Chinese Journal of Integrative Medicine

Available online at link.springer.com/journal/11655
Journal homepage: www.cjim.cn/zxyjhen/zxyjhen/ch/index.aspx
E-mail: cjim_en@cjim.cn

Review

Review on Antioxidants and Evaluation Procedures

Suresh Kumar[1,2], Sunil Sharma[1], and Neeru Vasudeva[1]

ABSTRACT Antioxidants are the substances that are capable of counteracting the damaging effects of the physiological process of oxidation occur in animal tissues. These may be nutrients (vitamins and minerals) as well as enzymes (proteins) in our body that assist in chemical reactions. They are believed to play a role in preventing the development of chronic diseases like cancer, diabetes, heart disease, stroke, Alzheimer's disease, rheumatoid arthritis, and cataracts etc. The present review article emphasizes on the various aspects of oxidants and antioxidants viz. definition, types, causes, mechanism, functions, adverse effects along with various *in vivo* and *in vitro* models of evaluation of antioxidant activity of new molecule, compounds or any plant or plant part. This review is one of its kinds which will be of great importance for researchers working in this area in search of antioxidant moieties and their biological evaluation.

KEYWORDS antioxidants, herbal antioxidants, *in vivo* antioxidant models, diphenyl-picryl-hydrazyl, vitamins

Oxygen in our body plays a very important role as most of the processes in our body take place in the presence of oxygen devoid of which life is not possible. Unfortunately, at the same time the life giving oxygen can create harmful side effects which cause cell damage and lead to chronic diseases like diabetes, cancer, arthritis, hypertension, obesity, inflammation etc. Oxidants or free radicals enter the body from various external sources such as pollution, intake of alcoholic beverages, unhealthy foods, cigarette, poor diet, and exposure to sun. Free radicals produced by this breakdown attack healthy cells, usually DNA as well as proteins and fats. This chain of events weakens immunological functions as well as speeding up the aging process, and is also linked to several diseases.[1]

Antioxidants, or anti-oxidation agents, reduce the effect of dangerous oxidants by binding together with these harmful molecules, decreasing their destructive power. These can also help in repairing the damage already sustained by cells.[2] Free radical is defined as any atom, group of atoms or molecules containing one unpaired electron within an outer orbit. Free radicals arise from sources both inside and outside our bodies. Based on the occurrence of oxidants these are classified into two ways i.e. endogenous oxidants and exogenous oxidants.[3] The oxidants that develop from processes within our bodies as a result of normal aerobic respiration, metabolism, and inflammation etc. are called as endogenous oxidants. Exogenous oxidants are the free radicals form from environmental factors such as pollution, sunlight, strenuous exercise, X-rays, smoking and alcohol.

An antioxidant is any substance or nutrient that when present at low concentration compared to that of an oxidizable substrate significantly delays or inhibits the oxidation of that substrate.[3] The physiological role of free radical and hydroxyl free radical-scavengers is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals. In recent years, a substantial body of evidence[4] has indicated a key role for free radicals as major contributors to aging and to degenerative diseases of aging, such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction. When the availability of antioxidants is limited, this damage can become cumulative and debilitating oxidative stress results. Antioxidants are capable of stabilizing, or deactivating free radicals before the latter attack cells and biological targets. They are therefore critical for maintaining optimal cellular and systemic health and well-being.[5] When our body cells use oxygen, they naturally produce free radicals (by-products) which can cause cell damage. Antioxidants act as "free radical scavengers" and

1. Department of Pharmaceutical Sciences, Guru Jambheshwar University of Science and Technology, Hisar (125001), India;
2. Laureate Institute of Pharmacy, Kathog, Distt. Kangra (177101), India
Correspondence to: Suresh Kumar, Tel: 91-9255577435, E-mail: pharm.vermasuresh@gmail.com
DOI: https://doi.org/10.1007/s11655-017-2414-z

hence prevent and repair damage done by these free radicals. They may also enhance immune defense and lower the risk of various disorders and infections.[6] Therefore, the use of antioxidants for the maintenance of health benefits, have yet to be identified. Thus the awareness and proper investigation regarding antioxidants and their evaluating procedures are the need of the hour in the management of various diseases.

Types of Antioxidants

Antioxidants can be classified by solubility and occurrence. According to the solubility, these can be divided into water soluble and fat soluble antioxidants. Based on the occurrence, they can be divided into natural antioxidants, synthetic antioxidants, antioxidants in body, and miscellaneous antioxidants.

Type Ⅰ Antioxidants Based on Solubility

Water Soluble Antioxidants

Water soluble antioxidants are also known as hydrophilic antioxidants. They react with the oxidants in the process of cell cytosol and the blood plasma. Some common water soluble antioxidants are ascorbic acid, glutathione, lipoic acid, and uric acid.

Ascorbic acid also known as vitamin C is a water-soluble vitamin. It scavenges free radicals that are in an aqueous (watery) environment, such as inside the cells. Vitamin C works synergistically with vitamin E to quench free radicals. Vitamin C also regenerates the reduced (stable) form of vitamin E. Vitamin C is an essential nutrient that is found in fruits and vegetables. Human beings can obtain ascorbic acid through diet. It is essential for maintaining blood vessels, bones and skin. It is required for the conversion of procollagen into collagen that is a protein and essential for maintaining healthy gums, cartilage, joints and blood vessels.[7]

Glutathione is a tripeptide that acts as a powerful antioxidant by virtue of which it maintains youthful skin and works against early sign of aging. It is produced by the liver. It is also considered as an immune booster.[7,8]

Lipoic acid or alpha-lipoic acid is a powerful antioxidant that is found in small amount in body cells. The source of lipoic acid is spinach, potatoes, carrots, red meat, etc. It reduces the effects of free radicals. It increases metabolism and found to treat nerve damage caused by radicals.[7]

Uric acid is an antioxidant that is found in higher concentration in blood. Uric acid is a metabolite of purine. The higher abundance of uric acid can cause gout.[7,8]

Fat Soluble Antioxidants

Fat soluble antioxidants play a key role in keeping healthy body. These antioxidants protect cell membrane from damage and also from lipid peroxidation. These antioxidants can be synthesized from the cells and tissues or it can be obtained from the food. The followings are some lipid soluble antioxidants are carotenes, ubiquinol, vitamin E.[8]

Vitamin A and beta-carotene are fat soluble antioxidants that are essential for bone formation, vision, and gastrointestinal health. It also promotes skin health and immune system. Carotene protect against the damage caused due to free radicals. Beta-carotene is the most widely studied of the 600 carotenoids identified till date. It is thought to be the best quencher of singlet oxygen (an energized but uncharged form of oxygen that is toxic to cells). Beta-carotene is also especially excellent at scavenging free radicals in low oxygen concentration.[8]

Ubiquinol is a potent antioxidant that is synthesized within the body. Studies[7,8] have found that ubiquinol protects from chronic disease and several heart related consequences. Ubiquinol is a form of coenzyme Q10, which is vitamin-like substance found in body cells and plays a key role in cell production and protect it from radical damage.[7,8]

Vitamin E is actually a generic term that refers to all entities (eight found so far) that exhibit biological activity of the isomer tocopherol. Alpha-tocopherol, the most widely available isomer, has the highest bio potency or strongest effect in the body. Because of its fat-solubility it is in a unique position to safeguard cell membranes largely composed of fatty acids from damage by free radicals. Alpha-tocopherol also protects the fats in low-density lipoproteins (LDLs, or the "bad" cholesterol) from oxidation.[7-9]

Type Ⅱ Antioxidants Based on Occurrence

Natural Antioxidants

Antioxidant agents are found in foods, such as

dark green leafy vegetables. Items high in vitamin A, vitamin C, vitamin E, and beta-carotene are believed to be the most beneficial. These nutrients are commonly found in fruits and vegetables, those with the strongest colours being healthiest. Orange and red peppers, tomatoes, spinach, and carrots are examples.[10]

Synthetic Antioxidants

Due to the inherent instability of natural antioxidants, several synthetic antioxidants have been used to stabilize fats and oils. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were originally developed to protect petroleum from oxidative gumming.[11] However, these compounds have been used as antioxidants in human foods since 1954[11] and are perhaps the most common antioxidants used in those foods today. BHT and BHA not only have similar names, but similar structures and antioxidant activity. Ethoxyquin (ETO) is another synthetic antioxidant which is widely used in the feed industry.[12,13] Like BHT and BHA, ETO has a benzene ring or phenol structure. On a molar basis, ETO appears to be more effective than both BHT and BHA as antioxidants.[12,13]

Natural antioxidants over synthetic have several advantages. The oils with higher content of unsaturated fatty acids, especially polyunsaturated fatty acids, are most susceptible to oxidation. In order to overcome the stability problems of oils and fats synthetic antioxidants, such as BHA, BHT, tertiary butyl hydroquinone (TBHQ) have been used as food additives.[12] But resent reports reveal that these compounds may be implicated by health risks, including cancer and carcinogenesis. Therefore the most powerful synthetic antioxidant (TBHQ) is not allowed for food application in Japan, Canada and Europe.[12] Similarly, BHA has also been removed from the generally recognized as safe (GRAS) list of compounds. Due to these safety concerns, there is an increasing trend among food scientists to replace these synthetic antioxidants with natural ones, which in general are supposed to be safer.[12]

Antioxidants in Body

Certain antioxidant enzymes are produced within the body. The most commonly recognized of these naturally occurring antioxidants are superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). SOD changes the structure of oxidants and breaks them down into hydrogen peroxide. CAT in turn, breaks down hydrogen peroxide into water and tiny oxygen particles or gases. GSH is a detoxifying agent, which binds with different toxins to change their form so that they are able to leave the body as waste.[8]

Miscellaneous Antioxidants

In addition to enzymes, vitamins, and above antioxidants, there appear to be many other compounds that have antioxidant properties which are discussed below.[9]

Selenium

Selenium is a trace element. It is a mineral that we need to consume in only very small quantity, but without which we could not survive. It forms the active site of several antioxidant enzymes including glutathione peroxidase. Similar to selenium, the minerals manganese and zinc are trace elements that form an essential part of various antioxidant enzymes.[9] Additionally, substances in plants called photochemical are being investigated for their antioxidant activity and health-promoting potential.[9]

Lycopene

Lycopene is a carotenoid, a substance our body uses to produce vitamin A. This substance occurs naturally in our colon, skin, liver and adrenal glands. It is also found in food sources, particularly tomatoes.[9] This carotenoid offers a very potent antioxidant benefits.

Zinc

Zinc is a trace mineral that is necessary for the synthesis of proteins from food sources. It may also speed the healing of skin wounds.[9] It may also promote the production of an enzyme called SOD, which may also provide antioxidant benefits.[9] Zinc is found in foods such as saltwater fish, beans, sunflower seeds, alfalfa, parsley, sage and whole-grain pastas and breads. It helps in strengthening the immune system, improves stress levels, prevents and shortens the duration of cold, reduce appearance of acne, and provide antimicrobial action in the gastro-intestinal tract.[9]

Causes of Oxidation

Oxidants, commonly known as "free radicals," are also introduced through external sources such as exposure to the sun or pollution. Other mediums

include stress, and intake of alcoholic beverages, unhealthy foods, and cigarette smoke. A poor diet also aids in the formation of free radicals.[11,13]

Mechanism of Oxidants and Antioxidants

Oxidative stress occurs when the production of harmful molecules called free radicals is beyond the protective capability of the antioxidant defences. Free radicals are chemically active atoms or molecular fragments that have a charge due to an excess or deficient number of electrons. Examples of free radicals are the superoxide anion, hydroxyl radical, transition metals such as iron, copper and nitric acid.[14] Free radicals containing oxygen, known as reactive oxygen species (ROS), are the most biologically significant free radicals. ROS include the radical's[14] superoxide and hydroxyl radical, plus derivatives of oxygen that do not contain unpaired electrons, such as hydrogen peroxide, singlet oxygen, and hypochlorous acid. Because they have one or more unpaired electrons, free radicals are highly unstable. They scavenge the body to grab or donate electrons, thereby damaging cells, proteins, and DNA (genetic material).[14] The same oxidative process also causes oils to become rancid, peeled apples to turn brown, and iron to rust.[14] It is impossible for us to avoid damage by free radicals. Antioxidants work by donating an electron to free radicals to convert them to harmless molecules. This protects cells from oxidative damage that leads to aging and various diseases.[14]

Side Effects of Antioxidants

Some antioxidants can become harmful if consumed in too large quantities as some examples are given below.[15,16]

Vitamin C

Stomach problems are one of the most noticeable side effects of excessive vitamin C. Regular consumption of high amounts of vitamin C has also been linked to the development of kidney stones.[15] Vitamin C is best known as a powerful immune system booster. It may help ward off colds and influenza, as well as bacterial and fungal infections.[15]

Vitamin A and D

Over-consumption of vitamin A and D can also cause serious problems. Hypervitaminosis A (vitamin A overdose) can result in headaches, hair loss, bone fragility, vomiting and dry peeling skin. Hypervitaminosis D can cause headaches, vomiting, lethargy, confusion and in severe cases, coma.[16]

Vitamin E

Vitamin E is also known to cause a multitude of side effects in large doses.[15,16] The concentration of vitamin that can cause negative effects is inconclusive.[15] Side effects that may occur include nausea, intestinal cramping, weakness, headache, blurred vision and gastrointestinal bleeding. It can increase the risk of a stroke. Vitamin E is a fat-soluble vitamin, which means that the body stores this vitamin in fat tissues.[15,16]

Selenium

Supplementing selenium over the long-term in areas where selenium is already adequate in the diet may increase the risk of diabetes and perhaps hypercholesterolemia. Selenium intake about 900 mcg daily can cause selenium toxicity.[16] Signs include depression, nervousness, emotional instability, nausea, vomiting, and in some cases loss of hair and fingernails.[16]

Zinc

Zinc taken orally seldom causes any immediate side effects other than occasional stomach upset are usually noticed when it is taken on an empty stomach. Some forms do have an unpleasant metallic taste. Long-term use of oral zinc at dosages of 100 mg or more daily can cause a number of toxic effects,[15,16] including severe copper deficiency, impaired immunity, heart problems, and anemia. Zinc at a dose of more than 50 mg daily might reduce levels of HDL ("good") cholesterol. In addition use of zinc supplements might increase risk of prostate cancer in men.[15]

Reported Role of Antioxidants

Antioxidants are believed to play a role in preventing the development of some chronic diseases such as cancer, diabetes, stroke, Alzheimer's disease, rheumatoid arthritis, cataracts and heart diseases. The followings are some of the reported examples in different disorders.[11,14]

In Oxidative Stress

Oxidation is a natural by product of cellular metabolism. Thus the process leads to the production of free radicals.[17] Free radicals are highly reactive and unstable molecules that attach themselves to cells and cause their death. The natural antioxidants

(e.g. vitamin A, C, and E from natural sources) present in many foods can provide a line of defence against the harmful effects of free radicals.[17] For example, vitamins E and C are two natural antioxidants that have been shown in the scientific research to fight free radical damage. Most of the data suggest that increased intake of vitamin E is protective against exercise induced oxidative damage.[17]

In Neurodegenerative Disorders

Alzheimer's disease is a severe disease that leads to progressively worsening mental decline and ultimately death. Polyphenols are potent plant antioxidants that are most abundant in fruits and vegetables such as apricots, tomatoes etc. currently used to treat this disorder as they improve the symptoms of this disease. Therefore, researchers have resorted to antioxidant compounds, such as polyphenols, to preserve neurological health.[18]

In Hypertension

Hypertension, or persistently elevated blood pressure, is a risk factor for heart disease. Natural antioxidants affect the body in a manner that can help reduce blood pressure.[2] Patients with high blood pressure who had not been treated with blood pressure-lowering prescription medication treated with tomato extract have shown reductions in blood pressure.[2] However, the researchers note that the long-term benefits should be further explored, since their study only assessed the short-term benefits.[19]

Skin Aging

Antioxidants affect the skin when applied topically. Oxidative damage is the main source of skin aging. Factors like sunlight, smoking, pollutants and a lack of exercise can exacerbate oxidative damage. As a result, skin cell turnover slows and leads to visible signs of aging such as wrinkles, dull appearance and brown pigmentation.[19] For example, creams[19] containing antioxidants including pycnogenol can improve pigmentation caused by sun exposure. Pycnogenol is derived from pine bark extract with antioxidant and anti-inflammatory properties.[19]

In Cancer Therapy

Antioxidants neutralize free radicals as the natural by-product of normal cell processes. Such damage may become irreversible and lead to disease including cancer.[20-22] Antioxidants are often described as "mopping up" free radicals, meaning they neutralize the electrical charge and prevent the free radical from taking electrons from other molecules which prevents the damage of DNA in the cell which ultimately prevent cancer.[20-22]

Sources of Antioxidants

Antioxidants are abundant in fruits and vegetables, as well as in other foods including nuts, grains, and some meats, poultry, and fish.[23] Beta-carotene is found in many foods that are orange in colour, including sweet potatoes, carrots, cantaloupe, squash, apricots, pumpkin, and mangos.[11] Some green, leafy vegetables, including collard greens, spinach, and kale, are also rich in beta-carotene. Lutein, best known for its association with healthy eyes, is abundant in green, leafy vegetables such as collard greens, spinach, and kale.[11] Lycopene is a potent antioxidant found in tomatoes, watermelon, guava, papaya, apricots, pink grapefruit, blood oranges, and other foods. Estimates[23] suggest 85 percent of American dietary intake of lycopene comes from tomatoes and tomato products. Selenium is a mineral, not an antioxidant nutrient. However, it is a component of antioxidant enzymes. Plant foods like rice and wheat are the major dietary sources of selenium in most countries. The amount of selenium in soil, which varies by region, determines the amount of selenium in the foods grown in that soil. Animals that eat grains or plants grown in selenium-rich soil have higher levels of selenium in their muscle. In the United States[23], meats and bread are common sources of dietary selenium. Brazil nuts[23] also contain large quantities of selenium. Vitamin A is found in three main forms, retinol (vitamin A1), 3, 4-didehydroretinol (vitamin A2), and 3-hydroxy-retinol (vitamin A3). Foods rich in vitamin A include liver, sweet potatoes, carrots, milk, egg yolks, and mozzarella cheese. Vitamin C is also called ascorbic acid, and can be found in high abundance in many fruits and vegetables and is also found in cereals, beef, poultry, and fish. Vitamin E, also known as alpha-tocopherol, is found in almonds, in many oils including wheat germ, safflower, corn, and soybean oils, and is also found in mangos, nuts, broccoli, and other foods.[23]

Models for Evaluating Antixidant Potential

There are mainly two types of models used for the evaluation antioxidant activity of a new molecule, compound or plant: *in vivo* models and *in vitro* models.

In Vivo Models

Cells are equipped with different kinds of mechanisms to fight against ROS and to maintain the redox homeostasis of cell. For example, antioxidant enzymes such as SOD, CAT and glutathione peroxidase (GPx) play important roles in scavenging the free radicals and preventing cell injury.[24]

In vivo methods are used for the evaluation of antioxidant activity of unknown compounds from organs like kidney, liver of laboratory animals maintained as per guiding principles in the care and use of animals. The liver and kidney are obtained from the anesthetized laboratory animals and tissue homogenates are prepared in 10 % normal saline solution and centrifuged at 2,000 *g* for 10 min. The supernatant liquid is assessed for enzyme activities of SOD, GSH, malondialdehyde (MDA) and CAT. The following methods are used for estimating anti-oxidant activity *in vivo*.[25]

Estimation of SOD

Superoxide activity can be estimated by the method of Beauchamp and fridovich (1971). The reaction mixture consists of 0.5 mL of hepatic PMS, 1 mL of 50 mmol/L Na_2CO_3, 0.4 mL of 25 μmol/L NBT and 0.2 mL of 0.1mmol/L hydroxylamine-hydrochloride, followed by the addition of 0.1 mL of liver homogenate (10% w/v). The change in absorbance is recorded at 560 nm over 3 min intervals.[5] The inhibitory effect of superoxide dismutase can be calculated as: inhibition (%) = [(Absorbance of control – Absorbance of test)/ Absorbance of control] × 100%[26,11]

Reduced GSH Activity

This can be determined by the Ellman method.[27] An aliquot of 1.0 mL of liver tissue supernatant is treated with 0.5 mL of Ellman's reagent (19.8 mg of DTNB in 100 mL of 0.1 % sodium nitrate) and 3.0 mL of phosphate buffer (0.2 mol/L, pH 8.0). The absorbance is measured at 412 nm.

Estimation of MDA

MDA, an index of free radical generation/ lipid peroxidation, which can be determined by colorimetrically by thiobarbituric acid reactive substance (TBARS) followed the Niehaus and Samuelson method.[28] 0.1 mL of tissue homogenate (10% w/v) is treated with 2 mL of TBA, TCA, HCl reagent in 1:1:1 ratio (TBA 0.37% and HCl 0.25N). The reaction mixture is stopped by the addition of 15% TCA. All the tubes are placed in a boiling water bath for 30 min and cooled. The amount of malondialdehyde formed in each of the samples is assessed by measuring the absorbance of clear supernatant at 535 nm against reference blank.[28]

Estimation of CAT

CAT can be estimated by assay method of Sinha.[29] 0.4 mL of hydrogen peroxide (0.2 mol/L) is added with 1 mL of 0.001 mol/L phosphate buffer (pH 7) followed by the addition of 0.1 mL clear supernatant of liver homogenate (10% w/v). Mixed the mixture gently at room temperature. The reaction mixture is stopped by adding 2 mL of dichromate acetic acid reagent (5% K_2Cr_2O7 in glacial acetic acid). The changes in the absorbance is measured at 620 nm and recorded after 3 min interval.[30]

In Vitro Models

A large number of *in vitro* methods have been developed to measure the efficiency of natural antioxidants either as pure compounds or as plant extracts. The detailed *in vitro* methods for the evaluation of antioxidant compounds are explained beow.[31]

Oxygen Radical Absorbance Capacity (ORAC) Assay

The capacity of a compound to scavenge peroxyl radicals, generated by spontaneous decomposition of 2, 2'- azo-bis, 2- amidinopropane dihydrochloride (AAPH), is estimated in terms of standard equivalents, using the ORAC assay.[32] The reaction mixture (4.0 mL) consist of 0.5 mL extract in phosphate buffer (75 mmol/L, pH 7.2) and 3.0 mL of fluorescein solution both are mixed and pre-incubated for 10 min at 37 ℃. Then, 0.5 mL of AAPH dihydrochloride solution is added and immediately the loss of fluorescence (FL) is observed at 1 min intervals for 35 min. The final results are calculated using the differences of areas under the FL decay curves between the blank and a sample and are expressed as micromole trolox equivalents (TE) per gram (μmol TE g-1).[33]

Total Antioxidant Activity

The antioxidant activity is determined by the conjugated diene method.[34] The test sample is mixed with 2.0 mL of 10 mmol/L linoleic acid emulsion in 0.2 mol/L sodium phosphate buffer (pH 6.6) in a test tube and kept in dark at 37 ℃ to accelerate oxidation.

After incubation for 15 h, 0.1 mL from each tube is mixed with 7.0 ml of 80% methanol in deionized water and the absorbance of the mixture is measured at 234 nm against a blank in a spectrophotometer. Ascorbic acid, BHA, or trolox can be used as a positive control.

Trolox Equivalent Antioxidant Capacity (TEAC) Assays

The TEAC assay is often used to measure the antioxidant capacity of foods, beverages and nutritional supplements.[25] The ABTS 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) free radical-scavenging activity of plants samples is determined by the method of Stratil et al.[25] The radical cation ABTS+ is generated by persulfate oxidation of ABTS. A mixture (1,1, v/v) of ABTS (7.0 mmol/L) and potassium persulfate (4.95 mmol/L) is allowed to stand overnight at room temperature in dark to form radical cation ABTS +. A working solution is diluted with phosphate buffer solution to absorbance values between 1.0 and 1.5 at 734 nm. An aliquot (0.1 mL) of each sample is mixed with the working solution (3.9 mL) and the decrease of absorbance is measured at 734 nm after 10 min at 37 ℃ in the dark. Aqueous phosphate buffer solution (3.9 mL, without ABTS + solution) is used as a control. Results can be expressed as TEAC in mmol of Trolox per kilogram (solid foods and oils) or per liter (beverages) of sample. Trolox, BHT, rutin, ascorbic acid or gallic acid can be used as a positive control.[35]

Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay is based on the ability of antioxidants to reduce Fe_3^+ to Fe_2^+ in the presence of 2,4,6-tri(2-pyridyl)- s-triazine (TPTZ), forming an intense blue Fe_2^+-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant content.[36] Then, 0.2 mL of the extract is added to 3.8 mL of FRAP reagent (10 parts of 300 mmol/L sodium acetate buffer at pH 3.6, 1 part of 10.0 mmol/L TPTZ solution and 1 part of 20.0 mmol/L $FeCl_3$. $6H_2O$ solution) and the reaction mixture is incubated at 37 ℃ for 30 min and the increase in absorbance at 593 nm is measured. $FeSO_4$ is used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of sample. BHT, BHA, ascorbic acid, quercetin, catechin or trolox can be used as a positive control.[36]

Diphenyl-picryl-hydrazyl Radical Scavenging (DPPH) Assay

This is the most frequently used method. The free radical scavenging activity can be measured by using 2, 2-diphenyl-1-picryl-hydrazyl or 1, 1- diphenyl-2-picryl-hydrazyl by the method of McCune and Johns.[37] The reaction mixture (3.0 mL) consists of 1.0 mL of DPPH in methanol (0.3 mmol/L), 1.0 mL of the extract and 1.0 ml of methanol. It is incubated for 10 min in dark, and then the absorbance is measured at 517 nm. In this assay, the positive controls can be ascorbic acid, gallic acid, BHA, quercetin, BHT, rutin, catechin or glutathione.[38]

Superoxide Anion Radical Scavenging Assay

The superoxide anion scavenging activity is measured as described by Robak and Gryglewski.[39] The superoxide anion radicals are generated in 3.0 mL of Tris- HCl buffer (16 mmol/L, pH 8.0), containing 0.5 mL of nitro blue tetrazolium (NBT) (0.3 mmol/L), 0.5 mL nicotinamide adenine dinucleotide (NADH) (0.936 mmol/L) solution, 1.0 mL test sample extract and 0.5 ml Tris-HCl buffer (16 mmol/L, pH 8.0). The reaction is started by adding 0.5 mL phenazine methosulfate (PMS) solution (0.12 mmol/L) to the mixture, incubated at 25 ℃ for 5 min and then the absorbance is measured at 560 nm against a blank sample. Gallic acid, BHA, ascorbic acid, curcumin, quercetin or trolox can be used as a positive control.[39]

Hydroxyl Radical Scavenging Assays

The scavenging ability for hydroxyl radicals is measured by the method of Kunchandy and Rao.[26] The reaction mixture (1.0 mL) consists of 100 μL of 2-deoxy-D ribose (28 mmol/L in 20 mmol/L KH_2PO_4 -KOH buffer, pH 7.4), 500 μL of the test sample, 200 μL EDTA (1.04 mmol/L) and 200 μmol/L $FeCl_3$ (1,1 v/v), 100 μL of H_2O_2 (1.0 mmol/L) and 100 μL ascorbic acid (1.0 mmol/L) which is incubated at 37 ℃ for 1 h. 1.0 mL of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8%) are added and incubated at 100 ℃ for 20 min. After cooling, absorbance is measured at 532 nm, against a blank sample. Gallic acid, mannitol, catechin, vitamin E, quercetin, BHA, rutin or ascorbic acid can be used as a positive control.[40]

Nitric Oxide Radical Scavenging Assays

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions are measured using

the Griess reaction reagent.[41] 3.0 mL of 10 mmol/L sodium nitroprusside in phosphate buffer is added to 2.0 mL of extract and reference compound in different concentrations (20–100 μg/mL). The resulting solutions are then incubated at 25 ℃ for 60 min. A similar procedure is repeated with methanol as blank, which serves as control. To 5.0 mL of the incubated sample, 5.0 mL of Griess reagent (1% sulphanilamide, 0.1% naphthyt ethylene diamine dihydrochloride in 2% H_3PO_3) is added and absorbance of the chromospheres formed is measured at 540 nm. Percent inhibition of the nitrite oxide generated is measured by comparing the absorbance values of control and test preparations. Curcumin, caffeic acid, sodium nitrite, BHA, ascorbic acid, rutin, BHT can be used as a positive control.[42]

Total Phenolic Content

The amount of total phenol content can be determined by Folin-Ciocalteu reagent method.[43] 0.5 mL of extract and 0.1 mL of Folin-Ciocalteu reagent (0.5 N) are mixed and incubated at room temperature for 15 min. Then 2.5 mL saturated sodium carbonate is added and further incubated for 30 min at room temperature and absorbance measured at 760 nm. Gallic acid, tannic acid, quercetin, chlorogenic acid, pyrocatechol or guaiacol can be used as positive controls. The total phenolic content is expressed in terms of standard equivalent (mg/g of extracted compound).[43]

Metal Chelating Activity

Ferrozine can quantitatively chelate with Fe^{2+} and form a complex with a red color. This reaction is limited in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe^{2+} complexes. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions.[35] The chelation of ferrous ions is estimated using the method of Dinis et al.[44] 0.1 mL of the extract is added to a solution of 0.5 mL ferrous chloride (0.2 mmol/L). The reaction is initiated by the addition of 0.2 mL of ferrozine (5 mmol/L) and incubated at room temperature for 10 min and then the absorbance is measured at 562 nm. EDTA or citric acid can be used as a positive control.

Hydrogen Peroxide Radical Scavenging Assay

The ability to scavenge hydrogen peroxide is determined according to the method of Ruch et al.[45] A solution of hydrogen peroxide (40 mmol/L) is prepared in phosphate buffer (50 mmol/L, pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. The test sample (20-60 μg/mL) in distilled water is added to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid, rutin, BHA or quercetin can be used as a positive control.[45]

Reducing Power

The reducing power can be determined by the method of Athukorala et al.[46] 1.0 mL extract is mixed with 2.5 mL of phosphate buffer (200 mmol/L, pH 6.6) and 2.5 mL of potassium ferricyanide (30 mmol/L) and incubated at 50 ℃ for 20 min. Thereafter, 2.5 mL of trichloroacetic acid (600 mmol/L) is added to the reaction mixture, centrifuged for 10 min at 3,000 r/min. The upper layer of solution (2.5 mL) is mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (6 mmol/L) and absorbance is measured at 700 nm. Ascorbic acid, BHA, tocopherol, trolox and BHT can be used as positive control.[46]

Total Flavonoid

The total flavonoid content can be determined by Aluminum chloride method.[47] The reaction mixture (3.0 mL) comprised of 1.0 mL of the test sample, 0.5 mL of aluminum chloride (1.2%) and 0.5 mL of potassium acetate (120 mmol/L) is incubated at room temperature for 30 min and absorbance measured at 415 nm. Quercetin or catechin can be used as a positive control. The flavonoid content is expressed in terms of standard equivalent (mg/g of extracted compound).

Xanthine Oxidase Methods

The xanthine oxidase activity with xanthine as the substrate is measured spectrophotometrically by the method of Noro et al.[48] The test sample (500 μL of 0.1 mg/mL) and allopurinol (100 μg/mL) (in methanol) is mixed with 1.3 mL phosphate buffer (0.05 mol/L, pH 7.5) and 0.2 mL of 0.2 units/mL xanthine oxidase solution. After 10 min of incubation at room temperature (25 ℃), 1.5 mL of 0.15 mol/L xanthine substrate solution is added to this mixture. The mixture is again incubated for 30 min at room temperature (25 ℃) and then the absorbance is measured at 293 nm using a spectrophotometer against blank (0.5 mL methanol, 1.3 mL phosphate buffer, 0.2 mL xanthine oxidase). The solution of

0.5 mL methanol, 1.3 mL phosphate buffer, 0.2 mL xanthine oxidase and 1.5 mL xanthine substrate is used as a control. BHT or catechin can be used as a positive control.[48]

Conjugated Diene Assays

This method allows dynamic quantification of conjugated dienes as a result of initial Polyunsaturated fatty acids (PUFAs) oxidation by measuring UV absorbance at 234 nm. The principle of this assay is that during linoleic acid oxidation, the double bonds are converted into conjugated double bonds, which are characterized by a strong UV absorption at 234 nm. The activity is expressed in terms of Inhibitory concentration (IC_{50}).[49,50]

Phosphomolybdenum Method

It is a spectroscopic method for the quantitative determination of antioxidant capacity, through the formation of phosphomolybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH.[51]

Peroxynitrite Radical Scavenging Activities

Peroxynitrite is now recognized by researchers as the culprit in many toxic reactions that were previously ascribed to its chemical precursors, superoxide and nitric oxide. Hence, an *in vitro* method for scavenging of peroxy radical has been developed to measure antioxidant activity. The scavenging activity is measured by monitoring the oxidation of dihydrorhodamine on a microplate fluorescence spectrophotometer at 485nm.[52]

N, N-dimethyl-p-phenylene Diamine Dihydrochloride (DMPD) Method

This method is based on the reduction of buffered solution of colored DMPD in acetate buffer and ferric chloride. The procedure involves measurement of decrease in absorbance of DMPD in presence of scavengers at its absorption maxima of 505 nm. The antioxidant activity of wines was measured by using this method. The activity was expressed as percentage reduction of DMPD.[53,54]

Beta Carotene Linoleate Model

This is one of the rapid method to screen antioxidants, which is mainly based on the principle that Linoleic acid, which is an unsaturated fatty acid, gets oxidized by ROS produced by oxygenated water.[53] The products formed will initiate the beta-carotene oxidation, which will lead to discoloration. The antioxidant activity of the extracts can be determined with this method. The β-carotene solution is prepared by dissolving 2 mg of β-carotene in 10 mL of chloroform. 40 mg of linoleic acid and 400 mg of Tween 20 is added for 1 mL of the solution. The chloroform being evaporated in a rotary evaporator, the solution is mixed with 100 mL of distilled water. This emulsion (4.8 mL) is placed into test tubes that contained 0.2 mg of the sample and 0.2 of the extract solvents. For control, 0.2 mg of solvent (methanol, ethanol, acetone or benzene) is put in the test tube instead of the extract. Immediately after the emulsion is placed in test tubes the initial absorbances is measured to at 470 nm with a spectrophotometer. The tubes are left for incubation at 50 ℃. The total antioxidant activity is calculated using the equation below.[55]

Cytochrome Tests

Superoxide anions were assayed spectro photometrically by a cytochrome reduction method described by McCord and Fridovich.[56] Xanthine oxidase converts xanthine to uric acid and yields superoxide anions and these radicals directly reduce ferri-cytochrome C to ferro-cytochrme C, having an absorbance change at 550 nm. When test compounds showed superoxide scavenger activity, there was a decrease in the reduction of ferri-cytochrome C.[1]

Erythrocyte Ghost Systems

This method involves isolation of erythrocytes ghost cells and the induction of lipid peroxidation using erythrocyte ghosts and the induction of tetra-butyl hydroxy peroxide (t-BHP). thio barbituric acid reactive substance (TBARS) produced during the reaction is measured at 535 nm.[57]

Natural Antioxidants

Synthetic antioxidants such as 2, 3-tert-butyl-4-methoxy phenol and 2, 6-di-tert-butyl-4-methyl phenol are widely used in the food industry. However there are serious concerns about the carcinogenic potential of these substances and there has been a general desire to replace synthetic food additives with natural alternatives.[10] Therefore, intensive research is being required on natural antioxidants that may serve as potent candidates in combating carcinogenesis and aging processes.

Plants Possessing Antioxidant Activity

Now a day there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing free radical-induced tissue injury. A lot of medicinal plants, traditionally used for thousands of years, are present in a group of herbal preparations of the Indian traditional health care system (Ayurveda) proposed for their interesting antioxidant activities. A large number of medicinal plants and their principle constituents have shown beneficial therapeutic potentials based on their antioxidant activity. Various herbs and spices have been reported to[11,13,18] exhibit antioxidant activity. Some of the plants that possess antioxidant activities are *Aegle marmelos*, *Allium cepa*, *Allium sativum*, *Aloe vera*, *Amomum subulatum*, *Andrographis paniculata*, *Asparagus racemosus*, *Azadirachta indica*, *Bacopa monniera*, *Cinnamomum verum*, *Cinnamomum tamala*, *Curcuma longa*, *Emblica officinalis*, *Glycyrrhiza glabra*, *Hemidesmus indicus*, *Momordica charantia*, *Nigella sativa*, *Ocimum sanctum*, *Picrorrhiza kurroa*, *Plumbago zeylanica*, *Syzigium cumini*, *Terminalia bellarica*, *Tinospora cordifolia*, *Trigonella foenumgraecum*, *Withania somnifera*, *Zingiber officinalis*, *Terminalia arjuna*, *Curcuma domestica*, *Cuscuta reflexa*, *Daucus carota*, *Foeniculum vulgare*, *Mangifera indica*, *Momordica charantia*, *Psoralea corylifolia*, *Santalum album*, *Solanum nigrum*, *Swertia chirayita*, *Baccharis coridifolia*, *Bryonia alba*, *Cichorium intybu*, *Cinnamomum zeylanicum*, *Crithmum maritimum*, *Cynara scolymus*, *Emilia sonchifolia*, *Eucalyptus camaldulensis*, *Eucalyptus rostrata*, *Eucommia ulmoides*, *Lavandula angustifolia*, *Lycium barbarum*, *Melissa officinalis*, *Murraya koenigii*, *Myrica gale*, *Panax ginseng*, *Piper nigrum*, *Plantago asiatica*, *Prunus domestica*, *Rhazya stricta*, *Rosmarinus officinalis*, *Salvia officinalis*, *Salvia triloba*, *Solanum melongena*, *Solanum tuberosum*, *Syzygium caryophyllatum*, *Eugenia caryophyllus*, *Thymus zygis*, *Tinospora cordifolia*, *Uncaria tomentosa*, *Centella asiatica*, *Morus Indica*.[58,59] The majority of the antioxidant activity is due to the flavones, isoflavones, flavonoids, anthocyanin, coumarin lignans, catechins and isocatechins.[46]

Side Effects of Antioxidants from Natural Sources

Along with the various positive effects of natural antioxidants on health, but they could also have harmful effects if taken in excess.[15] They can cause significant physiological effects like kidney stones, increased need for oxygen, excess uric acid excretion and erosion of dental enamel when vitamin C is taken in higher concentration.[15,24,51] Other complications may be like risk of bleeding with higher dose of vitamin E containing natural sources.[21,34,44] Natural sources of vitamin A overdoses for extended period can lead to symptoms such as fatigue, breast soreness, gastrointestinal stress, renal problems, vascular inflammation, and thyroid problems. Some dietary antioxidants like β-carotene, vitamin C, vitamin E and other produce complicated and conflicting results in cardiac health studied by different researchers. Therefore, the consumption of natural sources of antioxidants for health benefits without adverse effects, have yet to be identified. Thus the awareness and proper investigation regarding this is the need of the hour to minimize the toxic or additional effect of antioxidants.[15,16,51]

Discussion

This review emphasizes with the definition, types, mechanism, sources, side effects of antioxidants and various *in vitro* and *in vivo* models for the evaluation of antioxidants.[4,11] It is reported that oxidative damage caused by free radicals increases with age and result in many life-threatening diseases like diabetes, ulcer, cancer, inflammation, heart attack, hypertension, rheumatoid arthritis, retinopathy, nephropathy, HIV, Parkinson's, Alzheimer's and Huntington's disease as the physiology of these chronic and life-threatening diseases state the influential role of free radicals.[55] Thus the knowledge of oxidants and antioxidants plays an important role in the prevention of many disorders.

The new antioxidants from plants and plants products or from any other sources can be explored by using various *in vitro* and *in vivo* methods. The *in vitro* and in *vivo* methods reported are popular due to their high speed and sensitivity. However, it is essential to use more than one method to evaluate antioxidant capacity of plant materials because of the complex nature of phytochemicals.[1] The most commonly and uncommonly used antioxidant assays along with various standards that can be used as positive control are described in this review.

Thus a study on antioxidants should be promoted as they are of absolute value in preventing several diseases and exhibit a promising therapeutic approach. There should be more in-depth study and research in the area of free radicals and antioxidants to understand its properties and mechanism much better.

Assays for antioxidant status and oxidative damage are many and varied. The simplest ones are purely chemical *in vitro* reactions or tests in cell cultures. They can yield useful information about mechanisms of action, but extrapolation to effects of dietary antioxidants *in vivo* is dangerous, because uptake from the gastrointestinal tract and metabolism are not considered. Supplementation with antioxidants *in vivo* seems to be the best approach, at least in principle, and experiments should be performed with human subjects if possible. *In vitro* models animal experiments have not been considered, but generally the same assays and approaches as used with human subjects are applied. This review is one of its kinds that enlists and describes all the *in vitro* and *in vivo* antioxidant models which will be of great value for people in the search of antioxidant moieties and their biological evaluation.

Conflict of Interest

The authors declare that they have no conflict of interests.

Authors Contribution

Suresh Kumar collected the detailed study materials and write in the form of Review. Sunil Sharma and Neeru Vasudeva drafted and revise the manuscript. Neeru Vasudeva checked and corrected the English language. All authors read and approved the final manuscript.

REFERENCES

1. Halliwell B. Free Radicals, Antioxidants, and Human Disease: Curiosity, Cause, or Consequence? Lancet 1994;344:721-724
2. Mathew S, Abraham TE. Studies on the antioxidant activities of cinnamon (*Cinnamomum verum*) bark extracts, through various *in vitro* models. Food Chem 2006;94:520-528.
3. Hilton JW. Antioxidants, function, types and necessity of inclusion in pet foods. Can Vet J 1989;30:682-684.
4. Kumar S, Sharma S, Vasudeva N. GC-MS analysis and screening of antidiabetic, antioxidant and hypolipidemic potential of Cinnamomum tamala oil in streptozotocin induced diabetes mellitus in rats. Cardiovas Diabet 2012;11:95.
5. Podsedek A. Natural antioxidants and antioxidant capacity of brassica vegetables. LWT- Food Sci Technol 2007;40:1-11.
6. Ames BN, Shigenaga MK, and Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci 1993;90:7915-7922.
7. Gupta V, Sharma SK. Plants as natural antioxidants. Natural Product Radiance 2006;5:326-334.
8. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem 2005;53:1841-1856.
9. Omari BE, Fleck I, Aranda X, Abadía A, Cano A, Arnao MB. Total antioxidant activity in Quercus ilex resprouts after fire. Plant Physiology Biochemistry 2003;41:41-47.
10. Hennekens CH, Buring JE, Manson JE, Stampfer M, Rosner B, Cook NR, et al.: Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. N Engl J Med 1996;334:1145-1149.
11. Kumar S, Sharma S, Suman, Jitpal. *In vivo* Anti-hyperglycemic and antioxidant potential of Piper longum fruit. J Pharm Res 2011:4:1-4.
12. Pokorny J. Are natural antioxidants better and safer than synthetic antioxidants? Eur J Lipid Sci Technol 2007;109:629-642.
13. Kumar S, Sharma S, Vasudeva N, Ranga V. *In vivo* anti-hyperglycemic and antioxidant potentials of ethanolic extract from Tecomella undulata. Diabe Metab Syndrome 2012;4:33.
14. Neeraj, Pramod J, Singh S, Singh J. Antioxidants to the rescue of cell under invasion of free radicals – a review. Intern J Basic Applied Med Sci 2013;3:190-200.
15. Joseph K. Natural antioxidants in grapes & wines. J Agri food chem 1994:42:64-69.
16. Niehaus WG, Samuelson. Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem 1968;6:126-130.
17. Witt EH, Reznick AZ, Viguie CA, Starke-Reed P, Packer L. Exercise, oxidative damage and effects of antioxidant manipulation (review). J Nutr 1992;122:766-73.
18. Wang D, Wang LJ, Zhu FX, Zhu JY, Chen XD, Zou L, et al. *In vitro* and *in vivo* studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food). Food Chem 2008;107:1421-1428.
19. Ashok KJ. Imbalance in antioxidant defence and human diseases, multiple approach of natural antioxidant therapy. Current Science 2001;34:1179-1186.
20. Howell JC. Food antioxidants, international perspectives welcome and introductory remarks. Food Chem Toxicol 1986;24:997.
21. Lingnert H, Vallentin K, Eriksson CE. Measurement of antioxidative effect in model system. J Food Proc Pres 1979;3:87-103.
22. Ou B, Hampsch-Woodill M, Flanagan J, Deemer EK, Prior RL, Huang D. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. J Agric Food Chem 2002;50:2772-2777.
23. Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivatives (acetoaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxy radical scavengers. Arch Biochem Biophy 1994;315:161-169.
24. Kanner J. Natural antioxidants in grapes and wines. J Agric Food Chem 1994;42:64-69.

25. Vuotto ML, Basile A, Moscatiello V, Sole PD, Cobianchi RS, Laghi E, et al. Antimicrobial and antioxidant activities of Feijoa sellowiana fruit. Int J Antimicrob Agents 2000;13:197-201.
26. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power" the FRAP assay. Anal Biochem 1996;239:70-76.
27. Ellman GL. Tissue sulphydryl groups. Arch Biochem Biophys 1959;82:70-77.
28. Niehaus WG, Samuelson B. Formation of malondialdehyde from phospholipids arachidonate during microsomal lipid peroxidation. Eur J Biochem 1968;6:126-130.
29. Sinha KA. Colorimetric assay of catalase. Anal Biochem 1972;47:389-394.
30. Sinha AK. Colorimetric assay of Catalase. Anal Biochem 1972;42:389-394.
31. Hughes S, Samman S. The effect of zinc supplementation in humans on plasma lipids, antioxidant status and thrombogenesis. J Am Coll Nutr 2006;25:285-291.
32. Percival M. Antioxidants. Clin Nutrition Insight 1998;31:1-4.
33. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 2005;53:4290-4302.
34. Liu CZ, Murch SJ, EL-Demerdash M, Saxena PK. *Artemisia judaica* L. micropropagation and antioxidant activity. J Biotechnol 2004;110:63-71.
35. Soler-Rivas C, Espin JC, Wichers HJ. An easy and fast test to compare total free radical scavenger capacity of foodstuffs. Phytochem Anal 2000:11:330-338.
36. Cavalcantia FR, Resende MLV, Santos Lima JPM, Gomes Silveira JA, Abreu Oliveira JT. Activities of antioxidant enzymes and photosynthetic responses in tomato pre-treated by plant activators and inoculated by Xanthomonas vesicatoria. Physiol Mol Plant Path 2006;68:198-208.
37. McCune LM, Johns T. Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the indigenous peoples of the North American boreal forest. J Ethnopharmacol 2002;82:197-205.
38. McDonald S, Prenzler PD, Antolovich M, Robards K. Phenolic content and antioxidant activity of olive extracts. Food Chem 2001;73:73-84.
39. Robak J, Gryglewski RJ. Flavonoids are scavengers of superoxide anions. Biochem Pharmacol 1988;37:837-841.
40. Lee IM, Cook NR, Manson JE. Beta-carotene supplementation and incidence of cancer and cardiovascular disease, Women's Health Study. J Natl Cancer Inst 1999;91:2102-2106.
41. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and 15N nitrate in biological fluids. Anal Biochem 1982;126:131-138.
42. Ho KY, Huang JS, Tsai CC, Lin TC, Lin CC. Antioxidant activity of tannin component from *Vaccinium vitisidaea*. Pharm Pharmcol 1999;51:1075-1078.
43. Naika GH, Priyadarsinia KI, Satavb JG, Banavalikarc MM, Sohonic DP, Biyanic MK, et al. Comparative antioxidant activity of individual herbal components used in Ayurvedic medicine. Phytochem 2003;63:97-104.
44. Loo AY, Jain K, Darah I. Antioxidant and radical scavenging activities of the pyroligneous acid from a mangrove plant, Rhizophora apiculata. Food Chem 2007;104:300-307.
45. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogen 1989;10:1003-1008.
46. Bandoniene D, Pukalskas A, Venskutonis PR, Gruzdiene D. Preliminary screening of antioxidant activity of some plant extracts in rapeseed oil. Food Res Int 2000;33:785-791.
47. Chiaki S, Naomi O. Antioxidative polyphenols isolated from Theobroma cocoa. J Agric Food Chem 1998;46:454-57.
48. Okuonghae PO, Aberare LO, Mukoro N, Osazuwa F, Dirisu JO, Ogbuzulu J, et al. Total antioxidant status of zinc, manganese, copper and selenium levels in rats exposed to premium motor spirit fumes. Am J Med Sci 2011;3:234-237.
49. Athukorala Y, Kim KN, Jeon YJ. Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga Ecklonia cava. Food Chem Toxicol 2006;44:1065-1074.
50. Dimitrios B. Sources of natural phenolic antioxidants. Trends in Food Sci Technol 2006:17:505-512.
51. Kardosova, E, Machova. Antioxidant activity of medicinal plant polysaccharides. Fitoterapia 2006;77:367-373.
52. David GB, Erik EA, Rohini S, Alfins. Antioxidant enzyme expression and ROS damage in prostatic intraepithelial neoplasia and cancer. Cancer 2000;89:124-134.
53. Simonetti P, Pietta P, Testolin G. Polyphenol content and total antioxidant potential selected Italian wines. J Agric Food Chem 1997;45:1152-1155.
54. Vinson JA, Hontz BA. Phenol antioxidant index, comparative antioxidant effectiveness of red and white wines. J Agri Food Chem 1995;43:401-403.
55. Kamboj J, Sharma S, Kumar S. *In vivo* anti-diabetic and anti-oxidant potential of psoralea corylifolia seeds in Streptozotocin induced type- 2 diabetic rats. J health Sci 2011;57:1-11.
56. McCord JM, Fridovich I. Superoxide dismutase, an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244:6049-6055.
57. Choi HR. Peroxynitrite scavenging activity of herb extracts. Phytother Res 2002;16:364-367.
58. Scartezzini P, Speroni E. Review on some plants of Indian traditional medicine with antioxidant activity. J Ethnopharm 2000;71:23-43.
59. Varadacharyulu NC, Bondada Andallu, Vinay Kumar AV. Oxidative stress in streptozocin-diabetic rats: amelioration by Mulberry (*Morus Indica* L.) leaves. Chin J Integr Med 2012;1-6.

(Received March 27, 2013)
Edited by Chen Yiyu