Review of Literature

2. REVIEW OF LITERATURE

Free radicals and related species have attracted a great deal of attention of researchers. Free radicals can adversely alter lipids, proteins and DNA and have been implicated in aging and a number of human diseases. Nature has endowed us with protective antioxidant mechanisms (Genestra, 2007). In recent years, there has been an increased interest globally to identify antioxidant compounds that are pharmacologically potent and have low or no side effects for use in preventive medicine and in the food industry. As plants produce significant amounts of antioxidants to prevent the oxidative stress caused by photons and oxygen, they represent a potential source of new compounds with antioxidant activity (Ali *et al.*, 2008).

FREE RADICALS

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body's normal use of oxygen such as respiration and some cell-mediated immune functions. These free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust fumes, radiation, air pollutants, pesticides, etc. These exogenous pollutants generating free radicals have become part and parcel of our daily inhaling/ingesting life and in fact there appears no escape from them. Continuous interaction of the animal physiological systems with these free radicals generated either indigenously or inhaled/ingested from exogenous sources, therefore, lead to excess load of free radicals and cause cumulative damage of proteins, lipids, DNA, carbohydrates and membrane, resulting in the so-called oxidative stress (Valko *et al.*, 2007).

OXIDATIVE STRESS

Oxidative stress is correlated with a plethora of cellular alterations, including the accumulation of molecules damaged due to oxidation, increased levels of dysfunctional macromolecules and multiple compromises in cellular homeostasis. Oxidative stress occurs when there are insufficient levels of antioxidants to prevent reactive oxygen species (ROS) from promoting deleterious levels of oxidative damage. Examples of ROS include superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Each of these ROS is highly reactive and unstable due to the fact that they contain an unpaired electron in their outer electron shell. This conformation

promotes their ability to rapidly interact with cellular macromolecules such as proteins, lipids and nucleic acids. Thus, when cells are unable to sufficiently regulate the levels of ROS, or are unable to adequately remove or replace oxidized macromolecules, cellular dysfunction can occur via oxidative stress (Cecarini *et al.*, 2007).

Interestingly, the propensity or sensitivity of cells to undergo oxidative stress appears to be cell type specific, with cells exhibiting dramatic differences with regard to their sensitivity to accumulate oxidized molecules and undergo toxicity during periods of high ROS exposure. The basis for this cell type specificity is poorly understood but is clearly an important topic for aging, hepatic, cardiovascular, cancer and neuroscience research (Halliwell, 2006).

TYPES OF FREE RADICALS

SUPEROXIDE RADICAL ($O_2^{\bullet -}$)

Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalysed processes (Valko *et al.*, 2005). The production of superoxide occurs mostly within the mitochondria of a cell (Cadenas and Sies, 1998) and has been implicated in the pathophysiology of a variety of diseases (Kovacic *et al.*, 2005).

HYDROGEN PEROXIDE

Hydrogen peroxide (H_2O_2), although not a free radical by definition, is a biologically important oxidant because of its ability to generate the hydroxyl radical, an extremely potent radical. Further, because of its nonionized, low charged state and lipid solubility, H_2O_2 is able to diffuse across membranes (Yu, 1994). It is one of the most powerful oxidizers known in aqueous solution and can oxidize or reduce a variety of inorganic ions. H_2O_2 has a longer half-life than other ROS in biological systems (Genestra, 2007) and is even more toxic to cells than superoxide radicals, and must therefore be eliminated. The hydrogen peroxide is removed by various enzymes, such as catalase, several glutathione peroxidases (all of which are selenoenzymes) and cysteinyl peroxidase (Levander, 1987). Hydrogen peroxide (H_2O_2) can cause apoptosis in the cells of most animals from protozoa to mammals (Blanco *et al.*, 2005).

HYDROXYL RADICAL

The hydroxyl radical, $^{\bullet}OH$, is the neutral form of the hydroxide ion. The hydroxyl radical has high reactivity, making it a very dangerous radical with a very short *in vivo* half-life of approximately 10^{-9} seconds (Pastor *et al.*, 2000). Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions such as copper or iron.

$$O_2^{\bullet -} + H_2O_2 \rightarrow OH^{\bullet} + OH^{-} + O_2$$

In saturated compounds, a hydroxyl radical abstracts a hydrogen atom from the weakest C–H bond to yield a free radical. The resulting radicals can react with oxygen and generate other free radicals. Hydroxyl radicals react with lipid, polypeptides, proteins and DNA, especially thiamine and guanosine. Hydroxyl radicals also add readily to double bonds. The barrier to the addition of hydroxyl radicals to double bonds is less than that of hydrogen abstraction, so that in competition addition is often favoured. When a hydroxyl radical reacts with aromatic compounds, it can add on across a double bond, resulting in hydroxycyclohexadienyl radical. The resulting radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical, or decompose to phenoxyl-type radicals by water elimination (Lee *et al.*, 2004).

PEROXYL AND ALKOXYL RADICALS

Peroxyl radicals ($ROO^{\bullet}$) are formed by a direct reaction of oxygen with alkyl radicals ($R^{\bullet}$), for example, the reaction between lipid radicals and oxygen. Decomposition of alkyl peroxides (ROOH) also results in peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals. Irradiation of UV light or the presence of transition metal ions can cause homolysis of peroxides to produce peroxyl and alkoxyl radicals.

$$ROOH \rightarrow ROO^{\bullet} + H^{\bullet}$$

$$ROOH + Fe^{3+} \rightarrow ROO^{\bullet} + Fe^{2+} + H^{+}$$

Peroxyl and alkoxyl radicals can abstract hydrogen from other molecules with lower standard reduction potential. This reaction is frequently observed in the propagation stage of lipid peroxidation. Very often, the alkyl radical formed from this reaction can react with oxygen to form another peroxyl radical, resulting in a chain reaction. Some

peroxyl radicals break down to liberate superoxide anion or can react with each other to generate singlet oxygen. Aromatic alkoxyl and peroxyl radicals are less reactive than the respective open chain radicals because of the delocalization of electrons in the ring (Nishizawa *et al.*, 2005).

SINGLET OXYGEN

Singlet oxygen is a nonradical and excited status. Takayama and others (2001) reported that metastable phosphatidylcholine hydroperoxides present in the living organism produced singlet oxygen during their breakdown in the presence of Cu^{2+} in the dark. Singlet oxygen can be formed from hydrogen peroxide, which reacts with superoxide anion, or with HOCl or chloroamines in cells and tissues (Bhattacharjee, 2005).

Compared with other reactive oxygen species, singlet oxygen is rather mild and nontoxic to mammalian tissue (Tandon and Gupta, 2005). However, singlet oxygen has been known to be involved in cholesterol oxidation. Oxidation and degradation of cholesterol by singlet oxygen was observed to be accelerated by the co-presence of fatty acid methyl ester. In the human organism, singlet oxygen is both a signal and a weapon, with therapeutic potency against various pathogens such as microbes, viruses and cancer cells (Stief, 2003).

REACTIVE NITROGEN SPECIES (RNS)

Nitric oxide ($NO^{\bullet}$) is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation. Due to its extraordinary properties, in 1992 $NO^{\bullet}$ was acclaimed as the "molecule of the year" in Science Magazine (Koshland, 1992).

Since $NO^{\bullet}$ is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes. $NO^{\bullet}$ has effects on neuronal transmission as well as on synaptic plasticity in the central nervous system. In the extracellular milieu, $NO^{\bullet}$ reacts with oxygen and water to form nitrate and nitrite anions. Overproduction of reactive nitrogen species is called nitrosative stress (Klatt and Lamas, 2000; Ridnour

et al., 2004). This may occur when the generation of reactive nitrogen species in a system exceeds the system's ability to neutralise and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function (Agarwal *et al.*, 2005).

ANTIOXIDANT PROTECTION

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals. These components include:

- Nutrient-derived antioxidants like ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids and other low molecular weight compounds such as glutathione and lipoic acid
- Antioxidant enzymes, e.g., superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, which catalyze free radical quenching reactions
- Metal binding proteins, such as ferritin, lactoferrin, albumin and ceruloplasmin that sequester free iron and copper ions that are capable of catalyzing oxidative reactions
- Numerous other antioxidant phytonutrients present in a wide variety of plant foods (Valko *et al.*, 2007).

POSSIBLE MECHANISMS OF ANTIOXIDANT ACTION

Antioxidants function by several possible mechanisms as follows:

- Scavenging of free radicals involved in chain reactions (tocopherol acting in the lipid phase)
- Regeneration of other antioxidants (ascorbate reducing tocopheryloxy radical to tocopherol by donating an H atom)
- Reacting with initiating radicals or oxidants (catalase with hydrogen peroxide)
- Chelating or sequestering transition metal catalysts, which are pro-oxidants (albumin or polyphenols with cupric ion)

- Inhibiting or activating an enzyme (tocopherol and polyphenols inhibiting tyrosine kinase and ascorbate activating nitric oxide synthase) (Vinson, 2006).

ENZYMIC ANTIOXIDANTS

SUPEROXIDE DISMUTASE (SOD)

One of the most effective intracellular enzymatic antioxidants is superoxide dismutase (SOD) (EC 1.15.1.1). Superoxide dismutase is the antioxidant enzyme that catalyzes the dismutation of $O_2^{\bullet -}$ to O_2 and to the less-reactive species H_2O_2. While this enzyme was isolated as early as 1939, it was only in 1969 that McCord and Fridovich proved the antioxidant activity of SOD (McCord and Fridovich, 1969).

Superoxide dismutase exists in several isoforms, differing in the nature of the active metal centre and amino acid constituency, as well as their number of subunits, cofactors and other features. In humans, there are three forms of SOD: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD and extracellular SOD (EC-SOD) (Landis and Tower, 2005).

SOD destroys $O_2^{\bullet -}$ with remarkably high reaction rates, by successive oxidation and reduction of the transition metal ion at the active site in a "Ping-Pong" type mechanism (Nozik-Grayck *et al.*, 2005). Mn-SOD is one of the most effective antioxidant enzymes that has anti-tumour activity. A set of studies on different cell lines has confirmed that overexpression of Mn-SOD leads to tumour growth retardation (Behrend *et al.*, 2003).

CATALASE

Catalase (EC 1.11.1.6) is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria. Catalase is located in peroxisomes (Pryor, 1986). The enzyme very efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert ~6 million molecules of hydrogen peroxide to water and oxygen each minute.

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

The significantly decreased capacity of a variety of tumours for detoxifying hydrogen peroxide is linked to a decreased level of catalase (Manonmani *et al.*, 2005).

GLUTATHIONE PEROXIDASE/ REDUCTASE SYSTEM

This system forms an excellent protection against lipid peroxidation. It scavenges lipid peroxides, thereby arresting the progressive chain reaction of lipid peroxidation. It also scavenges hydrogen peroxide, which is responsible for the initiation of lipid peroxidation.

$$GSSG + NADPH + H^+ \xrightarrow{GRD} GSH + NADP^+$$

$$2GSH + R(OOH)\,COOH \xrightarrow{GPx} GSSG + R(OH)\,COOH + H_2O$$

Glutathione reductase (GR) stimulates the reduction of glutathione disulfide to reduced glutathione. This ensures a steady state supply of the reductive substrate (NADPH) to glutathione peroxidase. Glucose-6-phosphate dehydrogenase (G6PD) is required for the conversion of nicotinamide adenine dinucleotide phosphate (NADP+) to its reduced form (NADPH) (Agarwal and Prabakaran, 2005).

NON-ENZYMIC ANTIOXIDANTS

VITAMIN E

Vitamin E is a fat-soluble vitamin that exists in eight different forms. α-tocopherol is the most active form of vitamin E in humans and is a powerful biological antioxidant, which is considered to be the major membrane bound antioxidant employed by the cell. Its main antioxidant function is the protection against lipid peroxidation (Uneri *et al.*, 2006).

Vitamin E functions biologically as a scavenger of different free radicals by working as an antioxidant. As a consequence of its fat-solubility, vitamin E exerts its antioxidant activity in cell membranes, which predominantly contain lipids. (Isaac *et al.*, 2008).

VITAMIN C

Ascorbate is widely recognized as the principal endogenous biological antioxidant, able to repair radicals and to recycle other antioxidant compounds. An early

study demonstrated that ascorbate is the first endogenous antioxidant to be lost in human blood serum oxidized by peroxyl radicals (Moor *et al.*, 2006).

Ascorbic acid and tocopherol supplementation can substantially reduce oxidative damage. The antioxidant mechanisms of ascorbic acid are based on hydrogen atom donation to lipid radicals, quenching of singlet oxygen and removal of molecular oxygen (Domazou *et al.*, 2009). However, ascorbic acid can act as a prooxidant under certain conditions, including reduction of ferric iron to more active ferrous iron (Loke *et al.*, 2005). When protecting against the propagation of fatty acid peroxidation, vitamin E is oxidized to the α-tocopherol radical. *In vivo,* vitamin C and ubiquinol (Coenzyme Q) reduce the α-tocopherol radical and regenerate vitamin E (Bendich *et al.*, 1986).

CAROTENOIDS AND LYCOPENE

Carotenoids are lipid soluble pigments found in many vegetable crops, which possess reported health benefits of reducing cancers (lycopene), cardiovascular (lycopene), and age-related eye diseases (lutein and zeaxanthin) when regularly consumed in the diet. One of the most important physiological functions of carotenoids in human nutrition is as vitamin A precursors (β-carotene). Humans cannot synthesize carotenoids; therefore, fruits and vegetables are primary sources of carotenoids in human diets world-wide (Kopsell and Kopsell, 2009).

In part, the beneficial effects of carotenoids are thought to be due to their role as antioxidants. β-Carotene may have added benefits due its ability to be converted to vitamin A (Krinsky and Johnson, 2005). The effectiveness of carotenoids as antioxidants is also dependent upon their interaction with other co-antioxidants, especially vitamins E and C. They interact synergistically with other antioxidants; mixtures of carotenoids are more effective than single compounds (Stahl and Sies, 2003).

Lycopene, a non-provitaminic carotenoid, present in many fruits and vegetables, such as tomatoes and their processed products, has been associated with decreased risk of chronic diseases including cancer (Fornelli *et al.*, 2007). The antioxidant activity of lycopene is suggested to contribute to its efficacy as a chemoprevention agent (Erdman Jr. *et al.*, 2009).

Considerable evidence from several epidemiological studies suggests that lycopene has anti-carcinogenic and anti-atherogenic potential, the effects of which have been attributed primarily to its antioxidant properties (lycopene quenches singlet oxygen almost twice as well as β-carotene does) (Omoni and Aluko, 2005).

In addition to antioxidant activity, *in vitro* experiments indicate other mechanisms of chemoprevention by lycopene including the induction of apoptosis and antiproliferation in cancer cells, anti-metastatic activity and the upregulation of the antioxidant response element leading to the synthesis of cytoprotective enzymes (van Breemen and Pajkovic, 2008).

PHENOLICS

Phenolics, as secondary plant metabolites, are commonly found in various fruits and vegetables and they have been shown to give a defence against oxidative stress from endogenous ROS and free radicals (Choi *et al.*, 2006). Phenolics show various biological properties, such as antioxidative, antiproliferative, antibacterial, antiinflammatory and antiallergic effects (Heo *et al.*, 2007).

Research has also shown interest in phenolic compounds' anticarcinogenic activities. They could act as compounds preventing cancer either via interception of harmful free radicals, activating the detoxifying enzymes of the body, inhibiting the formation of ultimately carcinogenic metabolites and their binding to DNA, and modifying the immune response of the organism (Boudet, 2007).

FLAVONOIDS

Flavonoids are plant phytochemicals that cannot be synthesized by humans. The six classes of flavonoids (flavanones, flavones, flavonols, isoflavonoids, anthocyanins, and flavans) vary in their structural characteristics around the heterocyclic oxygen ring (Min and Ebeler, 2008).

Many studies are accumulating that report the neuroprotective, cardioprotective, and chemopreventive actions of dietary flavonoids (Williams *et al.*, 2004). The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including free radical scavenging, modifying enzymes that activate or detoxify

carcinogens, and inhibiting the induction of the transcription factor activator protein-1 (AP-1) activity by tumor promoters (Moon *et al.*, 2006).

CHLOROPHYLL

Chlorophyll is ubiquitous in all green plant parts. Chlorophyllins are derivatives of chlorophyll in which the central magnesium atom is replaced by other metals, such as cobalt, copper or iron (Hortensteiner and Matile, 2004). Chlorophyll has drawn significant attention as a cancer preventative agent. Biological activities attributed to chlorophyll derivatives consistent with cancer prevention include antioxidant and antimutagenic activity, mutagen trapping, modulation of xenobiotic metabolism and induction of apoptosis (Ferruzzi and Blakeslee, 2007).

GLUTATHIONE

GSH is a key molecule in redox body homeostasis. It is, an important water-soluble antioxidant, is synthesized from the amino acids glycine, glutamate and cysteine. GSH in the nucleus maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression. Oxidised glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Nogueira *et al.*, 2004).

The main protective roles of glutathione against oxidative stress are: (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g., glutathione peroxidase (GPx), glutathione transferase and others; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase; (iv) glutathione is able to regenerate the most important antioxidants, vitamins C and E, back to their active forms (v) glutathione can reduce the tocopherol radical of vitamin E directly, or indirectly, via reduction of semidehydroascorbate to ascorbate (Masella *et al.*, 2005; Valko *et al.*, 2007).

DISEASES CAUSED DUE TO FREE RADICAL DAMAGE

All the biological molecules present in our body are at risk of being attacked by free radicals. Such damaged molecules can impair cell functions and even lead to cell death eventually resulting in a diseased state.

CANCER

Oxidative stress induces a cellular redox imbalance, which has been found to be present in various cancer cells compared with normal cells; the redox imbalance, thus, may be related to oncogenic stimulation. Permanent modification of genetic material resulting from oxidative damage represents the first step involved in mutagenesis, carcinogenesis, and aging. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions have been noted in various tumours, strongly implicating such damage in the etiology of cancer. To date, more than 100 oxidised DNA products have been identified. ROS-induced DNA damage involves single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result in either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of which are associated with carcinogenesis (Marnett, 2000; Valko *et al.*, 2006).

Experimental as well as epidemiological data inidicate that a variety of nutritional antioxidants inhibit the process of cancer development, and reduce cancer risk. Therefore, the antioxidant phytochemicals can either scavenge constitutive H_2O_2 or paradoxically generate additional amounts of H_2O_2 to inhibit the proliferation of cancer cells and act as anticancer agents. Apart from these actions, antioxidants have also been advocated to impart anticancer activities by several other mechanisms: (i) trapping the ultimate carcinogen, (ii) blocking the metabolic activation of carcinogens, (iii) modulating xenobiotic metabolizing enzymes, (iv) scavenging free radicals, (v) inhibiting generation of free radicals, (vi) inhibiting promotion stage of carcinogenesis by inhibiting cell proliferation through blocking lipoxygenase/cycloxygenase pathway or by lowering ornithine decarboxylase activity, and (vii) by decreasing the bioavailability of ultimate carcinogen (Tiwari, 2004).

DIABETES

There is considerable evidence that hyperglycemia results in the generation of ROS, ultimately leading to increased oxidative stress in a variety of tissues. In the absence of an appropriate compensatory response from the indigenous antioxidant network, the system becomes overwhelmed (redox imbalance), leading to the activation of stress sensitive intracellular signalling pathways. One major consequence of this is the

expression of gene products that cause cellular damage and are ultimately responsible for late diabetic complications (Niedowicz and Daleke, 2005). The ability of antioxidant/free-radical scavengers to protect against the effects of hyperglycemia and free fatty acids along with clinical benefits following antioxidant therapy supports the causative role of oxidative stress in mediating and/or worsening these abnormalities (Sharma and McNeill, 2006).

Similarly, supplementation with antioxidants has also been shown to decrease oxidative stress and complications in animal models of diabetes (Sathishsekar and Subramanian, 2005) and in diabetic patients. Diabetes-induced defects in the homeostasis and the transport of intracellular calcium have been shown to decrease or recover by treatment of diabetic animals with some antioxidants (Arulselvan and Subramanian, 2006). Several studies have demonstrated that antioxidant supplementation prevents lipid peroxidation, haemoglobin glycation and inhibition of Na^+, K^+- ATPase and/or Ca^{++}-ATPase activity caused by hyperglycemia in various cells (Arulselvan *et al.*, 2006).

NEUROLOGICAL DISORDERS

The brain is particularly vulnerable to oxidative damage because of its high oxygen utilisation, its high content of oxidisable polyunsaturated fatty acids and the presence of redox-active metals (Cu, Fe). Oxidative stress increases with age and therefore can be considered as an important causative factor in several neurodegenerative diseases, typical in older individuals.

ALZHEIMER'S DISEASE

In Alzheimers disease (AD) biochemical and histological studies have provided evidence for increased levels of antioxidant enzymes such as catalase and Cu,Zn- and Mn-SOD in neurons in AD patients are consistent with their being under increased stress (Zhu *et al.*, 2007). Increased protein oxidation, protein nitration and lipid peroxidation occur in neurofibrillary tangles and neuritic plaques (Cappai and Barnham, 2008).

Lipid peroxidation is quiet extensive as indicated by increased levels of peroxidation products such as 4-hydroxynonenal (4-HNE) in the cerebrospinal fluid of AD patients (Sultana *et al.*, 2006). Iron (Fe^{2+}) likely contributes to increased lipid peroxidation in AD. Lipid peroxidation may promote neuronal death in AD by multiple

mechanisms that include impairment of the function of membrane ion-motive ATPase (Na^+/K^+-ATPase), glucose transporters and glutamate transporters. Lipid peroxidation leads to the production of the aldehyde 4-HNE that appears to play a central role in the neurotoxic action of amyloid β- peptide (Pratico, 2008).

PARKINSON'S DISEASE

A majority of studies have explored the effect of oxidative stress that contributes to the cascade of events leading to dopamine cell degeneration in PD (Tretter *et al.*, 2004). The occurrence of oxidative stress in PD is supported by both postmortem studies and by studies demonstrating the capacity of oxidative stress to induce nigral cell degeneration. There is evidence that there are high levels of basal oxidative stress in the substantia nigra pars compacta (SNc) in the normal brain, but that this increases in PD patients. However, other factors involving inflammation, excitotoxic mechanisms, toxic action of nitric oxide and mitochondrial dysfunction play roles in the etiology of PD (Andersen, 2004).

CATARACT

Cataracts are the leading cause of blindness worldwide. Opacity of the lens is a direct result of oxidative stress. Cataracts occur primarily due to age, but also are common in diabetes, where superoxide in the mitochondria is elevated as a result of hyperglycemia (Song *et al.*, 2005). With respect to the whole lens, the loss of glutathione from the nuclear region is probably the crucial feature that precedes cataract formation. Glutathione is the essential and primary lenticular antioxidant (Ferrigno *et al.*, 2005). The loss is apparently due to the oxidation of glutathione (GSH) to GSSG since its levels rise significantly once the cataract develops. The loss of GSH occurs in nearly all experimental cataracts (Truscott, 2005). It is interesting to note that large decreases in GSH occur in the nuclear region, while cortical GSH remains normal. The aqueous antioxidant vitamin C has more promise for cataract prevention. In humans, plasma and lens ascorbate were linearly related as was the intake of vitamin C and lens ascorbate (Jacques *et al.*, 2005).

AGING

Aging is the accumulation process of diverse detrimental changes in the cells and tissues with advancing age, resulting in an increase in the risks of disease and death (Bokov *et al.*, 2004). The genesis of aging starts with oxygen, occupying the final position in the electron transport chain (Stadtman, 2004).

Even under ideal conditions, some electrons "leak" from the electron transport chain. These leaking electrons interact with oxygen to produce superoxide radicals, so that under physiological conditions, about 1–3% of the oxygen molecules in the mitochondria are converted into superoxide. The primary site of radical oxygen damage from superoxide radical is mitochondrial DNA (mtDNA) (de Grey, 2004). The cell repairs much of the damage done to nuclear DNA (nDNA), but mtDNA cannot be readily fixed. Therefore, extensive mtDNA damage accumulates over time and shuts down mitochondria, causing cells to die and the organism to age (Kujoth *et al.*, 2005).

MOLECULAR DAMAGE INDUCED BY FREE RADICALS

OXIDATIVE DNA DAMAGE

Oxidative damage to DNA is a result of interaction of DNA with ROS or RNS. It has been estimated that one human cell is exposed to approximately 1.5×10^5 oxidative hits a day from hydroxyl radicals and other such reactive species (Devasagayam *et al.*, 2004). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone. Permanent modification of genetic material resulting from these oxidative damage incidents represents the first step involved in mutagenesis, carcinogenesis and aging. In fact, as is well established, in various cancer tissues free radical-mediated DNA damage has occurred (Gumaraes *et al.*, 2007).

To date, more than 100 products have been identified from the oxidation of DNA. ROS-induced DNA damage involves single- or double stranded DNA breaks, purine, pyrimidine or deoxyribose modifications and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis (Prashanth *et al.*, 2007).

LIPID PEROXIDATION

Membrane lipids present in subcellular organelles are highly susceptible to free radical damage. Lipids, when reacted with free radicals, can undergo the highly damaging chain reaction of lipid peroxidation (LP) leading to both direct and indirect effects. The damage caused by LP is highly deterimental to the functioning of the cell (Skrzydlewska *et al.*, 2005).

Lipid peroxidation is a free radical mediated process. ROS can attack lipids and extract a hydrogen atom from a methylene carbon in their side chain. The greater the number of double bonds in the lipid molecule, the easier will be the removal of the hydrogen atom. This explains why the polyunsaturated fatty acid residues of phospholipids are very sensitive to ROS (Sultana *et al.,* 2006). The peroxidation of lipids in plasmalemma or sub-cellular membranes can be very damaging because it can promote alterations in their biological properties (such as the degree of membrane fluidity) and lead to the inactivation of membrane-bound receptors or enzymes, which in turn may impair normal cellular function and increase cell permeability (Dalle-Donne *et al.*, 2006).

Lipid peroxidation is a self-propagating process that can proceed until the substrate is consumed or termination occurs, thus promoting extensive tissue injury (Jones, 2006). Moreover, various products of lipid peroxidation are chemically reactive and they covalently modify critical biomolecules like proteins and DNA, thus increasing cellular damage (Uchida, 2003a). Lipid peroxidation generates a variety of relatively stable end products, mainly aldehydic by-products, such as malondialdehyde and more reactive α,β-unsaturated reactive aldehydes, such as trans-4-hydroxy-2-nonenal, and 2-propenal (acrolein) (Uchida, 2003b; Carini *et al.*, 2004).

Carbonyl-crotonaldehyde is another highly reactive aldehyde recently studied in AD (Kawaguchi-Niida *et al.*, 2006). Other products, derived from the endocyclization of lipid hydroperoxyl radicals, are isoprostanes and neuroprostanes; more recently another class of compounds, named neurofurans, has been identified. All these substances have been extensively assessed in brain and biological fluids (CSF, plasma, urine), as an index of oxidative damage, in subjects with AD and myocardial infraction (Song *et al.*, 2008).

PROTEIN OXIDATION

Oxidation of proteins by ROS/RNS can generate a range of stable as well as reactive products such as protein hydroperoxides that can generate additional radicals particularly upon interaction with transition metal ions. The accumulation of oxidised proteins in living systems may be: (i) due to an increase in the steady state level of ROS/RNS and/or to a decrease in the antioxidant capacity of an organisms; (ii) a decrease in the ability to degrade oxidised proteins due to either a decrease in the protease concentrations and/or to an increase in the levels of protease inhibitors. Although most oxidized proteins that are functionally inactive are rapidly removed, some can gradually accumulate with time and thereby contribute to the damage associated with aging as well as various diseases (Chakravarti and Chakravarti, 2007).

APOPTOSIS

The term apoptosis had been coined in order to describe the morphological processes leading to controlled cellular self-destruction (Kerr *et al.*, 1972). Apoptosis is of greek origin, having the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. This analogy emphasizes that the death of living matter is an integral and necessary part of the life cycle of organisms. The apoptotic mode of cell death is an active and defined process, which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. It should be stressed that apoptosis is a well-defined and possibly the most frequent form of programmed cell death, but that other, non-apoptotic types of cell death also might be of biological significance (Koren, 2006).

Taken together, apoptotic processes are of widespread biological significance, being involved in development, differentiation, proliferation / homoeostasis, regulation and function of the immune system and in the removal of defective and therefore harmful cells. Thus, dysfunction or dysregulation of the apoptotic program is implicated in a variety of pathological conditions. Defects in apoptosis can result in cancer, autoimmune diseases and spreading of viral infections, while neurodegenerative disorders, AIDS and ischaemic diseases are caused or enhanced by excessive apoptosis (Lee and Lim, 2006).

Due to its importance in such various biological processes, programmed cell death is a widespread phenomenon, occuring in all kinds of metazoans (Tittel and Steller, 2000) such as in mammals, insects (Richardson and Kumar, 2002), nematodes (Liu and Hengartner,1999) and cnidaria (Cikala *et al.*, 1999). Apoptosis-like cell death mechanisms have also been observed and used as a model system in yeast (Madeo *et al.*, 2004).

MORPHOLOGICAL FEATURES OF APOPTOSIS

Apoptotic cells can be recognized by stereotypical morphological changes. The cell shrinks, shows deformation and looses contact with its neighbouring cells. Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies', which contain cytosol, the condensed chromatin and organelles. The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles (Arden and Betenbaugh, 2004).

ALTERNATIVE SYSTEMS TO THE USE OF LIVE ANIMALS

Globally, scientific research uses millions of animals in experiments every year. The main thrust of the ethical argument against animal experiments is that animals, at least vertebrates, can experience pain and suffering. Apart from that, they can be harmed by confinement, frustration, fear, isolation and loss of life (Wood *et al.*, 2008).

There is an increasing interest in developing alternatives to animal testing and the three R's of reduction, refinement and replacement (Replacement of experimental animals by alternatives, Refinement of housing, handling and experimental procedures to reduce discomfort, pain, fear, stress and suffering and Reduction of numbers of animals used) are the basis due to a variety of reasons including impending regulatory initiatives, new assessments of the performance of the accepted battery of *in vitro* tests and new knowledge in medical research (Tweats *et al.*, 2007).

There are several alternative approaches, such as tissue, cell and organ culture, survey of human studies, replacement with less sentient organisms, use of discarded human placentas for microsurgery, chromatography and mass spectrometry, computer simulation, audio visual aids, centralization of existing data with easy access and so on (Spardling *et al.*, 2006).

The use of *in vitro* systems (subcellular fractions, cell lines, primary cell cultures, tissue slices, organ cultures, etc.) as research tools in toxicology is widespread (Kniewald *et al.*, 2005). Alternative models, such as yeasts or invertebrates (Drosophila, Caenorhabditis), may also be used to develop rapid genetic or pharmacological screenings (Marin and Vallejo, 2005).

CELL CULTURE MODELS

In vitro assays are increasingly being used in drug metabolism studies to screen novel chemicals. Their advantages are twofold: first, they allow testing early in the drug discovery phase, providing important information on chemical characteristics; second, human cells or cell constituents can be utilized, increasing the relevance to man (van de Bovenkamp *et al.*, 2007).

Cell-based *in vitro* models are invaluable tools in elucidating the pharmacokinetic profile of a drug candidate during the drug discovery and development process. These models are useful tools to study the uptake of drugs across the barriers of the human body, like the intestine, the skin or the blood-brain barrier (Bock *et al.*, 2004). Cell-based *in vitro* models not only help to reduce the number of animals used but are also much faster to perform, more cost effective and give more reproducible data than animal studies (Vasudevan *et al.*, 2005).

Cell culture is highly desirable, as it provides systems for ready, direct access and evaluation of tissues. The use of tissue culture is a valuable tool to study problems of clinical relevance, especially those related to diseases, screening and studies of cell toxicity mechanisms (Vermeir *et al.*, 2005). Ready access to the cells provides the possibility for easy studies of cellular mechanisms that may suggest new potential drug targets and in the case of pathological-derived tissue, has an interesting application in the evaluation of therapeutic agents that potentially may treat the dysfunction (Allen *et al.*, 2008).

Various types of cells lines are used in pharmacotoxicology. Established cell lines are easily available, with few ethical restrictions. Some specific properties are preserved, although they have kept the phenotype of the original tissue, which is frequently a tumour phenotype. They are usually more resistant to toxic compounds than freshly isolated cells. Some drug-metabolizing enzymes are expressed and regulated in these cells (Hariparsad *et al.*, 2006).

TISSUE SLICES

Precision-cut tissue slices are an appropriate model of *in vitro* systems for many reasons, including simplicity and ease of preparation, retention of normal organ architecture, and the ability to obtain multiple slices from each organ (Vickers and Fisher, 2004).

The organ slice methodology is readily adaptable to various organs and various species, facilitating experimentation of cross-species comparisons (Naik *et al.*, 2004). Moreover, organ slices represent a multicellular three dimensional experimental *in vitro* model, possessing the biologically relevant structural and functional features of *in vivo* tissues. By the presence of various cell types in an architectural organization that supports both cell-cell and cell-extracellular matrix interactions, this model has tremendous potential for evaluating mechanisms of drug-induced tissue injury.

The liver is the predominant organ in which biotransformation of foreign compounds takes place, although other organs may also be involved in drug biotransformation. Liver is an organ of paramount importance as it plays an essential role in maintaining the biological equilibrium of vertebrates. The spectrum of its functions include: metabolism and disposition of chemicals (xenobiotics) to which the organ is exposed directly or indirectly; metabolism of lipids, carbohydrates and proteins; blood coagulation and immunomodulation (Moronvalle-Halley *et al.*, 2005). Liver slice retains tissue specific micro-architecture with maintained cell diversity, identity and functional heterogeny compared to hepatocytes and, therefore, resembles more closely the *in vivo* situation.

YEAST

Yeast is a collective term for unicellular ascomycetous and basidiomycetous fungi, containing organisms of relatively far relationship. *Saccharomyces cerevisiae* is the best-researched yeast and probably the best-known eukaryotic organism. It was the first eukaryotic organism with a completely sequenced genome, in 1996 (Frohlich *et al.*, 2007). Yeast cells have remarkable similarities to mammalian cells at the molecular and organelle level, and a number of yeast proteins have been found to be functionally interchangeable with the highly homologous human proteins. Thus, it is not that surprising that the use of yeast cells as a model system provides revelant contribution to understand the molecular mechanism underlying the oxidative stress and apoptosis (Kitagaki *et al.*, 2007).

Although *S. cerevisiae* is a unicellular organism, there are compelling evidences that yeast can undergo altruistic programmed cell death similar to apoptosis (Knorre *et al.*, 2005). The small genome, fast growth and easy handling have made *S. cerevisiae* a favourite tool for genome wide exploration. *S. cerevisiae* has been (and still often is) employed as "clean room" for investigating the interaction of proteins involved in apoptosis and programmed cell death in general (Ludovico *et al.*, 2005).

Drosophila melanogaster

Drosophila is a genus of small flies whose members are often called small fruit flies or more appropriately vinegar flies, wine flies, pomace flies, grape flies and picked fruit flies. Drosophila is an invertebrate genetic model system emerging as a powerful tool for analyzing the function of human disease genes (Vidal and Cagan, 2006). About 75% of known human disease genes have a recognizable match in the genetic code of fruit flies and 50% of fly protein sequences have mammalian analogues (http://superfly.ucsd.edu/homophila). These Drosophila genes include homologs of genes causing a broad spectrum of human diseases ranging from neurological disorders and cancer, to developmental defects, metabolic / storage disorders, cardiovascular disease as well as genes required for function of the visual, auditory, and immune systems (Taylor and Adler, 2008).

The fruit fly, *Drosophila melanogaster*, has a variety of features, making it a particularly useful model organism (Demir *et al.*, 2008). Included in the list are the fly's

relatively short life span, ease of maintenance, genetic and environmental manipulations that alter life span, storehouse of stocks available with altered genes, molecular genetic techniques, full Drosophila genomic sequence and its proven success in pioneering the understanding of complex biological phenomena such as development and behaviour (Zhao *et al.*, 2008).

MEDICINAL PLANTS

Plants and plant products are being used as a source of medicine since long. Among the most important constituents of edible plant products, low molecular weight antioxidants are the most important species. It is known that consumption of fruits and vegetables is essential for normal health of human beings (Son *et al.*, 2008).

Ayurvedic Indian and traditional Chinese system are living great traditions and have important roles in bioprospecting of new medicines from medicinal plants, which are rich sources of antioxidants. Current estimate indicates that about 80% of people in developing countries still rely on traditional medicine based largely on various species of plants and animals for their primary healthcare (Lee *et al.*, 2007a).

The medicinal properties of plants have been investigated in the recent scientific development throughout the world, due to their potent antioxidant properties, no side effects and economic viability. Flavonoids and phenolic compounds widely distributed in plants have been reported to exert multiple biological effects, including antioxidant, free radical scavenging, anti-inflammatory and anti-carcinogenic effects (Ranga *et al.*, 2005). By fusing ancient wisdom and modern science, India can create world-class products. Therefore, it has embarked on a fast track programme to discover new drugs by building on traditional medicines and screening the diverse plants of the country (Devasagayam *et al.*, 2004).

The candidate plant of the present study is *Zea mays*, which is commoly known as maize or makkacholam. A decoction of the leaves and roots is used in the treatment of strangury, dysuria and gravel. The corn silks are cholagogue, demulcent, diuretic, lithontripic, mildly stimulant and vasodilators. They also act to reduce blood sugar levels and so are used in the treatment of diabetes mellitus as well as cystitis, gonorrhoea and gout. A decoction of the cob is used in the treatment of nose bleeds and menorrhagia. The seed is diuretic and a mild stimulant. It is a good emollient poultice for ulcers, swellings

and rheumatic pains and is widely used in the treatment of cancer, tumours and warts. It contains the cell-proliferant and wound-healing substance allantoin, which is widely used in herbal medicine to speed the healing process. The plant is said to have anticancer properties and is experimentally hypoglycemic and hypotensive (Duke and Ayensu, 1985).

In spite of such reports being available, not many studies have concentrated on the type of antioxidant responses evoked by the *Zea mays* leaves on oxidative stress-induced events at the molecular level. The present study is an extensive search into the antioxidant, anti-apoptotic and anticancer activities of *Zea mays* leaves and their effects on cellular biomolecules.

The layout of the study, the materials used and the methodology adopted are explained, with appropriate references quoted, in the next chapter.