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Review Article

ANTIOXIDANT PROPERTIES OF SOME PHYTOCOMPOUNDS OF SELECTED MEDICINAL PLANTS

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Abstract

Free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed in living organisms and has both beneficial and deleterious role. Moderate concentrations of free radicals are involved in physiological functions whereas excess production is highly toxic to cells resulting in oxidative stress. Free radicals have been implicated in the pathology of various human diseases like diabetes, cancer, inflammation, atherosclerosis, neurodegenerative diseases, aging process, etc. The excess ROS/RNS formed are effectively removed from our body by antioxidant defense systems. Antioxidants are emerging as both prophylactic and therapeutic agents. They are produced either endogenously or received exogenously and include both enzymatic and non-enzymatic antioxidants. Antioxidants act in several ways including scavenging reactive oxygen species or their precursors, inhibiting ROS formation and lipid peroxidation, chelating metal ions needed for catalysis of ROS generation and reducing ability. Various *in vitro* methods have been developed to evaluate the antioxidant activity of compounds. The present article briefly reviews i) the generation and activities of ROS and RNS ii) oxidative damage and diseases caused by free radicals iii) defense mechanisms against free radicals iii) some medicinal plants reported to possess antioxidant activity and iv) the different *in vitro* antioxidant screening models.

Keywords: Free radicals, oxidative stress, antioxidants, human disease, medicinal plants, Phytocompounds, Structure activity relationship.

Introduction

Free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism, which are formed and degraded by all aerobic organisms. A free radical is defined as any atom or molecule possessing one or more unpaired electrons in atomic or molecular orbital. They are generally unstable and highly energized reactive chemical species which participate in hydrogen abstraction, radical addition, bond scission and annihilation reactions (Fang *et al.,* 2002). Free radicals can be formed in living organisms by both endogenous and exogenous sources. Endogenous sources of free radicals include mitochondrial electron transport chain, pro-oxidative enzyme systems, lipid peroxidation, inflammation, peroxisomes, glycoxidation, auto-oxidation of aminoacids, catecholamines, haemoglobin, ischemia-reperfusion injury and stimulation of polymorphonuclear leucocytes and macrophages due to respiratory burst. The exogenous sources include electromagnetic radiation, cosmic radiation, ultraviolet radiation, ozone, tobacco smoke, automobile exhaust fumes, air pollutants, industrial effluents, fungal toxins, organic solvents, pesticides, herbicides, etc. (Irshad and Chaudhuri, 2002).

Free radicals play a dual role as both beneficial and deleterious species, leading to either physiological concentration required for normal cell function, or in excessive quantities leading to oxidative stress. Vital beneficial cellular functions of ROS/RNS occur at

low/moderate concentrations and have been demonstrated in gene expression, activation of nuclear transcription factors, intracellular signal transduction, redox regulation, induction of mitogenic response, cellular responses to noxia and defense mechanism against microbial infections. Superoxide anion and hydrogen peroxide serves as cell growth regulators. Singlet oxygen induces physiological inflammatory response by attacking various pathogens. Nitric oxide acts as a neurotransmitter and mediator of immune response. It acts as a signaling molecule and participates in regulation of transcription factors, regulating the relaxation and proliferation of vascular smooth muscle cells, leucocyte adhesion, platelet aggregation, angiogenesis, thrombosis, vascular tone and hemodynamics (Droge, 2002). In contrast, the harmful effect due to the overproduction of ROS/RNS causing potential biological damage thus exceeding the total antioxidant activity in the body is termed as oxidative stress or nitrosative stress respectively. This occurs due to an imbalance between prooxidant/antioxidant system in living organisms where there is overproduction of free radicals on one side and deficiency of enzymatic/nonenzymatic antioxidants on the other side (Nordberg and Arner, 2001).

Excess ROS can damage cellular lipids, proteins and DNA, thus causing a wide range of toxic oxidative reactions like initiation of the peroxidation of the membrane lipids leading to the accumulation of lipid peroxides, direct inhibition of mitochondrial respiratory chain enzymes, fragmentation or random cross linking of molecules like DNA, enzymes and proteins which ultimately leads to cell death . In short, free radicals can attack most biological substrates from large macromolecules to smaller molecules. Oxidative stress has been increasingly recognized to play a pivotal role in influencing the pathophysiology of critical illness like cancer, diabetes, cardiovascular diseases, diabetes, neurodegenerative diseases, aging, etc. (Bandopadhyay *et al*., 1999). A delicate balance between beneficial and harmful effects of free radicals is achieved by a process called “redox regulation”. This mechanism protects living organisms from various oxidative insults and thus maintains “redox homeostasis” *in vivo.*

Reactive Oxygen Species (ROS)

Oxygen is vital for aerobic life processes. Molecular oxygen, O_2, is a biradical with two unpaired electrons. It has a unique electronic configuration, but does not exhibit extreme reactivity due to quantum-mechanical restrictions. Mitochondria consume more than 90% of inhaled oxygen and thus the mitochondrial electron transport chain is the main source of ATP in the mammalian cell which is essential for life. Mitochondria generate energy through chain reactions by reduction of oxygen with four electrons resulting in the formation of water, which can be depicted as follows,

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

The main source of reactive oxygen species in aerobic living organisms is mitochondria. About 5% of the oxygen consumed by mitochondria is reduced and converted to ROS. During energy transduction, a small number of electrons leak prematurely and directly react with oxygen to form reactive oxygen species. Free radicals derived from oxygen represent the most important class of radical species generated in living systems (Cadenas and Davies, 2000). They can be classified into oxygen-centered radicals and oxygen-centered non radicals. The primary oxygen-centered free radicals are superoxide anion ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), hydroperoxyl ($OOH^{\cdot}$), peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals. The oxygen-centered non free radicals are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($^1O_2$). These reactive intermediates are collectively termed as reactive oxygen species (ROS). The addition of one electron to an oxygen molecule results in the formation of superoxide anion radical ($O_2^{\cdot-}$) which is considered as the “primary ROS” which can react with other molecules to generate “secondary ROS”. The two electron reduction product of oxygen, when fully protonated, forms hydrogen peroxide (H_2O_2). This process can be represented as follows,

$$O_2 \xrightarrow{e^-} O_2^{\cdot-} \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} OH^{\cdot} \xrightarrow{e^-} H_2O$$

Superoxide anion radical ($O_2^{\cdot-}$)

Superoxide anion is a reduced form of molecular oxygen, created by the addition of an electron. It is the initial free radical produced within the inner mitochondrial membrane of a cell during electron transport system. It is formed spontaneously in the electron-rich aerobic environment of the respiratory chain. In spite of being a free radical, $O_2^{\cdot-}$ is not highly reactive due to its charged state and lacks the ability to penetrate a biological membrane, with the exception of erythrocyte membrane which has an ‘anion channel’ that helps in its penetration. It is therefore enclosed in the compartment where it is produced. Superoxide anion radical regulates metabolites capable of signaling and communicating important informations to the cellular genetic machinery. It is produced endogenously by flavoenzymes like xanthine oxidase activated in a number of different tissue injuries. Xanthine oxidase produced during ischemia-reperfusion, acts on xanthine/hypoxanthine to generate $O_2^{\cdot-}$ and H_2O_2. A deliberate high level of $O_2^{\cdot-}$ is produced from NADPH-dependent oxidase, a membrane associated-enzyme complex present in phagocytic cells such as neutrophils. Cytochrome P-450, P-450

reductase and cytochrome *b*-5 reductase in the endoplasmic reticulum and also enzymes like lipooxygenase and cyclooxygenase under certain conditions generates $O_2^{\cdot-}$ during their catalytic cycles. Over production of $O_2^{\cdot-}$ takes place in various chronic inflammatory cases, induced by drug, toxin, stress, tissue injury and heavy exercises (Valko *et al.,* 2006).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is a non-radical oxygen species which is the least reactive molecule among ROS. It acts as an intracellular signaling molecule. H_2O_2 is mainly generated from superoxide anion through a dismutation reaction by the enzyme superoxide dismutase. Other enzymes which produce H_2O_2 are peroxisomal oxidase, flavoproteins like xanthine oxidase, as well as NADPH oxidase, glucose oxidase, L-hydroxy acid oxidase, fatty acyl oxidase and D-aminoacid oxidase. It is relatively stable and hence long-lived under physiological pH and temperature in the absence of metal ions. It is poorly reactive because of its weak oxidizing and reducing property (McCord JM, 2000). In a transition-metal-free system, H_2O_2 shows limited toxicity. H_2O_2 is highly diffusible and easily crosses the cell membrane and plays a radical forming role as an intermediate in the production of free radicals via oxidation of transition metal ions. Hydrogen peroxide can generate the highly reactive hydroxyl radicals in the presence of superoxide anion and metal ions (Leonard *et al.,* 2004).

Hydroxyl radical ($OH^{\cdot}$)

The hydroxyl radical is the neutral form of the hydroxide ion. It is the most reactive and very dangerous oxy radical because of its highest 1-electron reduction potential. It has a very short half-life of approximately 10^{-9}s. Due to its strong reactivity, it can react with everything in living organisms and causes more damage to biological membranes than any other ROS. Hydroxyl radical is formed *in vivo* from superoxide anion and hydrogen peroxide in the presence of trace amounts of transition metal ions like iron or copper (Liochev *et al*., 2002). The highly toxic hydroxyl radical can cleave covalent bonds in lipid, polypeptides, proteins and DNA, especially thiamine and guanosine (Valko *et al*., 2005).

Peroxyl and alkoxyl radicals

Peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals are the additional reactive radicals derived from oxygen in the living systems (DeGrey, 2002). They are good oxidizing agents and are formed by decomposition of alkyl peroxides (ROOH), irradiation of UV light, homolysis of peroxides in the presence of transition metal ions and direct reaction of oxygen with alkyl radicals.

Singlet oxygen ($^1O_2$)

Singlet oxygen is a non-radical and rather mild oxidant compared with other ROS. It is highly reactive towards any molecule with π electrons or lone pairs of low ionization energy. $^1O_2$ can be formed from hydrogen peroxide, which reacts with either superoxide anion or HOCl in living tissues. It is involved in the oxidation and degradation of cholesterol and acts against various microorganisms and cancer cells (Stief, 2003).

Reactive Nitrogen Species (RNS)

Nitrosative stress is the overproduction of reactive nitrogen species (RNS) like nitric oxide ($NO^{\cdot}$), nitric dioxide ($NO_2^{\cdot}$) and peroxynitrite ($ONOO^{\cdot}$). This condition can initiate a series of nitrosylation reactions that can alter the structure of proteins leading to normal cellular dysfunction (Bergendi et al., 1999).

Nitric oxide ($NO^{\cdot}$)

Nitric oxide is a small lipophilic molecule with a single unpaired electron. It is synthesized in biological systems by a family of enzymes termed nitric oxide synthases (NOSs), which metabolize L-arginine to L-citrulline (Ghafourifar and Cadenas, 2005).

$$\text{L-arginine} + O_2 + \text{NADPH} \rightarrow \text{L-citrulline} + NO^{\cdot} + NADP^{+}$$

$NO^{\cdot}$ acts as an important biological signaling molecule as it rapidly undergoes addition, substitution, redox and chain terminating reactions. In the extracellular milieu, it reacts with oxygen and water to form nitrate and nitrite anions. Nitric oxide acts as a 'double edged sword' in health and disease (Bredt, 1999). Its main physiological role includes smooth muscle relaxation, blood pressure regulation, intracellular messenger by stimulating guanyl cyclase and protein kinases, defense mechanisms and immune regulation. Excess $NO^{\cdot}$ produced in conditions like ischemia-reperfusion, neurodegenerative and chronic inflammatory diseases is eliminated from our body by conjugation with low molecular weight thiols like glutathione and cysteine. Increased nitric oxide production occurs in septic shock, eclampsia, bronchial asthma, arthritis and ulcerative colitis. Excess $NO^{\cdot}$ is believed to be involved in various pathophysiological conditions like ischemia, stroke, gastrointestinal dysfunctions, achalasia, congenital hypertrophic pyloric stenosis, etc. (Brown and Borutaite, 2001)

Nitric dioxide ($NO_2^{\cdot}$)

Nitric oxide is mainly formed from the reaction of peroxyl radical and $NO^{\cdot}$, and in addition from tobacco smoke and polluted air. It initiates lipid peroxidation by abstraction of labile hydrogen atoms from the double bonds and thus generates free radicals (Ridnour et al., 2004).

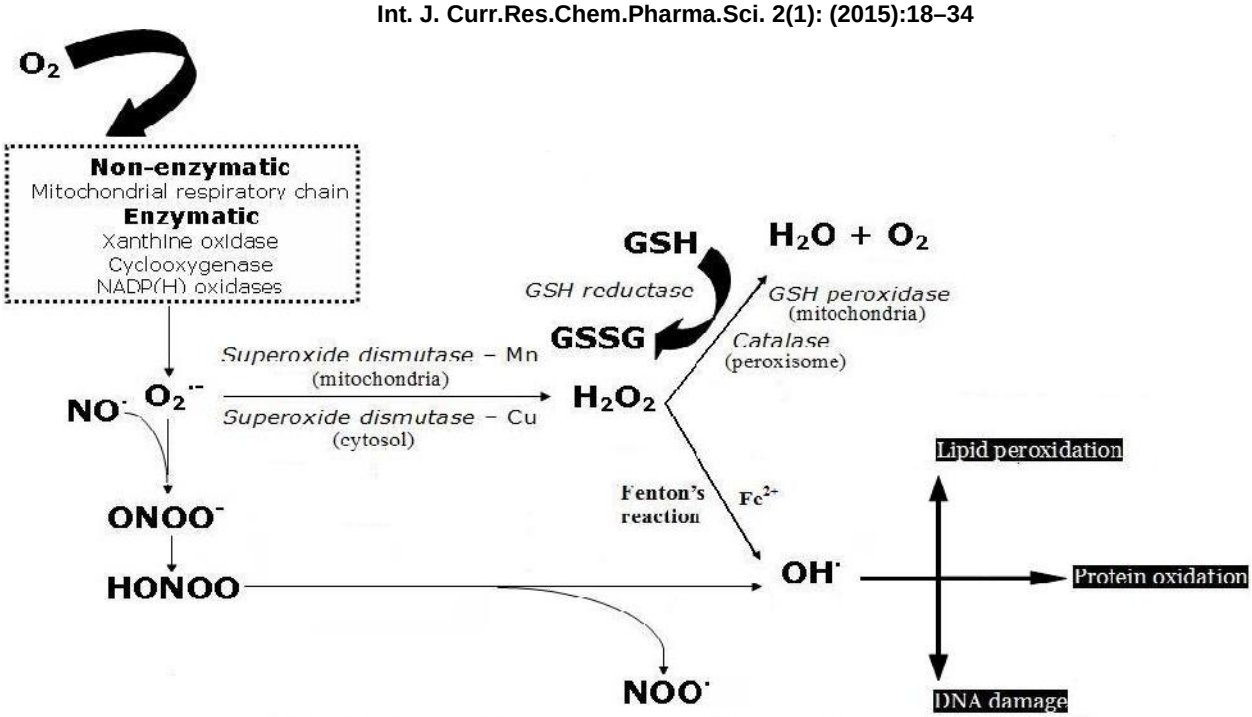

Fig. 1 Free radicals – generation and effects

Peroxynitrite ($ONOO^-$)

Peroxynitrite is a potent oxidizing agent similar to hydroxyl radical. It is highly cytotoxic due to its high diffusibility across cell membranes. It acts as an important tissue-damaging species generated during inflammation, neurodegeneration and renal disorders. During oxidative burst, nitric oxide and superoxide anion react together to produce significant amounts of peroxynitrite anion.

$$NO^{\cdot} + O_2^{\cdot -} \rightarrow ONOO^-$$

$ONOO^-$ can combine with aminoacids to form nitrotyrosine, which is associated with age-related disorders. Peroxynitrite can cause oxidation of proteins, LDL and DNA bases resulting in oxidative stress. This may cause cell death and tissue damage causing neurological disorders and stroke, arthritis, inflammatory bowel disease, toxic shock and ischemia-reperfusion injury (Virag et al., 2003).

Oxidative damage due to free radicals

Oxidative stress is defined as a disruption of the prooxidant-antioxidant balance in favour of the former, leading to potential damage. It is a result of one of the three factors: an increase in reactive oxygen species (ROS), an impairment of antioxidant defense system or an insufficient capacity to repair oxidative damage. Free radicals are known to play a definite role in a wide variety of pathological manifestations. Humans are constantly exposed to free radicals created by external sources from the environment or man made and by internal cellular metabolism (Jalili *et al*., 2007). These radicals can cause structural alterations and damage to cardinal cellular components by increasing the vascular permeability, enhancing the production of proinflammatory cytokines (TNF-α and cytokines), chemotactic factors like leukotrienes, provoke lipid peroxidation and oxidation of proteins and DNA. Polyunsaturated fatty acids are particularly vulnerable to free radical attack, because the double bonds within the cell membranes allow easy removal of hydrogen ion by ROS such as $OH^{\cdot}$ radicals (Ilavarasan *et al*., 2005). Free radicals can also damage proteins and nucleic acids leading to subsequent cell death by mode of necrosis or apoptosis. Cells normally have a number of mechanisms acting to defend against damage induced by free radicals. Problems occur when production of ROS exceeds their elimination by the antioxidant protective system or when the latter is damaged (Gilgun-Sherki *et al*., 2002).

Lipid peroxidation (LPO)

Lipid peroxidation is an autocatalytic, self-perpetuating and widespread process, which is a common cause of cell death (Bandhyopadhyay *et al*., 1999). It is a free radical chain reaction initiated by hydroxyl, alkoxy and peroxyl radicals and not by superoxide anion and hydrogen peroxide. The hydroxyl radical reacts with polyunsaturated fatty acid moieties (PUFA) of cell membrane phospholipids and produce lipid hydroperoxides (LH).

LH can be further decomposed to produce alkoxy and peroxy radicals. The peroxyl radicals are reaction initiators and undergo cyclisation reaction to yield endoperoxides (the precursors of malondialdehyde). The end products of lipid peroxidation are the toxic compounds like malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) whose involvement in various diseases has been suggested, mainly due to its cross linking ability (Nordmann, 1994). Malondialdehyde (MDA) is the major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acids (PUFA).

Protein oxidation

Protein oxidation is mainly initiated by the hydroxyl radicals leading to protein-protein cross linkage, oxidation of aminoacid side chains and protein fragmentation. Cysteine and methionine, the side chains of aminoacid residues of proteins are particularly susceptible to oxidation by the action of ROS/RNS. The thiol groups present in the proteins (-SH) undergoes reversible formation of mixed disulphides with the low molecular weight thiols (particularly GSH) due to the oxidation of cysteine residues. Free radical-mediated protein oxidation causes an increase in the concentration of reactive carbonyls, methionine sulfoxide, 2-oxohistidine and protein peroxides and a significant decrease in protein sulphydryls (Stadtman, 2000; Dalle-Donne et al., 2003).

DNA damage

Free radical-mediated reactions can cause structural alterations in DNA: both nuclear and mitochondrial by nicking, base-pair mutations, rearrangements, insertions and deletions (Marnett, 2000). Different free radicals affect DNA in different ways. Hydrogen peroxide does not react with DNA bases at all. The hydroxyl radical reacts with all four DNA bases and generates a multiplicity of products. Superoxide anion selectively attacks guanine. The most commonly produced DNA base lesion and extensively measured indicator of DNA damage is 8-hydroxyguanine, formed from guanine by reactive oxygen species. Altered DNA can be repaired by DNA glycosylase. However, increased concentrations of oxidized DNA bases occur under oxidative stress, leading to aging, mutagenesis and/or carcinogenesis (Valko *et al*., 2004).

Diseases caused by free radicals

Diseases caused by free radicals may be due to conditions like ‘mitochondrial oxidative stress’ or ‘inflammatory oxidative stress’. In addition, xanthine oxidase-induced formation of reactive oxygen species has been associated in ischemia-reperfusion injury. Oxidative stress has been implicated in the pathology of chronic inflammatory diseases such as rheumatoid arthritis as well as other diseases like diabetes, cancer, cardiovascular diseases, ischemia-reperfusion injury, neurodegenerative diseases, liver disorders, aging, etc. (Sati *et al*., 2010).

Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disorder linked predominantly with the formation of free radicals at the site of inflammation (Bauerova and Bezek, 1999). The enzyme xanthine oxidase (XO) catalyses the oxidation of hypoxanthine to xanthine and then to uric acid, the final reactions in the metabolism of purine bases. It causes chronic inflammation of the joints with infiltration of macrophages and deposition of monosodium urate crystals leading to gouty arthritis and uric acid nephrolithiasis. There is an increase in the intracellular levels of prostaglandins and other mediators and a decrease in glutathione level in the synovial fluid of patients with rheumatoid arthritis. This may be due to the abnormal induction of redox-signaling pathways (Liote, 2003).

Diabetes

One of the major causes of the long term complications of diabetes mellitus may be due to the occurrence of increased oxidative stress in diabetic individuals (Niedowicz and Daleke, 2005). A variety of sources like glucose autooxidation, oxidative phosphorylation, NAD(P)H oxidase, lipooxygenase and nitric oxide synthase stimulates the increased production of reactive oxygen species during hyperglycemia. Xanthine oxidase has also been proposed as one of the major source of ROS in diabetes. In addition, there is an increase in the reactive nitrogen species leading to nitrosative stress. Thus, elevated levels of ROS and RNS leads to the accumulation of MDA and isoprostanes (non-enzymatic product of arachidonic acid oxidation) causing depletion in both enzymatic and non-enzymatic antioxidants resulting in cell damage (Li and Shah, 2003).

Cancer

Oxidative stress induces a cellular redox imbalance in various cancer cells leading to permanent modification of genetic material by oncogenic stimulation. DNA mutation is a critical step in carcinogenesis and there is an elevated level of 8-hydroxy guanine (8-OH-G), the most extensively studied DNA lesion. ROS-induced DNA damage involves DNA strand break, base modification, replication errors and DNA cross-

links (Kovacic and Jacintho, 2001). In addition to ROS, RNS such as peroxynitrites and nitrogen oxides have also been implicated in DNA damage. Peroxynitrite reacts with guanine to form 8-nitroguanine, which causes stable RNA lesion. Apart from DNA damage, the end products of lipid peroxidation namely, malondialdehyde and 4-hydroxynonenal reacts with DNA bases to form adducts, which are proved to be mutagenic and carcinogenic in bacterial and mammalian cells (Marnett, 1999). A large number of studies have established an association between the incidence of cancer and depletion of antioxidant enzymes, most frequently being glutathione *S*-transferases (Pastore et al., 2003).

Cardiovascular diseases

ROS-induced oxidative stress has been linked with various cardiovascular diseases such as atherosclerosis, ischemic heart disease, cardiomyopathy, congestive cardiac failure, cardiac hypertrophy and hypertension (Dhalla et al., 2000). The main source of oxidative stress in heart involves the enzymes xanthine oxidase, NADPH oxidase and nitric oxide synthases. Increased formation of ROS modifies phospholipids and proteins leading to lipid peroxidation and oxidation of thiol groups. Thus there is a change in membrane permeability, lipid bilayer disruption and functional modification of various cellular proteins including abnormalities in myocyte function. Development of atherosclerosis involves the iron-catalysed formation of free radicals through the Fenton reaction. Another factor participating in atherosclerosis is calcium-overload induced production of ROS/RNS. Superoxide anion and peroxynitrite is known to initiate lipid peroxidation or lipoprotein oxidation. Studies suggest the etiology of ROS ($O_2^{\cdot-}$ and H_2O_2) -induced oxidative stress and thus a decrease in the levels of antioxidants in the pathogenesis of hypertension (Molavi and Mehta, 2004).

Ischemia-reperfusion injury

It is a problem which damages the myocardium following blood reperfusion after a critical period of coronary occlusion. Mitochondria serve as a source for the massive generation of ROS during ischemia/reperfusion, thus leading to serious tissue injury. Under these conditions, xanthine dehydrogenase is converted into xanthine oxidase, leading to the accumulation of purine metabolites, hypoxanthine and xanthine, which subsequently produce enormous amounts of superoxide anion radical and hydrogen peroxide (Becker, 2004).

Neurodegenerative diseases

Due to high oxygen consumption, low antioxidant status (particularly glutathione), high content of oxidisable polyunsaturated fatty acids (PUFA) and presence of redox active metals like iron and copper in the CNS, the brain appears to be particularly vulnerable to oxidative stress. This definitely plays an important role in the genesis and progression of Alzheimer's disease, Parkinson's disease, Down syndrome, brain tumour and other neurodegenerative diseases. Increased oxidative stress in brain may be due to increased i) free radical production ii) lipid peroxidation, iii) redox active metals iv) protein and DNA oxidation. On the other hand, a trigger caused by endogenous (genetic) and exogenous (environmental) factors results in oxidative and nitrosative stress, which in turn lead to cerebral damage (Sayre et al., 2001).

Liver disorders

Several studies suggest the involvement of free radicals in the pathogenesis of liver injury. ROS and lipid peroxidative metabolites formed during acute and chronic liver diseases damage hepatocytes leading to severe necrosis, sepsis or endotoxemia. During hepatotoxicity, superoxide anion may be formed from mitochondrial dysfunction and increased activity of NADPH-oxidase leading to tyrosine nitration. The toxicity is further mediated by oxidants such as peroxides, peroxynitrite, increased calcium, and decreased ATP. Also it has been suggested that the levels of enzymatic and non-enzymatic antioxidants are significantly reduced in liver diseases. Hepatotoxicity appears to be critically dependent on the depletion of cellular glutathione. A relatively high reduction in the intracellular level of reduced glutathione and an alteration in calcium homeostasis cause liver damage (Irshad et al., 2002).

Aging

Aging is the progressive decline in the physiological functions of an individual. It has been proved that free radical-mediated oxidative damage plays an important role in the process of aging and age-related diseases. Many animal studies suggest that aging is frequently associated with the accumulation of oxidized forms of proteins, lipids and DNA with increased levels of $O_2^{\cdot-}$, H_2O_2, hydroxyl and peroxyl radicals mediated oxidative damage. These radicals are capable of causing apoptosis, necrosis and cell death (Stadtman, 2004).

Defense mechanisms against free radicals

A series of defense mechanisms has been developed by living organisms against exposure to free radicals.

Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by protecting the antioxidant defense mechanisms or scavenging the reactive oxygen species . Catalytic removal of free radicals by antioxidant enzymes is the primary defense against ROS/RNS and free radical scavengers acts as second line defense and hence can be classified into enzymatic and non-enzymatic antioxidants respectively (Cadenas, 1997). The enzymatic antioxidants are produced endogenously and include superoxide dismutase, catalase, and glutathione peroxidase. The non-enzymatic antioxidants include reduced glutathione (GSH), antioxidant vitamins, minerals, co-factors and phytochemicals which are obtained from natural plant sources (Lee et al., 2004). Antioxidants can greatly reduce the damage due to oxidants by neutralizing the free radicals before they can attack the cells and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA (Fang et al., 2002). A wide range of antioxidants from both natural and synthetic origin has been proposed for use in the treatment of various human diseases (Cuzzocrea et al., 2001). There are some synthetic antioxidant compounds such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary butylhydroquinone which are commonly used in processed foods. However, it has been suggested that these compounds have shown toxic effects like liver damage and mutagenesis . Flavonoids and other phenolic compounds of plant origin have been reported as scavengers of free radicals (Rice-Evans *et al*., 1997). Hence, nowadays search for natural antioxidant source is gaining much importance (Kirthikar and Basu, 1987).

Enzymatic antioxidants

The term antioxidant has been defined as any substance that delays or inhibits oxidative damage to a target molecule. The first line antioxidant defense enzymes (primary enzymes) against free radicals are superoxide dismutase (SOD), glutathione peroxides (GPx), and catalase (CAT). The secondary antioxidant enzyme is glutathione reductase (GSSH) (Mates et al., 1999).

Superoxide dismutase (SOD)

Under normal circumstances, formation of superoxide anion is kept under tight control by SOD enzymes. SOD is a metalloprotein found in both prokaryotic and eukaryotic cells. It converts superoxide to hydrogen peroxide (H_2O_2) and represents the first line of defense against oxygen toxicity.

$$2O_2^{\cdot-} + 2H \rightarrow H_2O_2 + O_2$$

Three forms of SOD have been described, namely Cu-Zn-SOD, Mn-SOD, and Fe-SOD. The first isoform, containing copper and zinc at its active site (Cu/Zn SOD), is found in the cytoplasm of most eukaryotic cells. A different form of Cu-Zn-SOD is found in extra cellular fluids, where it is called EC-SOD. The second isoform, containing manganese at its active site is Mn SOD-2 which is located in the mitochondrial matrix and bacteria. The third isoform, Fe-SOD is present in extracellular fluids such as plasma and in many aerobic bacteria. It was found that the traces of transition metal like copper, zinc and manganese are essential for maintaining the antioxidant activity of SOD because the metals in the enzyme reacts with $O_2^{\cdot-}$ and takes its electron. (Ray and Husain, 2002). SOD is considered to be a stress protein, which is synthesized in response to oxidative stress. SOD has been detected in a large number of tissues and is thought to protect the cell from damage caused by $O_2^{\cdot-}$ and OH radicals generated from the metal-catalysed interaction of $O_2^{\cdot-}$ with H_2O_2. Superoxide anion is the only known substrate for SOD.

Glutathione peroxidase (GPx)

Glutathione peroxidase enzyme is a well-known first line defense against oxidative stress, which requires glutathione as a co-factor. It is one of the major enzymes responsible for the degradation of hydrogen peroxide and organic peroxides in the brain. GPx catalyses the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) at the expense of H_2O_2 (Zhu et al., 2006). By its selenium dependency, GPx can be divided in two isoforms, Se-dependent GPx and Se-independent GPx. The former is a tetramer of MW 84000 with very high activity towards both H_2O_2 and organic hydroperoxides. It is found in both cytosol (70%) and mitochondria (30%) of various tissues. Since selenium is an integral component of GPx, the measurement of this enzyme has been used as a functional index of selenium level (Rotruck et al., 1973). GPx activity is reduced in selenium deficiency (Muller *et al*., 1984; Wendel *et al*., 1984).

Catalase (CAT)

Catalase is a heme containing tetrameric enzyme. It is localized mainly in the mitochondria and in sub-cellular respiratory organelles of most mammalian cells, where it catalyses the dismutation of hydrogen peroxide to water and molecular oxygen,

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

CAT is found to act 10^4 times faster than peroxidase. It has a molecular weight of about 2,40,000 and consists of four protein subunits, each containing a heme

Fe(III)-protoporphyrin group bound to its active site. One of the main antioxidative functions of catalase is to reduce the formation of hydroxyl radicals from hydrogen peroxide, via the Fenton reaction (Aebi, 1984). Catalase binds NADPH, which protects the enzyme from inactivation and thus increases its efficacy. GPx and CAT were found to be important in the inactivation of many environmental mutagens (Ray and Husain, 2002).

Glutathione reductase (GSSH)

Glutathione reductase is a NADPH-dependent flavoenzyme that converts oxidized glutathione (GSSG) to reduced glutathione (GSH) by the oxidation of NADH to NAD^+ (Teoh and Davies, 2002).

Non-enzymatic antioxidants

The non-enzymatic antioxidants include mainly, i) low molecular weight antioxidants like glutathione ii) antioxidant vitamins like vitamin A, C, E and K iii) minerals like manganese, zinc and copper iv) antioxidant cofactors like ubiquinone/coenzyme Q10 and v) phytochemicals like polyphenols, carotenoids, etc.(Irshad and Chaudhuri, 2002).

Glutathione (GSH)

Glutathione (gamma glutamyl cysteinylglycine) is the most abundant intracellular thiol-based oxyradical scavenger, prevalent in millimolar concentrations in all living aerobic cells. It is a ubiquitous tri-peptide synthesized in the liver from three aminoacids glutamate, glycine and cysteine by two ATP-dependent enzymatic reactions. GSH functions mainly as a sulfhydryl buffer and also play a critical role in the detoxification of free radicals like peroxides and electrophilic toxins via conjugation reactions (Shen et al., 2005).

Antioxidant vitamins

α-Tocopherol (vitamin E) is a fat-soluble vitamin known to be one of the most potent antioxidant present in biological membranes. It contains a hydroxyl group by which it reacts with unpaired electrons and can reduce organic peroxyl radicals to form the corresponding organic hydroperoxides and α-tocopherol radicals. The metabolite -tocopherol quinine, which is capable of redox cycling, is normally present in very low amounts intracellularly, but may be elevated at high levels of -tocopherol intake . Vitamin E protects cells from peroxidation of PUFA in membrane phospholipids and from oxidative damage of VLDL, LDL, smooth muscle cell proliferation, proteins and DNA and thus provides protection against atherosclerosis, Alzheimer's disease and carcinogenesis. The antioxidant mechanism of vitamin E may be due to the direct scavenging of superoxide anion, upregulation of antioxidant enzymes and inhibition of lipid peroxidation. Deficiency of vitamin E induces lipid peroxidation and a reduction in the activities of enzymatic antioxidants causing various disorders. This can be reversed by dietary vitamin E supplementation.

Ascorbic acid (vitamin C), a water soluble vitamin plays an important antioxidant role in physiological concentrations. It plays a protective effect against the formation of 8-hydroxyguanine in DNA and other free radical-induced oxidative damage. Ascorbate prevents lipid hydroperoxide formation in LDL by reducing α-tocopherol radicals formed upon reaction with lipid peroxyl radicals, thus preventing atherosclerotic plaque formation. The antioxidant mechanisms of ascorbic acid are due to the donation of electrons to lipid radicals, quenching of singlet oxygen and removal of molecular oxygen. It scavenges superoxide anion radical by forming semihydroascorbate radical, which is subsequently reduced by glutathione. Thus ascorbate and GSH act together to protect the cell against oxidative damage (Kaur and Kapoor, 2001).

Minerals

Minerals like copper, zinc, manganese, magnesium, and selenium play an important role in enzyme functions. The enzymatic antioxidants like Cu, Zn-SOD and Mn-SOD requires copper and zinc and manganese respectively for their activities. Hence, dietary deficiency of these minerals significantly decreases the enzyme activities leading to lipid peroxidation and mitochondrial dysfunction.

The enzyme glucose-6-phosphate dehydrogenase requires magnesium as a co-factor for the production of NADPH from $NADP^+$. The activity of glutathione reductase is markedly reduced in the deficiency of dietary magnesium leading to oxidation of proteins with increased content of protein carbonyls (Rock et al., 1995).

Selenium has been identified as an essential co-factor for various selenoproteins and glutathione peroxidase is a selenium-dependent enzyme. The activity of glutathione peroxidase is markedly reduced by 90% in the dietary deficiency of selenium leading to peroxidative damage and mitochondrial dysfunction .

Antioxidant cofactors

In mitochondria, ubiquinone (Co-enzyme Q10) participates in the electron transport chain and in the

plasma and cell membranes it functions as an antioxidant. CoQ10 exerts its antioxidant mechanism by inhibition of lipid peroxidation and reduction in mitochondrial oxidative stress.

Medicinal plants

A number of extracts and isolated compounds from plants have been reported to afford protection against free radical-induced oxidative damage in various experimental models. They are effective antioxidants for improving human health and prevent carcinogenesis, aging, cardiovascular complications and other diseases. Among them, phenolic and polyphenolic compounds (flavonoids and epicatechins) and carotenoids exhibit potent antioxidant activities.

Phytochemical constituents

Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, antimutagenic, anticarcinogenic, free radical scavenging activities and also decreases cardiovascular complications (Yen et al., 1993; Benzie, 2003). The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. Total phenolic assay by using Folin-Ciocalteu reagent is a simple, convenient and reproducible method. It is employed routinely in studying phenolic antioxidants (Huang et al., 2005).

Flavonoids are a group of polyphenolic compounds, which exhibit several biological effects such as antiinflammatory, antihepatotoxic, antiulcer, antiallergic, antiviral, anticancer activities. They also inhibit enzymes such as aldose reducatse and xanthine oxidase. Polyphenolic compounds exert potent antioxidant activity by scavenging the reactive oxygen species because of their phenolic hydroxyl groups and have metal chelating ability (Cao et al., 1997; Freitas et al., 2004). In view of their wide pharmacological and biological actions, they have a greater therapeutic potential (Narayana et al., 2001; Nijveldt et al., 2001).

Epicatechins (tea polyphenols) effectively scavenge reactive oxygen and nitrogen species by one electron transfer or hydrogen abstraction mechanisms and chelate metal ions like iron and copper (Frei and Higdon, 2003). They inhibit the formation of superoxide anion and hydroxyl radicals and degrade the lipid hydroperoxides and thus prevent the peroxidation of membrane lipids. Dietary supplementation of tea polyphenols decrease the serum concentrations of LDL and total cholesterol and increase the HDL level. They also inhibit the growth of several cancer cells *in vitro* and induce apoptosis (Babich et al., 2005).

Carotenoids, a group of tetraterpenoids are potent antioxidants of plant origin. They are most effective singlet oxygen quenchers in biological systems . Carotenoids scavenge superoxide anion radicals by transfer of either hydrogen atoms or electrons to the free radicals. The potent antioxidant activity of carotenoids is helpful in the prevention of free radical-induced diseases like cataract, age-related neurodegeneration, atherosclerosis and multiple sclerosis.

***In vitro* antioxidant screening models**

Antioxidant activity can be measured using various *in vitro* methods (Umamaheswari and Chatterjee, 2008; Sivashanmugam and Chatterjee, 2011). The chemistry behind these antioxidant assays is the ready donation of electrons to free radicals by antioxidants, which then quenches the ROS and produces more stable and less damaging species. The ability of the antioxidant molecule to remove any source of oxidative initiation can also be tested by inhibition of enzymes and chelation of transition metal ions. Some *in vitro* antioxidant screening models are described below, which are very popular and simple to perform. They can be widely used to evaluate and compare the activity of medicinal plants (Khanam *et al*., 2004).

DPPH radical scavenging assay

The free radical scavenging activity can be assayed by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay. It is one of the most widely used methods for screening of antioxidant activity of plant extracts (Mensor et al., 2001). DPPH is a stable, nitrogen-centered free radical which produces violet color in ethanol solution. It is reduced to a yellow colored product, diphenylpicryl hydrazine, with the addition of antioxidants. The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl groups (Nanjo et al., 1996).

Reducing power ability

The reducing power ability can be investigated by the transformation of Fe^{3+} to Fe^{2+} in the presence of antioxidant compounds (Fejes et al., 2000). The formation of Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The reducing ability of a compound generally depends on the presence of reductones (antioxidants), which exert the antioxidant activity by breaking the free radical chain by donating a hydrogen atom (Meir et al., 1995). Increase in absorbance of the reaction mixture indicates an increase in the antioxidant activity.

Medicinal plants reported for antioxidant activities

Botanical name	Family	Common name	Parts used	Assay method	Indications	Active constituents	References
Allium sativum	Alliaceae	Garlic	Leaves, bulb	*In vitro*	Lowers blood pressure, anticancer	Kaemferol, methionine	Shobana and Naidu, 2000
Annona squamosa	Annonaceae	Custard apple	Whole plant	*In vitro*	Epilepsy, dysentery, cardiac problems, fainting, worm infestation, constipation, haemorrhage, dysuria, fever, thirst, malignant tumours, ulcers, abortifacient	Rutin, hyperoside	Shirwaikar *et al.,* 2004
Anogeissus latifolia	Combretaceae	Axle-wood tree	Bark	*In vitro*	Skin diseases. Snake and scorpion bite, stomach diseases. Colic, cough and diarrhoea	Leucocyanidin, ellagic acid glycosides	Govindarajan *et al.,* 2004
Anthriscus cerefolium	Apiaceae	Chervil	Whole plant	*In vitro*	Circulatory disorders, haematinic, tonic	Methyl cavicol, apiin	Fejes *et al.,* 2000
Artemisia abyssinica	Compositae	Aathir	Whole plant	*In vitro*	Diabetes mellitus, constipation, cold, rheumatism	Diterpenes, sterols, volatile oils	Burits *et al.,* 2001
Artemisia afra	Compositae	African worm wood	Leaves	*In vitro*	Cough, cold, chills, stomachic, dyspepsia, purgative	Essential oils	Burits *et al.,* 2001
Artemisia apiacea	Compositae	---------	Whole plant	*In vivo*	Dermatomycosis, jaundice, alopecia, debubitus	Arteminin, artemicapin C, dausterol, apeginin, cacticin	Kim *et al.,* 2003
Aster tataricus	Compositae	Tatarian aster	Roots Rhizome	*In vitro*	Cough, expectorant, diuretic, antitumour	Epifriedelinol, astertarone A, astins A,B, C & J	Ng *et al.,* 2003
Camellia sinensis	Theaceae	Green tea	Leaves	*In vitro*	Pancreatitis, cardiovascular diseases, lowers LDL cholesterol	Catechin, epicatechin, epigallocatechin	Khalaf *et al.,* 2008
Cassia fistula	Caesalpinaceae	Golden shower	Whole plant	*In vitro*	Skin diseases, inflammation,jaundice, rheumatism, anorexia	Chrsophanol, chrysophanein	Ilavarasan *et al.,* 2005
Catharanthus roseus	Apocyanaceae	Periwinkle	Whole plant	*In vitro*	Anticancer	Vincristine, vinblastine	Ferreres *et al.,* 2008
Centenella asiatica	Umbelliferae	Marsh penny	Whole plant	*In vitro*	Brain tonic, memory enhancer, blood purifier, diuretic, sedative spasmolytic	Asiaticoside, Asiatic acid, brahmoside, madecassoside	Hamid *et al.,* 2002
Chelidonium majus	Papaveraceae	Greater celandine	Whole plant	*In vitro*	Spasmolytic, anti-inflammatory, antimicrobial, antiviral, antifungal	Berberine, alkaloidal components	Then *et al.,* 2003
Citrus aurantifolia	Rutaceae	Lime	Fruit	*In vitro*	Immune modulatory, prevents oxidation of fats and cholesterol	Ascorbic acid, pinene, eugenol	Murcia *et al.,* 2001

Coccinia grandis	Cucurbitaceae	Ivy gourd	Leaves, fruits, roots	*In vitro*	Diabetes mellitus, bronchitis, gout, skin diseases	Polyprenol, triterpenoids	Umamaheswari & Chatterjee, 2008
Curcuma longa	Zingiberaceae	Turmeric	Rhizome	*In vitro*	Respiratory disorders, dermatophytosis, anticarcinogenic, antifungal, antibacterial	Curcumin	Jayaprakasha *et al.,* 2002
Curcuma longa	Zingiberaceae	Turmeric	Rhizome	*In vivo*	Anti-inflammatory, prevents premature ageing	Curcumin, curcuminoids, turmerone	Selvam *et al.,* 1995
Cydonia vulgaris	Rosaceae	Quince	Leaves and fruits	*In vitro*	Cardiovascular diseases, haemorrhoids, bronchial asthma, cough, tranqullizer		Yildrim *et al.,* 2001
Daucus carrota	Umbelliferae	Carrrot	Leaves, seeds, roots	*In vitro*	Antitoxin, edema, arthritis, rheumatism	Eugenol, camphene, terpinene	Ravindra and Narayan, 2003
Elletaria cardamomum	Zingiberaceae	Cardamom	Fruits	*In vitro*	Skin infections, indigestion, cooling properties	Myrcene, pinene, sabinene, limonene, zingberene	Khalaf *et al.,* 2008
Eugenia caryophyllus	Myrtaceae	Clove bud	Inflorescence	*In vitro*	Local anaesthetic, antimicrobial, anticonvulsant, ovicidal	Caryophyllene oxide, eugenol, isoeugenol	Shobana and Naidu, 2000
Fagopyrum esculentum	Polygonaceae	Buckwheat	Leaves, seeds	*In vitro*	Antihaemorrhagic, hypotensive, circulatory disorders, haemorrhagic retinopathy	Rutin	Quettier-Deleu *et al.,* 2000
Ficus bengalensis	Moraceae	Banyan tree	Bark	*In vivo*	Atherosclerosis	Leucopelargonidin, leucocyanidin	Daniel *et al.,* 1998
Foeniculum vulgare	Umbelliferae	Fennel	Seeds	*In vitro*	Tranquilliser, tonic, soporific drug	Anethole, limonene	Oktay *et al.,* 2003
Ginkgo biloba	Ginkgoaceae	Ginkgo	Whole plant	*In vitro*	Peripheral vascular disease, cerebrovascular insufficiency in elderly	Ginkgogolide	Maitra *et al.,* 1995
Jasminum grandiflorum	Oleaceae	Jasmine	Whole plant	*In vitro*	Ulcerative stomatitis, skin diseases, ulcers, wouds, corns	Oleacin, sambenceins	Umamaheswari *et al.,* 2007
Juniperus procera	Compositae	Sareda	Aerial parts	*In vitro*	Ulcer, head ache, stomach disorder, intestinal worms	Essential oils	Burits *et al.,* 2001
Lagenaria siceraria	Cucurbitaceae	Bottle gourd	Fruits, seeds	*In vivo*	Cardiotonic, aphrodisiac, general tonic, liver tonic, antiinflammtory, expectorant	Fucosterol, compesterol, cucurbitacins, ellagitannins	Despande *et al.,* 2008
Lithospermum erythrorhizon	Boraginaceae	Gromwell	Roots	*In vitro*	Wound healing, antiviral, antifungal, antitumour, contraceptive	Deoxyshikonin, β-sitosterol, caffeic acid esters	Han *et al.,* 2008
Myristica fragans	Myrtaceae	Nutmeg	Seed, leaf	*In vitro*	Protects nervous system	Camphene, eugenol, isoeugenol	Mila *et al.,* 2006
Ocimun sanctum	Lamiaceae	Basil	Leaves, seeds	*In vitro*	Arthritis, muscular pain, rheumatism	ascorbic acid, β-carotene, β-sitosterol,	Hakkim *et al.,* 2007

						histidine, palmitic acid,	
Olea europaea	Oleaceae	Olive	Leaves	*In vitro*	Immunomodulatory, maintains sugar and cholesterol levels	Luteolin, kaempferol	Benavente-Garcia *et al.,* 2000
Origanum vulgare	Lamiaceae	Oregano	Leaves	*In vitro*	Immune booster	Thymoquinone, carvacrol, eugenol, thymol	Milos *et al.,* 2000
Piper betle	Piperaceae	Betel	Leaves	*In vitro*	Astringent, antiseptic, anthelmintic, antifungal, antibacterial	Chavicol, eugenol, anethole	Dasgupta and De, 2004
Piper nigrum	Piperaceae	Black pepper	Fruit	*In vitro*	Neuralgia, arthritis, poor circulation	Piperine, myristic acid	Khalaf *et al.,* 2008
Polygonum aviculare	Polygonaceae	Common knotweed	Whole plant	*In vitro*	Diuretic, gingivitis	Phenolic compounds	Hsu 2006
Pulchea indica	Asteraceae	English Ivy	Root	*In vitro*	Astringent, antipyretic, anti-inflammatory, antiulcer		Ghosh *et al.,*2008
Smilex china	Smilacaceae	China root	Roots	*In vitro*	Prevents weight loss, anabolic, antimutagenic, anticarcinogenic, chronic rheumatism, gout, syphilis, skin diseases, leprosy, epilepsy, insanity	Steroidal saponins	Tripathi *et al.,* 2001
Spartium junceum	Fabaceae	Spanish broom	Flowers	*In vitro*	Peptic ulcers	Spartitrioside	Yesilada *et al.,* 2000
Sphaeranthus indicus	Asteraceae	East Indian Globe Thistle	Whole plant	*In vitro*	Aphrodisiac, anthelmintic, cough, piles	β-ionene, sphaerene	Shirwaikar *et al.,* 2006
Tachigalia paniculata	Leguminoseae	Bergantin	Leaves	*In vitro*	Gout, rheumatic swellings	Myricetin glycosides, methyl gallate, isovanillin	Cioffi *et al.,* 2002
Tagetes erecta	Compositae	Aztec marigold	Flower heads	*In vitro*	Colic, emmenagouge, fungal infections	Essential oils	Gutierrez *et al.,* 2006
Teucrium chamaedrys	Lamiaceae	Wall germander	Aerial parts	*In vitro*	Coronary heart disease, cancer	Luteolin, apigenin, disometin	Panovska *et al.,* 2005
Teucrium montanum	Lamiaceae	Mountain germander	Aerial parts	*In vitro*	Antipyretic	Luteolin, apigenin, disometin	Panovska *et al.,* 2005
Teucrium polium	Lamiaceae	Poley	Aerial parts	*In vitro*	Antispasmodic, diuretic, diaphoretic	Luteolin, apigenin, disometin	Panovska *et al.,* 2005
Thymus vulgaris	Labiatae	Thyme	Whole plant	*In vitro*	Sprains, muscular pain, arthritis	Carvacrol, lycopene, myrcene	Kulisic *et al.,* 2005
Vitex negundo	Verbenaceae	Chaste tree	Root, flowers, bark, leaves	*In vivo*	Parasiticidal, vermifuge, pain reliever, expectorant, diuretic	Negundin A and B, vitrofolal, lyoniresinol	Tandon and Gupta, 2005
Vitis vinifera	Vitaceae	Grapes	Fruits, seeds	*In vitro*	Reduce LDL, cholesterol, high blood pressure, strengthens blood vessels and capillaries, immuno	Alanine, α-tocopherol, ascorbic acid, β-carotene, β-sitosterol,	Baydar *et al.,* 2007

					moduatory	histidine, palmitic acid, selenium,	
Withania somnifera	Solanaceae	Ashwa-ganda	Roots, leaves, berries	*In vitro*	Blood tonifier, prevents premature ageing, immune booster	Withaferin A, glycowithanolides	Bhattacharya *et al.,* 1997
Zingiber officinalis	Zingiberaceae	Ginger	Leaves, rhizome	*In vitro*	Anti caner	Gingerol	Khalaf *et al.,* 2008

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity can be measured by the ability of the antioxidants to inhibit the degradation of deoxyribose by the free radicals generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) (Ilavarasan et al., 2005). The free radical damage imposed on the substrate, deoxyribose was measured using the thiobarbituric acid test. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituric acid.

Hydrogen peroxide scavenging assay

Hydrogen peroxide itself is not particularly reactive with most biologically important molecules, but is an intracellular precursor of hydroxyl radicals which is very toxic to the cell. Thus, scavenging of H_2O_2 is a measure of the antioxidant activity of the plant extracts, which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralising it into water.

Nitric oxide radical scavenging assay

In vitro inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs. Nitric oxide is a free radical which plays an important role in the pathogenesis of pain, inflammation, etc. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent .The absorbance of the chromophore formed can be measured at 546 nm. Antioxidants can decrease the amount of nitrite generated from the decomposition of sodium nitroprusside by competing with oxygen to react with $NO^{\cdot}$ (Sreejayan *et al.,* 1997).

Thiocyanate method

The peroxyl radical scavenging activity of antioxidants can be determined by thiocyanate method (Yildirim et al., 1999). The antioxidant compound can be incubated with linoleic acid emulsion in dark at 37^{o}C and the amount of formed peroxides can be determined spectrophotometrically by measuring the absorbance at 500 nm (Yen and Chen, 1995). A decrease in absorbance indicates the antioxidant activity of plant extracts which might be due to the inactivation of the free radicals and the presence of flavonoid like phytochemicals.

Phosphomolybdate method

The total antioxidant capacity can be determined by the phosphomolybdenum assay which has been routinely used to evaluate the antioxidant activity of plant extracts (Hong et al., 1996; Prieto et al., 1997). In the presence of antioxidants, the Mo(VI) will be reduced to Mo(V) and forms a green coloured phosphomolybdenum V complex which shows maximum absorbance at 695 nm (Jayaprakasha et al., 2002).

Ferrous chelating ability

The ferrous chelating ability of the compounds can be monitored by measuring the formation of the ferrous ion-ferrozine complex. Ferrozine combines with ferrous ions forming a red coloured complex which absorbs at 562 nm (Yamaguchi et al., 2000). It was repored that the chelating agents who form σ bonds with a metal, are effective as secondary antioxidants, because they reduce the redox potential thereby stabilising the oxidised form of the metal ion involved in the peroxidation of lipids (Duh et al., 1999).

-carotene bleaching assay

The β-carotene bleaching assay is a commonly used model to analyze the antioxidant activity of the plant extracts because β-carotene is extremely sensitive to free radical mediated oxidation of linoleic acid. In this assay, oxidation of linoleic acid, an unsaturated fatty acid occurs due to the production of reactive oxygen species formed from halogenated water. Plant extracts with antioxidant activity inhibit β-carotene oxidation,

which could be related to the high level of phenolic compounds.

Bleomycin-dependent DNA damage

Bleomycin-dependent DNA damage has been adopted as a sensitive and specific method to examine the potential pro-oxidant drugs. Degradation of DNA occur if the samples to be tested reduces the bleomycin-Fe^{3+} to bleomycin-Fe^{2+} resulting in the formation of a product similar to MDA which reacts with TBA to give pink colour (Liu and Ng, 2000). Antioxidants will decrease the absorbance and bleomycin-Fe^{3+} is not converted into bleomycin-Fe^{2+}, thus preventing the DNA degradation.

Conclusion

As diccussed above, it is clear that ROS and RNS are generated spontaneously in living cells during metabolism and in low amounts they act as signaling species in various normal physiological processes. Excessive production of free radicals has been shown to modify biological molecules, which result in various pathological conditions. In recent years, studies are increasing in the field of free radical-induced oxidative damage in the etiology of human diseases like cancer, diabetes, rheumatoid arthritis, inflammation, cardiovascular diseases, neurodegenerative diseases, etc. Oxidative stress is a deleterious process that can be an important mediator of damage to cell structures which consequently leads to lipid peroxidation, oxidation of proteins, DNA damage and cellular necrosis resulting in cell death. To counter the harmful effect of ROS/RNS, antioxidant defense mechanism in humans, such as enzymatic and non-enzymatic antioxidants operates to detoxify or scavenge these free radicals. They neutralize free radicals by their radical scavenging ability, inhibition of lipid peroxidation, metal ion chelating ability and reducing capacity. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases. They have been found to play an important role in the non-enzymatic protection against oxidative stress. Studies on free radicals and free radical-mediated disorders will be helpful in designing suitable antioxidant therapy to control the ROS-mediated diseases.

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