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Standard Review

Phytochemical antioxidants for health and medicine – A move towards nature

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Oxidants or reactive oxygen species are produced in our body during aerobic metabolism leading to many diseases such as cancer, cardiovascular diseases etc. Antioxidants are the chemicals that neutralize these oxidants. Natural antioxidants are the secondary metabolites of phytochemicals and are preferred over synthetic antioxidants, which are found to impose side effects. A review has been done on the effect of oxidants on human health and their neutralization by natural antioxidants. Major natural antioxidants and their plant sources, different recovery processes for antioxidants from plant matrices and the advantage of indirect supercritical fluid extraction over other processes are presented.

Keywords: Free radical, secondary metabolites, oxidative stress, recovery processes, process parameters.

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INTRODUCTION

Phytochemicals are the chemicals extracted from plants. These chemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's etc. Secondary constituents are the remaining plant chemicals such as alkaloids (derived from amino acids), terpenes (a group of lipids) and phenolics (derived from carbohydrates) (Walton et al., 1999).

Antioxidants are secondary constituents or metabolites found naturally in the body and in plants such as fruits and vegetables. An antioxidant can be defined in simple terms as anything that inhibits or prevents oxidation of a susceptible substrate. Plants produce a very impressive array of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of the susceptible substrate (Hollman, 2001). Common antioxidants include vitamin A, vitamin C, vitamin E, and certain compounds called carotenoids (like lutein and beta-carotene) (Hayek, 2000). These plant-based dietary antioxidants are believed to have an

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important role in the maintenance of human health because our endogenous antioxidants provide insufficient protection against the constant and unavoidable challenge of reactive oxygen species (ROS; oxidants) (Fridovich, 1998).

Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress (Mikulikova and Popes, 2001). Oxidative stress plays a role in heart diseases, malaria, neurodegenerative diseases, AIDS, cancer and in the aging process (Sian et al., 2003). This concept is supported by increasing evidence that oxidative damage plays a role in the development of chronic, age-related degenerative diseases, and that dietary antioxidants oppose this and lower risk of disease (Atoui et al., 2005; Alasalvar et al., 2005) and thus there arises a necessity to extract these antioxidants from the plant matrices. In a recent study (Grigonisa, 2005) different extraction techniques, such as dispersed-solids, percolation, Soxhlet, microwave assisted extraction and supercritical fluid extraction have been used to isolate antioxidants from the plants. Supercritical fluid extraction (SFE) is found to be the feasible and sophisticated technology for the extraction of the antioxidants (Nguyen et. al. 1994). The methodology of solid phase extraction (SPE) with super critical fluids such as CO_2 is found to yield higher purity antioxidants.

This review discusses the effect of oxidants on human health and their neutralization by antioxidants. Different types of antioxidants, their properties and their extraction processes from plant matrices have been dealt in detail. Recent advances in supercritical extraction of antioxidants are presented.

Formation of oxidants

Oxygen, an essential element for life, can also be a reason for the destruction of tissue and/or impair its ability to function normally (Kehrer et al., 1993). Oxidants or free radicals or oxygen-free radicals (OFR) or more generally called as reactive oxygen species (ROS) are formed due to various exogenous and endogenous factors. A free radical contains one or more unpaired electrons and is capable of independent existence. The formation of oxygen radicals could be the reason for the damaging effects of O_2. A class of enzymes called superoxide dismutases (SODS) is responsible for the catalytic removal of superoxide free radical, O_2^- (Lee et al., 2001). An average person has around 10,000–20,000 free radicals attacking each body cell every day. In some cases, ROS are produced specifically to serve essential biological functions, whereas in other cases, they are the byproducts of metabolic processes (Shigenaga et al., 1994).

Exogenous sources

Exposure to radiation from the environment and manmade sources is the exogenous source for formation of oxidants. Low-wavelength electromagnetic radiation such as gamma rays splits water in the body to generate hydroxyl radical, OH^-. The highly reactive OH^- thus formed begins to react vigorously with the nearby cells (Halliwell, 1994). Even though OH^- scavengers usually have rate constants more than 10^{10} M^{-1} sec^{-1} for reaction with OH^-, the most endogenous molecules react equally fast. The antioxidant systems that defend against damage by OH^- do so by preventing its formation and by repairing the damage it causes (Timothy et al., 2003).

It has been estimated that 1-3% of the oxygen we breathe in is used to make O_2^-. Since humans consume large quantities of O_2, a simple calculation shows that over 2 kg of O_2^- is made in the human body every year-people with chronic inflammations may make much more (Halliwell et al., 1994). These oxidants damage cellular macromolecules, including DNA, protein and lipid (Fraga et al., 1990) and accumulation of such damage may con-tribute to ageing and age related diseases.

Endogenous sources and characteristics of oxygen radicals

Other than the exogenous sources such as exposure to radiation, enzymatically or non-enzymatically mediated electron transfer reactions are the source of free radicals produced in the cells. Electron leakage that occurs from electron transport chains, such as those in the mitochondria and endoplasmic reticulum, to molecular oxygen are the major source of free radicals (Fridovich, 1986).

Oxidants are formed in the cells of our body mainly from the following four endogenous sources.

1. Consumption of O_2 by mitochondria during normal aerobic respiration to produce H_2O. Oxidants such as oxygen free radical, H_2O_2 and hydroxyl radical are the by products of this process
2. Destroying of bacteria and virus infected cells by phagogytic cells releases nitric oxide, hydrogen peroxide and oxygen free radical.
3. Degradation of fatty acids and other molecules by peroxisomes, the organelles produce hydrogen peroxide as byproduct, which is then degraded by catalase. The nondegraded peroxide gets into other compartments of nearby cell thereby leading to oxidative DNA damage (Ames et al., 1993). When two free radicals a nonradical is produced due to the formation of covalent bond between their unpaired electrons. But a radical is formed when a free radical reacts with a nonradical and thus can initiate a chain reaction in the body.
4. Oxidants produced during the course of p. 450 degra -dation of natural toxins.

Organisms have developed many defense mechanisms to limit the level of reactive oxidants and the damage inflicted by them (Sang et al., 2002). Despite the cell's antioxidant defense system to counteract oxidative damage from free radicals, radical-related damage of DNA and proteins have been proposed to play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis, eurodegenerative disorders and others (Ames, 1989). Reactive oxygen species interacts with cellular components including DNA bases and forms damaged bases or strand breaks (Atoui et al., 2005). Oxygen radicals oxidize lipids or proteins generating intemediates that react with DNA by forming adducts. Therefore, it is an utmost necessity to take antioxidants exogenously due to the changes in the environment for which man made activities such as deforestation, rise in carbon dioxide level in atmosphere etc., are also responsible factors.

Antioxidant and its mechanism

Antioxidants are defined as the substance that when prsent in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance (Halliwell and Guteridge, 1989). For the *in vivo* situation the concept of antioxidants includes antioxidant enzymes, iron binding and transport proteins and other compounds affecting signal transduction and gene expression (Gutteridge, 1989). In case of foods and beverages, antioxidants are related to the protection of specific oxidation substrates or the formation of specific oxidation.

Synergism, antagonism, co-antioxidants and oxidation retarders are the other useful concepts related to antioxidants. Synergism can be defined as the phenomenon in which a number of compounds, when present together in the same system, have a more pronounced effect than if they were alone (Uri, 1961). Antagonism can be defined likewise by substituting “more” with “less”, whereas co-antioxidants may be defined by substituting “more” with “same”. The compounds that reduce the rate of oxidation without showing a distinct lag phase of oxidation are retarders of oxidation. Antioxidant action is measured as a decrease in over-all rate of oxidation and as the length of the lag phase.

Antioxidants are divided into two classes: preventive antioxidants and chain breaking antioxidants. Preventive antioxidants inhibit oxidation by reducing the rate of chain initiation. In most cases hydroperoxide product, ROOH of the oxidation is the cause for the initiation process. Preventive antioxidants convert the hydroperoxides to molecular products that are not potential sources of free radicals (Burton et al., 1985). Most biological preventive antioxidants are also peroxide decomposers. Certain enzymes such as glutathione peroxidase can reduce H_2O_2 to H_2O and also lipid hyroperoxides to the corresponding alcohol as shown in the following equation (1).

$$ROOH \xrightarrow[\text{Glutathione peroxides}]{[2H]} ROH + H_2O \quad (1)$$

Commercial chain breaking antioxidants are generally phenols or aromatic amines. They owe their antioxidant activity to their ability to trap peroxyl radicals are as shown in equation (2).

$$O_2^- + \text{Antioxidant} \longrightarrow O_2 + \text{Antioxidant} + \text{heat} \quad (2)$$

Antioxidants can also be manufactured synthetically. These belong to the class of synthetic antioxidants. The main disadvantage with these antioxidants is their side effect when taken in vivo (Chen et al., 1992). Most of the natural antioxidants are found to have higher antioxidant activity when compared with that of the synthetic ones. Several arguments suggest that the antioxidant components of fruits and vegetables contribute in the defense effect. Epidemiological studies and intervention trials on prevention of diseases such as cancer and cardiovascular disease in people have shown the positive effects of taking antioxidant supplements (Ames et al., 1993; Enstrom et al., 1992; Rimm et al., 1993).

Carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols are some of the antioxidants produced by the plant for their sustenance. Some of the widely known antioxidants are beta-carotene, ascorbic acid and alpha tocopherol (McCall et al., 1999). Beta-carotene is known as a precursor to vitamin A; it is converted to vitamin A in the liver and the mucous membranes of the small intestine. Beta-carotene is found to be safer as it can be ingested in almost unlimited quantities without toxic effect to the body (Dagenais et al., 2000). Ascorbic acid has multi-functional properties. Based on conditions ascorbic acid can act as an antioxidant, pro-oxidant, a metal chelator, a reducing agent or an oxygen scavenger. Ascorbic acid can act as a pro-oxidant in aqueous systems containing metals, by reducing them, which become more active catalysts of oxidation in their lower valence state. In the absence of added metals, ascorbic acid is an effective antioxidant at high concentrations (Katsunari et al., 1999).

Vitamin E is a group of compounds with well known antioxidant functions. Among vitamin E compounds, tocopherol and especially alphtocopherol possesses the strongest biological activity. Tocopherol is prevalently found in mammalian tissue (Flohe et al., 2002)). Se is a naturally occurring antioxidant that preserves tissue elas-ticity by delaying oxidation of polyunsaturated fatty acids. Se is an essential component of glutathione peroxidase. Se deficiency has been implicated as contributing factor to the development of cardiovascular disease (congestive cardiomyopathy), accelerated atherosclerosis, skeletal muscle myopathy, increased cancer risk, aging, cataract and deranged immune function (Zima et al., 2004). Small molecule dietary antioxidants such as vitamin C (ascorbate), vitamin E (tocopherol), and cartoneoids have generated particular interest as anticarcinogens and as defenses against degenerative diseases (Leo, 1999). The

Table 1. Widely used antioxidants and their applications.

Antioxidant	Plant sources	Applications
Beta-Carotene $C_{40}H_{56}$	*Elaeis oleifera, Elaeis Guineensis Momordica Cochinchinnensis Spreng* *Eurycoma Longifolia* *Zanthoxylum Myriacanthum*	Reported to be anodyne, antidotal, aphrodisiac, diuretic, and vulnerary. Oil palm is a folk remedy for headaches, rheumatism and is used as a liniment for indolent tumors. Used as a coloring and flavoring agent in steamed glutinous rice, male aphrodisiac, stomach ache and antitumor agent.
Alpha-Tocopherol $C_{29}H_{50}O_2$	*Citrus Hystrix* *Calamus Scipronum* *Averrhoa Belimbi*	Fruit used as preservative, flavoring in both savory and sweet food. Leaves used as hair shampoo and as medicine. The buds of these canes are eaten as food and have medical and antiseptic properties. They are commonly used for treatment of fever and aches. The syrup of the fruit is useful in relieving thirst, febrile excitement, and also in some slight cases of hemorrhage from the bowels, stomach and internal hemorrhoids.
Ascorbic Acid $C_6H_8O_6$	*Apium Graveolens* *Sauropus Androgynous*	Arthritis, Back Pain (lower), Nervousness, Rheumatism. Insect and disease resistance.
Palmitic Acid $CH_3(CH_2)_{14}COOH$	*Elaeis Oleifera, Elaeis Guineensis*	Anodyne, antidotal, aphrodisiac, diuretic and vulnerary. Oil palm is source of palmitic acid and is a folk remedy for cancer, headaches, and rheumatism.
Beta Sitosterol $C_{29}H_{50}O$	*Morinda Citrifolia* *Alpinia Officinarum* *Sida Acuta*	Diabetes, high blood pressure, arthritis, skin afflictions, and conditions of aging Flatulence, dyspepsia, vomiting and sickness at stomach, and recommended as a remedy for stomach cancer. Entire plant for stomach ache.
Selenium	*Astragalus Membranaceus* *Valeriana Officinalis* *Achillea Millefolium*	Prevents severe side effects of chemotherapy in patients with cancer. Inhibits the growth of murine renal cell carcinoma. Activation of immune system. Sedative activity. General tonic for the cardio-vascular system, lowers blood pressure, and slows heartbeat.
Anthraquinone $C_{14}H_8O_2$	*Cassia Acutifolia*	Antihelminthic, antibacterial, laxative, diuretic, for treatment of snakebites and uterine disorders.
Tannic acid $C_{76}H_{52}O_{46}$	*Costus Spinosa*	Tanning of leather.
Quercetin$C_{15}H_{10}O_7$	*Blumea Balsamifera*	Treatment for the swelling of pancreas.

details of these antioxidants are shown in the Table 1 (Heinerman, 1996; Hashimoto et al., 2002; Cao, 1993; Nessa et al., 2004; Somchit, et al., 2003; Burton, et al., 1985; Halkes, et al., 2002; Burkill, 1993; Oomen and Grubben, 1998 and Zipser, et al., 1998).

Antioxidant and cancer

It has been estimated that one human cell is exposed to approximately 105 oxidative hits a day from hydroxyl radical and other such species of oxidants. ROS are normal oxidant by-products of aerobic metabolism, and under normal metabolic conditions about 2–5% of O_2 consumed by mitochondria is converted to ROS (Lopaczynsk and Zeisel, 2001 and Dreher et al., 1996). Oxidative stress thus created permanently modifies the genetic material leading to numerous degenerative or chronic diseases, such as atherosclerosis and cancer (Ames et al., 1993). Misrepair of DNA damage could result in mutations such as base substitution and deletion which could lead to carcinogenesis (Barcellos, 2005). Two different mechanisms are thought to play a role in oxidative damage and in the development of carcinogenesis.

The first mechanism is through the modulation of gene expression. Epigenetic effects on gene expression can lead to the stimulation of growth signals and proliferation (Crawford et al., 1995). Chromosomal rearrangements are thought to result from strand breakage misrepair, contributing to genetic amplifications, iterations in gene expression and loss of heterozygosity, which in turn may promote neoplastic progression (Bohr et al., 1995). Active oxygen species have been demonstrated to stimulate protein kinase and poly (ADP ribosylation) pathways, thus affecting signal transduction pathways. This further can lead to modulation of the expression of essential genes for proliferation and tumour promotion (Cerutti and Trump, 1991). There is a suggestion that free radical signal may be mediated through *ras* signal transduction pathways (Lander et al., 1995).

In the second mechanism, radicals induce genetic alterations, such as mutations and chromosomal rearrangements, which can play a role in the initiation of carcinogenesis (Guyton and Kensler, 1993; Cerda and Weitzman, 1997). Oxidative DNA damage results in a wide range of chromosomal abnormalities, causing a blockage of DNA replication and wide cytotoxicity (Bohr et al., 1995). Mutations can occur through misrepair or due to incorrect replication, while chromosomal rear-rangements can result from strand breakage misrepair.

It is known that repair mechanisms decay with age and thus DNA lesions accumulate with age (Jaruga and Dizdaroglu, 1996). The sequence specificity of DNA damage sites affects the mutation frequency (Dizdaroglu et al., 2002). Therefore, investigation of the sequence specificity of DNA damage would be beneficial for cancer prevention. Mutagenic potential is directly proportional to the number of oxidative DNA lesions that escape repair.

Antioxidant extraction processes

Plants contain a wide spectrum of metabolites, as many as 200,000 different compounds (Fiehn, 2002), although not every metabolite occurs in every species. These metabolites represent many different classes of compounds and their derivatives such as amino acids, fatty acids, carbohydrates, and organic acids. The physical-chemical properties of the metabolites are highly variable. Therefore appropriate extraction protocols have to be chosen, as the optimum extraction conditions differ widely for different types of compounds. The plant tissue must be homogenized properly in order to extract plant metabolites efficiently. Various techniques such as grind-ing with a mortar and pestle together with liquid nitrogen, milling in vibration mills with chilled holders, homogeni-zation with a metal pestle connected to an electric drill (Edlund et al., 1995) and ultra-turrax devices (Orth et al., 1999) are available. The degree of homogenization deter-mines the efficiency at which the solvent can penetrate the tissue, and therefore strongly influences the length of time required for solvent extraction. The most common way to extract metabolites is to shake the homogenized plant tissue at low or high temperatures in organic sol-vents, or mixtures of solvents (Fiehn et al., 2000). Methanol, ethanol, and water are the solvents mostly used for extracting polar metabolites, whereas chloroform is the most common solvent for non-polar ones. Alternative extraction techniques include subcritical water extraction (SWE) (Ozel, 2003), pressurized liquid extraction (PLE) (Rostagno et al., 2004), microwave-assisted extraction (MAE) (Shu et al., 2003) and supercritical fluid extraction (SFE) (Roger, 1999)

Different extraction techniques, such as Soxhlet, microwave assisted extraction (MAE) and supercritical fluid extractions (SFE) (Lopez-sebastian et al., 1998) have been used to isolate antioxidants from the plants. Each extraction has its own advantages and disadvantages. The main disadvantages of Soxhlet extraction are long extraction time, degradation of thermolabile compounds and limited solvent choice (Lao et al., 1996). Other conventional liquid–solid extraction procedures also are time-consuming, require large amounts of solvents that sometimes are hazardous compounds, and consequently require further clean-up and concentration steps. Recently MAE (Eskilson and Bjorklund, 2000) has been used as an alternative laboratory scale extraction method, which proved to be considerably faster. MAE also requires less solvent and provides higher recoveries compared to Soxhlet extraction. The major disadvantage with MAE is that it is usually performed at higher temperatures (110–150 ◦C). This temperature range may lead to the denaturation of the thermo labile compounds (Kaufmann and Christen, 2002).

Supercritical fluid extraction (SFE) with carbondioxide as supercritical fluid is a very attractive method for extraction (Guarise et al., 1994). This is because CO_2 is inert, non-flammable, non-explosive, inexpensive, odorless, colorless, clean solvent that leaves no solvent residue in the product. Moreover, the critical temperature of carbon dioxide being 304º K makes it attractive for the extraction of thermo labile compounds (Tonthubthimthong et al., 2001). However, carbon dioxide is restricted by its inadequate solvating power for highly polar analytes, which can, to some extent, be boosted by using an appropriate modifier (Zima et al., 2004). SFE modifiers such as ethanol are introduced at the levels of 1–10%; large modifier concentrations (10 – 50%) are also of interest in some applications. Optimization of the operating conditions such as pressure, percentage of modifier, the fluid pressure and temperature and the extraction time are generally considered as the most important factors for good recoveries (Goli et al., 2005).

The results of comparison of different extraction procedures for isolation of the antioxidant 5,8 dihydroxycoumarin from sweet grass (*Hierochlo¨e odorata*) were done by Grigonisa, et al., 2005. High yield of 0.58% and concentration of 40.4% were obtained for Soxhlet extraction. But this method of extraction is not always acceptable for Industrial applications due to long extraction time, large consumption of hazardous solvents. SFE extraction could be an alternative, as giving second best compound yield of 0.46% and a concentration of 20.3%. MAE was less successful because of lower 5, 8 dihydroxycoumarin extraction yield of 0.30%. When comparing extraction time it can be noted that MAE requires 15 min where-as SFE requires 1 to 2 h. However the former requires some time for extract to cool down; consequently total MAE and extract cooling time becomes longer. Soxhlet and microwave assisted extraction are unsuitable for thermolabile antioxidants. The major disadvantage of high modifier consumption in SFE can be significantly reduced by recycling. Therefore, SFE can be recommended as a suitable method to isolate antioxidants.

For medicinal plants the use of sampling techniques such as Soxhlet extraction, microwave assisted extraction (MAE), or pressurized liquid extraction (PLE) often results in non-selective extraction of relatively large amounts of undesirable components (e.g. lipids, sterols, chlorophylls), which can severely affect the quality of the product (Huie, 2002). Direct supercritical fluid extraction process, in which the chemicals are extracted directly from the plant matrices by the action of supercritical carbondioxide (Catchpole et al., 2002) is also in most cases is subjected to the additional clean-up procedures before the extract is made into useful product. Solid phase extraction is a popular and effective tool not only for the clean up, but also for the extraction and/or concentration of analytes.

Solid-phase extraction (SPE) is a simple preparation technique based on the principles used in liquid chromatography, in which the solubility and functional group interactions of sample, solvent, and adsorbent are optimized to affect the retention and elution (Sargenti and McNair, 1998). Moderately polar to polar analytes are extracted from non-polar solutions onto polar sorbents. Sorbents for normal phase are modified with cyano-, diol- or amino groups. Non-polar to-moderately polar analytes are extracted from polar solutions onto non-polar sorbents (Tekel and Hatrik, 1996). Chemically modified adsorbent materials such as silica gel and synthetic resins enable precise group separation on the basis of different types of physicochemical interaction. Methods combining solid phase extraction with supercritical fluid technology namely indirect supercritical fluid extraction (Khundker et al., 1995) have been employed to extract phytochemicals from aqueous matrices and are found to result in higher yield, concentration and purity of the antioxidant.

Future prospects

Cancer, cardiovascular diseases, diabetes etc., are the major diseases faced by humanity. Antioxidants are found to be preventive against these diseases. Plants are the major source of antioxidants. However, they can be manufactured synthetically. Synthetic antioxidant gives side effects when taken *in vivo*. The latest trend of returning to the natural sources for health and medicine has created a lot of development in the recovery of antioxidants from plants. Even though there are many extraction methodologies and techniques for the separation of antioxidants from plants, each one has its own advantage and disadvantage. Therefore, the choosing of an appropriate technology which is feasible at an industrial scale, economical and at the same time yielding higher concentration and purity of antioxidants is required. Indirect supercritical fluid extraction satisfies these criteria. Appropriate process parameters such as tempe-rature, pressure and exact modifier have to be chosen to get the desired results.

Conclusions

Reactive oxygen species (ROS) or oxidants formed in our body due to exogenous and endogenous factors are found to be responsible for many diseases. Day to day research is revealing the potential of phytochemical antioxidants as health benefactors. This is due to their ability to neutralize the free radicals or reactive oxygen species or oxidants responsible for the onset of cell damage. Synthetic antioxidants are found to be harmful to the health. Most of the natural antioxidants from plant source are safer to health and have better antioxidant activity. Various processes of extraction such as Soxhlet extraction, subcritical water extraction (SWE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE) are used to obtain antioxidants from plant matrices. Solid phase extraction with supercritical fluid technology or indirect supercritical fluid extraction is found to be an industrially feasible process.

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