increased in the placebo group [before: 74 ± 33 pg ml$^{-1}$; after: 88.2 ± 29 pg ml$^{-1}$ ($P = 0.03$)]. This difference between the groups was statistically significant ($P = 0.03$) (Fig. 3).

We found no significant difference in energy intake between the vitamin C and E supplementation group and placebo group (~10500 ± 3500 kJ in both groups), nor in macro- or micro-nutrients (data not shown). In their regular diet, the vitamin C and E supplementation group consumed 104 ± 72 mg of vitamin C and 11 ± 4 mg of vitamin E per day, whereas the placebo group consumed 102 ± 50 mg and 11 ± 4 mg of vitamin C and E, respectively ($P > 0.7$ between groups).

The vitamin C and E supplementation group reduced body mass by 1.0 ± 2.0% ($P = 0.02$) as a result of a 5.3 ± 8.6% ($P = 0.005$) loss of fat mass, but these changes did not differ from those in the placebo group (Table 3). Estimated muscle mass was stable in both groups.

All participants performed 38–45 exercise sessions during the 11 week intervention. The training diaries and heart rate data showed no differences in training intensity or perceived exertion between the groups (data not shown).

$\dot{V}_{O_2max}$ improved to the same degree in both groups (vitamin C and E supplementation group: from 52.9 ± 7.6 ml min$^{-1}$ kg$^{-1}$ to 57.2 ± 9.6 ml min$^{-1}$ kg$^{-1}$; placebo group: from 52.9 ± 8.6 ml min$^{-1}$ kg$^{-1}$ to 57.1 ± 7.4 ml min$^{-1}$ kg$^{-1}$), as did performance in the 20 m shuttle run test (vitamin C and E supplementation

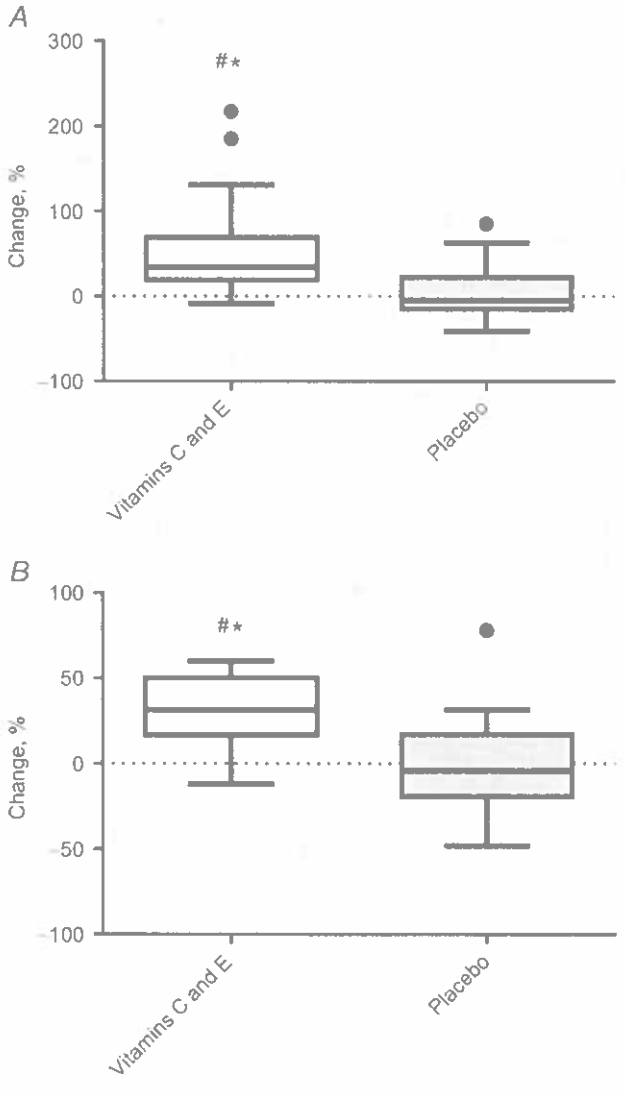



**Figure 2. Percentage changes in plasma levels of vitamin C and E in the vitamin C and E group and the placebo group**
*A*, vitamin C levels. *B*, vitamin E levels. Boxplots show maximum–minimum values, 25–75th quartiles, and medians •, outliers (Tukey's rule); #, difference between groups; *, within-group changes.

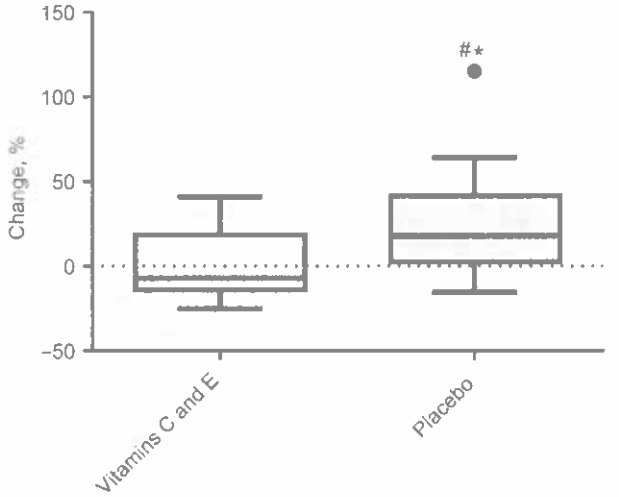



**Figure 3. Percentage changes in plasma 8-iso-prostane in the vitamin C and E group and the placebo group**
Boxplots show maximum–minimum values, 25–75th quartiles, and medians. •, outliers (Tukey's rule); #, difference between groups, *, within-group changes.

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**Table 3. Body composition before and after the 11 week intervention period**

| | Vitamin C and E group | | | Placebo group | | | |
|---|---|---|---|---|---|---|---|
| | Pre | Post | Change, % | Pre | Post | Change, % | *P*-value* |
| Body mass (kg) | 73.9 ± 14.2 | 73.1 ± 13.7 | −1.0 ± 2.0† | 70.2 ± 11.8 | 69.5 ± 12.5 | −1.1 ± 2.8 | 0.856 |
| Fat mass (kg) | 15.5 ± 7.1 | 14.6 ± 6.8 | −5.3 ± 8.9‡ | 12.6 ± 5.8 | 12.2 ± 5.9 | −3.3 ± 12.1 | 0.497 |
| Fat% | 20.8 ± 8.2 | 19.8 ± 7.9 | −4.6 ± 7.7‡ | 18.1 ± 7.1 | 17.6 ± 7.2 | −2.0 ± 11.0 | 0.324 |
| Muscle mass (kg) | 32.9 ± 7.2 | 33.0 ± 7.1 | 0.4 ± 2.2 | 32.4 ± 6.6 | 32.3 ± 6.8 | −0.4 ± 2.6 | 0.206 |

Values are mean ± SD. *P-values for between-group differences in percentage change. Within-group changes: †$P < 0.05$; ‡$P < 0.01$.

group: from 1660 ± 570 m to 1800 ± 540 m, placebo group: from 1670 ± 550 m to 1870 ± 530 m) (Fig. 4).

The subgroup of previously untrained participants increased their $\dot{V}_{O_2max}$ more than the trained participants (12.6 ± 6.2%; $P < 0.001$, untrained *versus* trained),

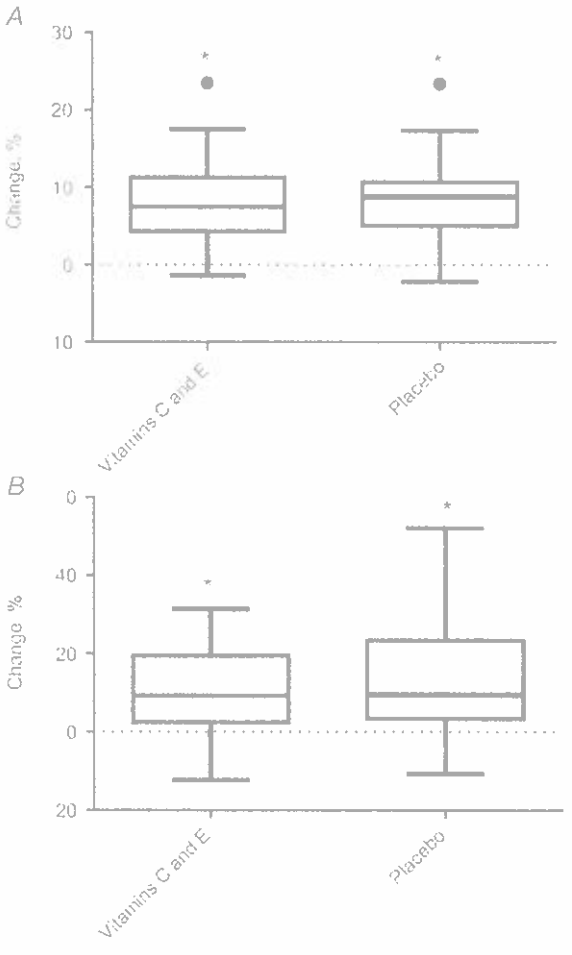



**Figure 4. Percentage changes in $\dot{V}_{O_2max}$ and the 20 m shuttle run test in the vitamin C and E group and the placebo group**
*A*, changes in $\dot{V}_{O_2max}$. *B*, changes in 20 m shuttle run. Boxplots show maximum–minimum values, 25–75th quartiles, and medians. •, outliers (Tukey's rule); #, difference between groups; *, within-group changes.

but there were no differences between the untrained participants in the vitamin C and E supplementation group and those in the placebo group ($P = 0.98$).

During submaximal running, corresponding to 58 ± 7% and 80 ± 7% of pre-intervention $\dot{V}_{O_2max}$, putative training effects were slightly larger in the placebo than in the vitamin C and E supplementation group, specifically for heart rate and RER values (Table 4). However, the group differences only tended towards statistical significance ($P = 0.08$–$0.09$; effect size = 0.5 for both variables).

The COX4 protein content in membrane fractions (including the mitochondrial components) of samples from the m. vastus lateralis increased with training only in the placebo group ($P = 0.01$). A similar trend was seen in COX4 mRNA levels from muscle biopsies (Fig. 5).

The PGC-1α mRNA levels increased during training only in the vitamin C and E supplementation group (Fig. 6), but no significant changes were found for PGC-1α protein content in either the cytosol or nuclear fractions in either group. However, a small but significant group difference was found for the change in PGC-1α protein levels in the cytosolic fraction ($P = 0.03$).

HSP60 and HSP70 did not change significantly during training at either the mRNA level (some data given in Fig. 5) or protein level in the cytosolic and nuclear fractions (Fig. 7).

The mRNA levels of CDC42 and MAPK1 decreased in the vitamin C and E supplementation group; these changes differed significantly from those in the placebo group ($P \leq 0.05$) (Fig. 8).

With no group differences in mRNA levels, vascular endothelial growth factor (VEGF) mRNA ($P = 0.018$) and CRYAB mRNA (αB-crystallin) ($P = 0.018$) decreased in the placebo group. (See online supporting information for this article for results for all analysed genes.)

No changes or group differences were found for fibre cross-sectional area or capillarisation (Table 5). When data for both groups were combined, a trend towards an increased proportion of type 2 fibres emerged ($P = 0.08$).

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**Table 4. Changes in oxygen uptake ($\dot{V}_{O_2}$), heart rate (HR), respiratory exchange rate (RER) and lactate during submaximal workloads at approximately 60% and 80% of $\dot{V}_{O_2max}$ at baseline**

| | Vitamin C and E group | | | Placebo group | | | |
|---|---|---|---|---|---|---|---|
| | Pre | Post | Change, % | Pre | Post | Change, % | *P*-value* |
| 60% of pre $\dot{V}_{O_2peak}$ | | | | | | | |
| $\dot{V}_{O_2}$ (ml·min$^{-1}$·kg$^{-1}$) | 30.9 ± 5.9 | 30.4 ± 6.3 | −1.4 ± 8.7 | 30.3 ± 4.5 | 29.1 ± 5.2 | −3.6 ± 11.3 | 0.430 |
| HR (beats·min$^{-1}$) | 140.8 ± 13.2 | 136.2 ± 12.7 | −3.0 ± 6.7 | 140.9 ± 17.3 | 131.7 ± 15.9 | −6.3 ± 7.2† | 0.095 |
| RER ($\dot{V}_{CO_2}$: $\dot{V}_{O_2}$) | 0.89 ± 0.05 | 0.89 ± 0.05 | 0.3 ± 5.4 | 0.91 ± 0.04 | 0.89 ± 0.04 | −1.7 ± 5.1 | 0.168 |
| Lactate (mmol·l) | 1.6 ± 0.9 | 1.3 ± 0.5 | −3.5 ± 33.4 | 1.5 ± 0.9 | 1.3 ± 0.7 | −6.1 ± 32.5 | 0.776 |
| 80% of pre $\dot{V}_{O_2peak}$ | | | | | | | |
| $\dot{V}_{O_2}$ (ml·min$^{-1}$·kg$^{-1}$) | 42.4 ± 7.9 | 42.5 ± 8.9 | −0.1 ± 6.7 | 41.7 ± 5.2 | 41.4 ± 6.0 | −0.4 ± 9.0 | 0.919 |
| HR (beats·min$^{-1}$) | 170.1 ± 11.1 | 165.0 ± 13.3 | −2.9 ± 5.9 | 169.8 ± 15.5 | 161.6 ± 14.6 | −4.7 ± 4.4† | 0.214 |
| RER ($\dot{V}_{CO_2}$: $\dot{V}_{O_2}$) | 0.93 ± 0.04 | 0.92 ± 0.05 | −1.5 ± 5.3 | 0.95 ± 0.04 | 0.91 ± 0.03 | −3.9 ± 4.5† | 0.083 |
| Lactate (mmol·l) | 3.8 ± 2.2 | 2.5 ± 1.3 | −27.4 ± 25.1‡ | 3.3 ± 2.2 | 2.4 ± 1.3 | −18 ± 26.0† | 0.270 |

Values are mean ± SD. *P-values for between-group differences in percentage change. Within-group changes: †$P < 0.05$; ‡$P < 0.01$.

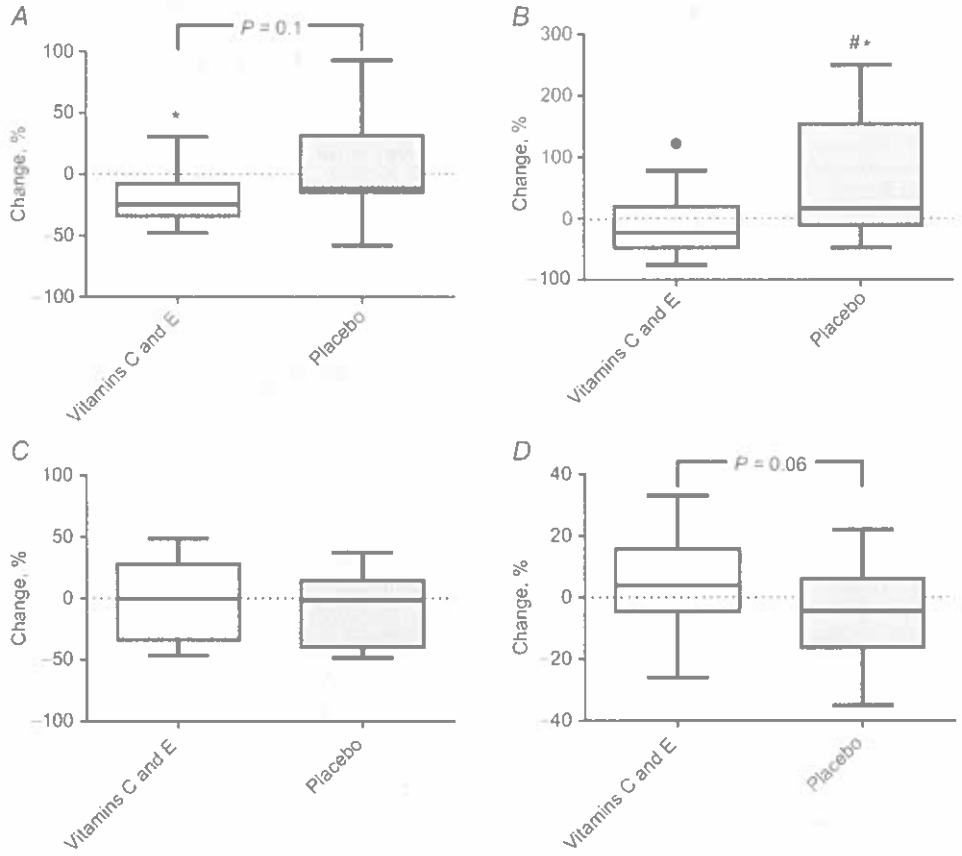



**Figure 5. Percentage changes in COX4 mRNA, COX4 (protein), HSP60 mRNA and HSP60 (protein) in the vitamin C and E group and the placebo group**
*A*, COX4 mRNA. *B*, COX4 (protein). *C*, HSP60 mRNA. *D*, HSP60 (protein). Boxplots show maximum–minimum values, 25–75th quartiles, and medians. •, outliers (Tukey's rule); *, within-group changes. Exact *P*-values denote tendencies for group differences.

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## Discussion

In the present study, we investigated the effects of vitamin C and E supplementation on adaptations to endurance exercise during an 11 week double-blind, randomised, controlled trial ($n$ = 54). The main findings were that the supplementation blunted the training-induced upregulation of cytosolic PGC-1$\alpha$ and the mitochondrial COX4 protein in m. vastus lateralis, without altering training-induced improvements in $V_{O_2max}$ or running performance. The supplementation decreased gene expression of the signalling proteins CDC42 and MAPK1, but did not alter stress proteins or capillarisation.

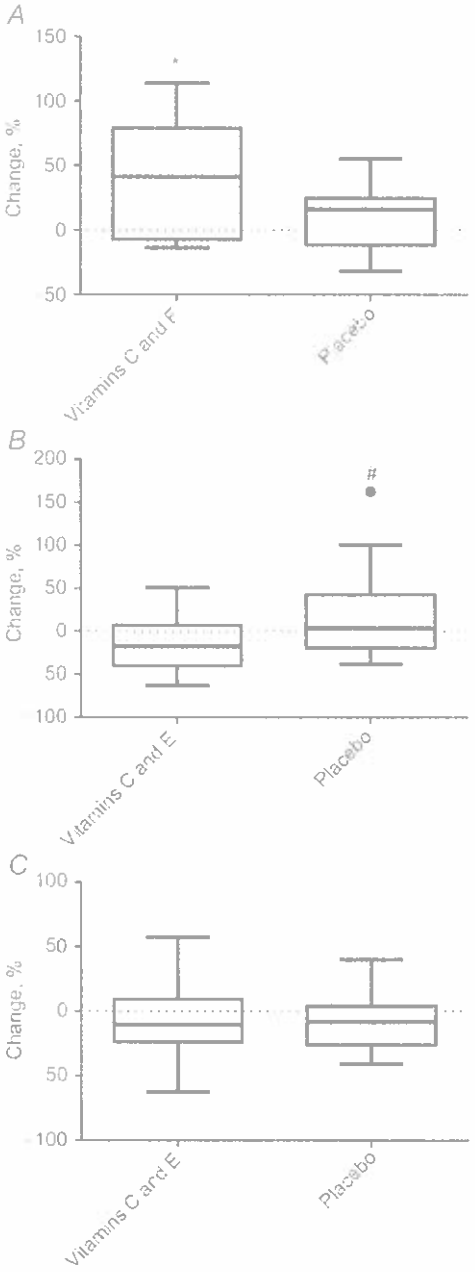



**Figure 6. Percentage changes in PGC-1α mRNA and PGC-1α in cytosol and nuclear fractions in the vitamin C and E group and the placebo group**

*A*, PGC-1α mRNA. *B*, PGC-1α in cytosol. *C*, PGC-1α in nuclei. Boxplots show maximum–minimum values, 25–75th quartiles, and medians. •, outliers (Tukey's rule); #, difference between groups; *, within-group changes.

### Cellular effects

Although conflicting results exist, animal models have demonstrated that high dosages of antioxidant supplements can shut down specific (redox-sensitive) cell signalling pathways and thereby decrease the synthesis of new muscle mitochondria and endogenous antioxidant production (Kang *et al.* 2009; Hawley *et al.* 2011; Strobel *et al.* 2011; Villanueva & Kross, 2012; Feng *et al.* 2013). Importantly, both the health benefits and improved athletic performance that occur in response to endurance training seem to depend on such cellular adaptations (Coffey & Hawley, 2007; Ristow & Zarse, 2010). **Using human participants, we herein provide novel evidence that high dosages of vitamin C and E reduce the endurance training-induced increase of COX4 (in the m. vastus lateralis), which suggests a blunted mitochondrial biogenesis.** It was not possible to decipher the exact mechanism behind this effect. However, as suggested by Ristow *et al.* (2009, 2010), we assume that the antioxidants attenuated the generation of reactive oxygen and/or nitrogen species (RONS), and thereby inhibited redox-sensitive signalling and blunted the induction of genes such as PGC-1α (as discussed further below).

Our observations conflict with findings in a recent human study by Yfanti *et al.* (2010), **who reported that supplementation with vitamin C and E did not alter training adaptations, as assessed by changes in CS and β-HAD activity in m. vastus lateralis.** A plausible explanation for this discrepancy could be that Yfanti *et al.* (2010) used a vitamin C supplement of 500 mg day$^{-1}$, rather than the 1000 mg day$^{-1}$ used in the present study. Furthermore, our participants were instructed to take the supplements in two doses (half dosage: 500 mg vitamin C and 117.5 mg vitamin E) 1–3 h before and within 1 h after each exercise session. **By contrast, participants in the study by Yfanti *et al.* (2010) consumed their vitamin supplement only at breakfast.** Given the pharmacokinetics of vitamin C in plasma [which decrease within a few hours; (Padayatty *et al.* 2004)], this might have caused a different cellular response to the supplementation.

We and others (Morton *et al.* 2009*a*; Feng *et al.* 2013) have used COX4 as a marker of mitochondrial content, and COX4 and total mitochondrial contents are found to correlate significantly (Larsen *et al.* 2012). Nevertheless, as a surrogate marker for mitochondrial content, COX4 content is not directly comparable with changes in enzyme

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activity, such as in CS as measured by Yfanti *et al.* (2010).

Mitochondrial biogenesis seems to be primarily regulated by PGC-1α, which controls the expression of both nuclear and mitochondrial gene transcription through proteins such as NFR1/2 and TFAM (Lanza & Sreekumaran, 2010). The upstream activators of PGC-1α comprise MAPK (p38 and ERK1/2) and AMPK (Lanza & Sreekumaran, 2010; Hawley *et al.* 2011). In the present study, we observed that vitamin C and E supplementation blunted any rise in muscle cytosolic PGC-1α levels and lowered gene expression of CDC42 and MAPK1 (ERK2). These responses are consistent with the changes observed for COX4. By contrast, PGC-1α mRNA was increased only in the vitamin C and E supplementation group, and nuclear PGC-1α protein levels were unchanged in both groups. Further complicating the issue, others have recently reported that PGC-1α is dispensable for exercise-induced mitochondrial biogenesis in mice (Rowe *et al.* 2012).

Notably, our biopsies were collected 2–4 days after the last training session and thus they do not reflect any immediate activation, subcellular movement of proteins (e.g. nuclear translocation of PGC-1α) or gene expression during exercise.

CDC42 is a member of the Rho family of small GTPases (Jaffe & Hall, 2005). Among various functions, CDC42 exerts certain effects via MAPKs (Maillet *et al.* 2009) and has been shown to be ROS-sensitive (Li *et al.* 2009). Nielsen *et al.* (2010) reported no changes in protein levels of CDC42 in response to 12 weeks of endurance training, but a decrease with cessation of training. Cessation of training is certainly strongly associated with a decrease in muscular fitness, including mitochondrial

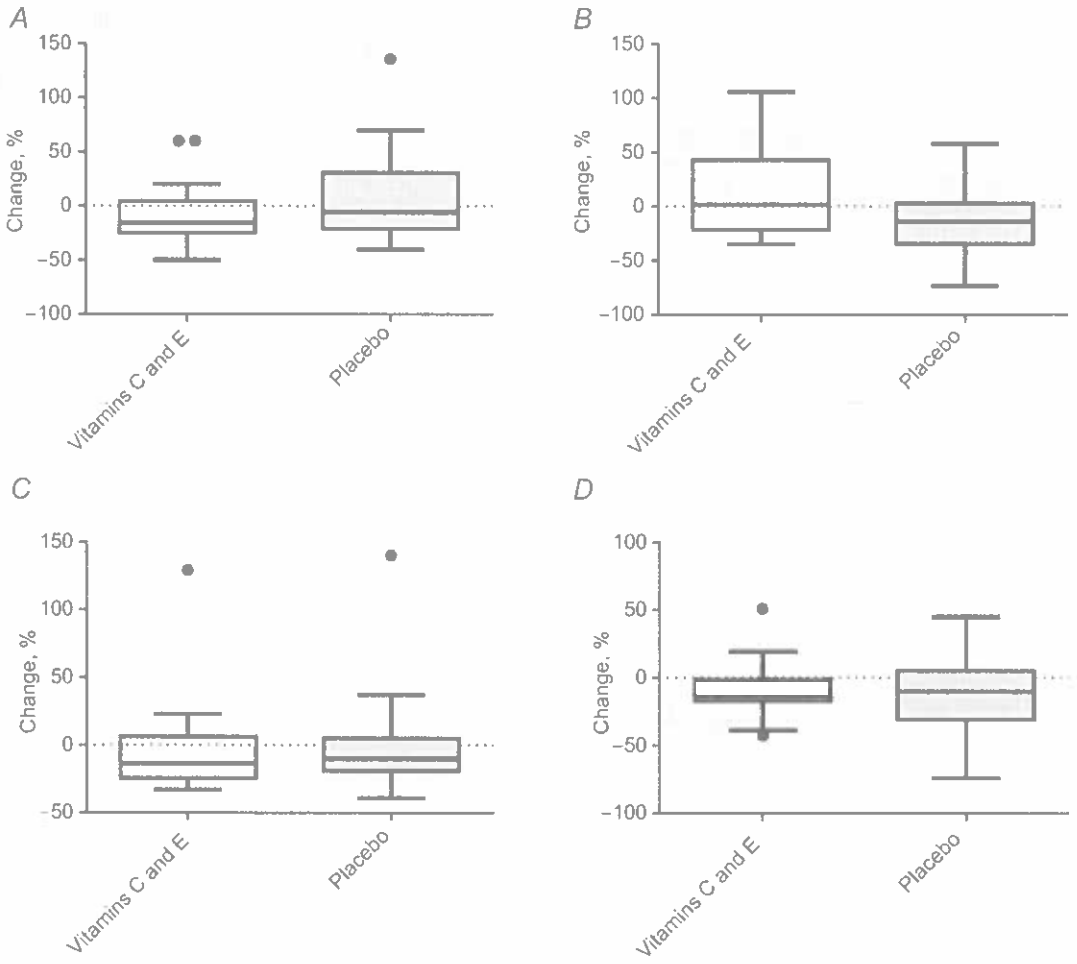



**Figure 7. Percentage changes in HSP60 and HSP70 levels in cytosol and nuclear fractions in the vitamin C and E group and the placebo group**
*A*, HSP60 in cytosol. *B*, HSP60 in nuclei. *C*, HSP70 in cytosol. *D*, HSP70 in nuclei. Boxplots show maximum–minimum values, 25–75th quartiles, and medians. •, outliers (Tukey's rule).

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capacity (Henriksson, 1992). Accordingly, the lower CDC42 gene expression may reflect an adverse effect of the vitamin C and E supplementation, further supporting the negative effect observed on COX4 levels, and shedding light on possible mechanisms for antioxidant interactions.

There were no significant changes in HSP60 and HSP70 levels (mRNA or cytosolic and nucleic protein). This suggests the absence of any accumulation of cellular stress during endurance training, with or without C and E vitamin supplementation (Morton *et al.* 2009*b*). Stable HSP levels contrast with the observations of previous studies (Liu *et al.* 2006; Morton *et al.* 2009*b*). This difference may reflect the fact that our participants (from whom we collected muscle biopsies) were recreational endurance athletes as they entered the study (Morton *et al.* 2009*b*). Similarly, the training status of the participants was probably the reason for the stability in capillary density.

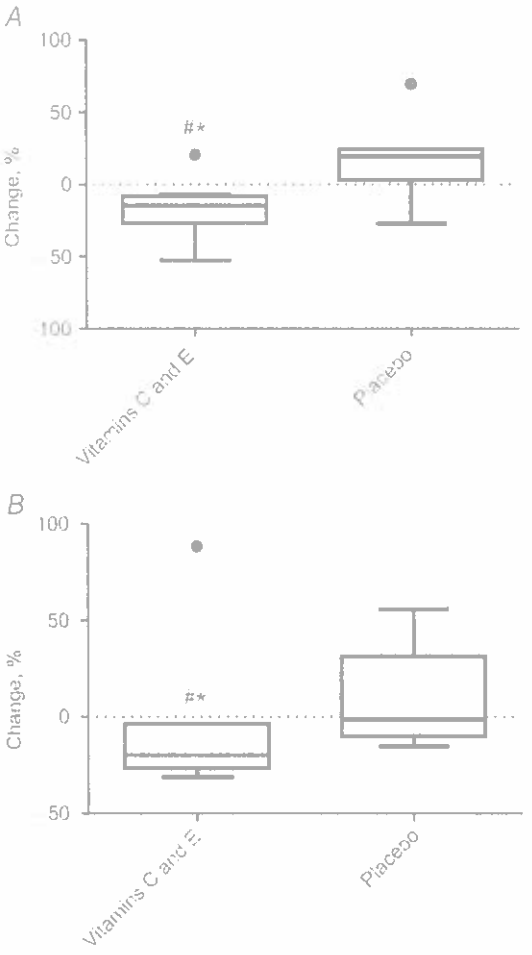



**Figure 8. Percentage changes in CDC42 mRNA and MAPK1 mRNA in the vitamin C and E group and the placebo group**
*A*, CDC42 mRNA. *B*, MAPK1 mRNA. Boxplots show maximum–minimum values, 25–75th quartiles, and medians. •, outliers (Tukey's rule); #, difference between groups; *, within-group changes.

## $\dot{V}_{O_2max}$ and performance

The various cellular effects of the vitamin C and E supplementation are interesting, but performance outcomes are more important for athletes. Thus, in contrast to the cellular observations, the increases in $\dot{V}_{O_2max}$ (~8%) and improvements in running performance (20 m shuttle run test; ~10–14%) were similar in both groups. This is in line with recent human studies in which an increase in $\dot{V}_{O_2max}$ attributable to endurance training was unaffected by vitamin C and E supplementation (Aguilo *et al.* 2007; Yfanti *et al.* 2010; Roberts *et al.* 2011). Interestingly, Gomez-Cabrera *et al.* (2008) reported that rats supplemented with vitamin C showed the same increases in $\dot{V}_{O_2max}$ as placebo-treated animals. **However, the vitamin C supplementation strongly suppressed improvements in endurance performance (running to exhaustion).** No group differences were detected in the present study, yet it is intriguing to note that the four participants with the largest improvements in running performance were all in the placebo group (effect size = 0.3 in favour of the placebo group). Although speculative, this may suggest that there are considerable inter-individual differences in the effects of vitamin C and E supplementation. However, subgroup analyses showed no effect of initial training status or gender on gains in $\dot{V}_{O_2max}$ or running performance during the training period (data not shown).

In further support of the (mild) negative effects of the vitamin C and E supplementation, we observed improved fat oxidation (indicated by reduced RER values) and reduced heart rates at submaximal workloads in the placebo group, but no significant changes were detected in the vitamin C and E group. The group differences were of moderate effect size and did not reach statistical significance ($P = 0.08$–$0.09$). Theoretically, improved fat oxidation at steady state submaximal workloads may reflect both a selective upregulation of enzymes, such as $\beta$-HAD, or a gross increase in mitochondrial mass, or both (Spina *et al.* 1996). Unfortunately, we did not measure cellular markers for fat oxidation; however, our observation of a group difference in COX4 levels, indicating increased levels of mitochondrial proteins, may be related to the RER findings.

Although we recruited a high number of participants, compared with similar studies (Nikolaidis *et al.* 2012), this study may have been insufficiently powered to detect small but potentially true biological effects (e.g. changes in RER values and running performance). For these variables, we had only 30–45% power to detect statistical group differences of the 3–4% observed.

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**Table 5. Fibre type distribution, fibre area, and capillarisation**

| | Vitamin C and E group | | | Placebo group | | | |
|---|---|---|---|---|---|---|---|
| | Pre | Post | Change, % | Pre | Post | Change, % | *P*-value |
| Fibre type 1 (%) | 54 ± 12 | 51 ± 12 | −3.9 ± 22.9 | 49 ± 13 | 44 ± 11 | −7.4 ± 28.5 | 0.124 |
| CSA ($\mu m^2$) fibre type 1 | 5070 ± 1614 | 5202 ± 1409 | 5.6 ± 22.1 | 5021 ± 1702 | 4893 ± 1206 | 3.7 ± 35.2 | 0.455 |
| CAF fibre type 1 | 4.4 ± 0.9 | 4.4 ± 0.9 | −0.6 ± 13.1 | 4.1 ± 0.8 | 4.2 ± 0.7 | 1.3 ± 13.6 | 0.774 |
| CAFA fibre type 1 | 0.9 ± 0.2 | 0.9 ± 0.2 | −1.6 ± 26.0 | 0.9 ± 0.2 | 0.9 ± 0.3 | 7.0 ± 35.9 | 0.746 |
| CSA ($\mu m^2$) fibre type 2 | 4831 ± 1646 | 5245 ± 2048 | 11.3 ± 34.3 | 5845 ± 2207 | 6019 ± 2368 | 4.5 ± 34.2 | 0.234 |
| CAF fibre type 2 | 3.8 ± 1.0 | 3.8 ± 1.0 | 3.1 ± 17.1 | 4.0 ± 0.7 | 4.0 ± 0.9 | 0.7 ± 14.4 | 0.730 |
| CAFA fibre type 2 | 0.8 ± 0.2 | 0.8 ± 0.2 | 0.0 ± 30.3 | 0.7 ± 0.2 | 0.8 ± 0.5 | 10.4 ± 57.0 | 0.579 |

Values are mean ± SD. CAF, capillaries around each fibre; CAFA, CAF/fibre area. **P*-value for between-group difference in percentage change.

### Vitamin C and E in plasma and changes in 8-iso PGF-2$\alpha$

Plasma measurements supported the efficiency of the vitamin C and E supplementation, albeit that vitamin C and E levels among our young, healthy participants were in the upper range of reference values at baseline (Karlsen *et al.* 2005; Gomez-Cabrera *et al.* 2008; Yfanti *et al.* 2010; Braakhuis *et al.* 2014).

It is interesting that vitamin C and E supplementation inhibited an elevation of 8-iso PGF-2$\alpha$, which is an established oxidative stress marker (Basu & Helmersson, 2005), that occurred in the placebo group. Vitamin C and E supplements (alone) have been found to reduce 8-iso PGF-2$\alpha$ levels (Basu & Helmersson, 2005), although, intriguingly, vitamin E has been shown to act as a pro-oxidant in certain experiments (Bowry *et al.* 1992; Abudu *et al.* 2004). Endurance training has been found to lower 8-iso PGF-2$\alpha$ plasma concentration, especially in individuals with initially high levels (Roberts *et al.* 2002; Campbell *et al.* 2010; Arikawa *et al.* 2013). Contrary to these training studies, we observed an increase in the placebo group. This increase may be explained by the intensive, high-frequency running programme for participants with normal baseline 8-iso PGF-2$\alpha$ levels.

### Supplement considerations

Our participants were supplemented with DL-$\alpha$-tocopherol acetate, the synthetic form of vitamin E. The bioavailability and biological action of natural D-$\alpha$-tocopherol/RRR-$\alpha$-tocopherol may differ (Traber *et al.* 1994; Burton *et al.* 1998). Thus, we must be careful when comparing our results with those of studies that have administered the natural form of vitamin E. Concerning vitamin C, there seem to be no differences in blood and tissue bioavailability of synthetic and natural or flavonoid-rich vitamin C (Carr *et al.* 2013).

### Conclusion

Vitamin C and E supplementation did not affect the endurance training-induced increases in $\dot{V}_{O_2max}$ and running performance (20 m shuttle test). However, at the muscle cellular level, the supplementation blunted the training-induced increase in mitochondrial COX4 protein content. Group differences in PGC-1$\alpha$ (cytosolic protein level), and CDC42 and MAPK1 mRNA levels provide further evidence that antioxidant supplementation may interfere with exercise-induced cell signalling in skeletal muscle. Moreover, the cellular results appeared to some degree to be reflected in physiological adaptations, as measured under submaximal workloads (heart rate and RER). Thus, supplementation with high dosages of vitamin C and E appears to diminish some of the endurance training-induced adaptations in human skeletal muscles. We suggest that high dosages of isolated antioxidants should be used with caution in individuals who are simultaneously engaged in endurance training.

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## Additional information

### Competing interests

None declared.

### Author contributions

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*Review*

# Synthetic or Food-Derived Vitamin C—Are They Equally Bioavailable?

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**Abstract:** Vitamin C (ascorbate) is an essential water-soluble micronutrient in humans and is obtained through the diet, primarily from fruits and vegetables. *In vivo*, vitamin C acts as a cofactor for numerous biosynthetic enzymes required for the synthesis of amino acid-derived macromolecules, neurotransmitters, and neuropeptide hormones, and is also a cofactor for various hydroxylases involved in the regulation of gene transcription and epigenetics. Vitamin C was first chemically synthesized in the early 1930s and since then researchers have been investigating the comparative bioavailability of synthetic *versus* natural, food-derived vitamin C. Although synthetic and food-derived vitamin C is chemically identical, fruit and vegetables are rich in numerous nutrients and phytochemicals which may influence its bioavailability. The physiological interactions of vitamin C with various bioflavonoids have been the most intensively studied to date. Here, we review animal and human studies, comprising both pharmacokinetic and steady-state designs, which have been carried out to investigate the comparative bioavailability of synthetic and food-derived vitamin C, or vitamin C in the presence of isolated bioflavonoids. Overall, a majority of animal studies have shown differences in the comparative bioavailability of synthetic *versus* natural vitamin C, although the results varied depending on the animal model, study design and body compartments measured. In contrast, all steady state comparative bioavailability studies in humans have shown no differences between synthetic and natural vitamin C, regardless of the subject population, study design or intervention used. Some pharmacokinetic studies in humans have shown transient and small comparative differences between synthetic and natural vitamin C, although these differences are likely

to have minimal physiological impact. Study design issues and future research directions are discussed.

**Keywords:** ascorbate; dietary vitamin C; bioavailability; animal studies; human studies; bioflavonoids

## 1. Introduction

Vitamin C (ascorbate) is an essential water-soluble micronutrient in humans and is obtained through the diet primarily from fruits and vegetables [1]. *In vivo*, it acts as a cofactor for numerous biosynthetic enzymes required for the synthesis of amino acid-derived macromolecules, neurotransmitters and neuropeptide hormones [2], and for various hydroxylases involved in the regulation of gene transcription and epigenetics [3,4]. Vitamin C is concentrated from the plasma into the body's organs and is found in particularly high concentrations in the pituitary and adrenal glands and in the corpus luteum [5], although skeletal muscle, brain, and liver comprise the largest body pools [6]. Most animals can synthesize vitamin C from glucose in the liver [7]; however, humans and a small selection of animal species have lost the ability to synthesize vitamin C due to mutations in the gene encoding L-gulono-γ-lactone oxidase, the terminal enzyme in the vitamin C biosynthetic pathway [8]. Therefore, an adequate and regular dietary intake is essential to prevent hypovitaminosis C and the potentially fatal deficiency disease, scurvy [9].

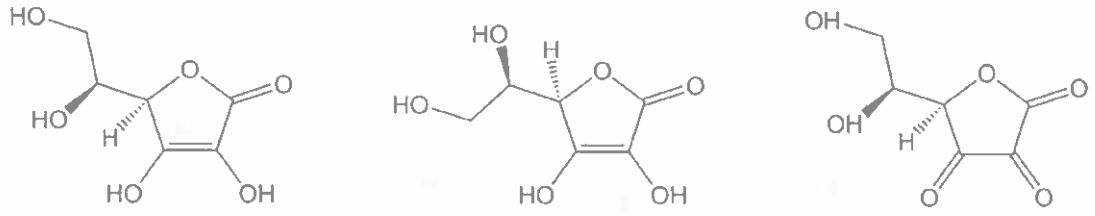
In the mid 1700s the Royal Navy surgeon James Lind carried out controlled dietary trials and determined that citrus fruit could cure individuals with scurvy (reviewed in [10]). However, it wasn't until the early 1900s that experimental scurvy was first produced in guinea pigs through dietary restriction and shown to be prevented by feeding the animals fresh fruits and vegetables. In the early 1930s vitamin C was isolated from fruit and vegetables and adrenal cortex and was named "hexuronic acid", which was shown to cure scurvy in guinea pigs and was subsequently renamed ascorbic acid to reflect its anti-scorbutic properties. Vitamin C was first chemically synthesized in 1933 [10] and, since the mid 1930s, the question of the comparative bioavailability of synthetic *versus* natural, food-derived vitamin C in animal models and human subjects has been a point of consideration.

The bioavailability of dietary vitamin C represents the proportion of the micronutrient that is absorbed by the intestines and is available for metabolic processes within the body. *In vivo* vitamin C levels are a function of uptake, metabolism, and excretion (see [11] for an excellent review of these processes). Vitamin C is actively transported into the body via two sodium-dependent vitamin C transporters, SVCT1 and SVCT2 [12,13]. These transporters exhibit different tissue distributions and uptake kinetics. SVCT1 is expressed in epithelial tissues and is primarily responsible for intestinal uptake and renal reabsorption of vitamin C, the latter helping to maintain whole body homeostasis [13]. SVCT2 is expressed in specialized and metabolically active tissues and is required for delivery of vitamin C to tissues with a high demand for the vitamin either for enzymatic reactions [2] and/or to help protect these tissues from oxidative stress [13]. Both of these transporters show significantly more affinity for the L- *versus* D-isoform of vitamin C (Figure 1) [12,14], and this selectivity likely explains earlier observations of significantly lower tissue accumulation and anti-scorbutic activity of D-ascorbic

acid in guinea pigs [15,16]. Although D-ascorbic acid is a commonly added food preservative [17], administration of D- and L-ascorbic acid together does not affect the bioavailability of the latter in humans [18].

Through its action as a reducing agent and antioxidant, ascorbate undergoes one and two electron oxidations to produce the ascorbyl radical and dehydroascorbic acid (DHA) (Figure 1). Recent research has shown that DHA can be taken up by the facilitative glucose transporters GLUT2 and GLUT8 in the small intestine [19]. Cells are also able to transport DHA via GLUT1 and GLUT3 [20,21], followed by intracellular reduction to ascorbate [22,23]. However, transport via the GLUTs is in competition with glucose which is at relatively high concentrations throughout the body and although different fruits and vegetables have been shown to contain relatively high amounts of DHA [24], the *in vivo* contribution of DHA is uncertain due to its minimal circulating and organ levels [25,26] (although white blood cells may be an exception to this) [27,28].

**Figure 1.** Vitamin C in its reduced form (ascorbic acid), shown as both its L- and D-isomers, and its two electron oxidation form (dehydroascorbic acid, DHA). DHA can be readily reduced back to ascorbic acid *in vivo* via both chemical and enzymatic pathways [23].




**L-Ascorbic Acid** **D-Ascorbic acid** **Dehydroascorbic acid**

Synthetic and food-derived vitamin C is chemically identical. However, fruit and vegetables are rich in numerous micronutrients (vitamins and minerals), dietary fiber, and phytochemicals (e.g., bioflavonoids), and the presence of some of these may affect the bioavailability of vitamin C. Vitamin C has long been known to interact with vitamin E by reducing the tocopheroxyl radical and regenerating native tocopherol [29]. Some fruit, such as kiwifruit, contain relatively high amounts of vitamin E and one animal study has indicated that vitamin E is able to preserve vitamin C *in vivo* [30]. Food-derived (and synthetic) vitamin C is well known to increase non-heme iron uptake and body status, likely via its ability to reduce iron from its ferric to ferrous state [31,32]. However, whether iron can affect vitamin C bioavailability is less clear [33–35]. Although iron has been shown to increase the uptake of vitamin C in cultured intestinal cells [33], human intervention studies have shown no effect of iron intake on vitamin C bioavailability [34,35]. One study has indicated that specific dietary fibers, such as hemicellulose and pectin, may affect the excretion of vitamin C [36], however, their influence on vitamin C uptake was not determined.

Plant-derived flavonoids have been of interest since the mid 1930s, when they were initially referred to as "vitamin P", primarily due to their effect on vascular permeability [37]. At the time, there was much debate in the literature regarding the role of "vitamin P" in experimental [38–42] and human scurvy [37,43–45]. Flavonoids can act as antioxidants via direct scavenging of free

radicals [46,47] and or chelation of redox-active metal ions [48,49]. As a result, it has been suggested that flavonoids may "spare" vitamin C and, thus, increase its bioavailability. Flavonoids have been shown to inhibit the *in vitro* oxidation of vitamin C [48–51]; however, the *in vivo* relevance of metal-ion mediated oxidation of vitamin C is likely to be minimal as free metal ions are largely sequestered in the body [52]. Whether flavonoids can affect vitamin C uptake *in vivo* is uncertain due to the low plasma bioavailability of these compounds [53]. Thus, any interaction of flavonoids with vitamin C would be expected to occur primarily in the intestinal lumen prior to active uptake.

Of note, several *in vitro* studies have shown that various flavonoids can inhibit vitamin C and DHA uptake by their respective transporters. The flavonoid quercetin can reversibly inhibit SVCT1 expressed in *Xenopus* oocytes [54] and limited data from an animal model indicates that this may occur *in vivo* [54]. Quercetin and myricetin can inhibit the uptake of vitamin C and DHA into cultured monocytic (HL-60 and U937) and lymphocytic (Jurkat) cells via inhibition of GLUT1 and GLUT3 [55] and possibly also SVCT2, which is expressed in leukocytes [56]. Quercetin and phloretin can also inhibit the intestinal GLUT2 and GLUT8 transporters [19]. Thus, based on the above *in vitro* studies, it is unclear whether flavonoids will enhance *in vivo* vitamin C bioavailability through a sparing action, or decrease its bioavailability through inhibiting vitamin C transporters.

The effect of various purified flavonoids or flavonoid-rich fruits and vegetables on vitamin C bioavailability in different animal models and human subjects is discussed below. To test comparative vitamin C bioavailability, both steady-state and pharmacokinetic models have been used. The former monitors ascorbate levels in blood and/or urine following a number of weeks of supplementation, while the latter monitors transient changes in plasma levels and/or urinary excretion over the hours following ingestion of the vitamin C-containing test substance. The gold standard for analysis of vitamin C is HPLC with coulometric electrochemical detection due to its sensitivity and specificity [57]. Early studies, however, were limited primarily to colourimetric methods based on reduction of ferric iron compounds and are prone to interference by numerous other substances [57].

## 2. Vitamin C Bioavailability Studies Using Animal Models

There are a number of benefits to the use of animal models to investigate vitamin C bioavailability, particularly the ease of diet control and the ability to obtain tissues not normally accessible in human studies. However, results can vary widely depending on the animal model used and the different treatment and analytical methodologies employed. It should also be noted that not all of the animal models that have been used are naturally vitamin C deficient. The animal models of choice are the naturally vitamin C deficient guinea pig, and genetically scorbutic animal models, such as the Osteogenic Disorder Shionogi (ODS) rat [58], the L-gulono-γ-lactone oxidase (*Gulo*$^{-/-}$) knockout mouse [59], and the spontaneous bone fracture (*sfx*) mouse [60]. Although animal studies can provide useful information, translation of the findings to humans should always proceed with caution.

Studies investigating the comparative bioavailability of synthetic *versus* natural vitamin C in animal models are shown in Table 1. Studies carried out in guinea pigs showed enhanced uptake of vitamin C into specific organs (e.g., adrenals and spleen) in the presence of flavonoid-rich juices/extracts or purified plant flavonoids (e.g., hesperidin, rutin, and catechin) [42,61–64]. Vinson and Bose [65] carried out a pharmacokinetic study in guinea pigs and found a 148% increase in the area under the

plasma ascorbate concentration-time curve when administered as citrus fruit media. They also noted that the citrus fruit group demonstrated delayed plasma vitamin C uptake compared with the synthetic vitamin C group [65]. Cotereau *et al.* [42] reported that animals given both vitmain C and catechin not only had four to eight-fold more vitamin C in the organs measured, but they were also the only group without scorbutic-type lesions. The latter finding was supported by a similar study showing fewer fresh hemorrhages in scorbutic guinea pigs receiving vitamin C with rutin or quercetin compared with vitamin C alone [66].

Several of the studies in Table 1, however, showed no differences in vitamin C accumulation in some organs (e.g., liver) [61–63,67]. Hughes *et al.* noted that the acerola cherry preparation they used was virtually flavonoid free due to dilution of the high vitamin C fruit extract, which they suggested may have accounted for its reduced efficacy compared with blackcurrant juice, which is flavonoid rich [64]. To account for the flavonoid-dependent differences in vitamin C uptake observed between the adrenals and livers of guinea pigs [62,63], Douglass and Kamp [62] noted that flavonols such as rutin are rapidly destroyed in liver tissue, but are relatively stable in adrenal homogenates. Papageorge *et al.* [63] also noted that when epinephrine oxidizes it can contribute to the destruction of vitamin C and thus the antioxidant effects of rutin may result in "sparing" of vitamin C in adrenals. A study by Levine's group [54] showed that the flavonoid quercetin can reversibly inhibit vitamin C intestinal transport and decrease plasma levels of the vitamin in the CD (Sprague-Dawley) rat, although it should be noted that this is not a vitamin C deficient animal model. Some of the variability observed in these different animal studies (Table 1) may be due to the varying ratios of flavonoid to vitamin C employed.

We recently carried out a comparative bioavailability study, using the *Gulo* mouse model, investigating the uptake of vitamin C from kiwifruit gel compared with synthetic vitamin C [68]. We found that the kiwifruit extract, which is rich in flavonoids [69,70], provided significantly higher serum, leukocyte, heart, liver, and kidney levels of vitamin C than the purified vitamin, suggesting some type of synergistic activity of the whole fruit in this model. As with Wilson *et al.* [61], we did not observe any difference between the two interventions with respect to vitamin C uptake into the brain. Indeed, there is significant retention of vitamin C in the brain during dietary depletion [64,68], suggesting a vital role for vitamin C in the brain. Thus, a significant proportion of animal studies show enhanced circulating and organ levels of vitamin C in the presence of food-derived or purified flavonoids.

### 3. Steady State Bioavailability Studies in Humans

An early report of several patients with scurvy whose plasma vitamin C levels did not increase with synthetic vitamin C alone, but only in the form of lemon juice [45], initially leant support to the "vitamin P"/flavonoid theory. However, in contrast to the animal studies, all steady state human studies (summarized in Table 2) have shown little difference in plasma and/or urine bioavailability between synthetic vitamin C and that from different fruits, fruit juices, and vegetables [35,71–76]. Mangels *et al.* [35] did observe a 20% lower plasma bioavailability of vitamin C from raw broccoli compared with cooked broccoli, however, this may have been due to differences in mechanical homogenization (chewing), a similar effect to that observed for carotenoid absorption from raw *versus* cooked carrots.

We recently carried out a steady state bioavailability study in young non-smoking men supplemented for six weeks with 50 mg/day vitamin C, in the form of a chewable vitamin C tablet or half a gold kiwifruit [77]. This dose was chosen as it lies on the steep part of the sigmoidal plasma uptake curve [78], thus enhancing the likelihood of detecting a difference between the two interventions. Although most steady state studies have used sequential or crossover study designs, we chose a randomized parallel arms design for a number of reasons. Block *et al.* [79] have previously observed a lower plasma vitamin C response to supplemental vitamin C in the second phase of a multiple depletion-repletion study. Furthermore, although washout of vitamin C could be monitored between the two phases of a cross-over study, it would not be possible to monitor washout of other kiwifruit-derived components, e.g., vitamin E, which may affect the second phase of a cross-over study due to potential *in vivo* interactions with the supplemental vitamin C [30].

Only one previous study has investigated the comparative bioavailability of synthetic *versus* natural vitamin C in leukocytes [71]. These investigators found no difference in leukocyte vitamin C uptake between synthetic vitamin C (in the presence or absence of rutin) and that in orange juice two hours after a single 75 mg dose [71]. Therefore, in addition to plasma, urine, and semen samples, we also isolated peripheral blood mononuclear cells and neutrophils before and after intervention. Due to ease of accessibility and isolation, peripheral blood leukocytes are often used as a marker for tissue vitamin C status, however, whether they are a good model for all organs and tissues is uncertain. In support of this premise our animal study indicated that different organs exhibited maximal vitamin C uptake at varying doses of the vitamin [68] and we have recently shown that human skeletal muscle exhibits greater relative uptake of vitamin C than leukocytes [80]. Therefore, we also carried out needle biopsies of skeletal muscle tissue (*vastus lateralis*), before and after intervention. In contrast to our earlier animal study [68], our human study clearly showed no differences in the steady-state bioavailability of kiwifruit-derived *versus* synthetic vitamin C to plasma, semen, peripheral blood leukocytes, and skeletal muscle tissue [77]. Thus, other nutrients and phytochemicals present in kiwifruit are neither enhancing nor inhibiting the uptake of vitamin C from the whole fruit in humans.

## 4. Pharmacokinetic Bioavailability Studies in Humans

Pharmacokinetic studies show transient changes in plasma vitamin C levels and urinary excretion over the hours following ingestion of the vitamin C-containing test substance (relevant studies are shown in Table 3). Supplemental vitamin C typically takes about two hours to reach maximal plasma levels following ingestion. An early animal study found that vitamin C provided in citrus fruit media took longer to reach peak plasma concentrations compared with a synthetic vitamin C solution and also provided a larger area under the plasma vitamin C concentration-time curve [65]. These same investigators observed a comparable trend in human subjects supplemented with 500 mg vitamin C in the presence or absence of a citrus fruit extract [81]. The citrus fruit extract delayed maximal plasma levels by one hour and provided a 35% increase in vitamin C bioavailability. Interestingly, the citrus fruit extract increased 24 h urinary vitamin C excretion in participants pre-saturated with vitamin C, but decreased excretion in non-saturated participants compared with synthetic vitamin C alone. This suggests that the baseline vitamin C status of the individual may affect the comparative bioavailability of vitamin C. Although two other studies showed increased urinary excretion in vitamin C pre-saturated

subjects in the presence of fruit juice [71,82], another pre-saturation study showed comparable plasma levels and 24 h urinary excretion in the presence of mixed bioflavonoids [83]. It should be noted that doses of 500 mg vitamin C have reduced intestinal bioavailability [78] and are significantly higher than would be obtained through a normal daily diet.

A number of pharmacokinetic studies have shown comparable bioavailability of vitamin C supplied in synthetic form or in the presence of foods or fruit juices [84–88]. Nelson *et al.* [88] used an intestinal triple lumen tube perfusion model to investigate the absorption of synthetic vitamin C and that from an orange juice solution. This method allows direct measurement of intraluminal events and showed no difference in the absorption of vitamin C from the two test solutions. A few pharmacokinetic studies have shown transient decreases in plasma vitamin C levels and/or urinary excretion at specific time points in the presence of food and fruit juices [34,71,84,85]. The physiological relevance of these transient differences is, however, likely minimal.

We recently carried out a pharmacokinetic bioavailability study of synthetic *versus* kiwifruit-derived vitamin C in nine non-smoking males (aged 18–35 years) who had "healthy" or "optimal" (*i.e.*, >50 μmol/L) baseline levels of plasma vitamin C [89]. The participants received either a chewable tablet (200 mg vitamin C) or the equivalent dose from gold kiwifruit. Fasting blood and urine were collected half hourly to hourly over the eight hours following intervention. Plasma ascorbate levels increased from 0.5 h post intervention, although no significant differences in the plasma time-concentration curves were observed between the two interventions. An estimate of the total increase in plasma ascorbate indicated complete uptake of the ingested vitamin C tablet and kiwifruit-derived vitamin C. There was an increase in urinary ascorbate excretion, relative to urinary creatinine, from two hours post intervention. There was also a significant difference between the two interventions, with enhanced ascorbate excretion observed in the kiwifruit group. Urinary excretion was calculated as ~40% and ~50% of the ingested dose from the vitamin C tablet and kiwifruit arms, respectively. Overall, our pharmacokinetic study showed comparable relative bioavailability of kiwifruit-derived vitamin C and synthetic vitamin C [89].

**Table 1.** Vitamin C comparative bioavailability studies in animal models.

| Animal Model | Intervention | Study Design | Vitamin C Analysis | Bioavailability Findings: Natural vs. Synthetic Vitamin C | Bioavailability Summary: Natural vs. Synthetic Vitamin C | Reference |
|---|---|---|---|---|---|---|
| Guinea pigs | 0.5–5 mg/day vitamin C solution<br>Kiwifruit gel | 4 weeks intervention | HPLC-ECD | Kiwifruit ↑ serum, leukocyte, heart, liver, and kidney, but not brain vitamin C | Enhanced uptake in 5/6 pools | [68] |
| CD rats | 60 mg/kg vitamin C gavage<br>15 mg/kg quercetin | Single dose; 4 h sampling | HPLC-ECD | Quercetin ↓ plasma vitamin C (at 4 h) | Decreased uptake in 1/1 pool | [54] |
| Guinea pigs | 50 mg vitamin C solution<br>Citrus fruit media | Single dose; 4 h sampling | Fluorometric (NQSA) | Citrus fruit media ↑ plasma AUC | Enhanced uptake in 1/1 pool | [65] |
| | 1 mg/kg vitamin C (low vitamin C diet)<br>50 mg/kg orange peel extract<br>50 mg/kg hesperidin | 26 days intervention | Colorimetric (DCPIP) | Orange peel extract ↑ adrenal, spleen and leukocyte, but not brain vitamin C; hesperidin ↑ adrenal and leukocyte, but not spleen vitamin C | Enhanced uptake in 3/4 pools | [61] |
| | 5 mg/kg vitamin C solution<br>Black current juice<br>Acerola cherry juice | 23 days intervention | Colorimetric (DCPIP) | Black current juice ↑ adrenal and spleen vitamin C; acerola cherry juice comparable adrenal and spleen vitamin C | Enhanced uptake in 2/2 organs | [64] |
| | 0, 5 and 10 mg/kg vitamin C<br>50 mg rutin | 5 weeks intervention | Colorimetric (DNPH) | Rutin ↑ adrenal, but not liver vitamin C | Enhanced uptake in 1/2 pools | [62] |
| | 4 mg/kg vitamin C (low vitamin C diet)<br>10 mg rutin tablet<br>18 mg/kg vitamin C (adequate vitamin C diet)<br>10 mg rutin tablet | 22 days intervention | Colorimetric (DNPH) | Rutin ↑ adrenal, but not liver or whole blood vitamin C of adequate animals | Enhanced uptake in 1/3 pools | [63] |
| | Basic diet<br>1 mg/animal catechin<br>10 mg/animal vitamin C<br>Vitamin C + catechin | 23 days intervention | Colorimetric (DCPIP) | Vitamin C + catechin ↑ liver, spleen, kidney, and adrenal vitamin C | Enhanced uptake in 4/4 organs | [42] |
| | 0.5 mg/day vitamin C solution<br>1 mL lemon juice | 20 days intervention | Colorimetric (DCPIP) | Lemon juice comparable plasma and adrenal vitamin C | Comparable uptake in 2/2 pools | [67] |

NQSA: 1,2-naphthoquinone-4-sulfonic acid; AUC: area under the concentration-time curve; DNPH: 2,4-dinitrophenylhydrazine; DCPIP: 2,6-dichlorophenolindophenol.

**Table 2.** Steady state comparative bioavailability studies in humans.

| Subjects | Intervention | Study Design | Vitamin C Analysis | Bioavailability Findings: Natural vs. Synthetic Vitamin C | Bioavailability Summary: Natural vs. Synthetic Vitamin C | Reference |
|---|---|---|---|---|---|---|
| 36 non-smoking males 18–35 years | 50 mg/day vitamin C tablet<br>Gold kiwifruit (50 mg vitamin C) | 6 weeks of supplementation; Parallel design | HPLC-ECD | Kiwifruit comparable plasma, urine, semen, mononuclear cell, neutrophil and muscle tissue vitamin C | Comparable uptake in 6/6 pools | [77] |
| 11 non-smoking women 21–39 years | 69 mg/day vitamin C capsule<br>Orange juice (66 mg vitamin C) | 2 weeks of supplementation; Crossover design (2 week washout) | Colorimetric (DNPH) | Orange juice comparable plasma vitamin C | Comparable uptake in 1/1 pool | [76] |
| 68 non-smoking males 30–59 years | 108 mg/day vitamin C tablet<br>Orange—pieces or juice<br>Broccoli—cooked or raw | 4 weeks of supplementation; Crossover design (4 week washout) | Colorimetric (DNPH) | Orange pieces/juice or cooked broccoli comparable plasma vitamin C; raw broccoli ↓ plasma vitamin C | Comparable uptake in 1/1 pool | [35] |
| 14 men and women | 75 mg/day vitamin C<br>Papayas (75 mg/day vitamin C)<br>Guava juice (75 mg/day vitamin C) | Sequential design | Colorimetric | Papayas and guava juice comparable plasma and urinary vitamin C | Comparable uptake in 2/2 pools | [72] |
| 4 healthy young subjects | 75 mg/day vitamin C tablets<br>Raw cabbage (75 mg/day vitamin C)<br>Tomato juice (75 mg/day vitamin C) | Pre-study saturation; Sequential design | Colorimetric | Raw cabbage and tomato juice comparable plasma and urinary vitamin C | Comparable uptake in 2/2 pools | [73] |
| 7 college women | 40 mg/day vitamin C solution<br>Red raspberries (40 mg/day vitamin C) | Pre-study saturation; Sequential design | Colorimetric (DCPIP) | Raspberries comparable blood and urinary vitamin C | Comparable uptake in 2/2 pools | [74] |
| 12 young adults | 100 mg/day vitamin C<br>Orange juice (100 mg/day vitamin C) | Sequential design | Colorimetric (DCPIP) | Orange juice comparable urinary vitamin C | Comparable uptake in 1/1 pool | [75] |

DNPH: 2,4-dinitrophenylhydrazine; DCPIP: 2,6-dichlorophenolindophenol.

**Table 3.** Pharmacokinetic comparative bioavailability studies in humans.

| Subjects | Intervention | Study Design | Vitamin C Analysis | Plasma Uptake | Urinary Excretion | Reference |
|---|---|---|---|---|---|---|
| 9 non-smoking males 18–35 years | 200 mg vitamin C tablet<br>Gold kiwifruit (200 mg vitamin C) | 8 h sampling;<br>Crossover design (3 week washout) | HPLC-ECD | Kiwifruit comparable plasma vitamin C and AUC | Kiwifruit ↑ urinary vitamin C and AUC (relative to creatinine) | [89] |
| 5 non-smoking males 22–27 years | 50 mg vitamin C solution;<br>282 g mashed potato (50 mg vitamin C)<br>87 g potato chips (50 mg vitamin C)<br>Placebo | 8 h sampling;<br>Crossover design (4 week washout) | HPLC-ECD | Mashed potatoes ↓ plasma vitamin C (at 1 to 2.5 h);<br>potato chips ↓ AUC | Mashed potatoes ↓ urinary vitamin C (at 3 h) | [84] |
| 5 non-smoking males 22–26 years | 50–500 mg vitamin C solution<br>100 mL acerola juice (50 mg vitamin C) | 6 h sampling;<br>Crossover design | HPLC-ECD | Acerola juice comparable plasma vitamin C and AUC | Acerola juice ↓ urinary vitamin C (at 1, 2 and 5 h) | [85] |
| 12 males 20–35 years | 284 mg vitamin C drink<br>590 mL orange juice (68 mg vitamin C)<br>Placebo (milk) | 4.5 h sampling;<br>Crossover design (1 week washout) | Colorimetric (TPTZ) | Orange juice comparable bioavailability (AUC concentration) | ND | [86] |
| 7 non-smoking females | 150 mg vitamin C solution<br>300 mL orange juice (150 mg vitamin C)<br>Placebo | 8 h sampling;<br>Crossover design (2 week washout) | HPLC-UV | Orange juice comparable plasma vitamin C | ND | [87] |
| 7 non-smokers 26–59 years | 30 mg vitamin C solution<br>200 mL red grape juice (30 mg vitamin C) | 4 h sampling;<br>Crossover design (3–4 week washout) | Fluorometric (phenylene diamine) | Grape juice ↓ plasma vitamin C (at 16 to 28 min) | ND | [34] |
| 9 healthy subjects 19–41 years | 500 mg vitamin C tablet<br>Mixed bioflavonoids<br>Placebo | 1 g day vitamin C for 2 weeks pre-study; 8 h sampling;<br>Crossover design (1 week washout) | Colorimetric (DNPH) | Bioflavonoids comparable AUC | Bioflavonoids comparable 24 h vitamin C excretion | [83] |
| 12 non-smoking subjects 18–41 years | 500 mg vitamin C solution<br>2 g citrus extract<br>Placebo | Subgroup had 1 g/day vitamin C for 2 weeks pre-study; 8 h sampling;<br>Crossover design (1 week washout) | Fluorometric | Citrus extract ↑ AUC | Citrus extract ↓ 24 h vitamin C excretion in non-saturated subjects and ↑ 24 h vitamin C excretion in saturated subjects | [81] |

**Table 3.** *Cont.*

| | | | | | | |
|---|---|---|---|---|---|---|
| 5 men<br>21–25 years | 500 mg vitamin C solution<br>500 mg vitamin C in blackcurrant juice | 100 mg/day vitamin C for 1 month pre-study; 8 h sampling; Crossover design (1 week washout) | Colorimetric (Indophenol dye) | ND | Blackcurrant juice slight ↑ 8 h vitamin C excretion in saturated subjects | [82] |
| 15 normal subjects<br>(4 smokers)<br>20–42 years | 70 mg/h vitamin C solution<br>Orange juice | Intestinal perfusion; Tandem design | Colorimetric (DNPH) | Orange juice comparable intestinal absorption | ND | [88] |
| 12 men<br>(6 smokers)<br>23–44 years | 75 mg vitamin C solution<br>400 mg rutin<br>Orange juice (75 mg vitamin C) | Pre- and post-saturation with 1 mg/day vitamin C; 2–24 h sampling; Crossover design (1 day washout) | Colorimetric | Orange juice and rutin ↓ plasma vitamin C (at 2 h) | Orange juice slight ↑ 24 h vitamin C excretion | [71] |

AUC: area under the concentration-time curve; ND: not determined; DNPH: 2,4-dinitrophenylhydrazine; TPTZ: 2,4,6-tris(2-pyridyl)-*s*-triazine.

## 5. Vitamin C Bioavailability from Different Tablet Formulations

Doses of vitamin C up to 2000 mg day are considered safe for general consumption [90]. However, pharmacokinetic studies indicate that ingestion of single doses of vitamin C greater than 200 mg have lower relative bioavailability [78], indicating that ingestion of several smaller doses each day is preferable to a single large dose. A number of studies have investigated the relative bioavailability of vitamin C from different tablet formulations and have shown that slow-release formulations provide superior vitamin bioavailability [91–94]. Salts of vitamin C, such as sodium and calcium ascorbate (Ester-C), have also been tested. Animal studies indicated that Ester-C (which contains calcium ascorbate, as well as DHA and calcium threonate) was absorbed more readily and excreted less rapidly than ascorbic acid [95] and had superior anti-scorbutic activity in ODS rats [96]. Johnston and Luo [83], however, found no significant differences between Ester-C and ascorbic acid bioavailability in humans. Nevertheless, Ester-C has been shown to be better tolerated in individuals sensitive to acidic foods [97].

## 6. Conclusions

Overall, a majority of animal studies have shown differences in the comparative bioavailability of synthetic *versus* food-derived vitamin C, or vitamin C in the presence of isolated bioflavonoids, although the results varied depending on the animal model, study design and body compartments measured. In contrast, all steady state comparative bioavailability studies in humans have shown no differences between synthetic and natural vitamin C, regardless of the subject population, study design or intervention used. Some pharmacokinetic studies in humans have shown transient and small comparative differences between synthetic and natural vitamin C, although these differences are likely to have minimal physiological impact. Thus, not only do the reviewed studies reiterate the injunction that the findings of animal studies should not be directly translated to humans [98,99], but it is also apparent that additional comparative bioavailability studies in humans are unwarranted.

Although synthetic and food-derived vitamin C appear to be equally bioavailable in humans, ingesting vitamin C as part of a whole food is considered preferable because of the concomitant consumption of numerous other macro- and micronutrients and phytochemicals, which will confer additional health benefits. Numerous epidemiological studies have indicated that higher intakes of fruit and vegetables are associated with decreased incidence of stroke [100], coronary heart disease [101], and cancers at various sites [102,103]. Vitamin C status is one of the best markers for fruit and vegetable intake [104], and food-derived vitamin C is associated with decreased incidence of numerous chronic diseases [1]; however, whether the observed health effects of fruit and vegetable ingestion are due to vitamin C and/or other plant-derived components is currently unknown. With respect to coronary heart disease, strong evidence exists for a protective effect of vegetables, moderate evidence for fruit and dietary vitamin C and insufficient evidence for supplemental vitamin C [105]. Some meta-analyses support the premise that dietary vitamin C is more protective than supplements [106], while others show reduced disease incidence with supplemental but not dietary vitamin C [107].

A major limitation with epidemiological studies is that they show only an association between dietary vitamin C intake and disease risk and cannot ascertain whether different sources of vitamin C (*i.e.*, food-derived *versus* supplement) are a cause, consequence, or simply a correlate of the particular end-point measured. Interpretations can also vary significantly depending on the input of different confounders [108]. Furthermore, epidemiological studies rely predominantly on food frequency questionnaires [109,110] and 24 h dietary recalls [111] to ascertain vitamin C intakes from foods and/or supplements [112]. This methodology has numerous limitations [113] and correlations with vitamin C status can vary depending on the methods employed as well as numerous other external factors [114]. Pooled or meta-analyses of epidemiological studies are particularly problematic due to the combining of variable study designs, cohorts and endpoints, often resulting in dilution or misinterpretation of study findings.

The gold standard for determining causality is the double-blind randomized placebo controlled clinical trial. Although this type of study design works well for comparing the effects of drugs against a placebo, it does not work for nutrients, such as vitamin C, which are already in the food chain and are required for life, *i.e.*, there is no true placebo. Numerous other methodological issues have been identified with the design of many clinical trials investigating the health effects of vitamin C [115]. For example, a major flaw with many vitamin C intervention studies is the use of study populations with already adequate or even saturating vitamin C levels, which significantly decreases the likelihood of observing any effects of the intervention. Thus, it is recommended that study populations are comprised of individuals with sub-optimal vitamin C status (*i.e.*, <50 μmol/L plasma vitamin C) or that sub-group analysis is carried out on the low vitamin C sub-populations [116]. With pharmacokinetic studies, both unsaturated and saturated individuals can be used, but comparative bioavailability studies have shown that results may vary depending on the baseline vitamin C status of the study subjects. Furthermore, the vitamin C doses chosen for intervention are critical since doses above 200 mg have decreased intestinal uptake [78], indicating that if higher doses are warranted then these should be provided as multiple doses of ~200 mg each to ensure complete bioavailability.

The comparative health effects of supplemental *versus* food-derived vitamin C will only be determined through the use of appropriate and well-designed studies. Determination of the physiological effects or health outcomes of intervention with synthetic *versus* natural vitamin C will depend largely on the endpoints measured. Only a handful of comparative intervention studies have been carried out to assess specific physiological or health endpoints. Guarnieri *et al.* [89] investigated potential protection of mononuclear leukocytes from supplemented individuals against *ex vivo* oxidative DNA damage. Although they found comparable vitamin C bioavailability between a single portion of orange juice (containing 150 mg vitamin C) and a synthetic vitamin C drink of the same dosage, they showed that only the orange juice protected the leukocytes from *ex vivo* oxidative DNA damage [89]. However, how closely *ex vivo* oxidation of DNA resembles events occurring *in vivo* is debatable and results could also vary significantly depending on the type of oxidative stress. Johnston *et al.* [76] compared plasma lipid peroxidation in individuals who had been supplemented with either orange juice or synthetic vitamin C (~70 mg/day) for two weeks. They found comparable vitamin C bioavailability and a similar reduction in lipid peroxidation with both interventions [76]. Several studies have assessed the effects of synthetic and natural vitamin C, or vitamin C in the presence of bioflavonoids, on the common cold. Two earlier studies showed a lack of an effect of

vitamin C (~200 mg/day), with and without purified bioflavonoids, on the prevention and cure of the common cold [117,118]. Another study indicated that synthetic vitamin C (80 mg/day) and orange juice both decreased the symptoms of the common cold compared with placebo, but there were no differences between the two interventions [119].

As alluded to in the introduction, vitamin C is known to enhance the bioavailability of other nutrients, such as vitamin E [30] and non-heme iron [31,32], which may enhance the health effects of vitamin C-containing foods. Bioflavonoids are also known to have numerous biological activities [120]. Recently vitamin C has been shown to modulate specific biological activities of quercetin and tea polyphenols [121,122]. Thus, future studies may elucidate the physiological relevance of these interactions.

## Conflicts of Interest

The authors declare no conflict of interest.

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*Review Article*

# Anticancer Properties of Graviola (*Annona muricata*): A Comprehensive Mechanistic Review

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Graviola (*Annona muricata*) is a small deciduous tropical evergreen fruit tree, belonging to the Annonaceae family, and is widely grown and distributed in tropical and subtropical regions around the world. The aerial parts of graviola have several functions: the fruits have been widely used as food confectionaries, while several preparations, especially decoctions of the bark, fruits, leaves, pericarp, seeds, and roots, have been extensively used in traditional medicine to treat multiple ailments including cancers by local communities in tropical Africa and South America. The reported therapeutic benefits of graviola against various human tumors and disease agents in in vitro culture and preclinical animal model systems are typically tested for their ability to specifically target the disease, while exerting little or no effect on normal cell viability. Over 212 phytochemical ingredients have been reported in graviola extracts prepared from different plant parts. The specific bioactive constituents responsible for the major anticancer, antioxidant, anti-inflammatory, antimicrobial, and other health benefits of graviola include different classes of annonaceous acetogenins (metabolites and products of the polyketide pathway), alkaloids, flavonoids, sterols, and others. This review summarizes the current understanding of the anticancer effects of *A. muricata* and its constituents on diverse cancer types and disease states, as well as efficacy and safety concerns. It also includes discussion of our current understanding of possible mechanisms of action, with the hope of further stimulating the development of improved and affordable therapies for a variety of ailments.

## 1. Introduction

Cancer is the second leading cause of mortality worldwide. Over 10 million new patients are diagnosed with cancer annually with over 6 million associated deaths, representing roughly 12% of worldwide mortality [1]. The occurrence of new cancer cases is expected to grow by about 70% over the next two decades and estimated to reach over 15 million

new cases diagnosed annually by the year 2020 [2]. This rapid increase is due to both an aging and growing population, along with carcinogens, infections, genetic mutations, hormones, immune conditions, and the adoption of behavioral and dietary risk factors, such as smoking, unhealthy diet, physical inactivity, and environmental pollutants [3]. The risk factors may act singly or in concert to cause mutation of normal cells [4]. Many of these mutations alter the expression or activity of key gene products, causing unregulated cell division leading to cancer. Currently, the main cancer treatment modalities are surgery, radiation based therapy, chemotherapy, gene therapy, and/or hormonal therapy, either singly or in combination [1]. The most commonly used chemotherapy drugs are antimetabolites, DNA-interacting agents, antitubulin agents, hormones, and molecular targeting agents, all of which work to destroy cancerous cells or limit their proliferation [5]. However, most cytotoxic drugs act on both cancerous and healthy cells and therefore elicit side effects such as hair loss, bone marrow suppression, drug resistance, gastrointestinal lesions, neurologic dysfunction, and cardiac toxicity [5]. Consequently, development of new anticancer agents with higher efficacy, selectivity, and little or no side effects is an urgent goal.

Natural products, especially phytochemicals, have been used to help mankind sustain health since the dawn of medicine [4]. Phytotherapy (also called herbalism or herbal medicine) has provided remedies for ailments, including cancer, to the present day [6]. Dietary phytochemicals have many built-in advantages over synthetic compounds due to their proven safety, low cost, and oral bioavailability [7]. However, it is only recently that researchers have begun to elucidate the mode of action of plant-derived agents at the molecular, cellular, and tissue level [8–10]. Many natural products have now been extensively researched, and numerous compounds have exhibited anticancer and other beneficial actions in modern controlled studies. Most anticancerous natural products interfere with the initiation, development, and progression of cancer by modulating various mechanisms including cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis [11].

Extracts from *Annona muricata* (also known as graviola) are among a myriad of botanical products which have shown promising medicinal value [12–14]. Studies have linked *A. muricata*-derived compounds (Table 1 and Figure 1) to a variety of anticancer effects including cytotoxicity [15–18], induction of apoptosis [19–27], necrosis [28], and inhibition of proliferation [25, 29–31] on a variety of cancer cell lines, including breast [32], prostate [29], colorectal [25], lung [16], leukemia [33], renal [34], pancreatic [15], hepatic [24], oral [35], melanoma [36], cervical [37], and ovarian cancers [38]. Moreover, all aerial parts of this plant, including the bark, fruit, leaves, root, and seeds, are used as natural medicines in the tropics [39]. However, there is a need for more rigorous studies to establish safe and effective care regimes. This review summarizes the recent advances in the application and mechanisms of *A. muricata* extracts against several cancers both *in vitro* and *in vivo*.

## 2. Botanical Description and Distribution

*Annona muricata* is a lowland tropical, fruit-bearing tree of the family Annonaceae found in the rainforests of Africa, South America, and Southeast Asia. *A. muricata*, commonly known as soursop, graviola, guanabana, or Brazilian paw-paw, has large, glossy, dark green leaves [4, 40], with edible, green heart-shaped fruits [4, 41]. Soft, curved spines cover the leathery skin of the fruits, each of which may contain 55–170 black seeds distributed in a creamy white flesh with a characteristic aroma and flavor [41, 42]. All portions (leaves [16, 18, 31, 38, 43, 44], pericarp [24, 45, 46], fruits [4, 30, 47], seeds [47–50], and roots [27]) of *A. muricata* have been used in traditional medicine, but the most widely used in the preparations of traditional medical decoctions are stem barks, roots, seeds, and leaves [51, 52]. Coria-Téllez et al. have reported 212 bioactive compounds in *A. muricata* extracts [41]. Reports in the literature indicate that seventy-four of these bioactive compounds exhibit a variety of anticancer effects in preclinical cell culture and animal model systems. Several dozen annonaceous acetogenins have been studied (39 of which are listed alphabetically in Table 2, with key structural features summarized in Figure 2). Moreover, at least ten solvent extracts (Table 1) in addition to an extract from fungi (*Periconia* sp.) collected on *A. muricata* that contains bioactive compounds (Figure 1) have been tested for their anticancer properties and other health benefits.

*A. muricata*-derived preparations have been utilized to treat numerous ailments, making this plant an ethnomedically important species. In developing tropical countries including Africa, different parts of *A. muricata* are being used to treat conditions such as diabetes [53, 54], coughs, skin diseases [55], and cancers [25–27, 56–58]. Furthermore, in both Jamaica [59] and Trinidad [60], *A. muricata* is the most prevalently used herbal remedy in the treatment of most cancers. For example, in Jamaica, a large proportion of cancer patients use medicinal plants in self-medicating practices, with *A. muricata* being commonly used (along with *Petiveria alliacea*) for treating breast and prostate cancers, respectively [59].

*A. muricata* has also been used, mainly in developing tropical countries, for the treatment of arthritis [61], hypertension [62], snake bite [63], diarrhea [59], headache [64], and malaria [65]. In addition, it has been mentioned as an antimicrobial [66], antidiabetic [51], anti-inflammatory [67], antiprotozoan [68], antioxidant, insecticide [69], larvicide [70], and anticancer [71]. Although these uses of *A. muricata* strongly imply the presence of bioactive compounds with medical benefits, a full insight into the potential of *A. muricata* in the treatment of disease will require the identification of specific bioactive compounds and a scientifically rigorous demonstration of their ability to improve health outcomes.

## 3. Anticancer Effects

More than 47% of current anticancer drugs on the market are natural products, their derivatives or natural product

Table 1: Different solvent extracts of *A. muricata* and their reported anticancer activities.

| Extract (solvent) | Cancers (cell lines) |
|---|---|
| *n*-Hexane | Cervical (HeLa) cancer [37] |
| Chloroform | Cervical (HeLa) cancer [37] |
| Pentane | Melanoma (A375) cancer [36] |
| *n*-Butanolic | (MDA-MB-435S) cancer [89], now known as a melanoma cell line [90] |
| DMSO | Pancreatic (Capan-1 [92], FG/COLO357, and CD18/HPAF [28]) cancer |
| Fungal strain | Breast (MCF-7) [38], colorectal (HTC-8) [38], lung cancer (A549) [38], hepatic (Bel-7402) [38], gastric (BGC-823) [38], and ovarian (A2780) [38] cancers |
| $H_2O$ | Squamous cell carcinoma (SCC-25) [91], melanoma (A375) [36], prostate (PC-3) [21], pancreatic (CD18/HPAF) [28], and breast cancer patients [118] |
| Hexane | Breast (MCF-7 and MDA-MB-231) [31], colorectal (HT-29 and HCT-116) [26], lung cancer (A549) [31], leukemic (U-937) [33, 46], pancreatic (Capan-1) [92], and hepatic (Hep G2) [31] cancers |
| Ethyl acetate | Breast (MCF-7 and MDA-MB-231) [31], colorectal (HT-29 and HCT-116) [25], lung (A549) [31], leukemic (U-937) [33, 46, 131], hepatic (Hep G2) [31], and cervical (HeLa) [37] cancers. |
| Ethanol | Ehrlich ascite carcinoma (EACC) [93], breast (MCF-7 [45], MDA-MB-231-BCRP clone 23 [77, 139], T47D [22], MDA and SKBR3 [93]), colorectal [20] [140] (COLO-205 and DLD-1) [94], lung (H-460) [45, 95], leukemic (K562 [19] [96], ECV304 [96] and HL-60 [27]), stomach (C-678) [95], melanoma (A375) [36], skin [141], glioma (SF-268) [45], and cervical (HeLa) [37] cancers |
| Methanol | Breast (MCF-7 and MDA-MB-231 [31], MDA-MB-231-pcDNA3, and MDA-MB-231-BCRP clone 23 [24]), colorectal (HT-29 and HCT-116 [26], HCT116 ($p53^{+/+}$), and HCT116 ($p53^{-/-}$) [24]), lung (A549 [31] and NCI-H292 [113]), leukemic (U-937 [33, 46], CCRF-CEM, and CEM/ADR5000 [24]), hepatic (Hep G2 [24, 31] and Hep 2,2,15 [31]), glioma (U87MG and U87MG $\Delta EGFR$) [24], and laryngeal (currently cervical HeLa; Hep-2) [113] cancers |

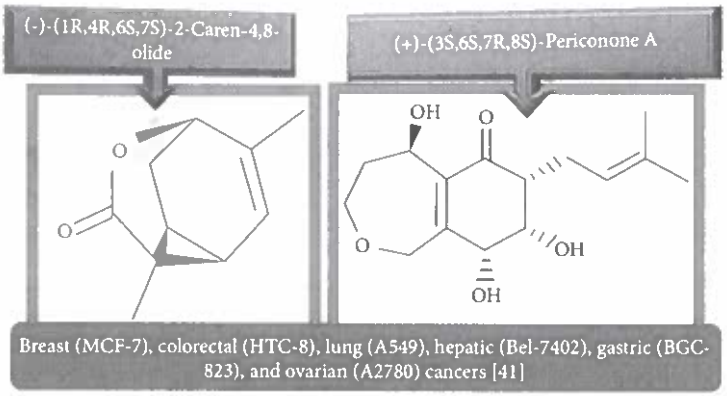



Figure 1: Chemical structure of compounds derived from fungal strain extracts and the cancers sensitive to them.

synthetic mimics, and more than 25,000 identified phytochemicals have been shown to possess potent anticancer activities [72, 73]. The aerial parts of graviola have been extensively studied with several reported in vitro and in vivo pharmacological activities, and have been shown to be effective in the management of several cancer types. The detailed molecular mechanisms of action of various graviola organs against various cancers are summarized in tabular format (Table 3 and Figure 3).

## 4. Cytotoxicity

There is no universal definition for the term "cytotoxic drug." Nonetheless, this term is commonly used in a variety of regulations for pharmaceutical development and manufacturing of drugs [74]. Simply put, a cytotoxic drug is an agent that has destructive actions on cells, often implying that these cells are targeted for destruction [75], a concept that certainly applies to many antineoplastic drugs [75].

The major bioactive components that have been extracted from various *A. muricata*'s parts are known as annonaceous acetogenins (AGEs). These are derivatives of long-chain (C32 or C34) fatty acids derived from the polyketide pathway, reviewed in [76]. Many of these derivatives are reported to be selectively toxic to cancer cells, including multidrug-resistant cancer cell lines [77]. Annonaceous acetogenins induce cytotoxicity, at least in part, by inhibiting mitochondrial complex I, which is involved in oxidative phosphorylation and ATP synthesis [78]. As cancer cells have a higher demand for ATP than the normal cells, mitochondrial complex I inhibitors have potential in cancer therapy [79].

Table 2: ACEs of *A. muricata* reported to have anticancer activities. Structures were drawn using ChemDraw, Arial, point 20.

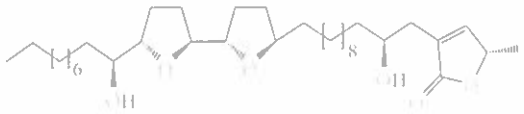
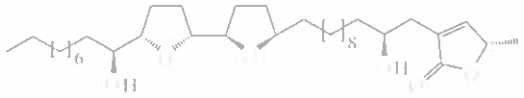
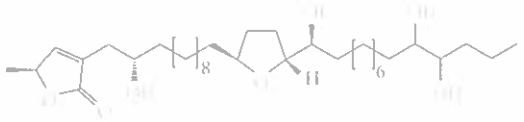
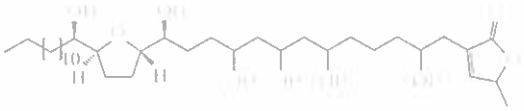
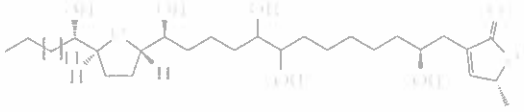
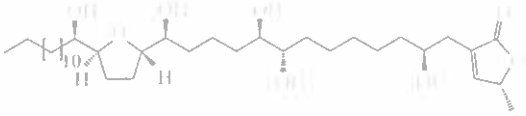
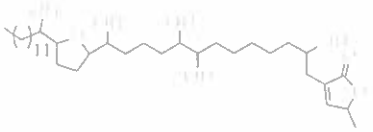
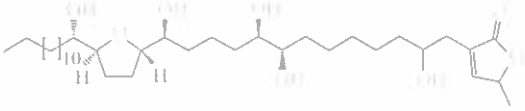
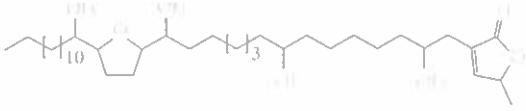
| Compound names | Structure | Molecular formula | MWT (g/mol) | Cancer cell lines tested on |
|---|---|---|---|---|
| Annocatacin A |  | $C_{37}H_{70}O_6$ | 578.88 | Hepatic (Hep G2 and Hep 2,2,15) cancer [80] |
| Annocatacin B |  | $C_{37}H_{70}O_6$ | 578.88 | Hepatic (Hep G2 and Hep 2,2,15) cancer [80] |
| Annocatalin |  | $C_{41}H_{80}O_5$ | 596.89 | Hepatic (Hep G2 and Hep 2,2,15) cancer [81] |
| Annohexocin |  | $C_{37}H_{68}O_6$ | 628.888 | Breast (MCF-7), prostate (PC-3), colorectal (HT-29), lung (A549), renal (A498) and pancreatic (PACA-2) cancers [34] |
| Annomuricin A |  | $C_{37}H_{68}O_8$ | 612.889 | Breast (MCF-7), colorectal (HT-29), lung (A549) [17], and leukemic (CCRF-CEM) [46] cancers |
| Annomuricin B |  | $C_{37}H_{68}O_8$ | 612.889 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [17] |
| Annomuricin C |  | $C_{37}H_{68}O_8$ | 612.889 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [86] |
| Annomuricin E |  | $C_{37}H_{68}O_8$ | 612.889 | Breast (MCF-7), prostate (PC-3), lung (A549), renal (A498), pancreatic (PACA) [16], and colorectal (HT-29) cancers [16, 25] |
| Annomutacin |  | $C_{37}H_{68}O_7$ | 624.944 | Breast cancer (MCF-7), colorectal (HT-29), and lung cancers (A549) [43] |

Table 2: Continued.

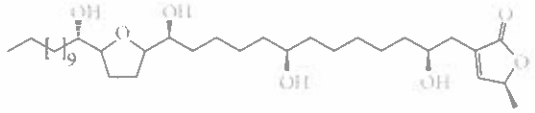
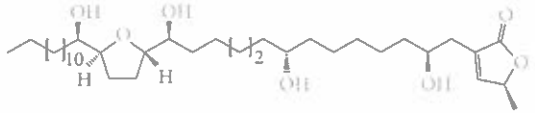
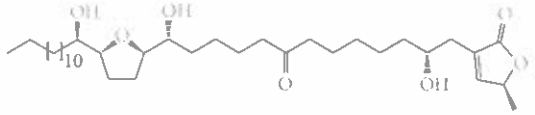
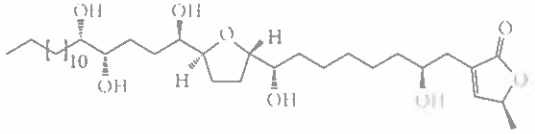
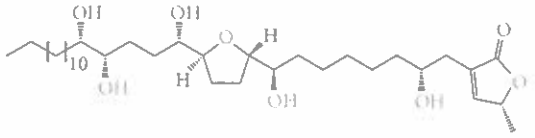
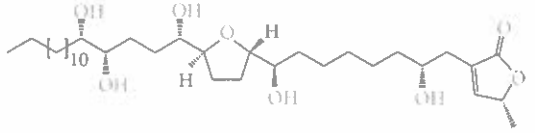
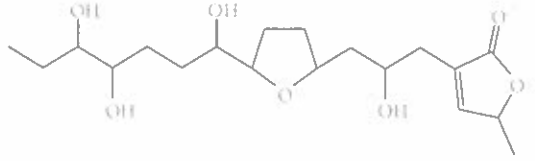
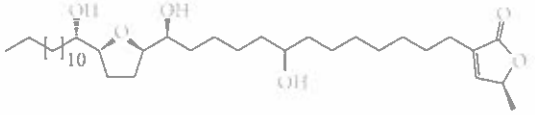
| Compound names | Structure | Molecular formula | MWT (g/mol) | Cancer cell lines tested on |
|---|---|---|---|---|
| Annonacin |  | $C_{35}H_{64}O_7$ | 596.89 | |
| Annonacin A |  | $C_{35}H_{64}O_7$ | 596.89 | Leukemia (U-937) [46] |
| Annonacinone |  | $C_{35}H_{62}O_7$ | 594.874 | Oral cancer (KB) [35] |
| Annopentocin A |  | $C_{35}H_{64}O_8$ | 612.889 | Breast (MCF-7), prostate (PC-3), colorectal (HT-29), lung (A549), renal (A498), and pancreatic (PACA-2) cancers [32] |
| Annopentocin B |  | $C_{35}H_{64}O_8$ | 612.889 | Breast (MCF-7), prostate (PC-3), colorectal (HT-29), lung (A549), renal (A498), and pancreatic (PACA-2) cancers [32] |
| Annopentocin C |  | $C_{35}H_{64}O_8$ | 612.889 | Breast (MCF-7), prostate (PC-3), colorectal (HT-29), lung (A549), renal (A498), and pancreatic (PACA-2) cancers [32] |
| Arianacin |  | $C_{35}H_{64}O_7$ | 596.89 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [85] |
| Corossolin |  | $C_{35}H_{64}O_6$ | 580.891 | Hepatic (Hep G2 and Hep 2,2,15) [82] and oral (KB) cancers [35] |

Table 2: Continued.

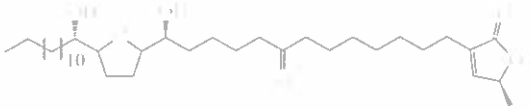
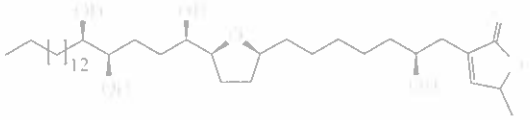
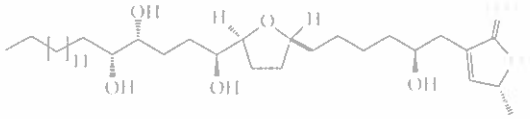
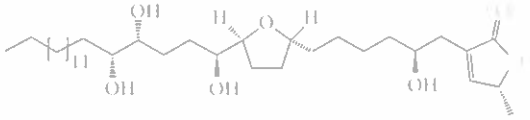
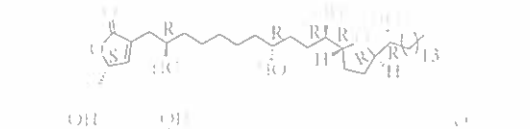
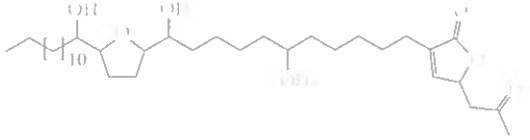
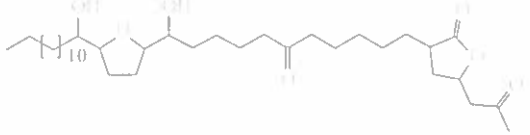
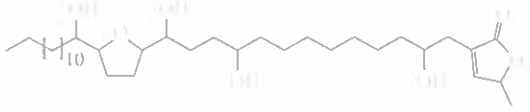
| Compound names | Structure | Molecular formula | MWT (g/mol) | Cancer cell lines tested on |
|---|---|---|---|---|
| Corossolone |  | $C_{35}H_{58}O_6$ | 578.875 | Hepatic cancer (Hep G2 and Hep 2,2,15) [82] and oral (KB) cancers [35, 83] |
| Gigantetrocin |  | $C_{35}H_{64}O_5$ | 596.89 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [49] |
| Gigantetrocin A |  | $C_{35}H_{64}O_7$ | 596.89 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [50] |
| Gigantetrocin B |  | $C_{35}H_{64}O_5$ | 596.89 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [17, 50] |
| Gigantetrocin |  | $C_{35}H_{64}O_5$ | 596.89 | Breast (MCF 7), colorectal (HT-29), and lung (A549) cancers [49] |
| Isoannonacin |  | $C_{35}H_{64}O_7$ | 596.89 | Breast (MCF 7), colorectal (HT 29), and lung (A549) cancers [48] |
| Isoannonacin 10-one |  | $C_{35}H_{62}O_7$ | 594.874 | Breast (MCF 7), colorectal (HT 29), and lung (A549) cancers [49] |
| Javoricin |  | $C_{35}H_{64}O_7$ | 596.89 | Breast (MCF 7), colorectal (HT 29), and lung (A549) cancers [85] |

Table 2: Continued.

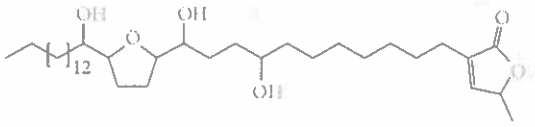
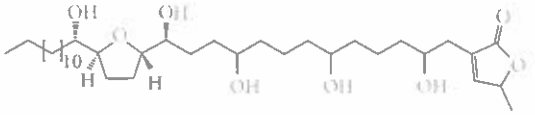
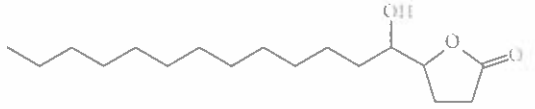
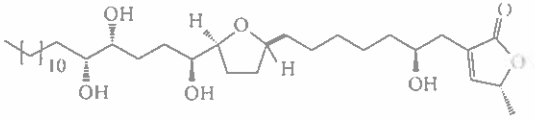
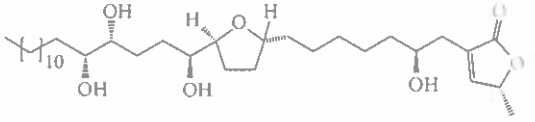
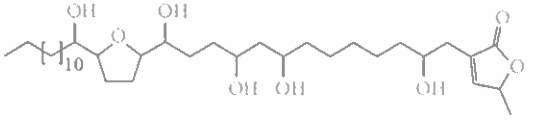
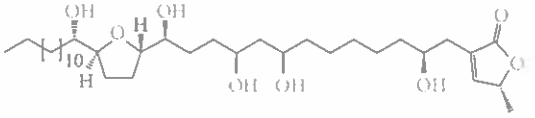
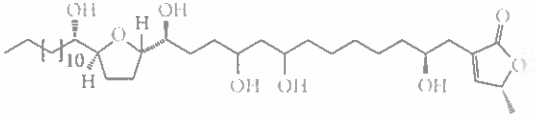
| Compound names | Structure | Molecular formula | MWT (g/mol) | Cancer cell lines tested on |
|---|---|---|---|---|
| Longifolicin |  | $C_{35}H_{64}O_6$ | 580.891 | Hepatic (Hep G2 and Hep 2,2,15) cancer [82] |
| Muricapentocin |  | $C_{35}H_{64}O_8$ | 612.889 | Breast (MCF-7), prostate (PC-3), colorectal (HT-29), lung cancer (A549), renal (A498), and pancreatic (PACA) cancers [16] |
| Muricatacin |  | $C_{17}H_{32}O_3$ | 284.44 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [48] |
| Muricatetrocin A |  | $C_{35}H_{64}O_7$ | 596.89 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [17, 50] |
| Muricatetrocin B |  | $C_{35}H_{64}O_7$ | 596.89 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [17, 50] |
| Muricatocin A |  | $C_{35}H_{64}O_8$ | 612.889 | Breast (MCF-7), colorectal (HT-29) and lung (A549) cancers [44] |
| Muricatocin B |  | $C_{35}H_{64}O_8$ | 612.889 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [44] |
| Muricatocin C |  | $C_{35}H_{64}O_8$ | 612.889 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [86] |

Table 2: Continued.

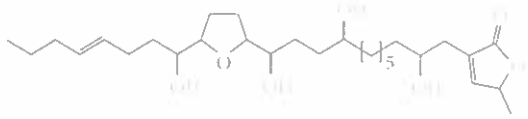
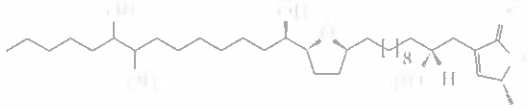
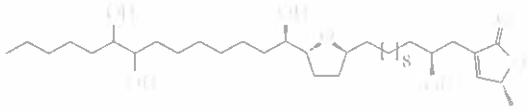
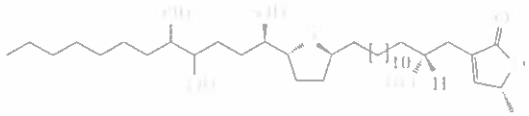
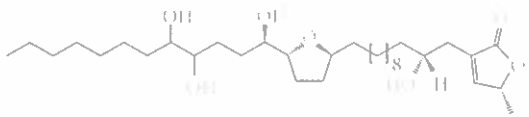
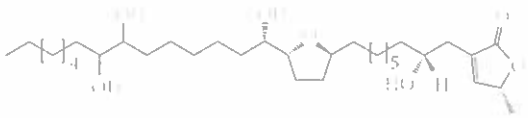
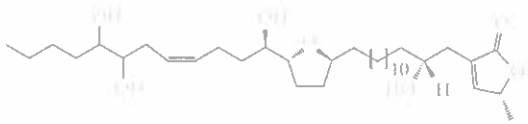
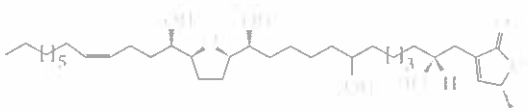
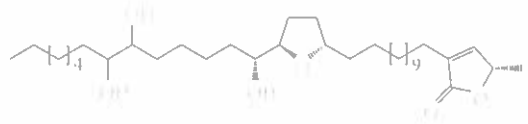
| Compound names | Structures | Molecular formula | MWT (g/mol) | Cancer cell lines tested on |
|---|---|---|---|---|
| Muricenin |  | | | Prostate (PC-3) cancer [30] |
| Muricin A |  | $C_{35}H_{64}O_7$ | 596.878 | Hepatic (Hep G2 and Hep 2,2,15) cancer [82] |
| Muricin B |  | $C_{35}H_{64}O_7$ | 596.878 | Hepatic (Hep G2 and Hep 2,2,15) cancer [82] |
| Muricin C |  | $C_{35}H_{64}O_7$ | 596.878 | Hepatic (Hep G2 and Hep 2,2,15) cancer [82] |
| Muricin D |  | $C_{34}H_{60}O_7$ | 568.836 | Hepatic (Hep G2 and Hep 2,2,15) cancer [82] |
| Muricin E |  | | | Hepatic (Hep G2 and Hep 2,2,15) cancer [82] |
| Muricin F |  | $C_{35}H_{62}O_7$ | 594.874 | Hepatic (Hep G2 and Hep 2,2,15) cancer [82] |
| Muricin G |  | | | Hepatic (Hep G2 and Hep 2,2,15) cancer [82] |
| Muricin H |  | $C_{37}H_{68}O_6$ | 580.891 | Hepatic (Hep G2 and Hep 2,2,15) cancer [81] |

Table 2: Continued.

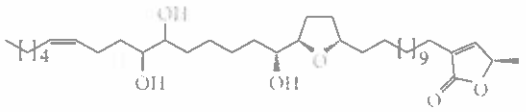
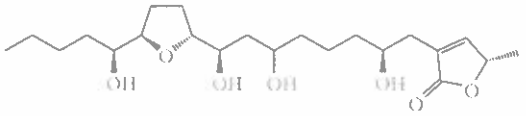
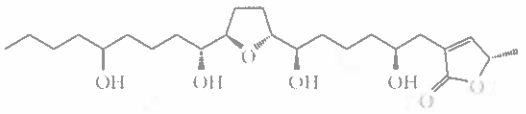
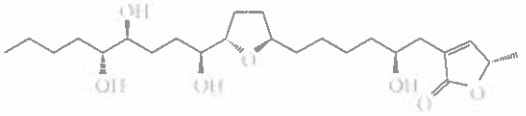
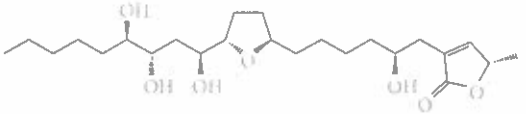
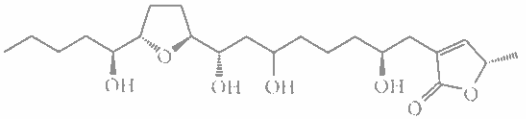
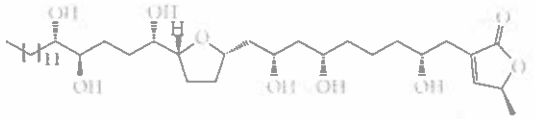
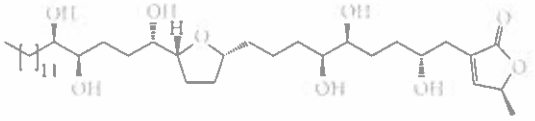
| Compound names | Structure | Molecular formula | MWT (g/mol) | Cancer cell lines tested on |
|---|---|---|---|---|
| Muricin I |  | $C_{37}H_{66}O_6$ | 606.929 | Hepatic (Hep G2 and Hep 2,2,15) cancer [81] |
| Muricin J |  | $C_{22}H_{38}O_7$ | 414.2618 | Prostate (PC-3) cancer [29] |
| Muricin K |  | $C_{24}H_{42}O_7$ | 442.2931 | Prostate (PC-3) cancer [29] |
| Muricin L |  | $C_{24}H_{42}O_7$ | 442.2931 | Prostate (PC-3) cancer [29] |
| Muricin M |  | $C_{24}H_{42}O_7$ | 442.2931 | Prostate (PC-3) cancer [30] |
| Muricin N |  | $C_{22}H_{38}O_7$ | 414.2618 | Prostate (PC-3) cancer [30] |
| Muricoreactin |  | $C_{35}H_{64}O_9$ | 628.4550 | Breast (MCF-7), prostate (PC-3), colorectal (HT-29), lung cancer (A549), renal cancer (A498), and pancreatic (PACA-2) cancers [47] |
| Murihexocin A |  | $C_{35}H_{64}O_9$ | 628.888 | Breast (MCF-7), prostate (PC-3), colorectal (HT-29), lung (A549), renal (A498), and pancreatic (PACA-2) cancers [15] |

Table 2: Continued.

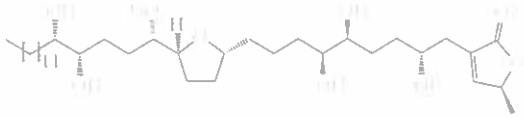
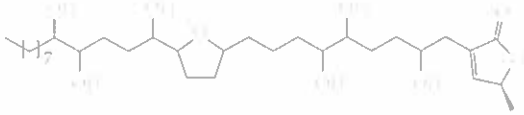
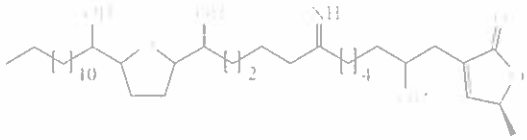
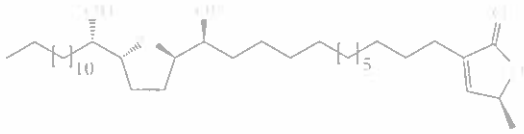
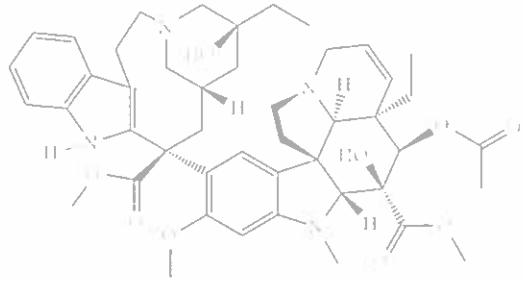
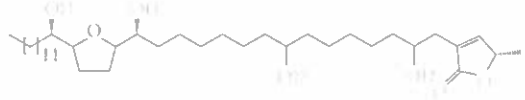
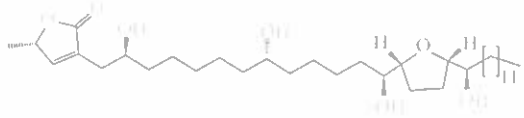
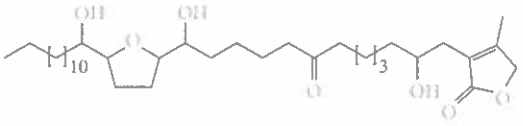
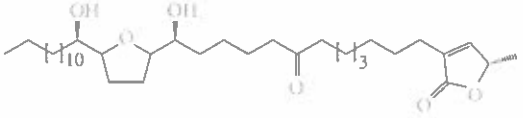
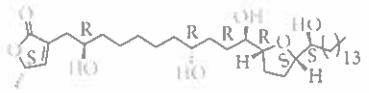
| Compound names | Structure | Molecular formula | MWT (g/mol) | Cancer cell lines tested on |
|---|---|---|---|---|
| Muricin B |  | $C_{35}H_{64}O_6$ | 628.4550 | Breast (MCF-7), prostate (PC-3), colorectal (HT-29), lung (A549), renal (A498), and pancreatic (PACA-2) cancers [15] |
| Muricin G |  | $C_{37}H_{72}O_6$ | 572.3924 | Breast (MCF-7), prostate (PC-3), colorectal (HT-29), lung cancer (A549), renal cancer (A498), and pancreatic (PACA-2) cancers [47] |
| Murisolin |  | $C_{37}H_{71}NO_6$ | 593.4655 | Oral (KB) cancer [35] |
| Solamin |  | $C_{35}H_{63}O_6$ | 564.892 | Oral (KB) cancer [35] |
| Vinblastine |  | $C_{46}H_{58}N_4O_9$ | 810.989 | Oral (KB) cancer [35] |
| *cis*-Annomontacin |  | $C_{37}H_{68}O_6$ | 624.4965 | Hepatic (Hep G2 and Hep 2,2,15) cancer [88] |
| *cis*-Annonacin |  | $C_{35}H_{64}O_7$ | 596.88 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [85] |

Table 2: Continued.

| Compound names | Structure | Molecular formula | MWT (g/mol) | Cancer cell lines tested on |
|---|---|---|---|---|
| *cis*-Annonacin-10-one |  | $C_{35}H_{62}O_7$ | 594.874 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [85] |
| *cis*-Corossolone |  | $C_{35}H_{62}O_6$ | 578.875 | Hepatic (Hep G2 and Hep 2,2,15) cancer [81] |
| *cis*-Goniothalamicin |  | $C_{35}H_{61}O_7$ | 596.89 | Breast (MCF-7), colorectal (HT-29), and lung (A549) cancers [85] |

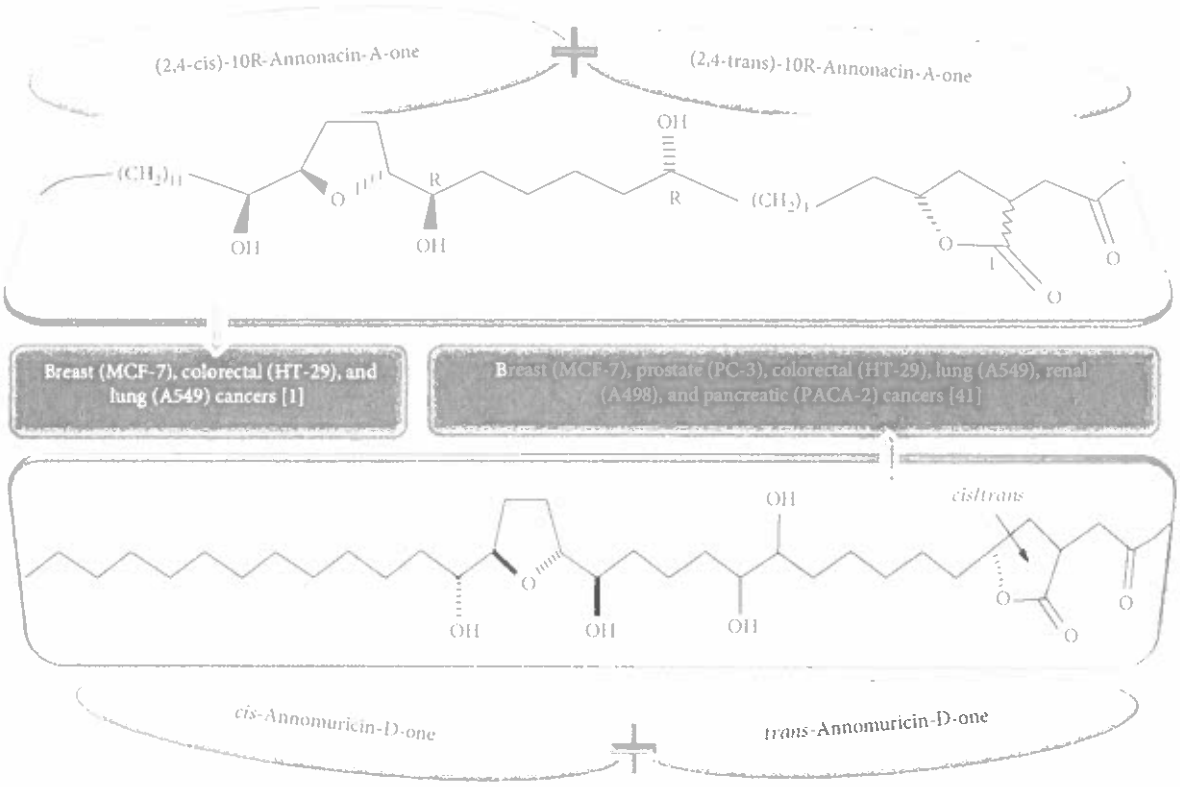



Figure 2: Chemical structures of two AGE combinations along with their targeted cancer phenotype.

Purified AGEs, such as annocatacin (A or B) [80] or annocatalin [81], have been found to induce significant cytotoxicity in Hep G2 and Hep 2,2,15 hepatic cancer cells *in vitro* [82, 83]. In breast cancer, cytotoxicity can be induced in MCF-7 cells using any of the following purified AGEs: annomuricin A, B [17], C [18], or E [16]; muricatocin A, B, or C [47]; muricapentocin [16]; annomutacin [43]; annohexocin [34]; annopentocin A, B, or C [32]; murihexocin A, B [15], or C [47]; muricoreacin [47]; muricatacin [48]; isoannonacin [49]; isoannonacin-10-one [49]; goniothalamicin [49]; gigantetrocin [49] A or B [50], muricatetrocin A or B [17, 84], *cis*-annonacin; *cis*-annonacin-10-one; *cis*-goniothalamicin; arianacin; or javoricin [85]. In addition, synergistic therapeutic effects have been shown with the combination of AGEs. For example, cytotoxicity in breast cancer (Figure 2) has been observed using a combination of (2,4-*cis*)-10R-annonacin-A-one and (2,4-*trans*)-10R-Annonacin-A-one [43], or a mixture of *cis*-annomuricin-D-one and *trans*-annomuricin-D-one [32]. Moreover, AGEs induce cytotoxicity in a variety of other cancers such as prostate [15, 16, 21, 22, 34, 47], colorectal [16, 17, 25, 32, 34, 44, 86], lung [15–17, 32, 34, 44, 47][5], leukemia [46], renal [15, 16, 32, 34, 47], pancreatic [87, 88], hepatic [35, 36], and oral [32, 43] cancers. Combinations of AGEs also exhibited cytotoxicity in colorectal (HT-29), lung (A549) [32], prostate (PC-3), renal (A498), and pancreatic (PACA-2) cancers [28].

Organic solvent extracts derived from the different parts of *A. muricata* (presumably containing multiple bioactive compounds) have also been shown to induce cytotoxicity in a variety of cancer cell lines. For example, leaf extracts induced cytotoxicity in human A375 melanoma [36], immortalized HaCaT keratinocytes, and MDA-MB-435S, previously cross-contaminated and mislabeled as breast carcinoma cells [89], but currently identified and authenticated as a melanoma cell line (M14) [56, 90], or head and neck squamous cell SCC-25 carcinoma [91], pancreatic (CD18/HPAF and EG/COLO357) [28], colorectal (HT-29 and HCT-116) [25], *Liver HepG2* [56], and lung A549 [31] cancer cell lines. Leaf extracts have also demonstrated reduced cell viability in pancreatic Capan-1 cancer cells [92]. Extracts derived from seeds are toxic to hepatic Hep G2 [31] cancer cells, while extracts from leaf, pericarp, seed, and stem have each shown cytotoxicity towards hematological malignant cells such as the leukemia U-937 cell line [56, 69].

The most commonly used solvents for the *A. muricata* extracts that induced cytotoxicity against cancer cells are ethanol and methanol (Table 3). Ethanolic leaf extracts induce cytotoxicity in breast MCF-7 [45] and MDA [93], colorectal COLO-205 and DLD-1 [94], lung H-460 [95], leukemic K562 [19] and ECV-304, also previously misidentified as a human umbilical vein endothelial cell line [96], but now re-authenticated as a T24-contaminated human bladder cancer cell line [90, 97, 98] and (see http://iclac.org/databases/cross-contaminations), stomach C-678 [95], melanoma A375 [36], and Ehrlich ascite EACC [93] cancer cells. However, according to available data, ethanolic

Table 3: Anticancer effects of AGEs and extracts derived from the different aerial organs of *A. muricata*.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| Breast cancer | MCF-7 | Annomuricin A<br>Annomuricin B | AGE | Leaf | $ED_{50} > 1.0\,\mu g/mL$ | Cytotoxic activity [17] |
| | | Annomuricin C | AGE | Leaf | — — — | Cytotoxic activity [86] |
| | | Annomuricin E | AGE | Leaf | $ED_{50} = 1.45\,\mu g/mL$ | Cytotoxic activity [16] |
| | | Muricatocin C | AGE | Leaf | — — — | Cytotoxic activity [86] |
| | | Muricapentocin | AGE | Leaf | $ED_{50} = 1.90\,\mu g/mL$ | Cytotoxic activity [16] |
| | | Annomutacin | AGE | Leaf | $ED_{50} > 1.0\,\mu g/mL$ | Cytotoxic activity [43] |
| | | (2,4-*cis*)-10R-Annonacin-A-one + (2,4-*trans*)-10R-annonacin-A-one | AGE | Leaf | $ED_{50} = 5.70 \times 10^{-1}\,\mu g/mL$ | |
| | | Annohexocin | AGE | Leaf | $ED_{50} = 2.26\,\mu g/mL$ | Significant cytotoxic activity [34] |
| | | Muricatocin A | AGE | Leaf | $ED_{50} = 1.23 \times 10^{-1}\,\mu g/mL$ | Cytotoxic activity [44] |
| | | Muricatocin B | AGE | Leaf | $ED_{50} = 1.03 \times 10^{-1}\,\mu g/mL$ | |
| | | Annopentocin A | AGE | Leaf | $ED_{50} = 17.93\,\mu g/mL$ | Cytotoxic activity [32] |
| | | Annopentocin B | AGE | Leaf | $ED_{50} = 3.56\,\mu g/mL$ | |
| | | Annopentocin C | AGE | Leaf | $ED_{50} = 2.97\,\mu g/mL$ | |
| | | *cis*-Annomuricin-D-one + *trans*-annomuricin-D-one | AGEs | Leaf | $ED_{50} = 6.11 \times 10^{-1}\,\mu g/mL$ | |
| | | Murihexocin A | AGE | Leaf | $ED_{50} = 12.54\,\mu g/mL$ | Significant cytotoxic activity [15] |
| | | Murihexocin B | AGE | Leaf | $ED_{50} = 6.95\,\mu g/mL$ | |
| | | Murihexocin C | AGE | Seed | $ED_{50} = 3.8\,\mu g/mL$ | Cytotoxic activity [47] |
| | | Muricoreacin | AGE | Seed | $ED_{50} = 1.3\,\mu g/mL$ | |
| | | Muricatacin | AGE | Seed | $ED_{50} = 9.8\,\mu g/mL$ | Cytotoxic activity [48] |
| | | Isoannonacin | AGE | Seed | $IC_{50} = 1.1 \times 10^{-2}\,\mu g/mL$ | Cytotoxic activity [49] |
| | | Isoannonacin-10-one | AGE | Seed | $IC_{50} = 1.4 \times 10^{-2}\,\mu g/mL$ | |
| | | Goniothalamicin | AGE | Seed | $IC_{50} = 5.7 \times 10^{-2}\,\mu g/mL$ | |
| | | Gigantetrocin | AGE | Seed and/or leaf | $IC_{50} = 2.3 \times 10^{-2}\,\mu g/mL$ | Cytotoxic activity [50] |
| | | Gigantetrocin A | AGE | Seed | $ED_{50} = 5.3 \times 10^{-1}\,\mu g/mL$ | |
| | | Muricatetrocin A | AGE | Seed and/or leaf | $ED_{50} = 1.03\,\mu g/mL$ | Cytotoxic activity [17, 50] |
| | | Muricatetrocin B | AGE | Seed | $ED_{50} = 1.86\,\mu g/mL$ | |
| | | Gigantetrocin B | AGE | Seed | $ED_{50} = 5.3 \times 10^{-1}\,\mu g/mL$ | |
| | | *cis*-Annonacin | AGE | Seed | $IC_{50} = 1.18\,\mu g/mL$ | Cytotoxic activity [85] |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | *cis*-Annonacin-10-one | AGE | Seed | $IC_{50} = 2.9 \times 10^{-1}$ *μg*/mL | |
| | | *cis*-Goniothalamicin | AGE | Seed | $IC_{50} = 1.05$ *μg*/mL | |
| | | Arianacin | AGE | Leaf | $IC_{50} = 4.0 \times 10^{-3}$ *μg*/mL | |
| | | Javoricin | AGE | Leaf | $IC_{50} = 2.3 \times 10^{-3}$ *μg*/mL | |
| | | Hexane | Extract | Leaf | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 *μg*/mL; $IC_{50} = 49.92 \pm 2.23$ *μg*/mL | |
| | | Ethyl acetate | Extract | Fruit | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 *μg*/mL; $IC_{50} = 6.39 \pm 0.43$ *μg*/mL | Significantly reduced cell proliferation in cancer cells [31] |
| | | Methanol | Extract | Leaf | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 *μg*/mL; $IC_{50} = 85.58 \pm 3.55$ *μg*/mL | |
| | | ——— | Extract | Leaf | 0, 50, 100, 150, and 200 *μg*/mL; $IC_{50} > 200$ *μg*/mL | Inhibited growth of cancer cells [23] |
| | | Ethanol (95%) | Extract | Leaf | $GI_{50} = 6.2$ *μg*/mL | Cytotoxic activity [45] |
| | | (+)-(3*S*,6*S*,7*R*,8*S*)-Periconone A<br>(−)-(1*R*,4*R*,6*S*,7*S*)-2-Caren-4,8-olide | Fungal strain Extract | Leaf | 0.01–10 *μ*mol/mL | Cytotoxic activity [38] |
| | | Hexane | Extract | Leaf | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 *μg*/mL; $IC_{50} = 38.72 \pm 0.99$ *μg*/mL | Significantly reduced cell proliferation in cancer cells [31] |
| | | Ethyl acetate | Extract | Fruit | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 *μg*/mL; $IC_{50} = 11.36 \pm 0.67$ *μg*/mL | |
| | MDA-MB-231 | Methanol | Extract | Leaf | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 *μg*/mL; $IC_{50} > 100$ *μg*/mL | Significantly reduced cell proliferation in cancer cells [31] |
| | | ——— | Extract | Seed | Doses: 50, 100, 150, and 200 *μg*/mL; $IC_{50} > 200$ *μg*/mL | Inhibited the growth of cancer cells [23] |
| | | Methanol | Extract | Pericarp | | |
| | MDA-MB-231-pcDNA3 | Methanol | Extract | Leaf | | |
| | | Methanol | Extract | seed | | |
| | | Methanol | Extract | Pericarp | $IC_{50} > 80$ *μg*/mL | Cytotoxic activity [24] |
| | MDA-MB-231-*BCRP* clone 23 | Methanol | Extract | Seed | | |
| | | Methanol | Extract | Leaf | | |
| | ——— | Ethanolic component (7,12-dimethylbenzeneanthracene (DMBA)) | Extract | Fruit | Three groups of albino mice treated intragastrically by gavage for 6 weeks: 20 mg/mL/week of DMBA + 200 mg/mL/day of extract, 20 mg/mL/week of DMBA + 100 mg/mL/day of extract and | Prevented DMBA-induced DNA damage [77, 139] |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | | | | 20 mg/mL/week of DMBA + 50 mg/mL/day of extract [139] | |
| | | Leaves boiled in water | Beverage | Leaf | A 66-year-old female who has been diagnosed with cancer used to boil 10–12 dry leaves in water for 5–7 minutes, 8 oz PO daily at that time | Her metastatic breast cancer is still stable after 5 years on graviola and Xeloda after previously progressing on multiple lines of therapy [118] |
| | MDA-MB-468 | ——— | Extract | Leaf | Doses: 5, 25, 50, or 100 $\mu$g/mL; $IC_{50}$ = 4.8 $\mu$g/mL *in vitro*. In addition 200 mg/kg/35 week injected into the back of athymic mice *in vivo* | Inhibited EGFR-overexpression and EGFR mRNA expression. Induced cell cycle arrest at the G0/G1 phase. Induced apoptosis through caspase-3 activation. *In vivo*, it inhibited the growth of MDA-MB-468 tumors implanted in athymic mice (32% growth inhibition). It also significantly reduced the protein expression of EGFR, p-ERK, and p-EGFR in tumors [23] |
| | MDA | Ethanol | Extract | Leaf | $IC_{50}$ = 248.77 $\mu$g/mL | Cytotoxic activity [93] |
| | SKBR3 | Ethanol | Extract | Leaf | $IC_{50}$ = 202.33 $\mu$g/mL | |
| | T47D | Ethanol | Extract | Fruit | $IC_{50}$ = 17.15 $\mu$g/mL | Induced cytotoxicity and apoptosis [22] |
| Bladder cancer | ECV-304 | Ethanol | Extract | Twing | 0.1–10 mg/mL *in vitro*, MIC = 2 mg/mL and 0.5 g/kg into albino mice *in vivo* [96] | Cytotoxic activity against cancer cells *in vitro* and within reduction of time reaction *in vivo* [96] |
| | | Muricin J, K, or L | AGEs | Leaf | Dose: 20 $\mu$g/mL (24 h) | Antiproliferative activity against human cancer cells [29] |
| | | Annomuricin E | AGE | Leaf | $ED_{50} = 1.46 \times 10^{-1}$ $\mu$g/mL | Cytotoxic activity [16] |
| | | Muricapentocin | AGE | Leaf | $ED_{50} = 4.50 \times 10^{-1}$ $\mu$g/mL | |
| | | Annohexocin | AGE | Leaf | $ED_{50}$ = 0.0195 $\mu$g/mL | Significant cytotoxic activity [34] |
| | | Annopentocin A | AGE | Leaf | $ED_{50}$ = 1.14 $\mu$g/mL | |
| Prostate cancer | PC-3 | Annopentocin B | AGE | Leaf | $ED_{50} = 2.12 \times 10^{-1}$ $\mu$g/mL | Cytotoxic activity [32] |
| | | Annopentocin C | AGE | Leaf | $ED_{50} = 2.28 \times 10^{-1}$ $\mu$g/mL | |
| | | *cis*-Annomuricin-D-one + *trans*-annomuricin-D-one | AGEs | Leaf | $ED_{50}$ = 1.32 $\mu$g/mL | |
| | | Murihexocin A | AGE | Leaf | $ED_{50} = 1.71 \times 10^{-2}$ $\mu$g/mL | Significant cytotoxic activity [15] |
| | | Murihexocin B | AGE | Leaf | $ED_{50}$ = 0.126 $\mu$g/mL | |
| | | Murihexocin C | AGE | Fruit | $ED_{50}$ = 0.86 $\mu$g/mL | Cytotoxic activity [47] |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | Muricoreacin | | | $ED_{50} = 0.025\ \mu g/mL$ | |
| | | Muricin M | AGE | fruit | | |
| | | Muricin N | AGEs | Leaf | Dose: 20 $\mu g$/mL | Antiproliferative activities against human prostate cancer cells [30] |
| | | Muricenin | | | | |
| | — | Water | Extract | Leaf | F344 male rats (≈200 g) were gavaged 30 mg/mL (10 rats) and 300 mg/mL (10 rats) and fed ad libitum alongside 10 control rats for two months | Reduced prostate size *in vivo*, possibly through apoptosis [21] |
| | | Annomuricin A | AGE | Leaf | $ED_{50} > 1.0\ \mu g/mL$ | Cytotoxic activity [17] |
| | | Annomuricin B | AGE | Leaf | $ED_{50} = 4.35 \times 10^{-1}\ \mu g/mL$ | |
| | | Annomuricin C<br>Muricatocin C | AGE | Leaf | — | Cytotoxic activity [86] |
| | | Annomuricin E | AGE | Leaf | Doses: 1, 2, 4, 8, and 16 $\mu g$/mL [25]; $ED_{50} = 6.68 \times 10^{-2}\ \mu g/mL$ [16]; $IC_{50}$: $3.72 \pm 0.41\ \mu g/mL$ (12 hr), $3.49 \pm 0.22\ \mu g/mL$ (24 hr), and $1.62 \pm 0.24\ \mu g/mL$ (48 hr) [25]. | Induced toxicity against cancer cells [16, 25]. Suppressed proliferation of cancer cells and induced lactate dehydrogenase leakage, cell cycle arrest at G1 phase, and apoptosis mediated through activation of caspases 3/7 and 9. Also induced a time dependent upregulation of Bax and downregulation of Bcl-2 at both the mRNA and protein level [25] |
| Colorectal cancer | HT-29 | Muricapentocin | AGE | Leaf | $ED_{50} = 7.10 \times 10^{-2}\ \mu g/ml$ | Cytotoxic activity [16] |
| | | Annomutacin | AGE | Leaf | $ED_{50} > 1.0\ \mu g/mL$ | Cytotoxic activity [43] |
| | | (2,4 *cis*)-10R-Annonacin-A-one + (2,4 *trans*)-10R-annonacin-A-one | AGE | Leaf | $ED_{50} > 1.0\ \mu g/mL$ | |
| | | Annohexocin | AGE | Leaf | $ED_{50} = 0.78\ \mu g/mL$ | Significant cytotoxic activity [34] |
| | | Muricatocin A | AGE | Leaf | $ED_{50} = 1.56\ \mu g/mL$ | Cytotoxic activity [44] |
| | | Muricatocin B | AGE | Leaf | $ED_{50} = 1.66\ \mu g/mL$ | |
| | | Annopentocin A | AGE | Leaf | $ED_{50} = 1.63\ \mu g/mL$ | |
| | | Annopentocin B | AGE | Leaf | $ED_{50} = 1.61\ \mu g/ml$ | Cytotoxic activity [32] |
| | | Annopentocin C | AGE | Leaf | $ED_{50} = 1.24\ \mu g/mL$ | |
| | | *cis*-Annomuricin D-one + *trans*-annomuricin D-one | AGEs | Leaf | $ED_{50} < 10^{-2}\ \mu g/ml$ | |
| | | Muricin A | AGE | Leaf | $ED_{50} = 3.00\ \mu g/ml$ | Significant cytotoxic activity [13] |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | Murihexocin B | AGE | Leaf | $ED_{50} = 2.30\ \mu g/mL$ | |
| | | Murihexocin C | AGE | Seed | $ED_{50} = 1.3\ \mu g/mL$ | Cytotoxic activity [47] |
| | | Muricoreacin | AGE | Seed | $ED_{50} = 0.57\ \mu g/mL$ | |
| | | Muricatacin | AGE | Seed | $ED_{50} = 14.0\ \mu g/mL$ | Cytotoxic activity [48] |
| | | Isoannonacin | AGE | Seed | $IC_{50} < 10^{-3}\ \mu g/mL$ | |
| | | Isoannonacin-10-one | AGE | Seed | $IC_{50} = 1.8 \times 10^{3}\ \mu g/mL$ | Cytotoxic activity [49] |
| | | Goniothalamicin | AGE | Seed | $IC_{50} = 1.1 \times 10^{-3}\ \mu g/mL$ | |
| | | Gigantetrocin | AGE | Seed and/or leaf | $IC_{50} < 10^{3}\ \mu g/mL$ | |
| | | Gigantetrocin A | AGE | Seed | $ED_{50} < 10^{-8}\ \mu g/mL$ | Cytotoxic activity [50] |
| | | Muricatetrocin A | AGE | Seed and/or leaf | $ED_{50} < 10^{-8}\ \mu g/mL$ | |
| | | Muricatetrocin B | AGE | Seed | $ED_{50} = 2.8 \times 10^{-5}\ \mu g/mL$ | Cytotoxic activity [17, 50] |
| | | Gigantetrocin B | AGE | Seed | $ED_{50} = 4.1 \times 10^{-5}\ \mu g/mL$ | |
| | | *cis*-Annonacin | AGE | Seed | $IC_{50} = 1.0 \times 10^{-8}\ \mu g/mL$ | |
| | | *cis*-Annonacin-10-one | AGE | Seed | $IC_{50} = 9.0 \times 10^{-4}\ \mu g/mL$ | |
| | | *cis*-Goniothalamicin | AGE | Seed | $IC_{50} = 5.3 \times 10^{-3}\ \mu g/mL$ | Cytotoxic activity [85] |
| | | Arianacin | AGE | Leaf | $IC_{50} = 4.4\ \mu g/mL$ | |
| | | Javoricin | AGE | Leaf | $IC_{50} = 1.8\ \mu g/mL$ | |
| | | Hexane | Extract | Leaf | Doses: 10, 20, 40, and 80 $\mu g/mL$, $IC_{50} = 14.93 \pm 0.6\ \mu g/mL$ (72 hr) | Significantly reduced cell proliferation in cancer cells [26] |
| | | Ethyl acetate | Extract | Leaf | Doses *in vitro*: 10, 20, 40, and 80 $\mu g/mL$ [26]; 0.62, 1.25, 2.5, 5, 10, 20, 40, and 80 $\mu g/mL$ [25]; $IC_{50} = 4.29 \pm 0.24\ \mu g/mL$ (72 hr) [26]. *Doses in vivo*: 250 or 500 mg/kg into male Sprague-Dawley rats [25]. | Induced significant cytotoxic effects, cell cycle arrest at G1 phase, and apoptosis. Treatment also caused excessive accumulation of ROS followed by disruption of MMP, cytochrome c leakage, and activation of the initiator and executioner caspases in cancer cells. In addition, it upregulated Bax and downregulated Bcl-2 proteins. Furthermore, treatment conspicuously blocked the migration and invasion of cancer cells [26]. In rats treated with azoxymethane to induce colorectal carcinogenesis. |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | | | | | This extract reduced colonic aberrant crypt foci formation by 72.5% *in vivo* via downregulation of PCNA and Bcl-2 proteins and upregulation of Bax protein as well as an increase in the levels of enzymatic antioxidants and a decrease in the malondialdehyde level of the colon tissue homogenates, suggesting the suppression of lipid peroxidation [25] |
| | | Methanol | Extract | Leaf | Doses: 10, 20, 40, and 80 $\mu g$/mL and $IC_{50} > 100\,\mu g$/mL (72 hr) | Significantly reduced the cell proliferation in cancer cells [26] |
| | | Hexane | Extract | Leaf | Doses: 10, 20, 40, and 80 $\mu g$/mL and $IC_{50} = 12.26 \pm 0.42\,\mu g$/mL (72 hr) | |
| | HCT-116 | Ethyl acetate | Extract | Leaf | Doses: 10, 20, 40, and 80 $\mu g$/mL and $IC_{50} = 3.91 \pm 0.35\,\mu g$/mL (72 hr) | In cancer cells, induced significant cytotoxic effects, cell cycle arrest at the G1 phase, and apoptosis as well as excessive accumulation of ROS followed by disruption of MMP, cytochrome c leakage, and activation of the initiator and executioner caspases. It also upregulated Bax and downregulated Bcl-2 protein. Furthermore, treatment conspicuously blocked the migration and invasion of cancer cells [26] |
| | | Methanol | Extract | Seed | Doses: 10, 20, 40, and 80 $\mu g$/mL and $IC_{50} > 100\,\mu g$/mL (72 hr) | Significantly reduced cell proliferation in cancer cells [26] |
| | HCT116 ($p53^{+/+}$) | Methanol | Extract | Pericarp | $IC_{50} > 100\,\mu g$/mL | |
| | | Methanol | Extract | Leaf | | |
| | | Methanol | Extract | Seed | | |
| | HCT116 ($p53^{-/-}$) | Methanol | Extract | Pericarp | $IC_{50} > 80\,\mu g$/mL | Cytotoxic activity [24] |
| | | Methanol | Extract | Leaf | | |
| | | Methanol | Extract | Leaf | | |
| | ——— | Ethanolic | Extract | Leaf | 300 mg/kg into Wistar albino rats | Showed potent anticancer activity through apoptosis and reduction of aberrant crypt foci formation [20] |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | Ethanol | Extract | Leaf | 100 mg/kg body weight/4 weeks are administrated into Wistar rats | In a rat model of *Cycas*-induced colorectal carcinogenesis, protected against some early events as monitored by histology and protein expression [140] |
| | COLO-205 | 96% Ethanol [112] or ethanol soluble fraction leaf water extract contains 0.36% acetogenin (*w*/*w*) or 3.6 mg/g, and a 10 g water extract is equivalent to a 2 g ethanolic fraction [94]. | Extract | Leaf | Doses *in vitro*: 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 mg/L, $IC_{50}$ = 189.6 $\mu$g/mL (48 hr) [112]. Ex vivo, the colorectal cancer patients consumed either 300 mg of the extract, or maltose as a placebo, in the form of a capsule after breakfast [94]. | Enhanced proapoptotic caspase-3 marker activity [112]. Ex vivo and clinical studies showed higher cytotoxicity in the supplemented group compared with the placebo group [94] |
| | DLD-1 | Ethanol soluble fraction leaf water extract contains 0.36% acetogenin (*w*/*w*) or 3.6 mg/g, and a 10 g water extract is equivalent to a 2 g ethanolic fraction | Extract | Leaf | Patients consumed either 300 mg of extract, or maltose as a placebo, in the form of a capsule after breakfast. | Ex vivo and clinical studies showed higher cytotoxicity in the supplemented group compared with the placebo group [94] |
| | HTC-8 | (+)-(3S,6S,7R,8S)-Periconone A | Fungal strain Extract | Leaf | Doses: 0.01–10 $\mu$mol/ml | Cytotoxic activity [38] |
| | | (−)-(1R,4R,6S,7S)-2-Caren-4,8-olide | Fungal strain Extract | Leaf | | |
| Lung cancer | A549 | Annomuricin A | AGE | Leaf | $ED_{50} = 3.30 \times 10^{-1}$ $\mu$g/mL | Cytotoxic activity [17] |
| | | Annomuricin B | AGE | Leaf | $ED_{50} = 1.59 \times 10^{-1}$ $\mu$g/mL | |
| | | Annomuricin C | AGE | Leaf | —— | Cytotoxic activity [86] |
| | | Muricatocin C | AGE | Leaf | | |
| | | Annomuricin E | AGE | Leaf | $ED_{50} = 1.12 \times 10^{-1}$ $\mu$g/mL | Cytotoxic activity [16] |
| | | Muricapentocin | AGE | Leaf | $ED_{50} = 1.93 \times 10^{-1}$ $\mu$g/mL | |
| | | Annomutacin | AGE | Leaf | $ED_{50} = 1.57 \times 10^{-1}$ $\mu$g/mL | Cytotoxic activity [43] |
| | | (2,4-*cis*)-10R-Annonacin-A-one + (2,4-*trans*)-10R-annonacin-A-one | AGEs | Leaf | $ED_{50} = 1.74 \times 10^{-1}$ $\mu$g/mL | |
| | | Annohexocin | AGEs | Leaf | $ED_{50} = 0.34$ $\mu$g/mL | Significant cytotoxic activity [34] |
| | | Muricatocin A | AGE | Leaf | $ED_{50} = 7.55 \times 10^{-2}$ $\mu$g/mL | Cytotoxic activity [44] |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{95}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | Muricatocin B | AGT | Leaf | $ED_{50} = 3.34 \times 10^{-1}$ *μg*/ml | |
| | | Annopentocin A | AGL | Leaf | $ED_{50} = 1.71 \times 10^{-1}$ *μg*/mL | |
| | | Annopentocin B | AGE | Leaf | $ED_{50} = 2.74 \times 10^{-2}$ *μg*/mL | |
| | | Annopentocin C | AGL | Leaf | $ED_{50} = 2.06 \times 10^{-2}$ *μg*/ml | Cytotoxic activity [32] |
| | | *cis*-Annomuricin-D-one + *trans*-annomuricin-D-one | AGLs | Leaf | $ED_{50} < 10^{-2}$ *μg*/ml | |
| | | Muribexocin A | AGT | Leaf | $ED_{50} = 1.32$ *μg*/ml | Significant cytotoxic activity [15] |
| | | Muribexocin B | AGT | Leaf | $ED_{50} = 1.08$ *μg*/ml | |
| | | Muribexocin C | AGT | Seed | $ED_{50} = 1.1$ *μg*/ml | Cytotoxic activity [47] |
| | | Muricoreacin | AGE | Seed | $ED_{50} = 0.23$ *μg*/ml | |
| | | Muricatacin | AGL | Seed | $ED_{50} = 23.3$ *μg*/ml | Cytotoxic activity [48] |
| | | Isoannonacin | AGL | Seed | $IC_{50} = 9.6 \times 10^{-1}$ *μg*/ml | |
| | | Isoannonacin-10-one | AGE | Seed | $IC_{50} = 9.7 \times 10^{1}$ *μg*/ml | Cytotoxic activity [19] |
| | | Goniothalamicin | AGT | Seed and/or leaf | $IC_{50} = 8.0 \times 10^{-3}$ *μg*/ml | |
| | | Gigantetrocin | AGT | Seed and/or leaf | $IC_{50} < 10^{-3}$ *μg*/ml | |
| | | Gigantetrocin A | AGE | Seed | $ED_{50} = 8.1 \times 10^{-1}$ *μg*/mL | |
| | | Muricatetrocin A | AGT | Seed and/or leaf | $ED_{50} = 1.1 \times 10^{-1}$ *μg*/mL | Cytotoxic activity [17, 50] |
| | | Muricatetrocin B | AGT | Seed | $ED_{50} = 1.9 \times 10^{-1}$ *μg*/mL | |
| | | Gigantetrocin B | AGE | Seed | $ED_{50} = 2.5 \times 10^{-1}$ *μg*/mL | |
| | | *cis*-Annonacin | AGT | Seed | $IC_{50} = 2.3 \times 10^{-1}$ *μg*/mL | |
| | | *cis*-Annonacin-10-one | AGE | Seed | $IC_{50} = 3.5 \times 10^{-1}$ *μg*/mL | |
| | | *cis*-Goniothalamicin | AGE | Seed | $IC_{50} = 1.3 \times 10^{-1}$ *μg*/mL | Cytotoxic activity [85] |
| | | Arianacin | AGL | Leaf | $IC_{50} = 4.7 \times 10^{-1}$ *μg*/mL | |
| | | Javoricin | AGE | Leaf | $IC_{50} = 1.7 \times 10^{-2}$ *μg*/mL | |
| | | Ethyl acetate component | Extract | Leaf | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 *μg*/mL; $IC_{50}$: 5.09 ± 0.41 *μg*/ml (72 hr) | Selective cytotoxic effect against cancer cells and significant lactate dehydrogenase leakage and phosphatidylserine externalization demonstrated by fluorescence analysis. Treatment also elevated ROS formation, while attenuating MMP via upregulation of Bax and downregulation of Bcl-2. This was |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | | | | | accompanied by cytochrome c release to the cytosol, which triggered activation of caspase-9 and caspase-3. These proapoptotic effects were accompanied by cell cycle arrest at the G0/G1 phase and suppression of NF-$\kappa$B translocation from the cytoplasm to the nucleus [31] |
| | | Hexane | Extract | Leaf | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 $\mu$g/mL; $IC_{50}$ = 21.05 ± 0.42 $\mu$g/mL | Significantly reduced cell proliferation in cancer cells [31] |
| | | Methanol | Extract | Leaf | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 $\mu$g/mL; $IC_{50}$ > 100 $\mu$g/mL | |
| | | (+)-(3S,6S,7R,8S)-Periconone A | Fungal strain Extract | Leaf | Doses: 0.01–10 $\mu$mol/ml. | Cytotoxic activity [38] |
| | | (−)-(1R,4R,6S,7S) 2-Caren-4,8-olide | Fungal strain Extract | Leaf | | |
| | H-460 | Ethanol | Extract | Tree/Leaf | $IC_{50}$ < 0.22 $\mu$g/ml. | Cytotoxic activity [95] |
| | | Ethanol (95%) | Extract | Pericarp | $GI_{50}$ = 4.0 $\mu$g/ml. | Cytotoxic activity [45] |
| | NCI-H292 | Methanol | Extract | Pericarp | $IC_{50}$: 24.94 ± 0.74 $\mu$g/ml. | Antiproliferative and cytotoxic activities towards cancer cells [113] |
| | | Annonacin A | AGE | Pericarp | Doses: 0.1, 0.46, and 1.0 mg/ml. | |
| | | Annomuricin A | AGE | Pericarp | | |
| | | Methanol | Extract | Pericarp | MEC > 1 mg/ml. | Cytotoxic activity [46] |
| | | Hexane | Extract | Leaf | MEC = 1 mg/ml. | |
| Leukemia (hematological malignancies) | U-937 | Ethyle acetate | Extract | Stem | MEC = 0.1 mg/ml. | |
| | | Ethyle acetate | Extract | Stem | $LC_{50}$ = 7.8 ± 0.3 $\mu$g/ml. | Cytotoxic activity [131] |
| | | Ethyle acetate | Extract | Stem | $IC_{50}$ = 10.5 ± 2.3 and/or 28.1 ± 13.0 $\mu$g/ml. | |
| | | Methanol | Extract | Stem | $IC_{50}$ = 60.9 ± 10.4 and/or 38.5 ± 8.6 $\mu$g/ml. | Cytotoxic activity [33] |
| | | Hexane | Extract | Stem | $IC_{50}$ = 18.2 ± 0.8 and/or 15.7 ± 5.1 $\mu$g/ml. | |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$ and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | K562 | Ethanol | Extract | Leaf | Doses *in vitro*: 0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, and 5.0 mg/mL [19]; 0.1–10 mg/mL [96]; MIC = 7 mg/mL [96]. Dose *in vivo*: 0.5 g/kg into albino mice [96]. | Showed cytotoxicity *in vitro* [19] and *in vivo* [96]. This was accompanied *in vitro* by significantly increased caspase-3 activity. Induction of apoptosis was confirmed by a terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay [19] |
| | HL-60 | Ethanol | Extract | Root | $IC_{50}$ = 14 ± 2.4 $\mu$g/mL | Induced apoptosis through loss of MMP and inhibited proliferation via G0/G1 cell cycle arrest [27] |
| | | Ethanol | Extract | Fruit/pericarp | $IC_{50}$ = 49 ± 3.2 $\mu$g/mL | |
| | | Ethanol | Extract | Leaf | $IC_{50}$ = 9 ± 0.8 $\mu$g/mL. | |
| | CCRF-CEM | Methanol | Extract | Seed | $IC_{50}$ = 4.58 ± 0.25 $\mu$g/mL. | Induced cytotoxic, apoptosis, and cell cycle arrest [24] |
| | | Methanol | Extract | Leaf | $IC_{50}$ = 0.57 ± 0.02 $\mu$g/mL. | |
| | | Methanol | Extract | Seed | $IC_{50}$ = 0.36 ± 0.03 $\mu$g/mL. | |
| | | Methanol | Extract | Pericarp | $IC_{50}$ = 5.25 ± 0.38 $\mu$g/mL. | |
| | CEM/ADR5000 | Methanol | Extract | Leaf | $IC_{50}$ = 6.65 ± 0.22 $\mu$g/mL. | |
| | | Methanol | Extract | Leaf | $IC_{50}$ = 25.70 ± 1.64 $\mu$g/mL. | |
| Renal cancer | A498 | Annomuricin E | AGE | Leaf | $ED_{50}$ = 1.41 $\mu$g/mL. | Cytotoxic activity [16] |
| | | Muricapentocin | AGE | Leaf | $ED_{50}$ = 1.72 $\mu$g/mL. | |
| | | Annohexocin | AGE | Leaf | $ED_{50}$ = 2.36 $\mu$g/mL. | Cytotoxic activity [34] |
| | | Annopentocin A | AGE | Leaf | $ED_{50} = 6.07 \times 10^{-1}$ $\mu$g/mL. | Cytotoxic activity [32] |
| | | Annopentocin B | AGE | Leaf | $ED_{50} = 3.79 \times 10^{-1}$ $\mu$g/mL. | |
| | | Annopentocin C | AGE | Leaf | $ED_{50} = 2.58 \times 10^{-1}$ $\mu$g/mL. | |
| | | *cis*-Annomuricin-D-one + *trans* annomuricin-D-one | AGEs | Leaf | $ED_{50} = 1.22 \times 10^{-1}$ $\mu$g/mL. | |
| Pancreatic cancer | PACA | Murihexocin A | AGE | Leaf | $ED_{50}$ = 2.51 $\mu$g/mL. | Significant cytotoxic activity [15] |
| | | Murihexocin B | AGE | Leaf | $ED_{50}$ = 4.92 $\mu$g/mL. | |
| | | Murihexocin C | AGE | Leaf | $ED_{50}$ = 2.5 $\mu$g/mL. | Cytotoxic activity [47] |
| | | Muricoreacin | AGE | Leaf | $ED_{50}$ = 0.71 $\mu$g/mL. | |
| | | Annomuricin F | AGE | Leaf | $ED_{50} = 2.42 \times 10^{-2}$ $\mu$g/mL. | Cytotoxic activity [16] |
| | | Muricapentocin | AGE | Leaf | $ED_{50} = 5.03 \times 10^{-2}$ $\mu$g/mL. | |
| | PACA-2 | Annohexocin | AGE | Leaf | $ED_{50}$ = 0.77 $\mu$g/mL. | Significant cytotoxic activity [34] |
| | | Annopentocin A | AGE | Leaf | $ED_{50} = 3.58 \times 10^{-1}$ $\mu$g/mL. | Cytotoxic activity [32] |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | Annopentocin B | AGE | Leaf | $ED_{50} = 1.62 \times 10^{-1}$ μg/mL | |
| | | Annopentocin C | AGE | Leaf | $ED_{50} = 4.28 \times 10^{-1}$ μg/mL | |
| | | *cis*-Annomuricin-D-one + *trans*-annomuricin-D-one | AGEs | Leaf | $ED_{50} < 10^{-2}$ μg/mL | |
| | | Murihexocin A | AGE | Leaf | $ED_{50} = 9.73 \times 10^{-2}$ μg/mL | Significant cytotoxic activity [15] |
| | | Murihexocin B | AGE | Leaf | $ED_{50} = 0.413$ μg/mL | |
| | | Murihexocin C | AGE | Leaf and/or stem | $ED_{50} = 0.49$ μg/mL | Cytotoxic activity [47] |
| | | Muricoreacin | AGE | Leaf and/or stem | $ED_{50} = 2.3$ μg/mL | |
| | FG/COLO357 | Powder without binders or fillers (capsule contents is suspended in DMSO (100 mg/mL DMSO) | Extract | Leaf | Doses: 10–200 μg/mL, $IC_{50} = 200$ μg/mL | Induced cytotoxicity and necrosis by inhibiting cellular metabolism. In addition, it downregulated the expression of molecules related to hypoxia and glycolysis (i.e., HIF-1α, NF-κB, GLUT1, GLUT4, HKII, and LDHA) in cancer cells. Also, the motility of pancreatic cancer cells was decreased [28] |
| | CD18/HPAF | DMSO *in vitro* and $H_2O$ *in vivo* | Extract | Leaf | Doses: 10–200 μg/mL, $IC_{50} = 73$ μg/mL *in vitro*. 50 mg/kg/35 days injected orthotopically in the pancreas of athymic nude mice | Induced cytotoxicity and necrosis and inhibited cellular metabolism. In addition, it downregulates the expression of molecules related to hypoxia and glycolysis (i.e., HIF-1α, NF-κB, GLUT1, GLUT4, HKII, and LDHA) in cancer cells. After treatment, the motility of pancreatic cancer cells was decreased. It also caused 59.8% growth inhibition of pancreatic tumor induced in mice orthotopically implanted with CD18/HPAF cells [28] |
| | Capan-1 | Hexane | Extract | Seed | $IC_{25}$~7.8 – 8 μg/mL | Inhibited cell proliferation and induced mild cytotoxicity in cancer cells [92] |
| | | DMSO | Commercialized Extract | Seed | $IC_{25}$~0.9 – 1.0 μg/mL | |
| | | Muricin H | AGE | Seed | $IC_{50} = 9.51 \times 10^{-2}$ μg/mL | Exhibited significant activity in *in vitro* and cytotoxic assays against human hepatoma cell line [81] |
| Hepatic cancer | Hep G2 | Muricin I | AGE | Leaf | $IC_{50} = 5.09 \times 10^{-2}$ μg/mL | |
| | | *cis*-Annomontacin | AGE | Leaf | $IC_{50} = 2.98 \times 10^{-1}$ μg/mL | |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | *cis*-Corossolone | AGE | Seed | $IC_{50} = 1.65 \times 10^{-1}$ *μg*/mL | |
| | | Annocatalin | AGE | Leaf | $IC_{50} = 5.70$ *μg*/mL | |
| | | Annocatacin A | AGE | Leaf | $IC_{50} = 12.11$ *μg*/mL | Significant *in vitro* cytotoxic activity [80] |
| | | Annocatacin B | AGE | Seed | $IC_{50} = 3.35 \times 10^{-2}$ *μg*/ml | |
| | | Methanol | Extract | Pericarp | | |
| | | Methanol | Extract | Seed | $IC_{50} > 80$ *μg*/mL | Cytotoxic activity [24] |
| | | Methanol | Extract | Seed | | |
| | | Muricin A | AGE | Seed | $IC_{50} = 5.04$ *μg*/ml | |
| | | Muricin B | AGE | Seed | $IC_{50} = 1.78$ *μg*/mL | |
| | | Muricin C | AGE | Seed | $IC_{50} = 1.99$ *μg*/ml | |
| | | Muricin D | AGE | Seed | $IC_{50} = 6.60 \times 10^{-1}$ *μg*/ml | |
| | | Muricin E | AGE | Seed | ——— | |
| | | Muricin F | AGE | Seed | $IC_{50} = 4.28 \times 10^{-2}$ *μg*/ml | Cytotoxic activity [82] |
| | | Muricin G | AGE | Seed | ——— | |
| | | Muricatetrocins A & B | AGE | Seed | $IC_{50} = 4.93 \times 10^{-2}$ *μg*/ml | |
| | | Longifolicin | AGE | Seed | $IC_{50} = 4.04 \times 10^{-3}$ *μg*/mL | |
| | | Corossolin | AGE | Leaf | $IC_{50} = 3.53 \times 10^{-1}$ *μg*/mL | |
| | | Corossolone | AGE | Leaf | $IC_{50} = 4.80 \times 10^{-1}$ *μg*/mL | |
| | | Hexane | Extract | Leaf | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 *μg*/mL; $IC_{50} = 77.92 \pm 2.23$ *μg*/mL | |
| | | Ethyl acetate | Extract | Seed | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 *μg*/mL; $IC_{50} = 9.3 \pm 0.91$ *μg*/mL | Significantly reduced cell proliferation in cancer cells [31] |
| | | Methanol | Extract | Seed | Doses: 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 *μg*/mL; $IC_{50} > 100$ *μg*/mL | |
| | Hep 2,2,15 (a Hep G2 cell line transfected with HBV) | Muricin H | AGE | Seed | $IC_{50} = 1.18 \times 10^{-2}$ *μg*/mL | |
| | | Muricin I | AGE | Leaf | $IC_{50} = 2.22 \times 10^{-1}$ *μg*/mL | Exhibited significant activity in *in vitro* and cytotoxic assays in a human hepatoma cell line [81] |
| | | *cis*-Annomontacin | AGE | Leaf | $IC_{50} = 1.62 \times 10^{-2}$ *μg*/mL | |
| | | *cis*-Corossolone | AGE | Leaf | $IC_{50} = 4.76 \times 10^{-2}$ *μg*/mL | |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose, $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | | Annocatalin | AGE | Leaf | $IC_{50} = 3.48 \times 10^{-3}$ $\mu$g/mL | |
| | | Annocatacin A | AGE | Seed | $IC_{50} = 8.17 \times 10^{-1}$ $\mu$g/mL | Significant *in vitro* cytotoxic activity [80] |
| | | Annocatacin B | AGE | Seed | $IC_{50} = 2.22 \times 10^{-1}$ $\mu$g/mL | |
| | | Muricin A | AGE | Seed | $IC_{50} = 5.13 \times 10^{-3}$ $\mu$g/mL | |
| | | Muricin B | AGE | Seed | $IC_{50} = 4.29 \times 10^{-3}$ $\mu$g/mL | |
| | | Muricin C | AGE | Seed | $IC_{50} = 3.87 \times 10^{-3}$ $\mu$g/mL | |
| | | Muricin D | AGE | Seed | $IC_{50} = 4.80 \times 10^{-2}$ $\mu$g/mL | |
| | | Muricin E | AGE | Seed | ——— | |
| | | Muricin F | AGE | Seed | $IC_{50} = 3.86 \times 10^{-3}$ $\mu$g/mL | Cytotoxic activity [82] |
| | | Muricin G | AGE | Seed | ——— | |
| | | Muricatetrocins A & B | AGE | Seed | $IC_{50} = 4.83 \times 10^{-3}$ $\mu$g/mL | |
| | | Longifolicin | AGE | Seed | $IC_{50} = 4.90 \times 10^{-3}$ $\mu$g/mL | |
| | | Corossolin | AGE | Leaf | $IC_{50} = 2.34 \times 10^{-1}$ $\mu$g/mL | |
| | | Corossolone | AGE | Leaf | $IC_{50} = 2.84 \times 10^{-1}$ $\mu$g/mL | |
| | Bel-7402 | (+)-(3S,6S,7R,8S)-Periconone A | fungal strain Extract | Seed and/or Leaf | Doses: 0.01–10 $\mu$mol/mL | Cytotoxic activity [38] |
| | | (−)-(1R,4R,6S,7S)-2-Caren-4,8-olide | fungal strain Extract | Seed | | |
| | | Corossolone | AGE | Seed | $ED_{50} = 0.1$ $\mu$g/mL | Toxicity against oral cancer cells *in vitro* [35, 83] |
| | | Corossolin | AGE | Seed | $ED_{50} = 0.003$ $\mu$g/mL | |
| | | Solamin | AGE | Seed | $ED_{50} = 0.3$ $\mu$g/mL | |
| Oral cancer | KB | Murisolin | AGE | Seed | $ED_{50} = 0.1$ $\mu$g/mL | Toxicity against oral cancer cells *in vitro* [35] |
| | | Annonacinone | AGE | Seed | $ED_{50} = 0.01$ $\mu$g/mL | |
| | | Annonacin | AGE | Leaf | $ED_{50} = 0.0001$ $\mu$g/mL | |
| | | Vinblastine | AGE | Leaf | $ED_{50} = 0.01$ $\mu$g/mL | |
| Stomach cancer | C-678 | Ethanol | Extract | Leaf | $IC_{50} < 0.22$ $\mu$g/mL | Cytotoxic activity [95] |
| | | $H_2O$ | Extract | Leaf | $IC_{50} > 500$ $\mu$g/mL (24 and 72 hr) | |
| Melanoma | A375 | Ethanol | Extract | Leaf | $IC_{50} = 20 \pm 6$ $\mu$g/mL (24 hr) and $20 \pm 7$ $\mu$g/mL (72 hr) | Cytotoxic activity [36] |
| | | Pentane | Extract | Leaf | $IC_{50} = 140 \pm 25$ $\mu$g/mL (24 hr) and $120 \pm 8$ $\mu$g/mL (72 hr) | |

Table 3: Continued.

| Cancers | Cell lines | Chemical compound or solvent | Class | Plant part | Dose; $IC_{50}$, $ED_{50}$, $GI_{50}$, $LC_{50}$, $IC_{25}$, and/or MIC | Anticancer effects |
|---|---|---|---|---|---|---|
| | MDA-MB-435S | *n*-Butanolic | Extract | Leaf | $IC_{50}$ = 29.2 $\mu$g/mL; 25, 50, 100, 200, and 400 $\mu$g/mL | Significant cytotoxic activity [89, 90] |
| Skin cancer | ——— | 80% aqueous ethanol | Extract | Leaf | 30 mg/kg body weight into ICR mice | Suppressed tumor initiation as well as tumor promotion even at lower dosage [141] |
| | SF-268 | Ethanol (95%) | Extract | Seed | $GI_{50}$ = 8.5 $\mu$g/mL | Cytotoxic activity [35] |
| | | Methanol | Extract | Pericarp | | |
| | U87MG | Methanol | Extract | Leaf | | |
| Glioma | | Methanol | Extract | Seed | $IC_{50}$ > 80 $\mu$g/mL | Cytotoxic activity [24] |
| | | Methanol | Extract | Pericarp | | |
| | U87MG.*EGFR* | Methanol | Extract | Leaf | | |
| | | Methanol | Extract | Leaf | | |
| | | Ethyl acetate | Extract | Leaf | $LC_{50}$ of (2000 $\mu$g/mL) = 131.89%; and $LC_{50}$ of (15.625 $\mu$g/mL) = 11.37% | |
| | | Ethanol-distillate water | Extract | Leaf | $LC_{50}$ of (2000 $\mu$g/mL) = 35.80%; and $LC_{50}$ of (15.625 $\mu$g/mL) = 3.97% | |
| Cervical cancer | HeLa | Chloroform | Extract | Leaf | $LC_{50}$ of (2000 $\mu$g/mL) = 65.20%; and $LC_{50}$ of (15.625 $\mu$g/mL) = 18.42% | Induced apoptosis [37] |
| | | *n*-Hexan | Extract | Leaf | $LC_{50}$ of (2000 $\mu$g/mL) = 106.53%; and $LC_{50}$ of (15.625 $\mu$g/mL) = 21.41% | |
| | HEp-2 (now HeLa) | Methanol | Extract | Leaf | $IC_{50}$ = 54.92 ± 1.34 $\mu$g/mL | Antiproliferative and cytotoxic activities [90, 113] |
| Ehrlich ascite carcinoma | EACC | Ethanol | Extract | Leaf | $IC_{50}$ = 335.85 $\mu$g/mL | Cytotoxic activity *in vitro* [93] |
| Gastric cancer | BGC-823 | (+)-(3*S*,6*S*,7*R*,8*S*)-Periconone A | Fungal strain Extract | Leaf | Doses: 0.01–10 $\mu$mol/mL | Cytotoxic activity [38] |
| | | (−)-(1*R*,4*R*,6*S*,7*S*)-2-Caren-4,8-olide | Fungal strain Extract | Leaf | | |
| Ovarian cancer | A2780 | (+)-(3*S*,6*S*,7*R*,8*S*)-Periconone A | Fungal strain Extract | Leaf | | |
| | | (−)-(1*R*,4*R*,6*S*,7*S*)-2-Caren-4,8-olide | Fungal strain Extract | Tree/Leaf | | |
| Head and neck squamous cell carcinoma (HNSC) | SCC-25 | $H_2O$ | Extract | Leaf | Doses: 2.5–160 $\mu$g/mL; $IC_{50}$ = 12.42 $\mu$g/mL | Displayed promising cytotoxic activity and inhibition of cell proliferation via G2M cell cycle arrest [91] |

ED50: median effective dose; $GI_{50}$: a concentration for 50% of maximal inhibition of cell proliferation; $IC_{25}$: a concentration causing 50% inhibition; $IC_{50}$: a concentration causing 50% inhibition; $LC_{50}$: a concentration causing 50% cell death; LD: lethal dose; MIC: minimum inhibitory concentration.

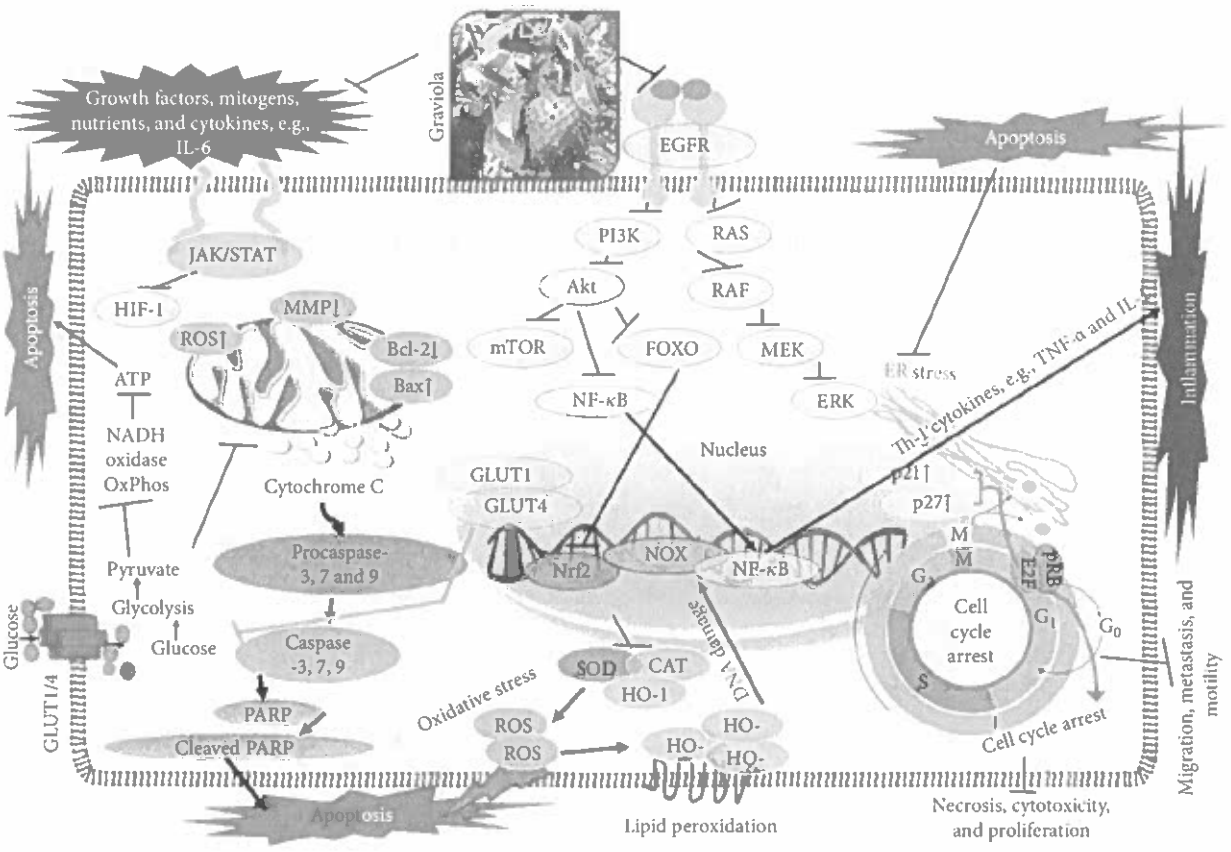



FIGURE 3: Overview of the molecular actions of *A. muricata* (graviola) leading to anticancer and other health benefits. Extracts of the different aerial parts of *A. muricata* using several solvents have been shown to induce cytotoxicity, cell cycle arrest, apoptosis, and necrosis and, conversely, to inhibit cancer cell motility, migration, metastasis, and proliferation. Other reported health benefits include antioxidant, anti-inflammatory, and immunomodulatory activities. Our current understanding is that graviola components modulate several cellular processes including inhibition of signaling pathways downstream of the epidermal growth factor receptor (EGFR), with others causing downregulation of phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K/Akt), RAS, NF-κB, and JAK/STAT [31]. Further actions include inhibition of HIF-1α, GLUT1, and GLUT4 [28]; proinflammatory cytokine expression (inflammation); and generation of reactive oxygen species (ROS) via upregulatoin of enzyme systems like catalase (CAT), superoxide dismutase (SOD), and heme-oxygenase (HO-1) expression [39, 54, 89, 124].

fruit extracts induce toxicity only against both breast T47D [22] and lung H-460 [95] cancer cells. In addition, while ethanolic twig extract shows cytotoxic activity against ECV-304 cancer cells *in vitro* [96], methanolic extracts of the leaves, pericarp, or seeds of *A. muricata* all exert toxicity against glioma U87MG, breast MDA-MB-231-pcDNA3 and MDA-MB-231-*BCRP* clone 23, colorectal HCT-116 ($p53^{+/+}$), and HCT-116 ($p53^{-/-}$) cancer cells [24]. Moreover, stem methanolic extract induces cytotoxicity towards leukemia U-937 cells [33].

## 5. Apoptosis

Apoptosis, or programmed cell death, is integral for normal development and tissue homeostasis in most multicellular organisms [99]. Apoptosis plays a vital role in destroying cells which are selectively unnecessary or that present a threat to the integrity of an organism, thereby limiting the development and/or spread of cancer [1]. In many cancers, however, the gene(s) regulating apoptosis are faulty which leads to uncontrolled proliferation [100]. The ability to induce cellular apoptosis in tumor tissue is the key to finding a successful natural product as an anticancer agent [27, 101].

Apoptosis displays characteristic morphological and biochemical changes which may include cell shrinkage, nuclear fragmentation, chromatin condensation, and membrane blebbing [99, 102, 103]. The major apoptotic pathways are intrinsic and extrinsic [104]. The intrinsic (or mitochondrial) pathway can be induced through intracellular stresses such as DNA damage or oxidative stress leading to the release of mitochondrial cytochrome c forming the apoptosome complex [105]. This complex is composed of cytochrome c, apoptotic protease activating factor, and procaspase [106], which activates different caspases [107]. The extrinsic pathway, also known as the death receptor pathway, can be induced by death ligands, tumor necrosis factor α, and tumor necrosis factor-related apoptosis inducing

ligand (TRAIL)) [108]. These ligands bind to their cell surface receptors (tumor necrosis factors), death receptors, and Fas causing sequential activation of caspase-8, caspase-3, and caspase-7 [109]. Moreover, apoptosis is regulated by several proteins such as BCL-2 [110], BAX [111], and PCNA [25].

Several studies examining the anticancerous properties in *A. muricata* extracts have observed the induction of apoptosis. According to the available data, there are about six extract types with regard to solvent extraction of *A. muricata* parts, including water [21], ethanol [20, 112], methanol, ethyl acetate [31], chloroform, and n-hexane [37] extracts. Leaf extracts of *A. muricata* induce apoptosis in breast MDA-MB-468 cancer cells through caspase-3 activation [23]. Similarly, *A. muricata* fruit extract induces apoptosis in breast T47D cancer cells [22]. An ethanolic extract of *A. muricata* leaves induces apoptosis [20] in COLO-205 colon cancer cells through upregulation of proapoptotic caspase-3 marker activity [112]. Similarly, in HT-29 colorectal cancer cells, annomuricin E derived from leaves of *A. muricata* induced apoptosis mediated through activation of caspases 3/7 and 9, upregulation of BAX, and downregulation of BCL-2 at the mRNA and protein levels [25]. In K562 leukemia cancer cells, an ethanolic leaf extract significantly enhanced caspase-3 activity to induce apoptosis, which was confirmed by a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay [19]. In addition, ethanolic extracts of roots, fruits, or leaves of *A. muricata* have been shown to induce apoptosis in HL-60 leukemia cancer cells through loss of MMP [27]. An aqueous leaf extract was shown to reduce prostate size which was suggested to have been due to apoptosis [21]. Leaf extracts prepared using various solvents were also able to induce apoptosis on HeLa cervical cancer cells [37]. Treatment of colorectal HT-29 and HCT-116 cancer cells with an ethyl acetate extract of leaves caused apoptosis through excessive accumulation of ROS followed by disruption of MMP, cytochrome c leakage, activation of the initiator and executioner caspases, upregulation of Bax, and downregulation of Bcl-2 protein [25]. An ethyl acetate *leaf* extract also elicited a 72.5% reduction in aberrant crypt foci inhibition in azoxymethane-induced colorectal carcinogenesis in rats [25]. This effect was associated with a downregulation of PCNA and Bcl-2 proteins and an upregulation of Bax protein as well as an increase in the levels of enzymatic antioxidants and a decrease in the malondialdehyde level of the colon tissue homogenates suggesting the suppression of lipid peroxidation [25]. Similarly, an ethyl acetate extract derived from leaves of *A. muricata* induced apoptosis in lung A549 cancer cells through elevating ROS formation, followed by attenuation of MMP via upregulation of Bax and downregulation of Bcl-2. These effects were accompanied by the release of cytochrome c into the cytosol, which triggered the activation of caspase-9 followed by caspase-3 activation. Moreover, the treatment also suppressed the induced translocation of NF-$\kappa$B from cytoplasm to nucleus [31]. Apoptosis also has been shown in HeLa cervical cancer cells after treatment with an ethyl acetate leaf extract [37]. Methanolic extracts of seeds or leaves were also shown to induce apoptosis in leukemic CCRF-CEM cells, while pericarp or leaf extracts induced apoptosis in CEM/ADR5000 leukemia cells [24]. Furthermore, leaf extracts using ethyl acetate and ethanol-distillate water, as well as *n*-hexane and chloroform leaf extracts, have also been shown to induce apoptosis in HeLa cervical cancer cells [37].

## 6. Modulation of Cellular Proliferation

Proliferation is a hallmark of cancer development and progression manifested by altered expression and/or activity of cell cycle-related proteins. In cancer, the normal cell cycle process is impaired, resulting in uncontrolled cell proliferation, growth, and tumor progression. *A. muricata* extracts, and ACEs have been shown to regulate the cell cycle machinery, leading to cell cycle arrest and inhibition of cell proliferation. According to the available data, there are about seven ACEs and five extracts which have demonstrated antiproliferative activity. Specific ACEs include muricins J, K, L [29], M, and N; muricenin [30]; and annomuricin E [25]. Extracts with antiproliferative properties include those using solvents such as hexane [30], ethyl acetate [77], methanol [113], ethanol [27], and water [91] as discussed below.

Muricins J, K or L, M, and N and muricenin leaf ACEs all have shown antiproliferative activities when tested on human prostate PC-3 cancer cells [29, 30]. Annomuricin E derived from leaves of *A. muricata* was reported to suppress proliferation of HT-29 colorectal cancer cells via cell cycle arrest at the G1 phase, which also induces leakage of lactate dehydrogenase [25].

Hexane, ethyl acetate, and methanol extracts all significantly reduced cell proliferation in hepatic Hep G2 and breast MCF-7 and MDA MB-231 cancer cells [4]. A hexane *leaf* extract significantly reduced cell proliferation in PC-3 colorectal cancer cells [30]. Hexane and methanol extracts of *A. muricata* suppressed the proliferation of HT-29 and HCT-116 colorectal adenocarcinoma cells [26] as well as A549 [4] lung cancer cells. Antiproliferative and cytotoxic activities were seen in NCI-H292 cancer lung cells after treatment with methanol pericarp extract [113]. Ethanol extracts of roots, fruits, or leaves inhibited proliferation via G0/G1 cell cycle arrest in leukemia HL-60 cancer cells [26]. Hexane or DMSO seed extracts inhibited cell proliferation in pancreatic cancer Capan-1 cells [92]. An aqueous *leaf* extract showed promising antiproliferative activity by arresting the cell cycle in the G2M phase in SCC-25 squamous cell carcinoma [91]. A methanol leaf extract inhibited proliferation of Hep-2 cancer cells, first reported as laryngeal cancer cell line [113], currently authenticated as a cross-contaminated HeLa cell line [90] (http://iclac.org/databases/cross-contaminations). Finally, *A. muricata* leaf extracts were reported to induce cell cycle arrest at the G0/G1 phase in MDA-MB-468 breast [23], HCT-116 [31], and HT-29 [25] colorectal and A549 [31] lung cancer cells, and our preliminary unpublished observations showed similar effects on two nonmelanoma skin cancer cell lines, namely a basal (UWBCC1) and a squamous (A431) carcinoma cell line [116].

## 7. Necrosis and Other Related Effects

The death of cells in a tissue due to chemotherapeutic agents is defined as "necrosis" which contributes to chemotherapy-induced cell death [115]. Necrotic cell death is distinguished from its counterpart, apoptosis, in that caspase activation is not required for cell death. Unlike apoptosis, chemotherapy-induced necrosis results in plasma membrane rupture, thus spilling the contents of the cell and triggering the immune system. This results in the inhibition of cellular metabolism and induces further necrosis through the downregulation of factors related to hypoxia and glycolysis (i.e., HIF-1$\alpha$, NF-$\kappa$B, GLUT1, GLUT4, HKII, and LDHA) in pancreatic FG/COLO357 and CD18/HPAF cancer cells [28].

Cancer cell motility, migration, and invasion also play fundamental roles in cancer metastasis [116]. Therefore, inhibiting either cancer cell motility, migration, or invasion impedes metastasis, which is the cause of over 90% of patient deaths [117]. After treatment of pancreatic FG/COLO357 and CD18/HPAF cancer cells with leaf extracts, the motility of cancer cells was decreased [28]. More dramatically, treatment of HT-29 and HCT-116 colorectal cancer cells with an ethyl acetate leaf extract conspicuously blocked the migration and invasion of cancer cells [25, 26]. In addition to the preclinical studies with cell lines cited above, a subset of these effects have been demonstrated in a clinical model system [118].

## 8. Other Potential Health-Related Benefits

In addition to cancer chemopreventive and chemotherapeutic effects, graviola extracts and their constituents, individually or in combination, have shown therapeutic properties for other ailments that afflict humankind including chronic inflammatory and oxidative diseases, wounds, and noninfectious and infectious microbial and parasitic diseases. Graviola organs have been used as herbal medications against cystitis, diabetes, headaches, hypertension, insomnia, and liver diseases as well as antidysenteric, anti-inflammatory, and antispasmodic agents [119]. Other benefits thus far reported for graviola constituents, in addition to those listed above, have included anxiolytic, anticancer, antitumorigenic, antidepressant, gastroprotective, antimalarial, antinociceptive, immunomodulatory, antistress, and wound healing activities [4, 120], some of which are reviewed below.

## 9. Anti-inflammatory, Antinociceptive, Antiarthritic, Immunomodulatory, and Wound Healing Activities

Over the last few decades, herbal remedies and natural phytochemicals have garnered scientific interest for their utility in managing pain and inflammation. Natural agents including *A. muricata* can mechanistically modulate these effects by impacting molecular targets, some of which are common to pain medications such as NSAIDs, but with reduced side effects. The following examples from the literature are provided.

An ethanol extract of graviola leaves was administered orally to rats by de Sousa et al., followed by various tests of nociception and inflammation [119]. These authors found the following dose-dependent effects: (a) reduction in abdominal contortions after ip injection of acetic acid, (b) increased time to paw licking after subplantar injections of 2.5% formalin, (c) increased reaction time in a hot plate test, (d) reduced edema after subplantar injection of 2% carrageenan, and (d) reduced exudate and leucocyte counts after carrageenan-induced pleurisy. Taken together, these observations were interpreted by the authors as a confirmation of the ethnomedical use of ethanol extracts of graviola leaves for therapeutic purposes. However, one caution was that the ethanol extract was toxic to animals at approximately 1.7 g/kg, leading the authors to call for further studies to ensure safe usage in humans.

Similar tests with an ethanol extract of graviola leaves were carried out by [121], who confirmed the antinociceptive properties of this extract. These authors extended these studies to include an ethanol-induced ulcer model in rats pretreated with N-nitro-L-arginine methyl ester (L-NAME), finding that the graviola extract dose-dependently reduced the size of the ulcerative lesions. This effect was inhibited by N-ethylmaleimide, prompting the authors to conclude that the protective effect of the graviola extract in this setting might be due, at least in part, to antioxidant properties that increase the sulfhydryl content of the gastric mucosa [121].

Hamid et al. also tested an ethanol extract of graviola leaves, administered orally to rodents, for its acute and chronic anti-inflammatory actions. These authors reported that the ethanol extract (a) reduced xylene-induced ear edema in mice, (b) attenuated arthritis in rats induced by complete Freund's adjuvant, and (c) reduced TNF-$\alpha$ and IL-1$\beta$ levels in the arthritis model, suggesting that the antiarthritic actions are at least partially due to a suppression of proinflammatory cytokines.

Ishola et al. used methodology similar to that described above (writhing, formalin, hot plate tests, carrageenan-induced rat paw edema, and xylene-induced ear edema tests) to test lyophilized fruit extracts of graviola [122]. These authors also found a dose-dependent inhibition of (a) writhes, (b) formalin-induced pain, (c) carrageenan-induced paw circumference, and (d) xylene-induced ear edema. This study also probed the possible involvement of the opiodergic, nitric oxide, and prostaglandin pathways, with the following results: (a) the anti-inflammatory actions of the fruit extracts were blocked by NG-nitro-L-arginine (a nitric oxide inhibitor) as well as by naloxone and (b) treatment with extract dose-dependently inhibited both COX-1 and COX-2. These authors therefore concluded that the analgesic and anti-inflammatory actions of a graviola fruit extract, as it is used in traditional African medicine, are confirmed and that these actions involve the opiodergic, prostaglandin, and nitric oxide systems [122].

Laksmitawati et al. tested an ethanol extract of graviola leaves in cultures of the LPS-stimulated murine macrophage cell line (RAW264.7) [123] and performed cell viability, cytokine ELISAs, and nitric oxide (NO) production assays. These authors reported that (a) cell viability was not affected

in doses up to 50 $\mu$g/mL and b) levels of TNF-a, IL-1$\beta$, IL-6, and NO were all reduced relative to untreated cells.

Oliveira et al. used an aqueous extract of graviola in a similar in vitro study of inflammatory markers using RAW264.7 cells along with cell-free assays in comparison with an aqueous extract from *Jasminum grandiflorium* [67]. In addition to examining NO production, these authors also performed HPLC with diode array detection (HPLC-DAD) to determine the phenolic compounds responsible for some of the effects. These authors reported that (a) graviola was superior to *J. grandiflorium* in inhibiting both NO production as well as phospholipase $A_2$ ($PLA_2$) and (b) aglycones from the extract especially quercetin and 3-O-caffeoylquinic acid were capable of inhibiting NO production and/or $PLA_2$ in low micromolar concentrations. Cytotoxicity was not noted at the concentrations tested in this study [67].

Related to graviola's anti-inflammatory effects are its ability to promote wound healing; indeed, graviola preparations are commonly used in folk medicine for skin diseases and abscesses. Moghadamtousi et al. used an ethyl acetate extract of graviola leaves in an excisional wound rat model [4]. In addition to examining antioxidant activity of this extract (see discussion above), these authors also performed histological and immunohistochemical analysis of wounds treated with ointments containing the extract using intrasite gel as a positive control. Their results showed that (a) both doses of extract showed significant acceleration of wound closure and tissue regeneration, with the higher dose being comparable to the intrasite positive control, and (b) both doses of extract upregulated heat shock protein 70 (Hsp70) to levels comparable to those seen in the intrasite-positive control as monitored by immunohistochemical staining. These authors concluded that there is a clear wound healing effect of the extract, even though the bioactive compounds responsible for the effect have not yet been identified [4].

## 10. Antioxidant Activity

Several reports have described the antioxidant properties of various graviola-derived extracts. These will be described in order of appearance in the literature.

Given that graviola leaves are used in Cameroon to manage diabetes, Florence et al. tested an aqueous extract of graviola leaves in streptozotocin-induced diabetic rats [54]. Although this extract had no effect on normal rats, the aqueous extract was found to reduce blood glucose levels in diabetic rats. These authors also found the following: (a) after 15 days of treatment with 100 mg/kg of extract (given 3 days prior to streptozotocin), animals showed significant (46%) reductions in blood glucose compared to diabetic controls not treated with extract, (b) immunostaining at the end of treatment showed preservation of pancreatic $\beta$-cells in treated animals compared to diabetic controls not treated with extract, (c) activity of superoxide dismutase (SOD) and catalase in diabetic animals treated with extract (100 mg/kg) were normalized up to levels seen in nondiabetic controls, and (d) levels of tissue malondialdehyde (a marker of lipid peroxidation) and nitrites were reduced down to levels seen in nondiabetic animals. These studies in rats support the use of graviola as an antidiabetic agent and suggest that at least part of its beneficial actions are antioxidant in nature. One cautionary observation was made, however: a higher dose (200 mg/kg) of extract was not only less effective but also resulted in 25% mortality among that treatment group [54].

Gavamukulya et al. tested the antioxidant potential of ethanolic and aqueous extracts of graviola found in Eastern Uganda using 2,2-diphenyl-2-picrylhydrazyl (DPPH•) and reducing power assays [93]. Their results indicated that (a) the ethanolic extract was superior to the aqueous extract with respect to both reducing power and in vitro antioxidant activity and (b) the ethanolic extract, but not the aqueous extract, was selectively cytotoxic to three tumor cell lines as opposed to no effect on normal spleen cells [93].

George et al. compared methanolic and aqueous extracts of graviola with respect to their free radical scavenging and DNA protective properties using several assays including a ferric reducing antioxidant property (FRAP) assay, a DPPH• radical scavenging assay, a hydroxyl scavenging activity assay (HRSA), and a DNA damage protective activity [124]. These authors also carried out HPLC analysis of phenolic compounds in each extract. Both graviola extracts were found to possess significant radical scavenging assays, and a strong positive correlation was seen between the total phenolic content and the radical scavenging activity of each extract. The methanolic extract was found to confer superior protection against hydrogen peroxide-induced DNA damage [124].

Moghadamtousi et al. applied an ethyl acetate extract of graviola leaves to skin wounds in rats [4]. Although wound healing was the main focus of this study (see above), these authors also measured levels of malondialdehyde as well as activities of catalase, glutathione peroxidase, and superoxide dismutase in wound tissue homogenates. After 15 days of treatment, analysis of tissue samples revealed a "significant surge in antioxidants activities and decrease in the MDA level of wound tissues compared with vehicle control," providing yet another example of the antioxidative potential of graviola extracts [124].

Finally, Son et al. studied the antioxidant properties of steam and 50% ethanol extracts of graviola leaves in HepG2 cells [125]. Their results, standardized in some cases to vitamin C equivalents, indicated that (a) the 50% ethanol extracts were superior than steam extracts in scavenging peroxy and nitrogen radicals, although both were effective, and (b) the 50% ethanol extract upregulated superoxide dismutase 1 (SOD1) and Nrf2 (an important transcriptional regulator of antioxidant enzymes), but not catalase or heme oxygenase 1 (HMOX1).

In summary, accumulating evidence suggests that components of graviola possess potent antioxidant properties, although these may vary depending on the method of extraction as well as the cells/tissues in which they are tested.

## 11. Hepatoprotective Effects Related to Antioxidation

As described above, Adewole and Ojewole observed hepatic benefits after administration of an aqueous leaf extract of

graviola to streptozotocin-induced diabetic rats [39]. The described benefits in liver consisted mainly of increases in antioxidant enzymes (catalase, SOD, and glutathione peroxidase) and levels of glutathione to reduce oxidative stress in this tissue. However, other positive effects of this treatment included improvements in blood lipid levels, specifically a decline in diabetes-induced levels of LDL, total cholesterol, and triglycerides and an increase in HDL [39].

Padma et al. tested the ability of an ethanol extract of graviola stem bark, in comparison with a similar extract from *Polyalthia cerasoides*, to ameliorate carbon tetrachloride liver toxicity in albino rats [126], using liver function tests as a measure of liver damage. Their results showed that the graviola extract was superior to the *P. cerasoides* extract in (a) reducing blood levels of liver transaminases (ALT/SGPT and AST/SGOT) and alkaline phosphatase released into the blood by the liver damage and (b) reducing lipid peroxidation in liver samples as measured by detection of MDA. While the protection of liver was not complete, the reduction in these measures of liver damage by graviola (but not by *P. cerasoides*) was highly significant ($P < 0.001$) in each case when compared to controls (number of animals in each group ranged from 7 to 10).

## 12. Antidiabetic and Hypotensive Properties

As already mentioned, graviola extracts have been used as antidiabetic agents in many parts of the world (see [127] above). Additional evidence for the presence of antidiabetic agents in graviola extracts has been reported as follows.

Adewole and Ojewole administered an aqueous leaf extract of graviola to streptozotocin-induced diabetic rats [39]. After four weeks of treatment, these researchers found that (a) glucose levels were reduced in treated diabetic rats along with an elevation in blood insulin, (b) levels of ROS and blood lipids also declined in treated diabetic rats, and (c) activities of antioxidant enzymes (catalase, SOD, and glutathione peroxidase) and levels of glutathione were increased in liver relative to untreated diabetic animals. This study mainly focused on the hepatic benefits of graviola treatment in the setting of diabetes, with the conclusion that graviola is protective against oxidative stress in this tissue.

In addition to antidiabetic properties of graviola (*Annona muricata*), it should also be noted that a closely related plant, *Annona montana* (also known as "false graviola"), has been tested for its effect on blood lipids and blood glucose. Treatment of Wistar rats for 40 days with juices prepared from either leaf or fruit not only showed reduced blood glucose and blood low-density lipoproteins but also increased levels of high-density lipoproteins, prompting these authors to propose that use of this plant may help prevent diabetes mellitus and dyslipidemia [128].

Finally, Nwokocha et al. administered an aqueous leaf extract by IV injection to normotensive Sprague-Dawley rats, and short-term measurements (10 minutes apart) were taken of systolic and diastolic blood pressure, after which animals were sacrificed and aortic rings were isolated and tested in an isometric force transducer system [12]. These authors reported that (a) both systolic and diastolic blood pressures were reduced in a dose-dependent manner by the graviola extract, but without an effect on heart rate; (b) further tests determined that these effects were apparently not due to muscarinic, endothelial, histaminergic, or adrenergic mechanisms; and (c) testing of aortic rings suggested that alkaloid components of the extract might be exerting these effects via blockade of calcium ion channels, speculating that reticuline, an alkaloid found in graviola leaves [129], might be contributing to these effects [12].

## 13. Antimicrobial and Antiparasitic Potentials

Graviola parts have been used as antimicrobial and antiparasitic agents in traditional medicine. Examples are provided according to the year published, except for antimalarials which will be discussed at the end of this section.

Bories et al. studied methanol extracts of two species of *Annona*, including muricata (graviola) and cherimolia, for their ability to inhibit growth of the parasites *Entamoeba histolytica*, *Trichomonas vaginalis*, *Nippostrongylus brasiliensis*, *Molinema dessetae*, and *Artemia salina* [130]. Results were expressed in terms of minimum inhibitory concentration (MIC), compared to reference compounds metronidazole (for *E. histolytica*) and ivermectin (for *N. brasiliensis* and *M. dessetae*). The results with crude extracts showed that (a) the *A. cherimolia* extract performed better overall in these tests and (b) both extracts showed only weak activity against the protozoans *E. histolytica* and *T. vaginalis* but strongly inhibited the *M. dessetae* filaria larvae, although at doses still an order of magnitude or so higher than the reference ivermectin compound. Nevertheless, this last result prompted the authors to perform fractionation to identify compounds with effectiveness against *M. dessetae*, leading to the identification of seven acetogenins with filaricidal activity, four of which displayed $LD_{50}$ values equal to or lower than the ivermectin reference [130].

Osorio et al. compared 36 different extracts from six different plants, including six extracts from graviola, namely, hexane, ethyl acetate, and methanol extracts from stem and from leaves, for their effects on three *Leishmania* species, as well as on *Trypanosoma cruzi* and *Plasmodium falciparum* [131]. Briefly summarized, an ethyl acetate extract of graviola leaves and stems was found to have potent activity against *Leishmania*, but the relatively high toxicity displayed by the leaf extract in cultures of U-937 cells led the authors to conclude that the effectiveness against the *Leishmania* parasite likely reflected its toxicity against the host mammalian cells [131].

Vila-Nova et al. tested specific compounds from both graviola and *Platymiscium floribundum* for their effectiveness against three *Leishmania* species (*donovani*, *mexicana*, and *major*) [132]. The most potent leishmanicidal compound against all three species was the acetogenin annonacinone ($EC_{50}$ approximately 7 $\mu$g/mL), although corossolone and the coumarin scoparone (the latter compound from *P. floribundum*) also displayed moderate activity, suggesting that these compounds merit further development for treatment of leishmaniasis [132].

Mathew et al. examined aqueous extracts of leaves from graviola and *Simarouba glauca* on the dental root canal pathogen *Enterococcus faecalis* [133]. These authors reported that the graviola extract, but not the *S. glauca* extract, was as effective against *E. faecalis* in vitro as the sodium hypochlorite (1% bleach) reference disinfectant, providing a possible alternative for root canal irrigation.

Mithun Pai et al. similarly tested an aqueous extract of graviola leaves against the oral pathogens *Streptococcus mutans*, *Streptococcus mitis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Candida albicans* using an agar disc method [66]. Their results showed that (a) graviola displayed effectiveness against all organisms except for *P. intermedia*, with the highest dose being the most effective and a trend toward dose dependence, and (b) compared to the "gold standard controls" of ciprofloxacin for the bacteria and fluconazole for the *Candida* yeast, the graviola extracts were far less effective, but may warrant further testing especially in view of (to paraphrase the authors) their combined antimicrobial and (reputed) anticancer effects [66].

Simo et al. prepared ethanol extracts of leaves, stems, and roots from several plants belonging to the Annonaceae family, including graviola, and tested them for phenolic and flavonoid content, as well as antioxidant and antifungal activities, using various strains of *Candida* yeast and *Cryptococcus neoformans* [134]. Briefly summarizing the results, these authors showed that (a) all three extracts from graviola possessed free radical-scavenging activity (root being highest) and (b) all three compounds showed "moderate" antifungal activity (approximately two orders of magnitude less potent on a mg/mL basis as compared with a nystatin control).

Several studies have investigated the ability of extracts from plants, including graviola, to treat malaria caused by *Plasmodium falciparum*, with particular interest in chloroquine-resistant strains of this organism. Menan et al. prepared aqueous, ethanol, and pentane extracts of 18 plants used in traditional medicine, including graviola leaves, and tested these in culture against two African strains of *P. falciparum*, one sensitive to chloroquine and the other resistant to the drug, to determine $IC_{50}$ values (defined as concentrations required to inhibit *P. falciparum* growth in human blood cell culture by 50%) [36]. These authors also tested cytotoxicity of each extract on A375 melanoma cells, using incorporation of radiolabeled hypoxanthine as a measure of cell growth. Their results showed that (a) $IC_{50}$ values in most cases were highest for the pentane extracts, with the graviola pentane extract showing not only "excellent" antiplasmodial activity but also a favorable ratio of antiplasmodial to cytotoxicity activity (ratio greater than 10), (b) activity against the chloroquine-resistant strain was just as good, if not better, than activity against the chloroquine-sensitive strain, and (c) in contrast to the antiplasmodial activities, the cytotoxic effects were greatest with the ethanol extract (6–7 fold lower $IC_{50}$ compared with the pentane extract; the aqueous extract was largely ineffective in this assay). It should be noted that extracts from *Uvaria afzelii* and *Cola caricaefolia* also displayed significant antiplasmodial activities [36].

Mohd Abd Razak et al. tested the antiplasmodial activity of 51 extracts from 14 medicinal plant species using an ELISA assay for *P. falciparum* histidine-rich protein; cytotoxicity was evaluated in Madin-Darby bovine kidney (MDBK) cells using an MTT assay [135]. These authors reported that 11 of these extracts possessed antiplasmodial activities with "negligible" toxicity (ratio of antiplasmodial to cytotoxicity activity greater than 10). Specific findings for graviola were that all three leaf extracts tested (aqueous, methanol, and dichloromethane) displayed "promising" FC50 values of between 0.1 and 1 μg/ml combined with low toxicity to MDBK cells, with a ratio of antiplasmodial to cytotoxicity activity of >750 for the aqueous extract [135].

Somsak et al. also tested an aqueous leaf extract from graviola for its antiplasmodial activity and acute toxicity using an in vivo, *Plasmodium berghei*-infected mouse model [13]. To test for antimalarial action, mice were injected ip with parasitized erythrocytes, followed by four consecutive days of oral treatment with 100–1000 mg/kg of extract, using chloroquine as a positive control. The results indicated a significant and dose-dependent inhibition of parasitemia (up to over 85% at the highest dose), along with prolonged survival from 7 days in untreated mice up to almost 29 days in mice treated with the highest dose, almost equaling the effectiveness of chloroquine (99% inhibition of parasitemia and 30 day survival). Toxicity studies showed no mortality doses up to 4000 mg/kg. The authors concluded that graviola extracts could potentially be the basis for the development of safe, effective, and affordable antimalarial agents [13].

Yamthe et al. tested a series of extractions and subfractions from both *A. muricata* (graviola) and the related *A. reticulata* for their activity against *P. falciparum* (strain W2 in culture) as well as cytotoxicity against human erythrocytes and foreskin fibroblasts [136]. Whereas all extracts showed low toxicity and some ability to inhibit growth of *P. falciparum*, thus supporting traditional use of these plants against malaria, the most potent extract was found by these authors to be a column chromatography subfraction from a methylene chloride stem bark extract of graviola, which displayed and $IC_{50}$ of 70 ng/mL, with a ratio of antiplasmodial to cytotoxicity activity of >140. The authors planned to continue characterizing active subfractions and individual compounds to improve antiplasmodial activity and minimize cytotoxicity [136].

## 14. Toxicological and Safety Information

As briefly discussed above, several studies have included cytotoxic assays and other means of assessing toxicity in their studies of graviola extracts. The following comments are intended to provide perspective into the possibility of toxicity in the use of graviola components. Caparros-Lefebvre and Elbaz in 1999 reported that consumption of teas and fruits of some tropical plants, including graviola, was associated with atypical parkinsonism, leading to speculation that graviola might contain neurotoxins reviewed by Gavamukulya et al. [120]. No safety studies have been found to assess the efficacy of the extracts of *A. muricata* on the various cancers. A study discussed the possible connection between tropical

fruit intake and the occurrence of atypical parkinsonism in the French West Indies [4, 120]. Another study in Guadeloupe Island publicized an association between intake of AGEs and the endemicity of a neurodegenerative disease [137], suggesting that AGEs are environmental neurotoxins accountable for neurodegenerative disorders. One of several follow-up studies focused on one of the AGE compounds, namely, annonacin, in causing tau-related neuropathology [137]. However, a consensus was reached in 2010 that consumption of species of Annonaceae was not directly related to occurrence of atypical parkinsonism (reviewed in [4, 120]). Other toxicologic findings discussed above in relation to the individual studies nevertheless merit serious consideration such that future studies of the use of graviola components must include rigorous safety testing since the content of potential toxins could vary according to the part of the plant, the extraction method, the location where the plant is grown, and even the time of harvest. Employing mesencephalic dopaminergic neurons, rat striatal neuronal cells, and laboratory rats, the neurotoxicity of seven acetogenins was evaluated, and the most abundant acetogenin (annonacin) and alkaloid (reticuline) from *A. muricata* were demonstrated to be neurotoxic [4]. Annonacin is a thousand times (1000x) more toxic to cultured neuronal cells than reticuline and a hundred times (100x) more potent than 1-methyl-4-phenylpyridinium, a known neurotoxin that effects parkinsonism in humans and animal models. Intravenous administration of isolated annonacin to laboratory rats was determined to estimate the amount of annonacin a human should consume via the ingestion of fruit daily for one year. In this regard, AVIS (l'Agence Francaise de Sécurité des Aliments) issued a conclusive statement that based on available data, it is not possible to link atypical parkinsonian syndrome cases identified in Guadeloupe to consumption of plan species belonging to the Annonaceae family (reviewed in [4]).

## 15. Conclusion and Future Prospects

Despite enhanced synthetic small molecule-based targeted anticancer therapies with improved patient prognosis, cancer remains a leading cause of death worldwide, as a result of challenges including increased toxicity and development of resistance to treatment agents. Natural products found in medicinal plants have great promise for the treatment of cancer [71]. This current review demonstrates *Annona muricata*'s anticancer potential and other health-related benefits by providing insights into its bioactive chemical constituents as well as the in vitro and in vivo studies that have been carried out in order to elucidate the molecular mechanisms of action of these constituents. Graviola not only is a sought-after tropical tree plant as an important foundation for the food industry and alternative traditional medicine but is also endowed with a wealth of phytochemicals with a wide variety of biological activities including its most prominent anticancer, antioxidant, and other properties not limited to those discussed herein.

Acetogenins and other secondary metabolites, including alkaloids, of this plant possess demonstrable ability to decrease growth of cancer that could be further comprehensively exploited. Acetogenins or other *A. muricata*-derived compounds could be tested as monotherapy or as sensitizers in combination with standard cancer treatments for cancer patients. Numerous studies have reported anticancer actions of *A. muricata*. However, more rigorous evaluation of various plant parts, their extracts, and ultimately isolated bioactive compounds is clearly warranted. Indeed, the most effective bioactive compounds from *A. muricata* could potentially serve as scaffold entities for design and synthesis of derivatives that may even be more efficacious in preclinical and clinical trials. Whereas earlier studies characterized the biological activities of extracts from different graviola organs, future studies pivotal for the development of pharmaceutical and agricultural product development should focus on investigating on the biochemical and physiological functions of active phytochemical compounds and their combinations, as well as the detailed molecular mechanisms causal to these activities, progressing from in vitro studies to in vivo rodent models and ultimately to clinical trials to assess safety as well as therapeutic efficacy of the most promising graviola components.

There is already one clinical case report which describes a 66-year-old female who was diagnosed with metastatic breast cancer and whose metastases had progressed even after multiple rounds of chemotherapy including anthracyclines and taxanes. This patient self-medicated by boiling 10–12 dry leaves of *A. muricata* in water for 5–7 minutes, then orally consumed an 8 oz daily dose of this aqueous extract. Her metastases remained stable for 5 years on graviola (together with Xeloda) [118]. This report, although anecdotal, hints of the untapped antimetastatic potential of *A. muricata* and its components.

In a rodent in vivo study, graviola leaf extract inhibited 59.8% of pancreatic cancer growth of cells and their metastasis induced by CD18/HPAF cells in a mouse model [28].

In order to exploit the full medicinal potential of *A. muricata*, existing gaps in our knowledge of annonaceous acetogenins and other bioactive compounds should be addressed. Currently, there are no target-based approaches for evaluating components derived from *A. muricata* in cancer therapy. With the rapidly expanding knowledge of the pathways and networks that control cell signaling, proliferation, metastasis, and cell death, the possibility of using these components in a targeted fashion is very appealing in order to expand our anticancer armamentarium. Numerous in vitro and preclinical in vivo studies have supported most of the traditionally acclaimed benefits, but these must be validated in human clinical trials. The more than 200 phytochemicals identified in graviola mainly including acetogenins, alkaloids, and phenols have shown a multitude of pharmacological activities as discussed herein, and it is hoped that future studies will identify novel phytochemical scaffolding entities which are yet to be identified from *A. muricata*.

While most have provided health benefits, some of the derived phytochemicals like acetogenins have demonstrated in vitro and in vivo neurotoxicity. Although current consumption does necessarily lead to acute toxicity, further research to identify and quantify the amount of toxic

phytochemicals as well as determine the doses to be exposed to humans to induce toxicity is urgently needed. For future directions and safety concerns in developing these plant aerial parts and their constituents, current studies indicate that phytochemical composition and anticancer properties vary with the geographical sources of *A. muricata* [38]. Because consistent results are difficult to achieve since processing and formulation may vary from the desired biological activity, there is therefore the need for systematic and robust screening to identify biochemical fractions as well as the physiological effects of all isolated constituents by detailing the investigation of underlying mechanisms. More importantly, for more safety, the toxicological profile is required to be documented [137, 138]. Another area previously neglected that needs to be intensely focused on is clinical trials concerning the rich pharmaceutical potential of *A. muricata*.

In conclusion, the authors intended this review to be a call to action for the development of better graviola-based pharmaceutical, agricultural, and food industrial agents, for the human diseases and conditions discussed herein, and possibly for other conditions for which graviola components have not even been tested. Given that graviola is already widely used in traditional medicine, these agents, if properly tested and produced, could potentially represent huge benefit by providing accessible and affordable agents against many of the conditions that plague humankind.

## Disclosure

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## Conflicts of Interest

The authors declare that they have no conflict of interests.

## Authors' Contributions

These authors, Islam Rady, Melissa B. Bloch, and Roxane-Cherille N. Chamcheu contributed equally to this work. Islam Rady gathered the information and wrote the initial draft of the manuscript. Melissa B. Bloch, Roxane-Cherille N. Chamcheu, Sergette Banang Mbeumi, and Md Rafi Anwar gathered and contributed new information, added figures and tables, and restructured the first draft of the manuscript. Jean Christopher Chamcheu revised it for intellectual content and coordinated the study, as well as all other authors. All authors edited and reviewed the different versions of the manuscript and approved the final version for submission.

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*Research Article*

# Immunomodulatory Efficacy of Standardized *Annona muricata* (Graviola) Leaf Extract via Activation of Mitogen-Activated Protein Kinase Pathways in RAW 264.7 Macrophages

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*Annona muricata*, commonly known as Graviola, has been utilized as a traditional medicine to treat various human diseases. The aim of this study was to examine the immune-enhancing activity of Graviola leaf extracts in RAW 264.7 macrophage cells. Active ingredients in Graviola leaf extracts (GE) were identified as kaempferol-3-O-rutinoside and quercetin-3-O-rutinoside by LC-MS/MS. When treated with steam or 50% ethanol GE, cell morphology was altered due to initiation of cell differentiation. While the cell viability was not altered by the steam GE, it was reduced by the ethanol GE. Both steam and ethanol GE induced the transcriptional expression of cytokines, including tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-1$\beta$, but only the steam extract upregulated inducible nitric oxide synthase (iNOS). In consistence with mRNA expression, the production of TNF-$\alpha$ and nitrite was elevated by both steam and ethanol extracts of Graviola leaves. This is mainly due to activation of mitogen-activated protein (MAP) kinase signaling pathways. These results suggest that Graviola leaves enhance immunity by activation of the MAP kinase pathways. These bioactive properties of Graviola indicate its potential as a health-promoting ingredient to boost the immune system.

## 1. Introduction

Medicinal natural products have been traditionally applied for treatment of diseases and their active constituents have been studied for elucidation of their mechanisms of action [1, 2]. *Annona muricata* L. is a medicinal plant, commonly known as Graviola, Soursop, or Guabana. Graviola is a tropical evergreen fruiting tree, which belongs to the Annonaceae family [3, 4]. All parts of the Graviola tree are known to be effective against various human diseases such as cancer and parasitic infections. In particular, Graviola leaves were found to be effective against cystitis, diabetes, headache, insomnia, and inflammation [5, 6]. In South America and Africa, Graviola leaves have been applied as an ethnomedicine to treat cancer. Besides its medicinal uses, Graviola fruits are used for development of various food products including beverages, candy, and syrups [7].

These traditional pharmacological uses of Graviola have evoked an interest for further elucidation of its effective components. Among various constituents in Graviola, acetogenins have been applied for cancer therapy. Annonaceous acetogenins are composed of 35- or 37-carbon long-chain fatty acids that form the polyketide skeleton. These acetogenins, derived from seeds, leaves, and pericarp have cytotoxicity against carcinoma cells of prostate, pancreas, and breast [6, 8, 9]. Despite their anticancer efficacy, some acetogenins in Graviola leaves are known to be highly cytotoxic [10]. In addition, chronic consumption of Graviola juice containing annonaceous acetogenins aggravates cerebral tau phosphorylation in mice and contributes to development of neuropathological changes observed in Parkinson's disease [11, 12]. Another important attribute about Graviola is its high content of antioxidant compounds. Phenolic acids and flavonoid glycosides in Graviola leaves are known to have

potent antioxidant activities [13]. Recently, we have reported that the total phenolic acid and total flavonoid contents were present in the steam and ethanol extracts of Graviola leaves in high concentrations, which is associated with their free radical-scavenging effects [14]. Additionally, the ethanol extract of Graviola leaves was found to upregulate the expression of superoxide dismutase-1 (SOD-1) and nuclear factor erythroid-2-related factor-2 (Nrf-2), which contributed to its reactive oxygen species-reducing activity.

The adaptive immune system is a defense mechanism to prevent the invasion by microorganisms or exogenous materials. Natural products, derived mainly from plants have been widely studied in immune-related diseases [15]. Stimulation of the immune system is particularly important in immunocompromised patients, such as those after surgery and HIV-infected and senile people, to prevent the occurrence of secondary infections. Besides its use as supplemental food, plant-derived products are being consumed as herbal medicines to prevent progression of chronic diseases including cancer and infections.

These previous reports prompted us to question whether the Graviola leaf extracts (GE) modulate the immune response aroused by inflammatory mediators. The findings that oral intake of ethanol GE significantly reduced carrageenan-induced edema in rat paws support our hypothesis [16]. However, the effects of GE on modulation of immune responses induced by inflammatory mediators have not been elucidated. In this study, we examined the role of GE in macrophage activation and regulation of cytokines production.

## 2. Materials and Methods

*2.1. Preparation of Standardized Graviola Leaf Extract.* Graviola leaves, grown in the Philippines, were obtained from Graviola and Nature Company (Korea). For the steam extract, air-dried Graviola leaves (92.5 g) were heated at 121°C for 6 h and then indirectly heated using boiled water (100°C) for 1 h. They were collected and condensed to powder by a rotary vacuum evaporator at 55°C at a speed of 70 rpm. The final extract powder was kept at −20°C until use for analysis. For the 50% ethanol extract, air-dried Graviola leaves (63.7 g) were blended with ethanol/distilled water (1 : 1, v/v) and then kept in a shaking water bath (BF-30SB; Biotree Co. Ltd., Seoul, Korea) at 55°C at a speed of 70 rpm for 24 h. It was filtered through an 8.0 $\mu$m filter paper (Whatman Inc., Piscataway, NJ, USA) and concentrated using the same method used for the steam extract. It was kept at −80°C for 8 h and then dried in a freeze-drier for 5 days. It was then stored at −20°C until use for analysis. Bioactive components in standardized GE were analyzed by LC-MS/MS as reported previously [14].

*2.2. Chemical Fingerprint Analysis of Graviola Leaf Extract by Using UPLC/ESI-MS Methods.* Bioactive components in the extracts were analyzed by using an ultra-performance liquid chromatography/electrospray ionization-mass spectrometry (UPLC/ESI-MS) coupled with Accela photo diode array (PDA) detector, Accela auto sampler, and Accela pump (Thermo Fisher Scientific, Waltham, MA, USA). The analytes were separated on a Zorbax Eclipse XDB-C18 (250 mm × 4.6 mm; 5 $\mu$m, Agilent technologies) column. Detailed analytical conditions were used as described in a previous study [14]. In detail, the mobile phases used were solvent A (1% formic acid in water, HPLC grade, Sigma-Aldrich) and solvent B (acetonitrile, HPLC grade, Sigma-Aldrich) with gradient elution as follows: 95–5% A at 0–60 min. The analysis was carried out at a flow rate of 1.0 mL/min. UV wavelength was measured at 340 nm. ESI-MS spectra were acquired in positive ion mode. For the full-scan MS analysis, the spectra were recorded in the range of mass to charge ration ($m/z$) 100–800. The source parameters were as follows: electrospray capillary voltage = 30 V, capillary temperature = 200°C, sheath gas flow = 30 arb, and auxiliary gas flow = 2.0 arb. The stability of extracts was evaluated using other three samples.

*2.3. Cell Culture and Cell Viability Assay.* RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin under 5% $CO_2$-humidified atmosphere at 37°C. Cell viability was measured with the help of a cell proliferation assay kit (Welgene Inc., Korea), using 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT).

*2.4. RNA Isolation and Real-Time RT-PCR.* RAW 264.7 cells were treated with 0, 50, 100, and 200 $\mu$g/mL GE for 8 h after $8 \times 10^5$ cells/well were seeded into a 6-well plate and incubated for 24 h. Total RNA was isolated from RAW 264.7 cells using RNA Extraction Kit (Intron Biotechnology Inc., Korea) according to the manufacturer's instructions. cDNA was synthesized in a PCR Thermal Cycler (TaKaRa, Japan), using the iScript cDNA synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). PCR conditions were 25°C for 5 min, 42°C for 30 min, and 8°C for 5 min and then kept at 4°C for maintenance. Real-time PCR was performed in Step-One Plus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA), using primers and SYBR Green Master Mix (TaKaRa). Real-time PCR conditions were initially maintained at 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec, 72°C for 45 sec, 95°C for 15 sec, and 60°C for 1 min. The sequences of the primers used in this study were as follows: IL-1$\beta$, forward-ATG GCA ACT GTT CCT GAA CTC AAC T, reverse-GTG CTG CCT AAT GTC CCC TTG AAT C; IL-6, forward-AGT TGC CTT CTT GGG ACT GA, reverse-CAG AAT TGC CAT TGC ACA AC, COX-2, forward-CCC CCA CAG TGA AAG ACA CT, reverse-CTC ATC ACC CGA CTC AGG AT; iNOS, forward-AAT GGC AAC ATC AGG TCG GCC ATC ACT, reverse-GCT GTG TGT CAC AGA AGT CTC GAA CTC; TNF$\alpha$, forward-ACA AGC CTG TAG CCC ACG, reverse-TCC AAA GTA GAC CTG CCG; $\beta$ actin, forward-GAC GTT GAC ATC CGT AAA G, reverse-CAG TAA CAG TCC GCC TT. Expression of the genes was normalized to that of $\beta$-actin.

*2.5. Western Blot Analysis.* RAW 264.7 cells were treated with various concentrations of GE for 30 min after $8 \times 10^5$ cells/well were seeded into a 6-well plate and incubated for 24 h. Cells were homogenized in a cell lysis buffer containing 10 mM Tris (pH 7.8), 1 mM EDTA, 150 mM NaCl, phosphatase inhibitor, and protease inhibitor. The lysates were then centrifuged to remove cell debris and unbroken cells. Proteins were quantified using Protein Assay kit (Bio-Rad Laboratories) and the absorbance was measured at 750 nm. Thirty micrograms of the assayed protein was separated by using 10% acrylamide gel and then the proteins in the gel were transported to a PDVF membrane. The protein-specific primary antibody was attached and the mouse-specific or rabbit-specific secondary antibodies were then added. Proteins of interest were visualized by Enhanced Chemiluminescence kit (Bio-Rad Laboratories) using a Chemiluminescence imaging equipment (Vilber Lourmat, France). Primary antibodies against pJNK, JNK, pERK1/2, ERK1/2, pp38, and p38 (Cell Signaling Technology, Danvers, MA, USA) and $\alpha$-tubulin (Sigma-Aldrich, St. Louis, MO, USA) were used.

*2.6. Nitrite Quantification Assay.* RAW 264.7 cells were treated with various concentrations of GE for 24 h after $8 \times 10^5$ cells/well were seeded into a 6-well plate and incubated for 24 h. Cells were also treated with 1 $\mu$g/mL lipopolysaccaride (LPS). Culture media, isolated, and the concentration of nitrite, an indicator of nitric oxide (NO) synthesis, were measured by using Griess reagent (1% sulfanilic acid in 5% phosphoric acid and 0.1% *N*-ethylenediaminedihydrochloride; Molecular Probes, Waltham, MA, USA). The supernatant of the treated cell culture media (150 $\mu$L) was mixed with Griess reagent (20 $\mu$L) and deionized water (130 $\mu$L) in a 96-well plate and incubated for 30 min. The absorbance was then measured at 548 nm in a microplate reader. NO concentration was determined using a dilution of sodium nitrite as a standard.

*2.7. Measurement of Cytokine (IL-1$\beta$ and TNF-$\alpha$) Levels.* RAW 264.7 cells were treated with various concentration of GE for 24 h after $8 \times 10^5$ cells/well were seeded into a 6-well plate and incubated for 24 h. LPS (1 $\mu$g/mL) served as a positive control. The concentration of TNF-$\alpha$ and IL-1$\beta$ was measured using ELISA kit (Enzo Life Sciences, Ann Arbor, MI, USA). Samples were put in a microtiter plate to which the monoclonal antibody was attached and then shaken at a speed of 500 rpm for 2 h. Polyclonal antibody was added to the wells after four washing cycles with the washing buffer. The conjugate solution was added to each well followed by a washing cycle. After 30 min, the stop solution was added and the absorbance was measured at 450 nm using a microplate reader (Infinite; Tecan, Switzerland). Cytokines concentration was determined using a dilution of kit's standard solution.

*2.8. Statistical Analysis.* All results are expressed as mean ± the standard error of mean (SEM). At least three independent experiments were performed. Results were analyzed by using Student's $t$-test and $p$ value less than 0.05 was considered statistically significant.

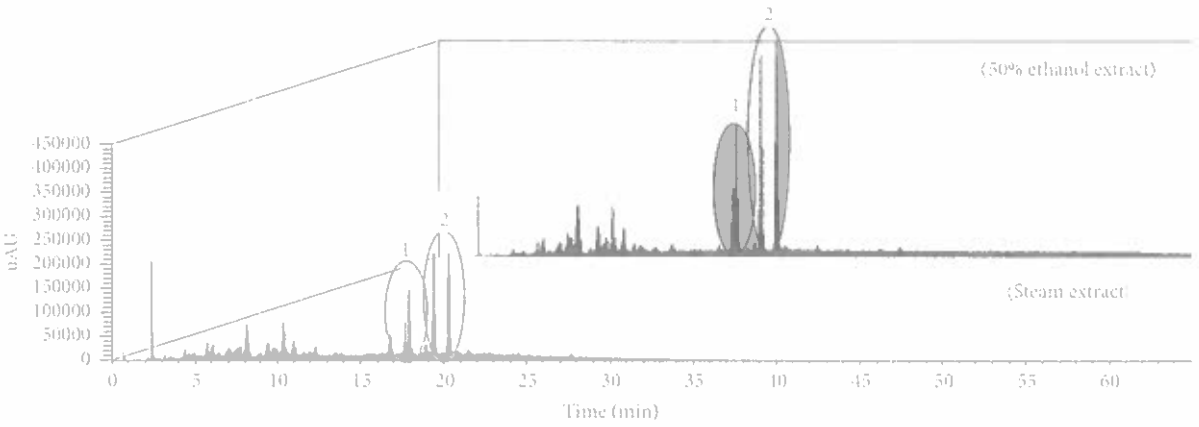
## 3. Results

*3.1. Standardized Graviola Leaf Extract (GE) Containing Flavonols.* To find out the major bioactive compounds in GE, we analyzed the components of the steam and 50% ethanol GE by UPLC/ESI-MS. As shown in the chromatograms (Figure 1(a)), two major peaks in standardized GE were eluted at retention time of 17.92 (peak 1) and 19.43 min (peak 2). Chemical fingerprints analyzed by ESI-MS with positive ion mode identified 610.93 and 594.96 $m/z$ for peak 1 and peak 2, respectively (Figure 1(b)). Based on the structural identity, the standardized GE contains two components of flavonols such as quercetin-3-O-rutinoside and kaempferol-3-O-rutinoside (Figure 1(c)). Quercetin-3-O-rutinoside has disaccharide binding with hydroxyl group of quercetin and its molecular weight is 610.52 g/mole. Another major flavonol identified in GE was kaempferol-3-O-rutinoside which forms a glycosidic bond with quercetin-3-O-rutinoside and its molecular weight is 594.52 g/mole.

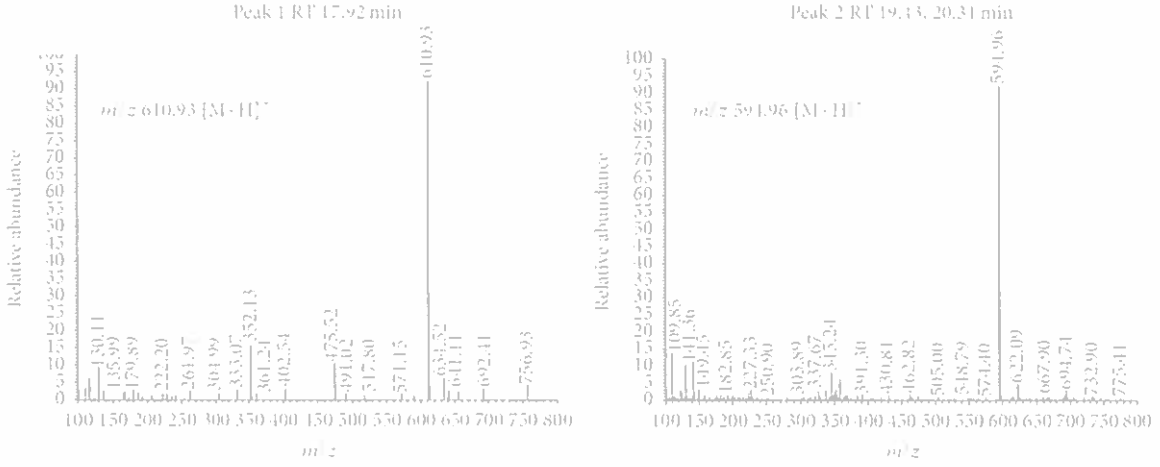
The two bioactive components in GE were evaluated with three duplicates extract batch during intraday in order to confirm the accuracy of amount of bioactive components in the extract by LC-MS/SM. The value of quercetin-3-O-rutinoside was 614.42, 608.38, and 623.61 $\mu$g per g dry weight in the steaming GE. Their Relative Standard Deviation (RSD) of accuracy was 1.02, 1.03, and 1.00%, indicating that they are within 5% of RSD range. The amount of kaempferol-3-O-rutinoside was 690.57, 706.97, and 703.83 $\mu$g per g dry weight that were calculated into 1.83, 1.78, and 1.79% of RSD, respectively. In contrast, the value of quercetin-3-O-rutinoside was 1428.82, 1431.5, and 1475.06 $\mu$g per g dry weight in the 50% ethanol GE. Their Relative Standard Deviation (RSD) of accuracy was 1.48, 1.48, and 1.44%. The amount of kaempferol-3-O-rutinoside was 1658.27, 1720.96, and 1686.32 $\mu$g per g dry weight, which were calculated into 1.55, 1.49, and 1.52% of RSD, respectively. The results from the current study confirmed that the steaming and 50% ethanol GE containing two flavonols were standardized. They were used for further study on evaluating immunomodulatory efficacy.

*3.2. GE Alters Macrophage Morphology and Induces Differentiation.* We questioned whether GE activates macrophage differentiation. When macrophages are exposed to endotoxins, such as LPS, a morphological change occurs, which is the first step to activate an inflammatory response [17]. RAW 264.7 macrophage cells were incubated with various concentrations of the steam or ethanol GE, respectively, and cell morphology was observed (Figure 2). When cells were cultured in an empty medium, most of the cells displayed a circular morphology and normal proliferation. However, when cells were treated with LPS, the cell morphology was changed to an extended form and proliferation was suppressed. In the presence of the steam or ethanol GE, the cell morphology was significantly altered in a similar manner as LPS-treated cells (arrows in Figures 2(a) and 2(b)).

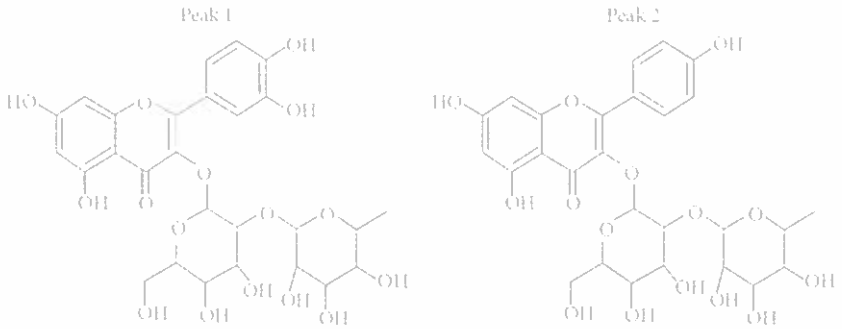
Moreover, we investigated whether GE affects the cell viability to determine its cytotoxicity. RAW 264.7 cells were incubated with the steam or 50% ethanol GE for 24 and




(a) Ultra-performance liquid chromatogram: UV spectrum




(b) ESI-MS$^1$ fingerprint




(c)

Figure 1: Identification of major components in the steam and 50% ethanol GE. GE were analyzed by UPLC/ESI-MS and the absorbance was measured at 340 nm (a). Major peaks were fingerprinted by identifying major $m/z$ (b) and found to be quercetin-3-O-rutinoside and kaempferol-3-O-rutinoside (c).

18 h, respectively. The steam GE did not affect the cell viability but rather increased cell proliferation after 24 h of incubation (Figure 3(a)) and the cell viability remained the same after 48 h incubation (Figure 3(b)). In contrast, the 50% ethanol GE decreased the cell viability after both 24 and 48 h incubation in a dose- and time-dependent manner (Figures 3(a) and 3(b)). These results suggest that both the steam and 50% ethanol GE induce macrophage activation but only the steaming GE does not have any cytotoxic effect on RAW 264.7 cells.

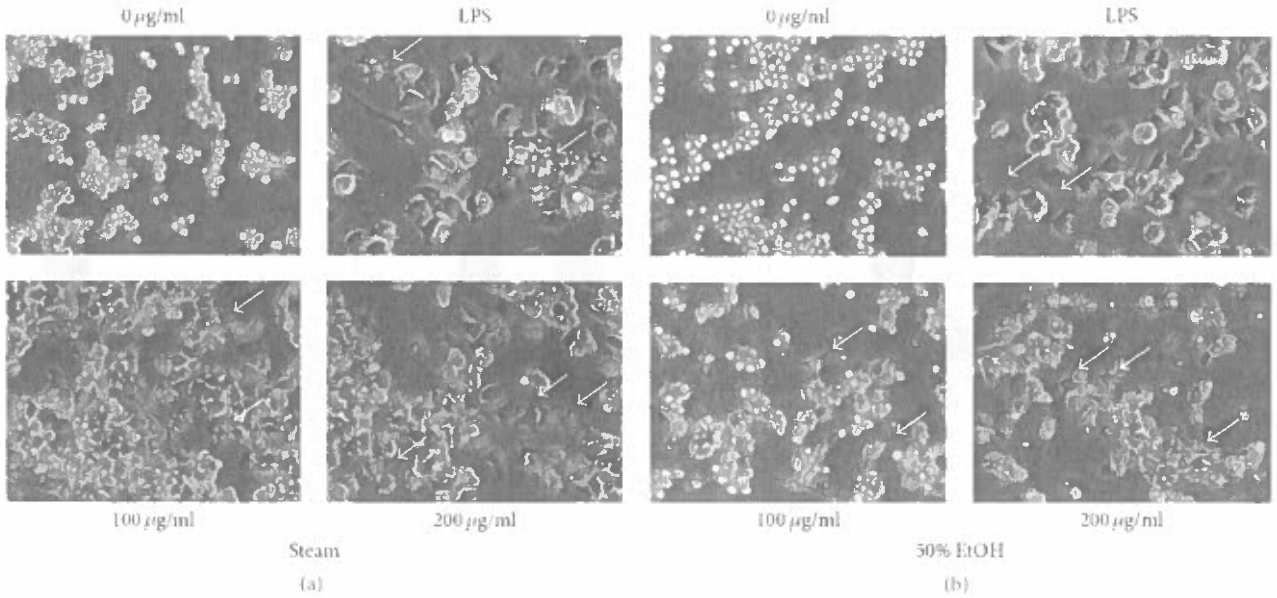



FIGURE 2: Altered morphology of RAW 264.7 cells after GE treatment. Cells were treated with the steam (a) or 50% ethanol (b) GE for 24 h at various concentrations.

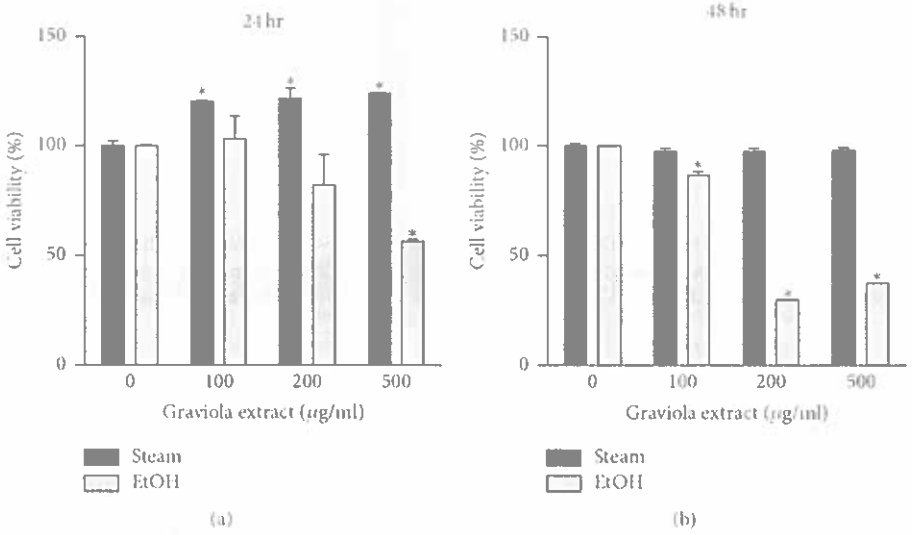



FIGURE 3: Cytotoxicity of GE in RAW 264.7 cells. Cells were treated with steam or 50% ethanol GE at various concentrations for 24 h (a) or 48 h (b). Cell viability was measured by XTT assay after incubation. The results were expressed as a percentage relative to the vehicle-treated control. The data were expressed as mean ± SEM. $^*p < 0.05$ versus vehicle-treated control.

*3.3. GE Activates Production of Cytokines and NO.* Our findings that GE induced macrophage activation led us to investigate its effect on the expression of cytokines and inducible nitric oxide synthase (iNOS). When the macrophages encounter infectious bacteria or endotoxins, their morphology is altered (Figure 2) and production of cytokines is activated due to activation of inflammatory signaling pathways. NO is synthesized in macrophages via the reaction converting l-arginine to l-citruline by iNOS [18]. NO is involved in a defense mechanism against cytosolic microorganisms and acts as a signaling molecule to mediate vasoconstriction [19]. We treated RAW 264.7 cells with various concentrations of GE and measured the mRNA expression of TNF-$\alpha$, IL-1$\beta$, and iNOS by real-time PCR. The steam GE upregulated the transcriptional expression of TNF-$\alpha$, IL-1$\beta$, and iNOS (Figure 4(a)). In addition, the 50% ethanol GE upregulated IL-1$\beta$ and iNOS but only a slight increase occurred in TNF-$\alpha$ which was statistically not significant (Figure 4(b)). However, the steam GE upregulated cytokines and iNOS expression to a lesser degree compared to LPS. To verify this, RAW 264.7 cells were treated with GE and the culture media were collected to measure cytokine concentrations. In consistence

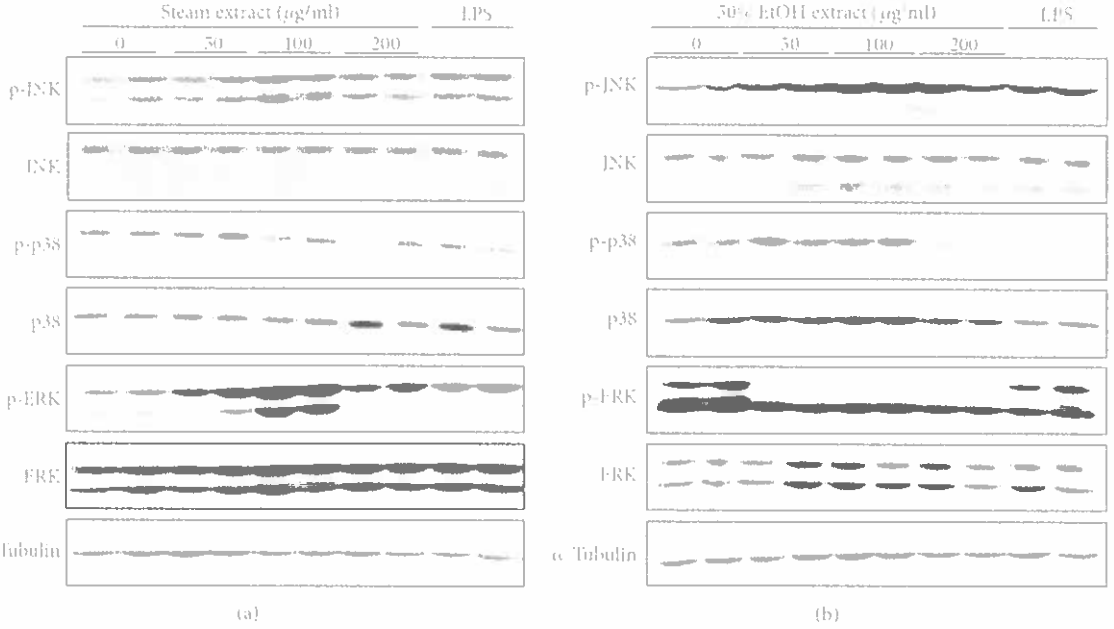



Figure 7: RAW 264.7 cells were treated with either steam (a) or 50% ethanol (b) extract at various concentrations for 30 min. Cells were harvested and protein extracts were analyzed by immunoblotting analyses.

[14]. In addition, Graviola reduced the production of reactive oxygen species (ROS).

The findings that GE has high contents of phenolic acids and flavonoids prompted us to investigate its probable proinflammatory effects on macrophage cells. Ishola et al. reported that Graviola fruit reduced carrageenan-induced rat paw edema and xylene-induced inflammatory edema [28]. Surprisingly, GE treatment of RAW 264.7 cells led to macrophage activation and altered morphology. As a result, transcriptional upregulation of inflammatory genes and increased production of cytokines and NO occurred. These conflicting results were probably due to endotoxin-like components in GE and TLR4-mediated activation of innate immune signaling pathways. It is worth noticing that the degree of upregulation and increased cytokine production was less than those induced by LPS. The reason for this is not clear but probably other components in GE may have an antagonizing anti-inflammatory effect against TLR-4-activating components, which can explain the lower efficacy of GE when compared to that of LPS. The major component in GE that induces macrophage activation and activates innate immune responses requires further study and the underlying mechanism should be elucidated.

When an exogenous microorganism invades a vertebrate host, the innate immune system is the first defense line against pathogens. This defense mechanism is mainly mediated by macrophages and dendritic cells. TLR4 on the surface of mammalian cells binds to the antigen on the invading microorganism, which initiates a signaling cascade to activate the immune system. In addition, phosphorylation of MAP kinases including JNK, ERK, and p38 induces innate immune response. We found that both the steam and 50% ethanol GE increased phosphorylation of JNK and p38. While the steam GE increased phosphorylation of p44 and p42 (ERK1/2), the 50% ethanol GE completely blocked the phosphorylation of p44 (ERK1). This finding indicates that regulation of ERK is differently modulated by the steam and 50% ethanol GE. In particular, the cytotoxic effect of the ethanol GE on RAW 264.7 cells (Figure 2) is indicative of this different mode of action. Interestingly, the degree of MAP kinase phosphorylation by GE is higher than those of LPS. This implies that GE is more effective in activating the innate immune signaling pathways than LPS but the interacting components may reduce this effect.

## 5. Conclusion

In conclusion, our findings suggest that GE can activate the innate immune system by inducing macrophage activation and production of cytokines and NO in mouse macrophage RAW 264.7 cells via activation of MAP kinases. These results indicate that GE has immunostimulatory potential and can be applied to boost the innate immune system in immunocompromised patients. In addition to its high contents of bioactive components and ROS-scavenging capacity, the immune-boosting ability of Graviola can be applied for the development of health-promoting functional foods.

## Abbreviations

SOD1: Superoxide dismutase-1
Nrf-2: Nuclear factor erythroid-2-related factor-2
GE: Graviola leaf extracts
XTT: 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
LPS: Lipopolysaccharide

NO: Nitric oxide
iNOS: Inducible nitric oxide synthase
TNF-$\alpha$: Tumor necrosis factor-$\alpha$
IL-1$\beta$: Interleukin-1$\beta$
MAPK: Mitogen-activated protein kinase
JNK: c-Jun N-terminal kinase
ERK: Extracellular signal-regulated kinase.

## Competing Interests

The authors declare no conflict of interests.

## Authors' Contributions

Goon-Tae Kim and Nguyen Khoi Song Tran contributed equally to this work.

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