2. REVIEW OF LITERATURE

Since ancient times, plants have been an exemplary source of medicine. Ayurveda and other Indian literature mention the use of plants in the treatment of various human ailments. India has about 45,000 plants species and among them, several thousands have been claimed to possess medicinal properties (Vermani, 2002). Traditional systems of medicine in most developing countries depend primarily on the use of plant products either directly or indirectly (Rao et al., 2004). Plants possess effective antioxidant defense system (Masella et al., 2005). Plant extracts and their isolated constituents have also been an important part of various therapeutic systems (Vanitha and Kathiravan, 2006).

India has increased the research in traditional Ayurvedic herbal medicine after observation that they are effective for the condition to which they have traditionally been applied (Mary et al., 2003). The use of different parts of medicinal plants not only help to decrease the cost of medication but are also locally available, with the least side effects as compared to chemical-based medication (Sidhu et al., 2007).

Free radical formation and oxidative stress is receiving considerable attention now-a-days. A great number of aromatic, spicy, medicinal and other plants contain chemical compounds exhibiting antioxidant properties (Mathew and Abraham, 2006). Therefore, testing their antiradical properties is of interest, primarily in order to find new promising sources for natural antioxidants, functional foods and nutraceuticals (Miliauskas et al., 2004).

Oxidative stress is fast becoming the nutritional and medical buzzword for the 21st century. Morphological, biochemical and molecular studies undertaken in recent years, both in experimental animals and in man, have shown that oxidative stress plays a primary role in the
development of degenerative changes in cells and tissues of our body (Dalle-Donne et al., 2006).

Oxidative stress is defined as the disturbance in the equilibrium state of prooxidants and antioxidants in the cell. When the prooxidants outbalance the antioxidants, it results in potential damage to lipids, proteins, carbohydrates and DNA, ultimately leading to cell death (Tau and Samson, 1993). Oxidative stress results from the increased generation of reactive oxygen species (ROS) and has been implicated in major human ailments like cardiovascular diseases, cancer and neural disorders and in the process of aging (Abresia and Golino, 2005).

**OXIDATIVE STRESS INDUCED CELLULAR DAMAGE**

Oxygen plays an important role in many of the metabolic processes associated with aerobic existence. When the overall generation of ROS and RNS exceeds the total antioxidant level in the body, the resulting condition is called oxidative stress.

Oxidative stress induced by ROS/RNS leads to oxidative damage to biological targets, including DNA, proteins and lipids. As a result, all aerobic organisms utilize a series of primary antioxidant defenses for protection against oxidative damage, including antioxidant compounds, metal chelaters, enzymes and proteins (Samuni et al., 2002). It plays an important role in chronic complication of diabetes and is postulated to be associated with increased lipid peroxidation (Elangovan et al., 2000).

The free radicals can attack any biological molecule causing large number of diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, epilepsy and trauma. These mechanisms employ low molecular weight, non-enzymatic antioxidants and antioxidant enzymes, which are inducible by oxidative stress (Dexda, 2004). Lungs are one of the
most vulnerable targets to oxidant damage. Oxidative stress has been implicated in a variety of lung disorders like chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis and lung cancer (MacNee, 2000).

**FREE RADICALS**

A free radical is an atom or a molecule with one or more unpaired electrons in its outermost orbit (Irshad *et al.*, 2002). It is highly unstable and attempts to achieve a more stable state by reacting with other atoms or molecules in the cell (Wu and Cederbaum, 2003). The most abundant radical in biological system is molecular oxygen and the important one is superoxide anion and the hydroxyl radical related with tissue injuries. Hydrogen peroxide is not a true free radical species, but constitutes a class of reactive oxygen metabolites that can also be highly toxic to tissue components (Terashima *et al.*, 2007).

Free radicals can be defined as any chemical species that contain unpaired electrons, which increase the chemical reactivity of an atom or molecule (Gutteridge and Halliwell, 2000). They have a short life and are formed by the following steps:

1. homolytic cleavage of covalent bonds where each fragment retains one electron, or
2. by the loss of single electron, or
3. by the addition of single electron for attaining the stability.

These free radicals abstract an electron from a stable molecule, converting it into a new free radical. This leads to a chain reaction, which can be divided into 3 steps.

a. Initiation
b. Propagation
c. Termination (Tripathi, 1997)
The free radicals damage the membrane and thereby change the alignment of the receptors and ion channels (Tripathi, 1998). The generation of free radicals may be endogenous or exogenous. Free radicals of importance in living organisms includes many such as hydroxyl, superoxide etc. (Lal et al., 1996). Nitric oxide, an example of a physiological radical, is of considerable interest through its role as a mediator of vascular tone (Gutteridge and Halliwell, 2000). Increased generation of free radicals like the superoxide ion and hydroxyl radical can produce cell membrane damage. Free radical mediated mitochondrial dysfunction can result in decreased production of ATP (Ravikumar et al., 2005). The scheme given below (Figure A) presents an overall picture of all metabolism of ROS and the mechanism of oxidative tissue damage leading to pathological conditions.

**Figure A**

**METABOLISM OF ROS AND MECHANISM OF OXIDATIVE TISSUE DAMAGE**
REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species (ROS) are generated through the process of oxidation by a variety of sources from the environment (e.g., photooxidation) and normal cellular functions (Frank and Gupta, 2005). ROS include free radicals such as superoxide (O$_2^-$) hydroxyl radical (OH$^*$) and non-free radical species, such as H$_2$O$_2$ and singlet oxygen (¹O$_2$) species. ROS can readily react with biomolecules such as carbohydrates, proteins, lipids and DNA (Gulcin et al., 2002). These molecules are deleterious factors inducing cellular injury and aging (Gulcin, 2006). The production of ROS and injury to biomolecules is shown in Figure B.

**Figure B**

PRODUCTION OF ROS AND INJURY TO BIOMOLECULES

![Diagram of ROS production and damage to biomolecules]

- Pancreatic β cell destruction
- DNA oxidation
- Protein oxidation
- Lipid peroxides/aldehydes
- Protein
- Lipo oxidation products
- Glycooxidation products
ADVERSE EFFECTS OF ROS

Reactive Oxygen Species (ROS) exert oxidative stress towards cell proteins, lipids, carbohydrates and DNA, and this leads to a number of physiological disorders (Mondal et al., 2006). ROS have been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, neurological disorders, congestive heart failure and ischemia reperfusion injury, which are characterized by an increased steady state concentration of reactive oxygen species (Lata and Ahuja, 2003; Bassenge et al., 2005).

ROS production depletes both enzymic and non-enzymic antioxidants, leading to additional ROS accumulation, causing cellular damage. Low levels of antioxidant enzyme expression and activity pose a greater risk of oxidative damage than in the tissue with higher antioxidant production (Ceriello and Imotz, 2004).

GENERATION OF FREE RADICALS

Ever since the origin of aerobic metabolism some 2.5 – 3.0 billion years ago, oxygen has occupied the central place in the evolution of life on this globe. The oxygen consumed by the body is reduced to generate ATP and water by an oxidative phosphorylation pathway involving a multienzyme process and the electron transport chain (ETC). The ETC is constituted by four complexes (C-I to C-IV) and comprise a series of reduction/oxidation reactions, involving NADP dehydrogenase (C-I), succinate dehydrogenase (C-II), cytochrome reductase (C-III) and cytochrome oxidase (C-IV). The reduced forms of the ubiquinone CVA and cytochrome c drive the electron transfer from one complex to another (Reiter et al., 2000).
TYPES OF FREE RADICALS

Reactive oxygen species

1. Superoxide anion radical (O$_2$•$^-$)

The superoxide anion is the first reduction product of oxygen. It regulates metabolites capable of signaling and communicating the cellular genetic machinery. O$_2$•$^-$ is non-reactive and dismutates to H$_2$O$_2$. It is unreactive, it can diffuse through a long way and at low pH, it becomes protonated (HO$_2$•), which is reactive (Devasagayam and Kamat 2002; Thomas and Kamat, 2002). Superoxide anion has a long lifetime, can move a long distance and thus, can be dangerous for the affected or associated system (Sun et al., 2005).

2. Hydrogen peroxide (H$_2$O$_2$)

H$_2$O$_2$ is formed during dismutation by superoxide dismutase (SOD) as well as via several other enzymatic reactions. The non-radical peroxide also generates the redox cycling of catecholamines from mitochondrial respiration, during the respiratory burst of phagocytes and from microsomal cytochrome P450. Intracellular concentration of H$_2$O$_2$ is maintained by its enzymatic degradation by catalase (CAT) and several glutathione peroxidases (Aguirre et al., 1998). H$_2$O$_2$ can easily cross the cell membrane and attack different sites by converting into OH$^•$ (Mikhailov et al., 2003).

3. Hydroxyl Radical (OH$^•$)

The OH$^•$ is normally generated via two primary mechanisms, i.e., either by homolytic cleavage of the H$_2$O$_2$ molecule during exposure to ionizing radiation and during the interaction of H$_2$O$_2$ with a transition metal referred to as Fenton’s reaction.

\[
\text{Metal}^{n+1} + \text{H}_2\text{O}_2 \rightarrow \text{Metal} + \text{OH} + \text{OH}^- 
\]
Under *in vivo* conditions, the metal involved is iron but it can also be a number of other metals, eg., copper, chromium and vanadium. The OH• may also be a degradation product of ONOOH and may be formed by the reaction of O2•− with HCl. It reacts at a diffusion controlled rate with every molecule it encounters and discriminately details DNA, RNA, lipids, proteins and carbohydrates, which are highly reactive species, and has an estimated half life within organisms in the order of 10⁻⁹ seconds (Hore, 2004).

It is highly reactive and short lived. Lipid molecules are very susceptible to hydroxyl radical attack and initiate lipid peroxidation (LPO) (Johri *et al.*, 2002). Hydroxyl radical also induces conformational changes in DNA and enhances the expression of protooncogenes (Ohinata *et al.*, 2003).

4. Malondialdehyde (MDA)

MDA is a byproduct of prostaglandin biosynthesis. It is a secondary product of LPO and an indicator of tissue damage (Scalera, 2003). It is mutagenic and genotoxic, forming adducts with cytosine and adenine, and is believed to lead to cancer (Altunas *et al.*, 2003).

5. Peroxyl radical (ROO•)

These radicals are formed from lipids, proteins, DNA and sugars, during oxidative damage (Park, 1992). Damages are induced by ionizing radiation in biological systems in an indirect manner and they are mediated by products of radiolysis of water including hydrogen radical (H•), OH•, hydrated electron (e− aq), H2O2, peroxyl radical (ROO•), O2•− and 1O2 (Devasagayam and Kesavan, 1996).

6. Singlet oxygen

It is a reactive oxygen species that is formed during photosensitization and chemical reactions (Devasagayam, 1993). These can convert O2 into H2O2 via a process that has been postulated to involve
dihydrogen trioxide which could be shown to be involved in antigen specificity (Wentworth et al., 2003).

7. Hypochlorous acid

These are chlorinating and oxidizing agents, attacking primary amines and sulfhydryl groups in proteins and chlorinated bases in DNA, which are genotoxic to human cells (Whiteman et al., 1997).

Reactive Nitrogen Species

8. Nitric oxide (NO)

Nitric oxide is generated by specific nitric oxide synthases (NOSs) in biological tissue, which metabolize arginine to citrulline with the formation of nitric oxide via a fine electron oxidative reaction (Ghajourifas and Cander, 2005). Nitric oxide can react with superoxide forming peroxynitrite, a highly reactive oxidant linked with many disease states including diabetes (Zou et al., 2002).

Nitric oxide (NO) acts as a reactive radical that acts as an important oxidative biological signal. It has been suggested that NO can stimulate $O_2^• / H_2O_2 / OH^•$ induced LPO (Rubbo et al., 1994). NO plays an active role in free radical and tumour biology (Tamir and Tannenbaum, 1996).

NO acts as a “double edged sword” in health and diseases. NO reacts with superoxide to form peroxynitrite, a strong oxidant (Korkmaz et al., 2005).

$$\text{NO} + O_2^• \rightarrow \text{ONOO}$$

9. Peroxy nitrite

Peroxy nitrite is a powerful oxidant, leading to tyrosine nitration, thiol oxidation, lipid peroxidation, DNA strand break, guanosine nitration / oxidation and finally cell death. The reaction of ONOO with excess NO
generates NO$_2$, which combines with more NO to form N$_2$O$_2$ to cause nitrosative stress (Koppenol et al., 1998).

**Others**

**10. Ethanol**

DNA integrity and repair mechanisms due to ethanol metabolism play a significant role in the process of transformation by experimental approach, establishing that oxidative stress is a central factor involved in alcohol-induced liver injury (Arteel, 2003). Cytochrome P450 2E1 (CYP2E1) leads to the metabolism of ethanol to acetaldehyde, producing effects like acetaldehyde and free radical induction of CYP2E1. Ethanol metabolism leads to increase in cellular damage through peroxidation (Morimoto et al., 1993; Dupont et al; 1998).

**11. CCl$_4$**

CCl$_4$ is a potent hepatotoxin and its hepatotoxicity depends on the reductive dehalogenation of CCl$_4$ catalyzed by CYP2E1 in the liver cell endoplasmic reticulum, leading to the generation of the unstable complex CCl$_3^\cdot$ radicals. These readily interact with molecular oxygen to form the trichloromethyl peroxyl radical (CCl$_3$OO$^\cdot$). This radical undergoes oxidative and reductive biotransformation (Figure C). The isoenzymes implicated in this process are CYP2E1 and CYP2B1/CYP2B2 (Gruebel et al., 1996).
Figure C

BIOTRANSFORMATION OF CCl₄

(Anders and Jacobsons, 1985; McGregor and Lang, 1996)

\[ CH_3COCl \xrightarrow{CYP} CCl_4 \xrightarrow{CYP-Cl} CCl_3 \xrightarrow{CYP} Cl_2C\cdot\cdot\cdot \xrightarrow{H_2O, HOCl} CO \]

Hexachloro ethane \( \rightarrow \) (Trichloromethyl radical)

\[ R^\bullet \xrightarrow{RH} Cl_3HC \xrightarrow{Cl_3COO^\bullet} \xrightarrow{Cl_3COO^\bullet} + O_2 \xrightarrow{Binding to tissue components} \]

Trichloro methyl peroxyl radical

\[ PUFA \xrightarrow{LPO} \]
The most important pathway in the elimination of trichloromethyl radical is the reaction with molecular oxygen, resulting in the formation of trichloromethyl peroxyl radicals (CCl₃OO⁺) (McCay et al., 1984; Pohl et al., 1984). This intermediate is more reactive than trichloromethyl radical, which may interact with lipids, causing LPO, along with the production of 4-hydroxy alkenals (Comporti et al., 1984). The association between CCl₄ metabolites with lipids occurs mostly in the liver and kidney cortex and medulla (Villaruel et al., 1977). Binding of trichloromethyl radical to membrane lipids leads to cross linking (Cheeseman et al., 1985).

Oral administration of CCl₄ inhibited the Ca-Mg-ATPase of the endoplasmic reticulum (Srivatsava et al., 1990). Disturbances in the hepatocellular Ca²⁺ homeostasis may be involved in the pathological changes elicited by CCl₄ (Recknagel, 1983). Glende and Recknagel (1991) suggested that depression of the Ca²⁺-sequestration capacity of the endoplasmic reticulum results in a rise in concentration of Ca²⁺ in the cytosol, leading to CCl₄-induced hepatotoxicity.

**POTENTIATION OF CCl₄ TOXICITY BY ALCOHOL**

Many reports show that ethanol, methanol and other alcohols potentiate the hepatotoxicity of CCl₄ (Ray and Mehendale, 1990; Simko et al., 1992). Micronodular cirrhosis was observed in rats administered with ethanol and CCl₄, while no animal treated with either ethanol or CCl₄ alone developed cirrhosis (Hall et al., 1994).

Pretreatment with various homologous alcohols in rats with CCl₄ potentiated liver injury but did not affect lethality. A combination of t-butanol, pentanol, hexanol and octanol potentiated liver injury and decreased animal survival significantly (Ray and Mehendale, 1990).
OXIDATIVE DAMAGE TARGETS AND TYPES

The continuous production of ROS from endogenous and exogenous sources results in oxidative damage to cellular components and alters many cellular functions (Gracy et al., 1999). Among the biological targets most vulnerable to oxidative damage are the lipid membranes (Gutteridge, 1994), proteinaceous enzymes (Levine and Stadtman, 2001) and DNA (Beckman and Ames, 1997).

LIPIDS AND LIPID PEROXIDATION

All cellular membranes are especially vulnerable to oxidation due to their high concentrations of unsaturated fatty acids. The major damage to lipids is lipid peroxidation. The damage caused by LPO is highly detrimental to the functioning of the cell and its survival (Devasagayam et al., 2003).

Lipid peroxidation is a free radical mediated process. Initiation of a peroxidative sequence is due to the attack by any species that can abstract a hydrogen atom from a methylene group (CH₂), leaving behind an unpaired electron on the carbon atom (CH°). The resultant carbon radical is stabilized by molecular rearrangement to produce conjugated dienes, which then react with an oxygen molecule to give a lipid peroxyl radical (LOO°). These radicals can further abstract hydrogen atoms from other lipid molecules to form lipid hydroperoxides (LOOH) and at the same time propagate lipid peroxidation further. The peroxidation reaction can be terminated by a number of reactions. The major one involves the reaction of LOO° or lipid radical (L°) with a molecule of antioxidant such as vitamin E or α-tocopherol (α-TOH), forming more stable tocopherol phenoxy radical that is not involved in further chain reactions. This can be "recycled" by other cellular antioxidants such as vitamin C or GSH.
LH + •OH $\longrightarrow$ L• + H₂O
L• + O₂ $\longrightarrow$ LOO•
LOO• + LH $\longrightarrow$ L• + LOOH
LOO• + 2TOH $\longrightarrow$ LOOH + 2TO•

Lipid peroxidation gives rise to many products of toxicological interest like malondialdehyde (MDA), 4-hydroxyenonenal (4-HNE) and various 2-alkenals (Saygili et al., 2003).

LPO consists of lipid peroxide dependent and independent systems. Primary LPO involves the interaction of initiator with lipid aryl chain and secondary LPO involves pre-existing lipid peroxides and initiator reaction, which take place with the help of transition metal ion (Upasani and Balaraman, 2003). It results in the formation of several toxic byproducts that can attack cellular targets, including DNA away from the site of generation (Devasagayam et al., 2003).

PROTEINS

Proteins, also major constituent of membranes, can serve as targets for attack by ROS. Oxidation of proteins by ROS/RNS can generate a range of stable as well as reactive products such as protein hydroperoxides, which can generate additional radicals, particularly by interaction with transition metal ions. Among the amino acids, histidine, tryptophan, methionine and tyrosine are more reactive towards ROS. Oxidation of these amino acids results in sulphoxides, which are short lived endoperoxides, that may be toxic to other cells (Headlam and Davies, 2003).

Proteins that are damaged by oxidative stress have decreased biological activity, leading to disturbances in energy metabolism, cell signaling, transport and ultimately cell death (Vincent et al., 2004). Thus, the presence of carboxyl content of the tissues is associated with a number of
pathological disorders (Cohen et al., 2003). The human diseases associated with protein carboxylation include Alzheimer's disease, chronic lung disease, chronic renal failure and diabetes (Dalle – Donne et al., 2003).

DNA

Though DNA is a stable, cell protected molecule, ROS can interact with it and may cause single strand breaks, double strand breaks, and sister chromatid exchanges, which lead to complete change in genome type (Dzeja et al., 2003). Oxidative stresses also cause shortening of telomerase (Von Zglinicli, 2002). The C4-C5 double bond of pyrimidine is particularly sensitive to attack by OH•, generating a spectrum of oxidative pyrimidine damage products, including thymine glycol, uracil glycol and 8-hydroxyl deoxyguanosine (8-OHdG). Several repair pathways repair the DNA damage (Helbock et al., 1999). 8-OHdG has been implicated in carcinogenesis and is considered a reliable marker for oxidative DNA damage (Valko et al., 2004). The endogenous reactions that are likely to contribute to DNA damage are oxidation, methylation, depurination and deamination (Shackelford et al., 2000).

CARBOHYDRATES

Free radicals such as OH• react with carbohydrates by randomly abstracting a hydrogen atom from one of the carbon atoms, producing a carbon centered radical. This leads to chain breaks in important molecules like hyaluronic acid. In the synovial fluid surrounding the joints, an accumulation and activation of neutrophils during inflammation produces significant amounts of oxyradicals that are being implicated in rheumatic arthritis (Devasagayam et al., 2004).
DISEASES CAUSED BY FREE RADICALS

Excessive production of free radicals causes damage to biological material and is an essential event in the etiopathogenesis of various diseases and conditions like aging and cancer (Jurank and Bezek, 2005).

AGING

The role of free radicals in the process of aging has been studied (Sohal et al., 2002). Proteins, nucleic acids and lipids undergo various oxidative modifications, resulting in irreversible loss of function. The age dependent accumulation of oxidatively modified and dysfunctional macromolecules provides the free radical theory of aging.

Lipofuscin, an age associated pigment deposited intracellularly represents the complexes of lipids and proteins derived from peroxidation of PUFA of subcellular organelle membrane (De la Funet, 2002). Prooxidants, however, are also capable of catalyzing reversible modification of proteins. These reactions participate in redox dependent regulation of cell metabolism and response to stress. Disruption of redox regulation contributes to the exponential age related rise in the level of oxidized protein, occurrence of disease and precipitation of death (Kenneth et al., 2006).

CARDIOVASCULAR DISEASES

Endogenous supplementation of antioxidants reduces the chances of developing atherosclerosis and associated coronary heart diseases, including acute myocardial infarction (Miyamoto et al., 2003). Oxidized LDL is the important contributor to the development of atherosclerosis (Siems et al., 2002). Oxidized LDL is taken up more readily by macrophages, resulting in atherosclerotic plaque (Thomas et al., 2002).

ROS induced oxidative stress plays a role in various cardiovascular diseases such as atherosclerosis, ischemic heart diseases, hypertension, and
congestive heart failure. The major sources of oxidative stress in the cardiovascular system involve the enzymes xanthine oxidoreductase (XOS), NAD(P)H oxidase (multi subunit membrane complexes) and NOS as well as mitochondrial cytochrome and hemoglobin (Berry and Hare, 2004; Hare and Stamler, 2005).

CANCER

Cancer is one of the leading causes of disease-related human death. Free radicals and oxidative processes have been implicated in both the initiation and the promotion of carcinogenesis (Weber et al., 1996). Damage to DNA by ROS has been widely accepted as a major cause of cancer. In patients associated with a risk of cancer, an increased rate of oxidative DNA damage is observed (Waris and Ahsan, 2006).

Herbal preparations have been increasingly used for cancer therapy in an attempt to assist in killing tumour cells and reducing the toxicity of combined chemotherapeutic agents (Wollschalpeger, 2003). Higher intake of vitamin E, β-carotene and vitamin C have been found to lower the incidence as well as mortality of stomach cancer. Low levels of antioxidants in the body are associated with high incidence of various cancers. Free radicals and oxidative stress are implicated in the pathogenesis of many disorders including cancer. These oxyradicals attack DNA causing a change in genomic sequences, leading to mutation, deletion, gene amplification or rearrangement (Montovani et al., 2003).

ALZHEIMER’S DISEASE

Alzheimer’s disease is characterized by progressive decline in memory, language and other cognitive functions, accompanied by concomitant behavioral, emotional, interpersonal and social deterioration (Rao and Balachandran, 2002). The cause of neuronal loss of Alzheimer’s
disease is related to increased oxidative stress from excessive free radical generation (Bush, 2000; Sayre et al., 2000). This involves numerous pathways, including oxidative damage pathway (Doraiswamy, 2002). Antioxidant therapy neutralizes highly reactive molecules and may therefore be of therapeutic value in Alzheimer’s disease (Frank and Gupta, 2005).

**DIABETES**

The endothelium-dependent vascular smooth muscle relaxation is impaired by high blood glucose, which is reversed by the administration of SOD and catalase (Maritima et al., 2003). Thus, antioxidant treatment is shown to influence experimental diabetes mellitus (Anjaneyulu and Chopra, 2005). The supplementation of vitamins E, C and β-carotene to diabetic patients helps in scavenging free radicals and retard the glycooxidation process (Peponis et al., 2002).

Diabetes represents a state of increased oxidative stress based on increased peroxidation (Collier et al., 1996). Glycated proteins produce free radicals and hydrogen peroxide in diabetes mellitus (Wolff, 1987). The increased glycation of proteins may induce an increase in free radical production and affect several enzyme activities (Zhao et al., 1998) and accelerate atherogenesis, resulting in modification of vascular membrane lipids. Increased peroxidation and reduced antioxidant reserves are observed in diabetes. Oxidative stress may play an important role in the pathogenesis of diabetic vascular complications such as neurovascular dysfunction (Cameron and Cotter, 1995). Therefore, the generation of ROS may contribute to the overall complication of diabetes (Lal et al., 1996). Diabetic oxidative stress seems mainly to be caused by both an increased production of plasma free radical concentrations and sharp reduction in antioxidant dienes (Dursun et al., 2005).
Among the causes of enhanced free radical production, hypoglycemia (Ceriello, 1997), hyperinsulinemia (Paolisso and Guigliano, 1996) and insulin resistance (Ceriello and Pirisi, 1995) seem to play a major role. Hyperglycemia and insulin resistance are accompanied by reduced insulin action (Ceriello, 2000).

**CATARACT**

Cataract is a leading cause of visual disability and blindness all over the world. LPO within the lens may contribute to the development of cataract. The LPO in the eye may be due to impaired enzymic defenses, leading to the accumulation of ROS, which induce LPO in lenticular membrane, which leads to the formation of lipid hydroperoxides in the hydrophobic barrier of the lipid bilayer of cellular membranes of the lens, which initiates human cataract (Goswami *et al.*, 2003).

The development of cataract can be protected by dietary intake of micronutrients such as zinc, copper and manganese (Goralska *et al.*, 2003). Prevention of cataract may be achieved through a mechanism involving free radical scavenging that prevents LPO (Yagi, 1987). Vitamins C, E and β-carotene play an important role in preventing cataract (Vander Pols, 1999).

**ANTIOXIDANTS**

Aerobic life is characterized by the continuous production of oxidants, balanced by the equivalent synthesis of antioxidants (Rice-Evans and Diplock, 1993). The improper balance between reactive oxygen metabolite production and antioxidant defenses results in oxidative stress, which deregulates the cellular functions, leading to various pathological conditions (Bandopadhyay *et al.*, 1999).
The human body is equipped with an antioxidant defense system that deactivates the highly reactive free radicals. Antioxidant enzymes (made in the body) and antioxidant nutrients (found in foods) soak up all the excess energy that these free radicals have, turning them into harmless particles that can be metabolized; so these antioxidant nutrients are functional components of foods that have extra health benefits in the body (Oboh, 2005).

ANTIOXIDANT ENZYMES

The first line defense against superoxide radical and \( \text{H}_2\text{O}_2 \) mediated injury are the antioxidant enzymes, SOD, catalase, (CAT), and GPx. Antioxidant enzymes, together with the substances that are capable of either reducing reactive oxygen metabolites or preventing their formation, form a protective mechanism inside the cell (Sies, 1997).

**Superoxide dismutase**

SOD is an antioxidant enzyme that catalyzes the dismutation of highly reactive superoxide anion to \( \text{O}_2 \) and to the less reactive species, \( \text{H}_2\text{O}_2 \). SOD dismutates superoxide to hydrogen peroxide (Kang and Kim, 2003).

\[
\text{O}_2^- + \text{O}_2^- \xrightarrow[2H\text{ SOD}]{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2
\]

SOD belongs to metalloenzymes and there are four families of SOD namely Mn-SOD, Fe-SOD, Cu-SOD and Cu-Zn-SOD. Prokaryotic SOD exists in two forms with Fe and Mn respectively in their active sites (Arouma, 1999). In tissues like the liver, the activity of SOD is high when the oxygen utilization is high (Krishnaswamy et al., 1998). SOD activity is increased in cells in response to diverse environmental and xenobiotic stress (Me Cord, 1990).
Catalase

Catalase (CAT) is a tetrameric enzyme that reacts very efficiently with \( \text{H}_2\text{O}_2 \) to form water and molecular oxygen and with H donors (like methanol, ethanol, formic acid or phenols) with peroxidase activity.

\[
\begin{align*}
2\text{H}_2\text{O}_2 & \xrightarrow{\text{CAT}} 2\text{H}_2\text{O} + \text{O}_2 \\
\text{ROOH} + \text{AH}_2 & \xrightarrow{\text{CAT}} \text{H}_2\text{O} + \text{ROH} + \text{A}
\end{align*}
\]

Catalase acts about 10\(^4\) times faster than peroxidase. It is located in mitochondria and in subcellular respiratory organelle (Pryor, 1986). It plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells (Mates et al., 1999). The therapeutic use of an antioxidant complex containing SOD and CAT has been proposed in the treatment of several diseases in which oxidative injury has an important role (Greenwald, 1990). Catalase contains four protein subunits, each containing heme Fe (III)-protoporphyrin group bound to its active site. It has antioxidant function such as promotion / transformation inhibitor of carcinogenesis. It also prevents chromosomal aberration caused by hypoxanthine / xanthine oxidase in Chinese hamster cells (Tauler et al., 2002).

Glutathione S-transferase

GSTs are a group of detoxifying enzymes that catalyze the conjugation of reduced glutathione with a variety of compounds bearing electrophilic centers in them. These enzymes are dimeric in nature with molecular weights between 40,000 and 50,000 daltons. GSTs function to protect against compounds that might be toxic or carcinogenic (Xiao et al., 2006).

GSTs are present in higher concentrations in the liver, constituting 5% of all cytosolic proteins. Thus, GST is a highly sensitive and rapidly
responding marker of liver damage (Beckett and Hayes, 1993; Tredger and Sherwood, 1997).

\[ \text{Rx} + \text{GSH} \xrightarrow{\text{GST}} \text{Hx} + \text{RSG} \quad \text{(Price and Stevens, 1999)} \]

**Glutathione peroxidase**

Glutathione peroxidase (GPx) is a selenoenzyme that catalyzes the reduction of hydroperoxides at the expense of GSH. Organic hydroperoxides are reduced to alcohols (Lou et al., 2003).

\[ \text{ROOH} + 2\text{GSH} \quad \text{-----------------} \quad \text{ROH} + \text{H}_2\text{O} + \text{GSSG} \]

\[ \text{H}_2\text{O}_2 + 2\text{GSH} \quad \text{-----------------} \quad 2\text{H}_2\text{O} + \text{GSSG} \]

This enzyme is present in the cytosol and mitochondrial matrix. It protects biomembranes from oxidative attack and prevents lipid peroxidation by scavenging \( \text{H}_2\text{O}_2 \) and slowing down \( \text{H}_2\text{O}_2 \) dependent free radical attack on lipids (Prabhu, 2002).

There are five different GPx isoenzymes found in mammals. Cytosolic and mitochondrial glutathione peroxidases reduce fatty acid hydroperoxides and hydrogen peroxide at the expense of glutathione. Although GPx shares the substrate, \( \text{H}_2\text{O}_2 \) with CAT, it alone can react with lipid and other hydroperoxides, and thus is a major source of protection against low levels of oxidant stress (Mates et al., 1999).

**Polyphenol oxidases**

Polyphenol oxidases (PPO) play an important role in the quality of food of plant origin because they are the enzymes responsible for browning of certain fruits and vegetables and mushrooms (Belitz and Grosch, 1999). Polyphenols are phenolic compounds containing several hydroxyl groups (Kuenzing et al., 1984). Catechol oxidase specifically oxidizes catechol to o-quinone by incorporating oxygen (Dinckaya et al., 1998).

\[ \text{Catechol} + \frac{1}{2} \text{O}_2 \xrightarrow{\text{catechol oxidase}} \text{o-quinone} + \text{H}_2\text{O} \]
RADICAL SCAVENGING ANTIOXIDANTS

These antioxidants belong to the second line defense and include glutathione (GSH), vitamin C, uric acid, albumin, bilirubin, vitamin E, carotenoids, flavonoids and ubiquinol. Antioxidant vitamins have a number of biological activities such as immune stimulation, inhibition of nitrosamine formation and an alteration of metabolic activities of carcinogens (Van Poppel and Van den Berg, 1997). The major protective function of the vitamins against cancer is the scavenging of ROMs (Tomenson et al., 1995).

Ascorbic acid

Ascorbic acid is a water soluble antioxidant present in both intracellular and extracellular compartments. It is an essential micronutrient required for normal metabolic functions (Satake et al., 2003). Ascorbic acid is a powerful scavenger and quencher of singlet oxygen (Rehim et al., 2003). It is also a cofactor for several enzymes involved in the biosynthesis of collagen, carotenoids and neurotransmitters (Takahasi et al., 2003).

Vitamin C is considered to be a ubiquitous water soluble antioxidant. Many antioxidant effects are associated with it. Ascorbic acid acts as an effective scavenger of free radicals including $\text{O}_2^-$, $\text{OH}^\cdot$, $\text{RO}_2$, RS and other nitrogen radicals (Ueta et al., 2003). Vitamin C prevents the formation of RCOO' by reaction with $\text{O}_2$ and help to release NO from endothelial cells, thus preventing LDL oxidation (Brown and Goodman, 1998). It is also responsible for replenishing the major lipid soluble antioxidant, vitamin E (Chen et al., 2003).

Humans cannot synthesize vitamin C due to a mutation in the gene encoding L-gluconolactone oxidase, which is required for the biosynthesis of vitamin C. It also reduces oxidative DNA damage and genetic mutations (Dorlochter et al., 2002). Ascorbic acid acts as a powerful scavenger and
quencher of singlet oxygen (Rehim et al., 2003). It is also reported that vitamin C enhances oxidative reactions in the system (May and Qu, 2004).

Many biochemical, clinical and epidemiological studies have indicated that the vitamin C supplementation could benefit in chronic diseases such as CND and cataract (Carr and Frei, 1999). Vitamin C protects non-smokers against the harmful effects of ROS from passive smoking (Jacob, 2000). Ascorbate in lung tissue protects against smoke-derived oxidants, suggesting that ascorbate can protect against oxidant damage from activated neutrophils in the lung (Cross et al., 1990). Vitamin C also protects indirectly by regenerating or sparing glutathione and tocopherol (Jacob, 1995).

Vitamin E

Vitamin E (α-tocopherol) is the most important lipid soluble antioxidant, protecting membranes, lipids and lipoproteins (Van Bakel et al., 2000). Tocopherol protects against LPO (Serdar et al., 2003). The protection of LDL from oxidation by vitamin E in vitro might be the same mechanism for the in vivo effect of vitamin E in reducing the risk of cardiovascular disease as reported in several observational studies (Gey et al., 1993).

Vitamin E, commonly comprising of tocopherol and its derivatives, is a lipid soluble vitamin. It occurs in the plasma and in the membranes of cells. Vitamin E comprises of a group of eight naturally occurring related tocopherols, and the most prominent is α-tocopherol (Suleyman et al., 2002).

Vitamin E is the only antioxidant within the membrane bilayer and is therefore responsible for protecting the membrane from oxidative damage, because it is responsible for maintaining the membrane integrity against oxidative assault (Noaman et al., 2002). It is also helped by vitamin C,
which is involved in replenishing vitamin E for sustained antioxidant action within the membrane (Pavlovic et al., 2001).

**Vitamin A and carotenoids**

Vitamin A (retinoic acid and other retinoid derivatives) is also a lipid soluble vitamin stored in the body and is necessary for clear vision in dim light (Gopalan et al., 2000). It occurs in the plasma as the alcohol derivative (retinol). It is synthesized from the provitamin forms that include α-, β- and γ-carotenes and cryptoxanthine (Liu et al., 2003). A significant inversion associated between vitamin A precursor, β-carotene, and carotenoids and the risk of breast cancer has been established (Russel, 2002). Retinoids and vitamin A related compounds are used in the prevention and treatment of dermatologic conditions such as acne and psoriasis (Klaasen and Braakhwin, 2002). Vitamin A has been implicated as a biological factor in reducing the incidence of cancer in humans. Vitamin A and β-carotene reverse precancerous leucoplakia and reduce the occurrence of pathological micronuclei even on continuous exposure to mutagens from betel nuts and chewing tobacco (Rao et al., 2003).

**Reduced glutathione**

Glutathione, in its reduced form, is a tripeptide, responsible for the protection of the cell against oxidative stress. In the cell, glutathione is found as a mixture of reduced thiol form (GSH) and an oxidized form (GSSG), where a disulphide bond links two glutathione molecules (Sen, 1997). Glutathione is a predominant tripeptide thiol compound in many prokaryotes and eukaryotes.

Glutathione is a major substrate in enzyme catalyzed antioxidant reactions. It is involved in thiol transfer reactions that protect cell membranes and proteins from oxidative damage (Dasgupta et al., 2003).
GSH maintains ascorbate and α-tocopherol in their reduced form, which also exerts an antioxidant effect by quenching free radicals. GSH acts as a substrate for GPx to scavenge peroxides. GSH/GSSG pair forms the major intracellular redox system, whose concentrations are mainly maintained by GR and NADP. GSH maintains redox cycle as antioxidant armoury. GSH upholds membrane integrity, enhances immune function, and facilitates transport of amino acids, gluconeogenesis and DNA synthesis (Upadhyay et al., 2003). GSH also plays an important role in the regulation of gene expression and apoptosis (Hammond et al., 2001).

**Natural antioxidants – Silymarin**

Silymarin is a polyphenolic mixture of flavonoids (silydiannin, silychristin and silybin) derived from the plant *Silybum marianum*. Results from pharmacokinetic studies demonstrate good bioavailability of silymarin after oral administration (Zhoa and Agarwal, 1999).

Silymarin is reported to reduce hepatic injury caused by CCl₄, radiation, ethanol and acetaminophen. Its antioxidant property has the ability to inhibit inflammatory cytokine action in normal and transformed cell line (Bhatia et al., 1999; Dhanalakshmi et al., 2002).

**MEDICINAL PLANTS**

Plants and plant derived compounds have been well known to play a dominant role in healthcare of humans and more than 50% of all modern drugs in clinical use are of natural product origin (Rosangkima and Prasad, 2004). Medicinal plants, as a group, comprise approximately 8000 species and account for 80% of all the higher flowering plant species in India (Thapliyal and Thapliyal, 2005). A number of Indian medicinal plants have been used for thousands of years in the traditional system of medicine (Auddy et al., 2003). Plant-based drugs are being increasingly preferred in
medicinal sciences. The use of various parts of several medicinal plants to
cure specific ailments has been in vogue from ancient times in our
indigenous medicine (Bhattacharjee, 2004).

Medicinal plants provide an inexhaustible source of anticancer
drugs in terms of both variety and mechanism of action. Induction of
apoptosis is the key to the success of plant products as anticancer agents
(Taraphdar et al., 2001).

The use of plant extracts especially the whole plant extracts are
effective cytostatic agents against cancer cells (Li et al., 2000; Vickers,
2002). *Rhodiola rosea*, used in Chinese and Russian folk medicine was
shown to induce apoptosis as well as necrosis in HL-60 cells and caused
marked reduction in their survival (Majewska et al., 2006).

An ethanol extract of *Physalis peruviana*, a popular folk medicine
used in treating cancer, leukemia and hepatitis, inhibits the growth and
induces apoptotic death of human HepG2 cells in culture. The extract was
found to trigger apoptosis through the release of cytochrome c, smac/
DIABLO and Omi / HtrA2 from mitochondria to cytosol and consequently
resulted in caspase-3 activation (Wuab et al., 2004).

*Linum persicum* and *Euphorbia cheiradenia* were found to induce
apoptosis in two leukemic lines KSB2 and Jurkhat (Amirghofran et al.,
2006). Curcumin, the major ingredient of turmeric, is known to induce
apoptosis in several cancer cells, like colon (Chen et al., 1999),
hepatocarcinoma (Jiang et al., 1996) and breast carcinoma (Simon et al.,
1998) cells.

A crude aqueous whole plant extract of *Sutherlandia frutescens*
induced apoptosis in neoplastic cells like cervical carcinoma cell line
(Chinkwo, 2005). Phytochemicals from *Anoectochilus formosamus*, a
popularly used folk medicine in the treatment of cancer, induced apoptosis in MCF-7 human breast cancer cells (Shayur et al., 2004).

The plant used in the present study, Clitoria ternatea is a perennial twinning herb with terrated stems and branches with compound leaves. The flowers are blue or white (Plate 1). The propagation is through seeds. The parts used in medications include roots, leaves and seeds. The roots are bitter, refrigerant, ophthalmic, laxative intellect promoting, diuretic, antihemintic and depurative. The leaves are useful in otalgia, hepatopathy and eruptions. The seeds are cathartic and useful in visceralgia (Ramesh, 2005).

Clitoria ternatea is widely used in traditional Indian system of medicine as a brain tonic and is believed to promote memory and intelligence (Taranalli and Cheeramkuzhy, 2003). The plant is also useful for throat and eye infections, skin diseases, urinary troubles and ulcer, and have antidotal properties (Malabadi and Nataraja, 2001). Besides its medicinal property, Clitoria ternatea is also a good source of phytochemical substances. It contains antifungal proteins and has been shown to be homologous to plant defense in Ct-AMP-1 (Thevissen et al., 2000).

In spite of such studies reported, there are not many studies that have concentrated on the type of responses evoked by Clitoria ternatea leaves on oxidative stress imposed systems, as a whole and at the molecular level. The present study is an elaborate probe into the antioxidant and hepatoprotective activities of Clitoria ternatea leaves (from plants bearing blue and white flowers) (Plate 1) 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.