# *Review of Literature*

# 2. REVIEW OF LITERATURE

Oxygen is essential for survival. However, its univalent reduction generates several harmful Reactive Oxygen Species (ROS), inheritable to living cells and highly associated with a wide range of pathogenesis (Tripathi and Kamat, 2007). About 5% or more of the inhaled oxygen is converted to ROS such as ($O_2^{\bullet-}$), $H_2O_2$ and ($^{\bullet}OH$) by univalent reduction of oxygen (Gupta and Sharma, 2006).

A number of physiological processes in the human body lead to the generation of a series of oxygen-centered free radicals and other ROS as by-products. During stressful situations, the energy requirement of the organism is increased, resulting in enhanced generation of free radicals. In oxygen involving metabolisms, ROS are natural by products in eukaryotic organisms (Marxen *et al.*, 2007).

An inherent problem during oxygen utilization in the mitochondrial electron transport chain is the oxygen slippage, which is estimated to be from 3-5%. The oxygen slippage results in the generation of the superoxide radical ($O_2^{\bullet-}$), which in turn can lead to the generation of other ROS. The oxygen consumption rates and metabolic activities of the tissues differ considerably. Hence, it may be anticipated that the quantum of oxygen slippage and ROS generation from mitochondria and cytosolic systems can show tissue specific differences. This, in turn, may be reflected in terms of the mitochondrial and cytosolic defense mechanisms involved in the elimination of oxygen and ROS (Patel and Katyare, 2006).

## REACTIVE SPECIES

ROS is a collective term that includes both oxygen radicals and certain non radicals that are oxidizing agents and are easily converted into radicals (HOCl,

HOBr, $O_3$, $ONOO^-$, $O_2^{\bullet-}$, $H_2O_2$). In other words, all oxygen radicals are ROS, but not all ROS are oxygen radicals. Reactive Nitrogen species is a collective term including nitric oxide and nitrogen dioxide radicals, as well as non radicals such as $HNO_2$ and $N_2O_4$ (Halliwell and Whiteman, 2004). Living beings have evolved over the past two billion years through adaptation, to an increasing atmospheric oxygen concentration, by both taking advantage of oxygen activating function and developing a complex control network. In these regards, potentially damaging species including reactive oxygen, nitrogen and chlorine species arise as by-products of metabolism and also work as physiological mediators and signalling molecules (Vertuani *et al.*, 2004).

## REACTIVE OXYGEN SPECIES

Reactive oxygen species are highly reactive oxidizing agents belonging to the group of free radicals. They are compounds with one, or more unpaired electrons (Bhutia *et al.*, 2006). Reactive oxygen species such as superoxide anions ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and (NO) inactivate enzymes and damage important cellular components causing injury through covalent bonding and lipid peroxidation (Baskar *et al.*, 2007).

ROS play a positive role in energy production, phagocytosis, and regulation of cell growth, intercellular signalling and synthesis of biologically important compounds. However, overproduction of ROS is also harmful to the body because the oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases. The ROS are dangerous, and, when present in excess, can attack biological molecules such as lipids, proteins, enzymes, DNA and RNA, leading to tissue or cell injury associated with degenerative diseases (Muchuweti *et al.*, 2006).

## SOURCES OF ROS

ROS have aroused significant interest among scientists. In living organisms, various ROS can form in different ways. Normal aerobic respiration stimulates polymorphonuclear leukocytes, macrophages and peroxisomes, which appear to be the main endogenous sources of most of the oxidants produced by cells (Kumar *et al.*, 2006).

Oxygen free radicals or ROS, the by-products of cell metabolism, are also produced in the body on exposure to sunlight, X-rays, ozone, tobacco smoke, automobile exhaust and other environmental pollutants, which are the exogenous sources of ROS. There is increasing evidence to show the involvement of free radicals and ROS in a variety of diseases. They can cause damage to cellular biomolecules such as nucleic acids, proteins, lipids and carbohydrates and consequently may adversely affect immune function (Prakash *et al.*, 2007).

## REACTIVE NITROGEN SPECIES (RNS)

RNS include free radicals like nitric oxide (NO) and nitrogen dioxide ($NO_2$), as well as non radicals such as peroxynitrite ($ONOO^-$), nitrous oxide ($HNO_2$) and alkyl peroxynitrates (RONOO). NO easily reacts with superoxide, generating the highly reactive molecule $ONOO^-$ and triggering a cascade of harmful events. Nitrogen dioxide is a highly toxic reactive nitrogen species recently discovered as an inflammatory oxidant with great potential to damage tissues (Shrivastava *et al.*, 2004).

## REACTIVE CHLORINE SPECIES

Hypohalous acid can halogenate or oxidize pyrimidines and purines. It has been reported that HOCl-derived chlorine chlorinates 2-deoxycytidine to generate 5-chloro-2-deoxycytidine (5-CldC) as the major product. Eight chlorinated products of 2-deoxyadenosine and 2-deoxyguanosine have also been identified in

the reaction with HOCl. Alternatively, in contrast to these stable carbon-chlorinated products, the formation of semistable or unstable nitrogen-chlorinated products, chloramines (RNHCl and RR'NCl), was also observed. Unstable nucleoside chloramines, such as thymidine chloramine, are suggested to initiate DNA single and double strand breaks via nitrogen centered radicals and to transfer their chlorine atoms to other nucleosides (Hawkins and Davies, 2002).

*In vitro* studies suggest that hypohalous acid derived halogenation of DNA bases may provide one pathway for mutagenesis and cytotoxicity at sites of inflammation. Both carbon and nitrogen halogenation of nucleobases endogenously occurred *in vivo* and the halogenated nucleobases are implicated in the mutagenesis and cytotoxicity of target cells at sites of inflammation (Kawai *et al.*, 2004).

## FREE RADICALS

Free radicals are highly reactive species produced in the body during normal metabolic functions or introduced from the environment. Free radicals have been reported to play an important role in more than sixty different health conditions, including the aging process, cancer, and atherosclerosis. Therefore, it is beneficial for our health to scavenge these harmful free radicals. Nowadays, scientists have been exploring the biosensors to evaluate their antioxidant status to prevent some diseases and control the quality of foods (Mello and Kubota, 2007), which has lead to a revolution that is promising a new paradigm of healthcare (Lan, 2007).

Free radicals cause oxidation of biomolecules, including lipids, nucleic acids and proteins. Free radicals also damage biomembranes, reflected by increased peroxidation, thereby compromising cell integrity and function. Supplementation with various macro and micronutrients and herbal preparations

has been evaluated for their adaptogenic activity during exposure to stressful environment (Kenjale *et al.*, 2007).

## FORMATION OF FREE RADICALS

Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions. Enzymatic reactions, which serve as source of free radicals, include those involved in the respiratory chain, phagocytosis, and prostaglandin synthesis and in cytochrome P450 system. Free radicals arise in non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiations (Hu *et al.*, 2002).

Generally bonds will not split in a way that leaves a molecule with an odd, unpaired electron. But when a weak bond splits, free radicals are formed. Free radicals are very unstable and react quickly with other compounds, trying to capture the needed electron to gain stability. Free radicals attack the nearest stable molecule, "stealing" its electron. When the "attacked" molecule loses its electron, it becomes a free radical itself, beginning a chain reaction. Once the process is started, it can cascade, finally resulting in the disruption of a living cell. Sometimes the body's immune cells purposefully create them to neutralize viruses and bacteria. However, environmental factors such as pollution, radiation, cigarette smoke and herbicides can also spawn free radicals (http://www.healthcheck systems.com/antioxid.htm).

## TYPES OF FREE RADICALS

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

Superoxides are produced from molecular oxygen due to oxidative enzymes of the body as well as via non-enzymatic reactions such as autooxidation by catecholamines (Khanam *et al.*, 2004). In addition, superoxide is generated upon uncoupling of the mitochondrial respiratory chain, oxidizes endothelial

derived nitric oxide and thus impairs endothelial function (Baldus and Meinertz, 2006). Superoxide radicals are known to be very harmful to cellular components as a precursor of more reactive oxygen species (Anandjiwala *et al.*, 2007). Superoxide is biologically important, since it can form more toxic hydrogen peroxide, by the SOD reaction and can be decomposed to form stronger oxidative species such as hydroxyl radical and singlet oxygen (Devi *et al.*, 2007).

## HYDROXYL RADICAL ($OH^{\bullet}$)

Hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage. The hydroxyl radical is an extremely reactive oxidizing radical that reacts with molecules at diffusion controlled rates (Soni *et al.*, 2006). Hydroxyl radicals have been implicated as highly damaging species in free radical pathology. This radical cause DNA strands breakage, which results in mutagenesis, carcinogenesis and cytotoxicity. Hydroxyl radical scavenging capacity is directly related to the antioxidant activity of test compounds and extracts (Babu *et al.*, 2001).

## HYDROGEN PEROXIDE ($H_2O_2$)

Exogenous and endogenous sources of hydrogen peroxide are believed to play an important role in the generation of free radical mediated DNA damage. Hydrogen peroxide contains one or more atoms of oxygen than does water. The cells responsible for fighting infection and foreign invaders in the body make hydrogen peroxide and use it to oxidize any offending culprits (Vaughan, 2005).

## SINGLET OXYGEN ($^1O_2$)

It is not a free radical and is formed as a result of spin reversal of electron in the outer orbital of oxygen molecule. It is considered a highly potent oxidant with short half-life causing tissue damage (Tandon and Gupta, 2005). Singlet

oxygen can be generated by a range of enzymatic and non-enzymatic reactions including processes mediated by heme proteins, lipoxygenases and activated leukocytes, as well as radical termination reactions (Davies, 2003).

## HYPOCHLOROUS ACID (HOCl)

HOCl formed from $H_2O_2$ and chloride in a reaction catalysed by myeloperoxidase activated by neutrophils, is also a potent oxidizing agent that produces DNA damage (Azad *et al.*, 2008). It is an effective biological oxidant produced by activated neutrophils. It plays a role of the major inflammation mediator in mammalian tissues (Lapshina *et al.*, 2006).

## NITRIC OXIDE (NO)

Nitric oxide is a free radical generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Oxygen reacts with the excess NO to generate nitrate and peroxy nitrite anions, which act as free radicals (Shivaprasad *et al.*, 2008). Nitric oxide is recognized as a mediator and regulator of inflammatory response. NO can also interact with molecular oxygen and superoxide anion to produce Reactive Nitrogen Species (RNS) that can modify various cellular functions (Tatiya *et al.*, 2008).

## PEROXY NITRITE ($ONOO^-$)

Peroxynitrite, formed from the reaction of superoxide and nitric oxide, is one of the most potent cytotoxic species known to oxidize cellular constituents including essential lipids, DNA and protein. $ONOO^-$ induces cellular and tissue injury, resulting in several human diseases such as Alzheimer's disease, atherosclerosis and stroke (Huang *et al.*, 2007).

## OXIDATIVE STRESS

Oxidants or free radicals are atoms or molecules capable of independent existence that contain one or more unpaired electrons, making these species highly reactive. Oxidant mediated injury or oxidant stress may occur through the reactions of these radicals and also those of non radicals. Oxygen and related species (oxidants) may contribute to the pathogenesis of a number of diseases (Rai and Phadke, 2006).

Oxidative stress is a state of imbalance between the generation of ROS like hydroxyl and superoxide radicals and the level of antioxidant defense system. Hydrogen peroxide formed as a natural by product of enzymatic oxidase action, is an endogenous source of hydroxyl free radicals that contributes to the background level of cellular oxidative stress. Exogenous $H_2O_2$ can elevate oxidative stress beyond the protective capacity of endogenous antioxidant defenses and induce apoptotic cell death. Oxidative stress and apoptosis play important roles in the pathogenesis of neurodegenerative disorders (Yu *et al.*, 2008).

Free radicals or oxidative injury now appears to be the fundamental mechanism underlying a number of human neurologic and other disorders. For instance, in diabetes, increased oxidative stress, which co-exists with reduction in the antioxidant status, has been postulated. Oxygen free radicals can initiate peroxidation of lipids, which in turn stimulates glycation of proteins, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes and play a role in the long term complication of diabetes (Atawodi, 2005). Oxidative stress has been implicated in the pathology of many diseases such as cancer, diabetes, aging and inflammatory conditions. Free radical damage to DNA induces mutations leading to uncontrolled proliferation of cells that characterizes cancer (Shrikumar *et al.*, 2007).

## LIPID PEROXIDATION (LPO)

Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids and involves ROS and transition metal ions. ROS and free radical-mediated processes have also been implicated in the pathogenesis of a variety of diseases. They damage cells by chemical chain reactions such as LPO or formation of DNA adducts that could cause cancer-promoting mutations or cell death. To prevent free radical damage, the body has a defense system of antioxidants (Sreejith *et al.*, 2007). Lipid peroxidation is regarded as one of the basic mechanisms of cellular damage caused by free radicals. Free radicals react with lipids, causing peroxidation and resulting in the release of products such as malondialdehyde, $H_2O_2$ and hydroxyl radicals (Ramprasath *et al.*, 2006).

## DISEASES ASSOCIATED WITH ROS

ROS induced oxidative damage has been implicated in the pathogenesis of several disorders and diseases. ROS/RNS are the key players in inflammation and cancer as their increased production leads to genetic mutations, predisposing individuals to cancer.

## CANCER

Cancer is a major health problem worldwide and one of the most important causes of morbidity and mortality in children and adults (Antony *et al.*, 2007). DNA damage, mutations and altered gene expression are key players in the process of carcinogenesis. The involvement of oxidants appears to be the common denominator of all these events (Valko *et al.*, 2004).

Cancer is a multifactorial heterogeneous disease characterized by the multistage nature of pathogenesis (Surch and KyungNa, 2007). Damage to DNA by ROS has been widely accepted as a major cause of cancer. In patients with diseases associated with a risk of cancer, an increased rate of oxidative DNA

damage or, in some instances, deficient repair system such as Fanconi anemia, chronic hepatitis, cystic fibrosis and various autoimmune diseases, are observed. ROS can damage DNA and the division of cells with unpaired or misrepaired damage can lead to mutations. Indeed, these species can act at several steps in the multistage carcinogenesis. It is now assumed that ROS are involved both in the initiation and the progression of cancer (Waris and Ahsan, 2006).

## DIABETES MELLITUS

Diabetes mellitus is a chronic, systemic, metabolic disease defined by hyperglycemia and characterized by alterations in the metabolism of carbohydrate, protein and lipid. Diabetes mellitus is a serious metabolic disorder and it is estimated that its annual incidence rate will continue to increase in the future world wide (Pavana *et al.*, 2007). Hypertension induced increase in glucose causes autoxidation, protein glycation and the subsequent oxidative degradation of glycated protein, which leads to enhanced production of reactive oxygen species. Deleterious effects of diabetes on the central nervous system are related to the oxidative imbalance set forth by hypertension (Devi *et al.*, 2007). In recent years, the oxidative stress-induced free radicals have been implicated in the pathology of insulin dependent diabetes mellitus (IDDM) (Ramakrishna and Jailkhani, 2007).

## ATHEROSCLEROSIS

Oxidative stress has been recognized as a key mechanism in the development of vascular damage, particularly atherosclerosis (Minuz *et al.*, 2006). Atherosclerosis is a very complex disease involving both genetic and environmental risk factors and their interactions. In the general population, genetic polymorphisms of many genes in the pathways of lipid metabolism, inflammation and thrombogenesis are likely to be responsible for the wide range of susceptibilities to myocardial infarction, the most deadly consequence of atherosclerosis (Chen *et al.*, 2007).

## CIRRHOSIS

Cirrhosis is commonly associated with abnormalities in the systemic circulation and impaired primary homeostasis. Gastrointestinal and oesophageal bleeding are the major causes of death in patients with cirrhosis. In general, the oxidative stress and the cell membrane integrity play an important role in the cell lysis. Many reports showed the defective function of platelets as the cause of bleeding in cirrhosis. But the information regarding the functional status of RBC in cirrhosis is limited. A recent study demonstrated the level of oxidative stress to be responsible for the membrane integrity of RBC in patients with liver cirrhosis (Geetha *et al.,* 2007).

## HYPERTENSION

Although the etiology of essential hypertension has genetic components, lifestyle factors such as diet also play an important role. Insulin resistance and glucose intolerance are common features of hypertension in humans and in animal models. Altered glucose metabolism leads to an increased production of the reactive aldehyde methylglyoxal. Methyl glyoxal binds sulfhydryl and amino groups of proteins forming conjugates / advanced glycation end products (AGES). This alters protein structure and function that can affect vascular calcium channels, enzymes, and tissue proteins leading to increased oxidative stress. These alterations impair endothelial function leading to an increase in intracellular free calcium, peripheral vascular resistance, and hypertension. Supplementation with antioxidants, including vitamins C, E or B6, thiols such as lipoic acid and cysteine and the quinine coenzyme Q10, have been shown to lower blood pressure in animal models and humans with essential hypertension (Vasdev and Vicki, 2005).

## ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a complex, multifactorial, heterogeneous mental illness, which is characterized by an age-dependent loss of memory and impairment of multiple cognitive functions (Reddy, 2006). The main characteristics of this disease are difficulties in household handling, routine and cognitive and emotional disturbance in the elderly (Neto *et al.,* 2006). Several oxidative stresses progressively lead to cell dysfunction and ultimately cell death. Oxidative stress is an imbalance between prooxidants and / or free radicals on one hand, and anti-oxidising systems on the other. ROS is hypothesized to be the main etiologic factor for progressive and specific neuronal degeneration, which is observed in the Alzheimer's disease (Vaisi- Raygani *et al.*, 2007).

## PARKINSON'S DISEASE

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra and the accumulation of proteinaceous intraneuronal inclusions known as Lewy bodies. The mechanisms responsible for neurodegeneration in PD are largely unknown, although previous work suggests that mitochondrial complex I dysfunction, oxidative stress and aberrant proteolytic degradation may contribute to pathogenesis (Whitworth *et al.,* 2005).

## RHEUMATOID ARTHRITIS (RA)

Rheumatoid arthritis is a very common chronic illness that affects around 1% of people in developed countries. It is caused by an abnormal immune reaction to various tissues within the body, as well as affecting joints and causing an inflammatory arthritis, it can also affect many other organs of the body. Severe rheumatoid arthritis can be life-threatening, but even mild forms of the disease cause substantial illness and disability (Kurreeman *et al.,* 2007). Free radicals have

long been implicated as mediators of tissue damage in RA patients, which are released in large amounts into the surrounding tissue. When the endogenous antioxidant defenses are overcome, the resulting production of free radicals induces impairment and destruction of the affected joint constituents such as synovial fluid, cartilage and other articular constituents. LPO is considered as a critical mechanism of the injury that occurs during RA (Narendhirakannan *et al.*, 2005).

## AGING

Aging is a process of bodily changes with time, leading to increased susceptibility to diseases, and ultimately death. Because reactive oxidant species and immune dysfunction are major causes of age-related diseases, the maintenance of antioxidant and immune fitness is a rational approach to preventive healthcare. Accumulation of ROS–induced oxidative damage to DNA, proteins, and other macromolecules have been regarded as a major endogenous cause of aging (Ko and Leung, 2007).

## ANTIOXIDANTS

The antioxidant system is important for cells in the defense against endogenous and exogenous ROS insult. The antioxidant enzymes work coordinately to achieve an equilibrium between pro-oxidant and antioxidant systems (Chowdhury *et al.,* 2007). Antioxidants, both enzymic as well as non-enzymic, provide protection against oxidative damage caused by several toxic reactants. Under oxidative stress, antioxidant enzymes such as catalase and SOD protect cells by maintaining low $O_2$ and $H_2O_2$ levels (Ghosh *et al.*, 2007).

Ideally, antioxidants should be odorless, colourless and non-toxic, should not interfere or alter the nutritional value of food products and should be effective at low doses. They are classified into 2 major groups of primary and secondary

antioxidants. The former category includes those substances that act as free-radical scavengers by donating a hydrogen atom to the free radical, thus creating a less reactive and less toxic product. On the contrary, secondary antioxidants inhibit the effects of pro-oxidants as well as enhance the action of the primary antioxidants (Sarkardei and Howell, 2007).

Antioxidants are potent scavengers of free radicals and serve as inhibitors of neoplastic process (Srinivasan *et al.*, 2007). Antioxidants are physiologic substances that are derived from both endogenous and exogenous sources that act against oxidant stress. Antioxidants may delay or prevent direct oxidation of oxidizable substrates or scavenge oxidant free radicals and thus neutralize the physiologic oxidant burden created by both exogenous and endogenous free radicals (Rai and Phadke, 2006).

An antioxidant is any substance that, when present at low concentration, significantly delays or prevents the oxidation of cell contents like proteins, lipids, carbohydrates and DNA. Antioxidants can be classified into 3 main types: First line, Second line and Third line defense antioxidants. SOD, CAT, GPx, glutathione reductase and some minerals like Se, Mn, Cu, Zn come under first line defense antioxidants. Glutathione, vitamin C, uric acid, albumin, bilirulin, vitamin E (mainly $\alpha$ - tocopherol), carotenoids, flavonoids etc., come under second line defense antioxidants. Third line antioxidants are a complex group of enzymes for repairing damaged DNA, damaged protein, oxidized lipids and peroxides and also to stop chain propagation of peroxyl lipid radical. The enzymes repair the damaged biomolecules and reconstitute the damaged cell membrane, e.g. lipases and DNA repair enzymes (Gupta and Sharma, 2006).

## ENZYMIC ANTIOXIDANTS

An array of non-enzymic antioxidants [vitamin E, vitamin C and reduced glutathione (GSH)] and enzymic antioxidants (SOD, CAT and GSH-Px) are

involved in the protection of free radicals-induced oxidative damage. The levels of ROS are regulated by a variety of cellular defense mechanisms consisting of enzymatic and non enzymatic systems (Sivakami *et al.*, 2003).

## SUPEROXIDE DISMUTASE (SOD)

SOD mainly acts by quenching superoxide ($O_2^{-\bullet}$) radical. SOD is an inbuilt defense mechanism to fight back peroxidative stress (Bet *et al.*, 2006). SODs are a class of closely related enzymes that catalyse the breakdown of the superoxide anion into oxygen and hydrogen peroxide. SOD enzymes are present in almost all aerobic cells and in extracellular fluids. Superoxide dismutase enzymes contain metal ion cofactors that, depending on the isozyme, can be copper, zinc, manganese or iron. In humans, the copper/zinc SOD is present in the cytosol, while manganese SOD is present in the mitochondrion. There also exists a third form of SOD in extracellular fluids, which contains copper and zinc in its active site. The mitochondrial isozyme seems to be the most biologically important of these three, since mice lacking this enzyme die soon after birth. In contrast, the mice lacking copper/zinc SOD are viable but have lowered fertility, while mice without the extracellular SOD have minimal defects. In plants, SOD isozymes are present in the cytosol and mitochondria, with an iron SOD found in chloroplasts that is absent from vertebrates and yeast (Grayck *et al.*, 2005).

## CATALASE (CAT)

Catalase brings about the decomposition of hydrogen peroxide to water and oxygen. Catalase is an enzymic antioxidant widely distributed in all animal tissues and the highest activity is found in the red cells and in the liver. CAT decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals. Therefore, the reduction in the activity of these enzymes may result in a number of

deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide (Dash *et al.*, 2007).

## PEROXIDASE

Peroxidases are a large family of enzymes. For many of these enzymes, the optimal substrate is hydrogen peroxide, but others are more active with organic hydroperoxides such as lipid peroxides. Peroxidases can contain a heme cofactor in their active sites or redox-active cysteine or seleno cysteine residues (http://en.wikipedia.org/wiki/Peroxidase).

Glutathione peroxidase is a selenium containing enzyme which catalyses the reduction of $H_2O_2$ and lipid hydroperoxide, generated during lipid peroxidation, to water using reduced glutathione as substrate. It catalyses the breakdown of inorganic and organic peroxides, prevents lipid peroxidation and protects the cell membrane from oxidative damage (Geetha *et al.*, 2007).

## GLUTATHIONE S-TRANSFERASE (GST)

Glutathione S-transferase consists of a large family of GSH utilizing enzymes that play an important role in the detoxication of xenobiotics in mammalian systems (Sohini and Rana, 2007). Glutathione-S-transferase and glutathione peroxidase are the most abundant and ubiquitous detoxification enzyme families in the plant system. These enzymes play a pivotal role in inhibiting the cellular damage produced by a wide variety of stresses. GST plays an essential role in liver by eliminating toxic compounds by conjugating them with glutathione. In our physiological system, glutathione and the related enzymes play a key role in the defense against oxidative stress (Ray *et al.*, 2007).

## GLUTATHIONE REDUCTASE (GR)

Glutathione reductase, a flavo enzyme, catalyses the reduction of oxidized glutathione to reduced glutathione, an important endogenous antioxidant (Chai *et al.*, 2005). GR also plays a key role in the antioxidant defense process, by reducing oxidized glutathione reaction, which consumes NADPH, thus allowing high GSH/GSSH ratio to be maintained (Ali *et al.*, 2006). GR is important in maintaining the level of thiol groups in the cells, the absence of which provokes cellular lysis. The GSH produced by GR combines with hydrogen peroxide in the presence of GPx to regenerate GSSG, thus preventing the harmful build up of $H_2O_2$. Erythrocyte GR deficiency in man has been reported to be associated with clinical syndromes of hemolysis and neurological disorders (Pretsch, 2002).

## NON-ENZYMIC ANTIOXIDANTS

### VITAMIN C

Vitamin C, a major ubiquitous non-enzymatic antioxidant, has a crucial role in scavenging several reactive oxygen species. It is an important antioxidant in humans, capable of scavenging oxygen-derived free radicals (Ardekani and Ardekani, 2007). At physiological concentrations, vitamin C is a potent free radical scavenger in the plasma, protecting cells against oxidative damage caused by ROS. The antioxidant property of ascorbic acid is attributed to its ability to reduce potentially damaging ROS formation, prevention of DNA mutation induced by oxidation, protection of lipids against peroxidative damage and repair of oxidized amino acid residues to maintain protein integrity (Li and Schellhorn, 2007).

### VITAMIN E

Vitamin E is one of the major chain breaking lipophilic antioxidants within the cell membrane, where it protects membrane fatty acids from peroxidation.

Vitamin E scavenges peroxyl radical intermediates in lipid peroxidation and is responsible for protecting PUFA (polyunsaturated fatty acids) present in cell membrane and low density lipo protein (LDL), against peroxidation (Pavana *et al.*, 2007). Singlet oxygen quenching by tocopherols is highly efficient and it is estimated that a single α-tocopherol molecule can neutralize upto 120 singlet oxygen molecules *in vitro* before being degraded (Shao *et al.*, 2008). As a redox active compound, α-tocopherol can react anti or pro-oxidatively depending on the reaction partner, thus α-tocopherol repairs co-antioxidants to become beneficial (Flohe, 2007).

## TOTAL CAROTENOIDS

Carotenoids are biochemically active pigments. They scavenge free radicals and decrease immuno suppressive peroxides, enhance the production of lymphocytes, the phagocytic ability of neutrophils and macrophages and tumor immunity (Horak *et al.*, 2004). β-carotene, an excellent scavenger of singlet oxygen, which is a precursor to vitamin A (retinol), is present in liver, egg yolk, milk, butter, spinach, carrots, squash, broccoli, yams, tomato, cantaloupe, peaches, and grains (http://www.rice.edu/~jenky/sports/antiox.html).

## LYCOPENE

Lycopene, a lipid soluble carotenoid antioxidant, is synthesized by many plants and microorganisms but not by animals and humans. It is a highly unsaturated open straight chain hydrocarbon consisting of 11 conjugated and 2 unconjugated double bonds (Rao *et al.*, 2006). It is responsible for the red color of many fruits and vegetables such as the tomatoes. Unlike some other carotenoids, lycopene lacks the terminal β-ionic ring in its structure and provitamin A activity. In nature, lycopene is present primarily in the all trans isomeric forms (Rao and Ali, 2007).

Antioxidants, such as lycopene, can interact with ROS at an early stage and prevent the cellular oxidation, which can either delay or prevent the progression of human diseases. The evidence to date for the beneficial role of lycopene in human diseases comes primarily from epidemiological studies. However, tissue culture studies utilizing several human cancer cell lines and animal studies have also been reported in the literature (Rao and Agarwal, 1999).

## REDUCED GLUTATHIONE

The non-enzymic antioxidant, glutathione is one of the most abundant tripeptides in the liver. Its functions are mainly concerned with the removal of free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals and maintenance of membrane protein thiols and as a substrate for glutathione peroxidase and GST. Glutathione is a major non-protein thiol in living organisms which plays a central role in co-ordinating the body's antioxidant defense processes (Venukumar and Latha, 2002).

GSH is a scavenger of many free radicals like (•HO) and various lipid hydroperoxides, may help to detoxify many oxidizing air pollutants like ozone and free radicals in cigarette smoke. GSH constitutes a major reducing substance of the cytoplasm and is known to protect the cellular system against the toxic effects of lipid peroxidation. GSH and vitamins C and E exist in the interconvertible reduced and oxidized forms and thus participate in neutralizing free radicals as and when they are formed. There is a well-established synergism between vitamin E, vitamin C and glutathione through the antioxidant network (Devi *et al.*, 2007).

## POLYPHENOLS

Polyphenols have three defense functions as radical scavengers, as biocides and metal chelators (Pietarinen *et al.*, 2006). Polyphenols are known to exert anti-ulcerative, anti-carcinogenic, anti-inflammatory, anti-arthritic and anti-viral

activities, prevent skin aging, scavenge oxygen-free radicals and inhibit UV radiation-induced peroxidation activity. Polyphenols are a wide class of substances, which contain over 8,000 compounds, from those with simple structure (e.g., phenolic acids) to the polymeric substances, such as tannins. They occur naturally in fruits, vegetables, nuts, seeds, flowers and bark and are an integral part of the human diet. They have been reported to exhibit a wide range of biological effects, including antibacterial, antiviral, antioxidant, anti-inflammatory, antiallergic, and vasodilatory actions (Bravo, 1998; Balasundram *et al.*, 2006; Koleckar *et al.*, 2007; Rehakova *et al.*, 2007).

## ANTIOXIDANT THERAPY

During the functioning of the immune system such as phagocytosis, reactive oxygen and nitrogen species are generated. If they are left unchecked, they can affect the components of the immune system by inducing oxidative damage. This is more so in the elderly or during inflammation where there is excess generation of these reactive species that can be taken care of by the defenses in the form of "Antioxidants". Free radical oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from deficient natural antioxidant defenses. Potential antioxidant therapy should therefore be including either natural free radical scavenging antioxidant enzymes and molecules, or agents which are capable of augmenting the activity of the antioxidant enzymes (Selvi *et al.*, 2007).

## APOPTOSIS

Programmed cell death or apoptosis is a genetically controlled process with important roles during development of multicellular organisms. Molecular genetic studies have revealed that the basic principles of regulation and execution of apoptosis are conserved. One common feature in the cell death program is the activation of caspases, a highly specialized class of cell death proteases. These

enzymes are generally divided into two distinct classes: initiator and effector caspases. Upon activation, initiator caspases activate downstream effector caspases via proteolytic processing, and activated effector caspases cleave key cellular substrates to promote apoptosis (Fan and Bergmann, 2008).

Apoptosis is a fundamental form of cell death that plays a major role in the development and homeostasis of multicellular organisms. Many lines of evidence have indicated that apoptotic process requires specialized machinery. One of the main regulatory steps of apoptotic cell death is controlled by the ratio of anti-apoptotic (Bcl-2, Bcl-XL, Bag-1 and Bcl-W) to pro-apoptotic (Bax, Bad, Bak and Bcl-Xs) members of the Bcl-2 family. There is a growing consensus that oxidative stress and the redox state of a cell play a pivotal role in regulating apoptosis. Oxidative stress induces a number of downstream events in apoptosis, including caspase activation and DNA fragmentation (Liu *et al.*, 2005a).

In addition to an essential role of caspases in apoptosis, recent findings have demonstrated that caspases also have important functions in non-apoptotic processes. One such non-apoptotic process is the induction of compensatory proliferation in apoptotic tissue. Coordination of cell death and cell proliferation is critical for the maintenance of tissue homeostasis. Excessive cell loss in a developing tissue can be compensated for by additional divisions of the remaining cells. This suggests that apoptotic cells induce compensatory proliferation of neighboring cells through the secretion of mitogens (Kuranaga and Miura, 2007; Lamkanfi *et al.*, 2007).

## APOPTOSIS INDUCING FACTOR

Cells undergoing apoptosis usually exhibit a characteristic morphology, including apoptotic body formation, fragmentation of the cellular proteins, nuclear and cytoplasmic condensation and chromosomal DNA cleavage. These characteristics of apoptosis comprise both caspase-dependent and caspase-

independent pathways (Kim *et al.*, 2006). Apoptosis Inducing Factor (AIF) is a ubiquitous FAD-binding flavoprotein comprised of 613 aminoacids and plays an important role as caspase-independent apoptotic effector, which translocates via cytosol to nuclei, where it causes chromatin condensation and large scale DNA fragmentation, and subsequently leads to apoptotic cell death (Henshall and Murphy, 2008).

## MORPHOLOGICAL CHANGES

Apoptotic cell death was initially defined by characteristic morphological features including condensation of cytoplasm and nucleus with cell shrinkage. Additionally, there is internucleosomal cleavage of chromatin and blebbing of the plasma membrane. Importantly, during apoptosis the cell membrane remains intact, preventing the release of cellular contents, and there is exposure of a number of surface molecules that trigger rapid engulfment by neighboring or phagocytic cells. Consequently, there is little inflammation during apoptosis (Winn and Harlan, 2005).

## NUCLEAR EFFECTS

Fluorescent nuclei can be scored and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei show non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei are identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin (Ramalho *et al.*, 2006).

## DNA FRAGMENTATION DURING APOPTOSIS

Apoptosis results from a collapse of cellular infrastructure through internal proteolytic digestion, which leads to cytoskeletal disintegration, metabolic derangement and genomic fragmentation. DNA fragmentation, the typical hall mark of apoptosis, can be visualized as DNA laddering on agarose gels and by the

appearance of a subdyploid cell population with lower DNA content by flow cytometry. The apoptotic process leads to fragmentation of cellular DNA in characteristic oligonucleosomal fragments as multiples of 200 base pairs (Barbini *et al.*, 2006).

## FUNCTIONS OF APOPTOSIS

Apoptosis serves as the physiological response to exogenous and endogenous death signals (Eberle *et al.*, 2007). Apoptosis is vitally important for tissue homeostasis and development in multicellular organisms. Specific cell populations are eliminated by apoptosis at different stages of embryogenesis and also in adult tissues. For example, during gut development, the innermost cells lining the lumen undergo apoptosis and exfoliate, while cells adjacent to the basement membrane become polarized epithelial monolayer along the length of the intestine. Apoptosis is also involved in the differentiation of fingers in the developing embryo as well as in the morphogenesis of ducts in the development of mammary gland (Zahir and Weaver, 2004).

Apoptosis is known to play an important role in the development of many diseases associated with aging, such as Alzheimer's disease, Parkinson's disease and stroke. It can be suggested that during aging, apoptosis is enhanced and the protection level of cells from programmed death decreases (Bazhanova, 2005).

## ALTERNATIVES TO THE USE OF LIVE ANIMALS

At present, safety evaluation studies depend predominantly on animal testing, as only a limited number of non-animal methods are validated. Thus, there is a dire need for non-animal, alternative methods in place of animal procedures wherever possible, which would minimize the animal use and suffering (Anon, 2002). In this direction, alternative methods such as *in vitro* cell-based assays, genomics and computational analysis have gained more attention (Suggitt and

Bibby, 2005). Replacement, Reduction and Refinement of animal procedures (the Three Rs) are implemented wherever possible. Over the last decade, there has been substantial progress with applying *in vitro* and *in silico* methods to both drug efficacy and safety testing (Combes *et al.*, 2003).

Alternatives to the use of live animals include *in vitro* model systems, which in turn minimize stress and suffering to animals. More commonly, *in vitro* systems are thought of as cell, tissue and organ cultures. The source of the tissue may be primary tissue or cell lines, obtained invasively or non-invasively. Tests have been developed using *in vitro* systems for the evaluation of cytotoxicity, inflammation and genotoxicity (http://compliance.vpr.okstate.edu/acuc/alternatives.htm).

## *In vitro* MODELS

To reduce the number of animal studies, other methods may also be utilized using cell lines or simple cell cultures in the laboratory under *in vitro* conditions (http://idw-online.de/pages/de/news219496). Some of the alternative methods gaining popularity are listed below.

## ORGAN SLICES

Precision-cut organ slices represent an *in vitro* model that mimics closely the multicellular complexity and extracellular interactions of the intact organ. The architectural composition of the various cell types is retained, as well as the cell-matrix and cell-cell interactions. Through the application of this model to various organs and species, mechanistic pathways leading to organ injury can be investigated *in vitro*. Furthermore, the identification of biologically relevant markers of human response to drug-induced injury can be compared (Vickers *et al.*, 2004).

## PRIMARY CULTURE AND CELL LINES

Primary cultures of human cells provide an increasingly important alternative to using virally transformed or otherwise immortalized cell lines or to using cloned cell lines derived from human or animal tumors. Advances in primary cell culture techniques, media formulations, and other reagents have enabled routine culture of primary cells derived from human tissues for biomedical research and drug discovery approaches (Marshak and Greenwalt, 2006). *In vitro* model systems with human cells is increasingly used to investigate the mechanism of chemical-induced toxicity and for toxicity screening of new drug families (Braga *et al.*, 2007).

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. 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, it has an interesting application in the evaluation of therapeutic agents that potentially may treat the dysfunction (Allen *et al.*, 2005).

Cell lines are widely used in many aspects of laboratory research, particularly as *in vitro* models in cancer research. They have a number of advantages, as they are easy to handle and represent an unlimited self replicating source that can be grown in almost infinite quantities. In addition, they exhibit a relatively high degree of homogeneity and are easily replaced from frozen stocks, if lost through contamination (Burdall *et al.*, 2003).

## YEAST CELLS

*Saccharomyces cerevisiae*, because of its well-defined genetic system, robust viability, and ease of handling, is an ideal model organism for studying eukaryotic cells (Purevdorj-gage *et al.*, 2006).

Yeast has proved to be a valuable tool for investigating basic molecular mechanisms in eukaryotes, e.g., cell cycle progression. Recent research revealed the presence of a regulatory network of apoptosis in yeast containing many of the crucial steps of mammalian apoptosis. The finding that factors relevant for apoptosis in animals are present in yeast and in lower eukaryotes suggests that apoptosis developed in unicellular organisms long before the evolutionary separation between fungi, plants and metazoan animals occurred (Madeo *et al.*, 2002). Yeast cells have been an informative model system for human cells, revealing many conserved aspects of cell biology. *Saccharomyces cerevisiae* was found to undergo apoptosis, showing characteristic markers such as DNA cleavage and phosphatidyl serine externalization (Du *et al.*, 2007).

## MEDICINAL PLANTS

Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject for very intense pharmacological studies, this has been brought about by the acknowledgement of the value of medicinal plants as potential sources of new compounds of therapeutic value and as sources of lead compounds in drug development. In developing countries, it is estimated that about 80% of the population really depends on traditional medicine for their primary healthcare. There arises a need to screen medicinal plants for bioactive compounds as a basis for further pharmacological studies. Medicinal plants are considered to be an important source of antioxidant compounds, and the therapeutic benefit of many medicinal plants often attributes to their antioxidant properties (Hasan *et al.*, 2007).

Medicinal plants are sources of important therapeutic aids for alleviating aliments. Advanced biotechnological methods of culturing plant cell and tissues should provide new means of conserving rapidly propagating valuable, rare and endangered medicinal plants (Ingale, 2006).

## PHYTOCHEMICALS

Numerous epidemiological studies have shown that free radicals are the leading causes of oxidative stress-related diseases like cancer, cardiovascular diseases and neurodegenerative disorders. Recently, naturally occuring antioxidants which are radical scavengers such as phenolic phytochemicals, vitamins C and E have received growing attention, because they are known to function as chemopreventive agents against oxidative stress-mediated diseases. However, the contribution of vitamin C to the total antioxidant activity of fruits is generally <15%. Therefore, many efforts have been directed towards elucidating the potential health benefits of dietary phenolic phytochemicals that have stronger antioxidant activities than vitamin C (Pakhale *et al.*, 2007).

A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials. Various herbs and spices have been reported to exhibit antioxidant activity. A majority of the antioxidant activity is attributed to the flavones, isoflavones, flavonoids, anthocyanin, coumarin, lignans, catechins and isocatechins. Antioxidant based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer (Khalaf *et al.*, 2007).

Phytochemicals mediate the interaction of plants with their environment functioning as feeding deterrents, pollination attractants, and protective compounds against pathogens or various abiotic stresses, antioxidants or signaling molecules. Phytochemicals, such as polyphenols and carotenoids, are present in a wide range of fruit and vegetable crops, whereas some phytochemicals are

distributed only among limited taxonomic groups. For example, glucosinolates are only found in the cruciferous vegetables crops, whereas the occurrence of sulfides is restricted to the *Liliaceae*. Additionally, each fruit and vegetable species has a distinct profile of phytochemicals, which is also within a special phytochemical group (Schreiner and Huyskens-keil, 2008)

Cancer chemoprevention by phytochemicals may be one of the most feasible approaches for cancer control. Phytochemicals obtained from vegetables, fruits, spices, teas, herbs and medicinal plants, such as alkaloids, terpenoids and other phenolic compounds, have been proven to suppress experimental carcinogenesis in various organs in pre-clinical models. Recent studies have indicated that mechanisms underlying chemopreventive potential may be a combination of antioxidant, anti-inflammatory, immune-enhancing, and hormone modulation effects, with modification of drug metabolizing enzymes, influence on cell cycle and cell differentiation, induction of apoptosis, suppression of proliferation and angiogenesis, playing roles in the initiation and secondary modification stages of neoplastic development (Rabi and Gupta, 2008).

## ALKALOIDS

Alkaloids are pharmacologically active compounds, which are widely used as pharmaceuticals and are synthesised as secondary metabolites in plants. Many of these compounds are strongly toxic. Therefore, they are often the subject of scientific interest and analysis (Petruczynika and Waksmundzka-Hajnos, 2006). Alkaloids represent a large and diverse group of compounds that are related by the occurrence of a nitrogen atom within a heterocyclic backbone. Unlike other types of secondary metabolites, the various structural categories of alkaloids are unrelated in terms of biosynthesis and evolution (Facchini and De Luca, 2008).

## PHENOLS

Phenolic compounds exhibit a wide range of biological and physiological properties due to their ability to act as antioxidants, free radical scavengers and chelators of divalent cations (Balasundram *et al.*, 2005) and the antioxidant activity of phenolics is mainly because of their redox properties that allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Kaur and Kapoor, 2002).

The flavonols and phenolic acids are particularly attractive, as they are known to exhibit various beneficial pharmacological properties such as the vasoprotective, anticarcinogenic, antineoplastic, antiviral, anti-inflammatory, as well as antiallergic and antiproliferative activity on tumor cells. Some of these properties have been related to the action of these compounds as antioxidants, free radical scavengers, quenchers of singlet and triplet oxygen and inhibitors of peroxidation. Antioxidant activity of phenolic compounds is correlated to some structure-activity relationships, such as redox properties and the number and arrangement of hydroxyl groups (Carr *et al.*, 2000).

## FLAVONOIDS

Flavonoids are phenolic compounds, present in several plants that inhibit lipoxygenases. Flavonoids are a group of polyphenolic compounds. Most of their beneficial health effects are attributed to their antioxidant and chelating properties. Flavonoids can interfere with not only the propagation reactions of the free radicals, but also with the formation of the radicals, either by chelating the transition metal, or by inhibiting the enzymes involved in the initiation reaction (Liu *et al.*, 2008). In addition, flavonoids inhibit lipid peroxidation (LPO), platelet aggregation, decrease capillary permeability, and the activity of enzyme systems, including cyclo-oxygenase and lipoxygenase. According to structural features, flavonoids are divided into various subclasses, such as flavones, flavonols,

flavanones, or flavan-3-ols (catechins). All subtypes can also occur as flavonoid glycosides. Flavonoids exhibit several biological effects such as anti-inflammatory, hepatoprotective and anti-dermatitic (Prabhu *et al.*, 2008).

In India, Ayurveda has often been proposed as a good alternative system of medicine. However, due to the lack of credible experimental evidences, it is not as popular as other systems. The need of the hour, therefore, is to subject the traditional system of medicine to vigorous experimental test and to fine tune treatment schedules for unambiguous results. We selected an herb that is often used by the tribal as well as native people for the treatment of several human complications. The proposed research activity was to characterize the antioxidant efficacy of *Rhinacanthus nasutus* leaf extracts, and its protective effect on oxidant induced damage. Antioxidant potential was measured using various *in vitro* systems that are alternatives to the use of live animals in experimentation.

***Rhinacanthus nasutus***

*Rhinacanthus nasutus* is an important medicinal plant, which possesses anticancer, antifungal and antiviral properties (Sudhakar *et al.*, 2006). This plant is widely distributed in some parts of the subcontinent of India, China and some parts of South East Asia (Puncturee *et al.*, 2005). It is a shrub, about 2 to 3 feet tall (Gotoh *et al.*, 2004). Various parts of this plant have been used for the treatment of many diseases such as eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension, and various skin diseases. Some of the bioactive components of the plant are known to be naphthoquinones such as rhinacanthins (A-D, G-Q), rhinacanthone and lignan groups (Yahuafai *et al.*, 2006).

It is extensively used in traditional medicine, to treat liver disorders, skin diseases, peptic ulcers, helminthiasis, scurvy, inflammation and obesity. The methanolic root extract of *Rhinacanthus nasutus* was studied for its hepatoprotective effect. *R.nasutus* helped to preserve an almost normal structure of

the liver, following $CCl_4$ – induced liver damage, indicating its hepatoprotective effects. *R. nasutus* possesses a significant hepatoprotective activity, comparable to that of silymarin (Suja *et al.,* 2003).

In spite of such reports being published, not many studies have concentrated on the type of antioxidant responses evoked by the *Rhinacanthus nasutus* leaves on oxidative stress induced events at the molecular level, its radical scavenging abilities and its anti-apoptotic effects. The present study is an extensive search into the antioxidant and anticancer activities of *Rhinacanthus nasutus* leaves.

The materials used and the methodology adopted in the present study are explained in the next chapter.