# 2. REVIEW OF LITERATURE

Oxygen is an element obligatory for all aerobic living organisms. The oxidative properties of oxygen are essential for diverse biological phenomena. Oxygen has high chemical reactivity and hence some of its by-products produced from cellular metabolism are toxic for living organisms. All aerobic living cells utilize oxygen to generate energy, in the form of adenosine triphosphate (ATP) in the mitochondria (Strzelczyk and Wiczkowski, 2012).

Free radicals are produced as by-products from the cellular redox process. These by-products are of two types, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS). These reactive species have double-edged properties in such a way that, being essential for life, they also can aggravate the damage within the cell by oxidative events. The balance between the two antagonistic effects of reactive species, called the redox homeostasis, is an important aspect of life (Husain and Kumar, 2012).

Under normal physiological processes and conditions, ROS play critical roles in various physiological processes, such as regulation of cell growth, apoptosis and other signaling cascades at the cellular level. They also contribute to complex functions such as blood pressure regulation, cognitive function, hormone production, mitosis induction and immunocompetance against infectious agents. ROS enable the response to growth factor stimulation and the generation of the inflammatory response (Murphy *et al*., 2011). Moderate levels of reactive molecules enable a reversible oxidative modification of lipids, proteins and DNA, that play a pivotal role in differentiation, maturation and trafficking of the intracellular vesicles (Pourova *et al*., 2010).

Free radicals, when produced in excess, cause irreversible oxidation to biomolecules, thereby causing damage to cells and tissues. A free radical is defined as a molecule (or molecular fragment) that contains one or more unpaired electrons in its outermost atomic or molecular orbit and is capable of independent existence (Singh *et al*., 2009). Free radicals are formed from molecules through the homolytic cleavage of a chemical bond and via redox reactions. Once formed, they become highly reactive and initiate a chain of reactions (Sen *et al*., 2010).

Oxidative stress represents an imbalance between the production of reactive species and the ability of a biological system to either detoxify the reactive intermediates or to repair the resulting damage. Oxidative stress plays a crucial role in the pathogenesis of various neurodegenerative diseases, cardiovascular diseases and cancer. Elimination of reactive

radicals is done by many complex mechanisms whereas the detoxification is mediated by antioxidants (Li *et al*., 2012).

Cells have evolved defence mechanisms for protection against ROS mediated oxidative damage, which include antioxidant defences. Antioxidants either prevent the generation of oxidants or inactivate the already formed oxidants, thereby inhibiting the chain of the reactions produced by the oxidants (Kunwar and Priyadarsini, 2011).

## 2.1. REACTIVE OXYGEN SPECIES (ROS) AND REACTIVE NITROGEN SPECIES (RNS)

Under aerobiosis, the production of reactive oxygen (ROS) and nitrogen (RNS) species is inevitable, especially at the level of electron transfer chain reactions for ROS and through enzymatic processes for RNS (Mittler *et al*., 2011). ROS are oxygen centered radicals that include superoxide radical, hydrogen peroxide, hydroxyl radical and singlet oxygen. RNS include nitric oxide and derived molecules such as nitrogen dioxide, dinitrogen trioxide and peroxynitrite. Some reactive species exist as intermediates between ROS and RNS; for example, peroxynitrite is formed from the reaction between nitric oxide and singlet oxygen (Schippers *et al*., 2012).

Another category of molecules that are sulfur centered, called reactive sulfur species (RSS), which includes thiyl radicals, is also found. The major form of RSS that is currently under investigation is hydrogen sulfide, which has physiological effects in animals (Lisjak *et al*., 2013).

## 2.2. SOURCES OF ROS AND RNS

In cells, ROS and RNS are generated through both enzymatic and non-enzymatic reactions. The enzymatic reactions producing free radicals include the reactions involved in the respiratory chain, the phagocytosis and the cytochrome P450 system. These reactions are mediated by NADPH oxidase, xanthine oxidase, nitric oxide synthase and peroxidases (Tsutsui *et al*., 2011).

ROS are also produced from non-enzymatic reactions of oxygen with organic compounds and from those initiated by ionizing radiations (Brennan and Kantorow, 2009). They are continuously produced in the cell and the environment, both at exogenous and endogenous levels. Endogenously, free radicals are produced from immune cell activation, inflammation in neutrophils and basophils, ischemia and infections (Venkatesh *et al*., 2009). The exogenous sources of reactive species include pollutants, alcohol, cigarette smoke, environmental toxins, metal-catalyzed reactions and ionizing radiations (UV, X-rays, gamma

rays and microwave radiation). These exogenous compounds are metabolized into free radicals inside the body (Demirci and Hamamcı, 2013).

## 2.3. TYPES OF ROS AND RNS

### 2.3.1. Superoxide radical ($O_2^{\bullet-}$)

Superoxide anion radical ($O_2^{\bullet-}$) is formed by the addition of one electron to dioxygen (Gutowski and Kowalczy, 2013). Superoxide anion is considered as the primary ROS, which arises either through metabolic processes or through oxygen activation by physical irradiation. In mammalian cells, the electron transport chain of the mitochondria is the main source of superoxide radical (Djordjevic *et al*., 2010). $O_2^{\bullet-}$ radical causes lipid peroxidation and also decreases the activity of antioxidant defence system enzymes such as catalase (CAT) and glutathione peroxidase (GPx). The protonated form of $O_2^{\bullet-}$ is $H_2O_2^{\bullet}$, which is more reactive and is able to cross the membrane and cause damage to tissues (Wu *et al.*, 2010).

### 2.3.2. Hydroxyl radical ($OH^{\bullet}$)

The neutral form of the hydroxide ion is the hydroxyl radical ($OH^{\bullet}$). The hydroxyl radical is a highly reactive free radical with a very short *in vivo* half-life of approximately $10^{-9}$ seconds and causes damage to cells (Mokudai *et al*., 2012). When produced *in vivo*, $OH^{\bullet}$ reacts close to its site of formation. In a cell, the redox state is associated with iron, which is maintained under physiological limits (Whalley *et al*., 2010). Under stress conditions, the *in vivo* production of $OH^{\bullet}$ radical is induced by superoxide radical which releases more free irons and acts as an oxidant of [4Fe–4S] cluster-containing enzymes, thus facilitating $OH^{\bullet}$ production. In the Fenton reaction proposed by Haber–Weiss, superoxide reacts with $H_2O_2$ to produce $OH^{\bullet}$ in the presence of iron ions (Lipinski, 2011).

### 2.3.3. Hydrogen peroxide ($H_2O_2$)

$H_2O_2$ is a non-radical that can diffuse across membranes through aquaporins. Under physiologic conditions, $H_2O_2$ is produced in peroxisomes. Oxygen consumption in the peroxisome leads to $H_2O_2$ production, which is then used to oxidize a variety of molecules (Miller *et al*., 2010).

Once activated, it causes cytotoxicity through DNA damage, membrane disruption and release calcium ions within cells, resulting in the activation of calcium-dependent proteolytic enzymes. The catalase present in the peroxisomes converts hydrogen peroxide into water and oxygen and thereby prevents the accumulation of $H_2O_2$. During peroxisome damage, $H_2O_2$ consuming enzymes are downregulated, resulting in the release of $H_2O_2$ into the cytosol,

leading to oxidative stress (Karuppanapandian *et al*., 2011). At high concentrations, $H_2O_2$ is involved in inflammatory diseases and plays an important role in defence against bacterial pathogens in higher animals (Shen *et al*., 2010).

### 2.3.4. Singlet oxygen ($^1O_2^{\bullet}$)

Singlet oxygen ($^1O_2^{\bullet}$) is produced when energy is transferred from one or more excited donors to molecular oxygen (Nam *et al*., 2013). Singlet oxygen is also generated by a range of enzymatic and non-enzymatic reactions that include processes mediated by lipoxygenases, activated leukocytes, heme proteins and radical termination reactions (Terao *et al*., 2011). Singlet oxygen stimulates lipid peroxidation and also decreases the activity of antioxidant enzymes such as catalase and glutathione peroxidase (Farmer and Mueller, 2013). It also causes damage to the ribonucleotide, thereby inhibiting DNA synthesis. The protonated form of $^1O_2^{\bullet}$ is $HO_2^{\bullet}$ which is more reactive and is able to cross the membrane and cause damage to tissue (Zou *et al*., 2013).

### 2.3.5. Nitric oxide (NO)

Nitric oxide is a small molecule that has one unpaired electron on the 2π*y orbital (Munakata, 2012). In biological tissues, it is synthesized by specific nitric oxide synthases (NOSs), which metabolize arginine to citrulline to form $NO^{\bullet}$ through electron oxidative reaction (Maron and Michel, 2012). Oxygen reacts with nitric oxide, resulting in the production of nitric oxide, nitrate and nitrite anions. On homolytic dissociation, the conjugate acid of nitric oxide (ONOOH) produces reactive $NO_2$ and $OH^{\bullet}$ radicals. In the presence of carbon dioxide, nitrosoperoxycarbonate anion ($ONOOCO_2^-$) is formed from nitric oxide, which on homolytic dissociation produces $CO_3^{\bullet-}$and $NO_2^{\bullet}$ free radicals (Jena, 2012).

### 2.3.6. Hypochlorus acid (HOCl)

Hypochlorous acid (HOCl) is a strong oxidant that plays a crucial role in host defence system against bacterial pathogens. It is produced by myeloperoxidase (MPO) of activated neutrophils and monocytes. HOCl reacts with nucleic acid bases, forming chlorinated and oxidised nucleosides such as 8-chloroguanosine (Nakano *et al*., 2012). HOCl initiates lipid peroxidation and promotes post-translational modification of target proteins. It also causes injury to normal tissues by bleaching the heme moieties of hemoproteins, and oxidatively destroying electron transport chains (Souza *et al*., 2011).

### 2.3.7. Peroxynitrite ($ONOO^-$)

The peroxynitrite ($ONOO^-$) anion is a short-lived oxidant that is produced by the

diffusion-limited reaction of nitric oxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet-}$) radicals. It can initiate both *in vitro* and *in vivo* toxic oxidative reactions (Esposito and Cuzzocrea, 2009). The cytotoxic effect of peroxynitrite includes initiation of lipid peroxidation and oxidative protein modifications. It also induces DNA strand breaks and activates nuclear enzyme poly (ADP-ribose) polymerase, thereby promoting cellular energetic collapse and cell death (Szabó and Módis, 2010).

## 2.4. DELETERIOUS EFFECTS OF FREE RADICALS AND OXIDANTS

A deleterious phenomenon called the oxidative stress is caused by excessive production of free radicals and oxidants beyond the antioxidant defence. Oxidative stress induces alterations in the cell membranes and other structures such as proteins, lipids, lipoproteins and DNA (Elahi *et al*., 2009). Such progressive adverse changes accumulate with age throughout the body. Genetics and environment factors influence these changes and modulate the free radical damage, thereby causing various pathological conditions such as diabetes, cardiovascular disease, neurological disorders, ischemia, aging and cancer (Hulsmans *et al*., 2012).

In the human body, all the biological molecules are susceptible to attack by free radicals. At high concentrations, ROS causes damage to cell structures, nucleic acids, lipids and proteins. Such biomolecular damage causes impairment of cell functions and eventually cell death.

### 2.4.1. Oxidative Damage to Lipids

Membrane lipids that are present in the plasma membrane and subcellular organelles are highly susceptible to free radical damage. Hardening of lipids, the major composition of all cell membranes, due to lipid peroxidation, leads to cell death (Chorvatova *et al*., 2013). During lipid peroxidation, various reactive species oxidize membrane lipids, especially the polyunsaturated fatty acids, thereby initiating a free radical chain reaction and leading to the formation of a number of aldehyde by-products, including acrolein, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (Singh *et al*., 2010). The α and β-unsaturated aldehydes are highly reactive and promote atherosclerosis by injuring the blood vessel and carcinogenesis by forming DNA adducts (Lee and Park, 2013). Oxidized phospholipids have been implicated in neurodegeneration and inflammation (Usatyuk and Natarajan, 2012).

### 2.4.2. Oxidative Damage to Proteins

Oxidative modification of proteins leads to protein dysfunction or tissue damage and disease progression. Oxidative modifications of proteins include carbonylation, nitrotyrosine

formation, S-sulfenation, S-nitrosylation, S-glutathionylation and disulfide formation (Cai and Yan, 2013). Protein carbonyls can be formed on several amino acid residues such as arginine, histidine, lysine, proline, threonine and cysteine (Fedorova *et al*., 2009). Protein carbonyls are the most widely used biomarkers for the measurement of protein oxidation and oxidative stress in aging and diseases. Oxidation of proteins affects signal transduction, enzyme activity and proteolysis susceptibility that leads to ageing (Naito *et al*., 2010).

### 2.4.3. Oxidative Damage to DNA

In aerobic organisms, ROS cause multiple oxidative DNA lesions that include oxidation of DNA bases, sugar fragments and abasic sites (Zaremba and Oliński, 2010). ROS also cause DNA single-strand breaks and double-strand breaks, which are lethal. Oxidized base lesions have both mutagenic and cytotoxic effects (Hegde *et al*., 2012a). The highly reactive hydroxyl radical reacts with all components of the DNA molecule and damages both the purine and pyrimidine bases, and the deoxyribose backbone. The most abundant products of oxidative DNA lesions are oxidized purines namely 8-oxoguanine (8-oxoG) and formamidopyrimidines (Hegde *et al*., 2012b). ROS-induced DNA damage has been implicated in a variety of diseases such as cardiovascular dysfunction, inflammatory diseases, metabolic disorders, arthritis, cancer, aging and age-related neurodegenerative disorders (Lee and Pervaiz, 2011).

## 2.5. OXIDATIVE STRESS AND HUMAN DISEASES

Oxidative stress has been implicated in the pathogenesis of many diseases and important biological processes including atherosclerosis, cancer, neurological disorders, hypertension, acute respiratory distress syndrome, ischemia, diabetes, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease and asthma (Birben *et al*., 2012).

### 2.5.1. Atherosclerosis

Oxidative stress plays an important role in the pathogenesis of atherosclerosis. Oxidative stress and inflammation in the vascular wall are the essential mechanisms underlying atherosclerosis and vascular dysfunctions (Yang and Ming, 2013). Activation of the vascular endothelial cells in the presence of the risk factors promotes oxidative stress and vascular inflammatory responses that accelerate atherosclerotic vascular disease (Hansson and Hermansson, 2011). Vascular dysfunction is caused due to the uncoupling of endothelial nitric oxide synthase (eNOS) that results in the massive production of superoxide anion instead of the vasoprotective nitric oxide (Förstermann and Sessa, 2012).

ROS production through myeloperoxidase (MPO)-mediated respiratory burst and raft-associated NAD(P)H-oxidase in macrophages of vascular endothelial cells have been observed in vascular inflammation. Activation of nuclear transcription factors, like NF-κB, that regulate gene expression for proinflammatory and adhesion molecules by ROS have been implicated in the pathogenesis of atherosclerosis (Wang *et al*., 2011). ROS also promotes lipid oxidation, which amplifies foam cell formation through oxLDL uptake (Gu *et al.*, 2013). Advanced glycation end products (AGEs) produced from oxidative stress also contribute to accelerated atherosclerosis (Ando *et al*., 2013).

### 2.5.2. Diabetes mellitus

Many experimental and clinical studies have confirmed the role of ROS in the pathogenesis of both diabetes mellitus type 1 and type 2 (Stadler, 2012). Oxidative stress is one of the major factors involved in long-term diabetic complications. Hyperglycemia, the main feature of diabetes induces dysregulation of reactive oxygen and nitrogen generating pathways that lead to the disruption of vascular endothelium and produce nitric oxide ($NO^{\bullet}$). Superoxide combines with $NO^{\bullet}$ of the endothelial cells to form the cytotoxic peroxynitrite (Zatalia and Sanusi, 2013).

Hyperglycemia also increases the superoxide production through mitochondrial electron transport chain, which, in turn, activates the five pathways involved in the pathogenesis of diabetes complications, namely activation of polyol pathway flux, increased AGEs production, increased AGEs receptor expression, protein kinase C (PKC) activation and excessive activity of the hexoamine pathway (Ceriello and Testa, 2009; Giacco and Brownlee, 2010)

Lipid peroxidation of unsaturated fatty acids present in plasma and mitochondrial membranes are known to cause impaired insulin secretion (Henriksen *et al.*, 2010). Taylor-Fishwick (2013) showed that the NADPH oxidases in beta cells stimulate the superoxide generation that results in beta-cell dysfunction (Szabo, 2009). Oxidative stress has also been implicated in the pathogenesis of diabetic atherosclerosis, where increased ROS concentration was shown to be associated with high glucose-induced apoptosis of endothelial cells resulting in endothelial dysfunction (Li *et al.*, 2013).

### 2.5.3. Neurodegenerative disorders

Oxidative stress is involved in the etiology and pathogenesis of neurological disorders including Alzheimer's, Parkinson’s disease, Huntington disease and Friedreich's ataxia (Jones *et al*., 2013).

### i) Alzheimer's disease

Free radical damage in the pathogenesis of Alzheimer's disease involves the formation AGEs, nitration products, adduction products of lipid peroxidation, carbonyl-modified neurofilament proteins and free carbonyls, all of which induce neuronal death (Nakamura and Lipton, 2011). A strong correlation between deletion of mitochondrial DNA and 8-hydroxyguanosine (8-OHG) formation with Alzheimer's disease was found (Aliev *et al*., 2013). Increased level of oxidized protein in Alzheimer's disease is associated with loss of the 20S proteasome activity, a major enzyme that degrades oxidized proteins (Yen, 2011). Protein oxidation and lipid peroxidation have been identified in the hippocampus and neo cortex of patients with Alzheimer's disease (Honma *et al.,* 2013). ROS production by NADPH oxidase proteins through oxidation of DNA, proteins, lipids, amino acids, metals and through activation of redox-sensitive signaling pathways occurs in Alzheimer's disease (Hernandes and Britto, 2012).

### ii) Parkinson's Disease

Oxidative stress mechanisms play a vital role in the neurodegenerative pathogenesis of Parkinson's disease (PD). The major factors of oxidative stress associated with PD are the abnormalities in iron metabolism with increased iron concentration and low ferritin concentration, somatic mitochondrial DNA mutations with impaired respiratory transport chain function, decreased production of reduced glutathione and increased amount of oxidized glutathione in the substantia nigra (Nikolova, 2012).

Production of free radicals within the basal ganglia resulting in progressive damage and death of neurons in the substantia nigra was found. Lipid peroxidation products such as 4-hydroxynonenal and N-(carboxymethyl) lysine have been found in Lewy bodies (Jiang *et al*., 2013). Two AGEs namely pentosidine and pyrraline have also been detected in the cortex of the brain (Friedlich *et al*., 2009). Elevated levels of oxidized DNA products (8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-OHG) in the serum and cerebrospinal fluid and impaired oxidative phosphorylation due to mitochondrial DNA deletions in neurons of substantia nigra were observed in patients with Parkinson's disease (Ciccone *et al*., 2013).

## 2.5.4. Hypertension

Hypertension is characterized by increased peripheral resistance of small-resistance arteries due to endothelial dysfunction, vascular remodeling and inflammation (Virdis *et al*., 2011). Many mechanisms for the role of oxidative stress in the pathophysiology of hypertension have been implicated. Increased production of superoxide by NAD(P)H oxidase

in vascular endothelial cells, with reduced bioavailability of the vasodilator nitric oxide due to oxidative stress, was observed in hypertension (Urso and Caimi, 2011). ROS induce proliferation and hypertrophy of vascular smooth muscle cells, thereby leading to increased vascular resistance, which characterizes the hypertensive disease. Reduced level of SOD and GPx were observed in newly diagnosed and untreated patients with idiopathic pulmonary arterial hypertension (Mata *et al*., 2012). A significant correlation between increased $H_2O_2$ levels and systolic blood pressure was observed in hypertensive patients (Perez-Vizcaino *et al*., 2010).

### 2.5.5. Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation of the joints and tissues surrounding the joints with infiltration of macrophages and activated T-cells. The formation of free radicals in the inflammation sites play a key role in the pathogenesis of this disease (Wruck *et al*., 2011). Association of oxidative injury in rheumatic diseases was confirmed for various factors such as elevated isoprostanes and prostaglandins levels in serum and synovial fluid and decreased GSH levels in the T cells of the synovial fluid. High incidence of TP53 mutations, increased level of malondialdehyde and decreased SOD and GPx activities due to oxidative stress were observed in rheumatoid arthritis patients (Hassan *et al*., 2011). Abnormal induction of redox-sensitive signaling pathways, resulting in abnormal expression of several adhesion molecules (ELAM-1, VCAM-1, ICAM-1, ICAM-2), which mediate the migration of monocytes and lymphocytes into the arthritis synovium was also found (Prakash *et al*., 2010).

### 2.5.6. Cancer

Carcinogenesis is a multistep process that involves mutation and the subsequent selective clonal expansion of the mutated cell. Chronic oxidative stress, especially from chronic inflammation, is associated with carcinogenesis (Pan *et al*., 2009). Oxidative stress has been implicated in the three major events of carcinogenesis namely, oxidative DNA damage, signal transduction abnormalities and extra cellular matrix remodelling. In radiation-induced carcinogenesis, the highly reactive hydroxyl radical causes oxidative DNA damage and produces mutagenic purine, pyrimidine and deoxyribose oxidation products (Dawane and Pandit, 2012).

Oxidative DNA damage promotes single- or double-strand breakage, DNA cross-linking, base modifications and deoxyribose modifications. Such oxidatively damaged DNA, when undergoes replication without repair, causes genomic instability and point mutations in

tumour suppressor genes (Schetter *et al*., 2010). 8-hydroxydeoxy guanosine is the most extensively studied and most abundant oxidative DNA lesion, whose levels are elevated in various cancers (Visconti and Grieco, 2009).

Alteration in the functions of GSH-related enzymes and decreased GSH/GSSG ratio have been reported in patients with colon and breast cancer. Lipid peroxidation also has been implicated in the mechanism of carcinogenesis (Zitka *et al*., 2012). Lipoperoxyl radicals ($ROO^{\bullet}$) are rearranged to endoperoxides to produce malondialdehyde via a cyclisation reaction. These lipid peroxidation products react with DNA and form mutagenic malondialdehyde deoxyguanosine adducts, which are widely used as biomarkers for oxidative stress in breast cancer (Peluso *et al*., 2011).

### 2.5.7. Infertility

Recent studies have reported the association of oxidative stress in the pathophysiology of infertility (Agarwal *et al*., 2012a). In sperm, ROS are mainly produced by both spermatozoa and circulating leucocytes. Excess ROS production causes a decrease in sperm viability and motility with subsequent onset of infertility (Lavranos *et al*., 2012). Toxic levels of ROS induce mitochondrial membrane damage in the sperm, cytochrome-c release and the caspase cascade activation to stimulate excess apoptosis, which, in turn, cause germ cell degeneration. Toxic levels of $NO^{\bullet}$ have also been implicated in the lipid peroxidation within the sperm plasma membrane (Makker *et al*., 2009). Oxidative stress in the male germ line also affects male fertility and normal embryonic development (Gharagozloo and Aitken, 2011). In females, the redox imbalance has been implicated in various reproductive diseases such as endometriosis, polycystic ovary syndrome and unexplained infertility. Oxidative stress also induces pregnancy complications such as spontaneous abortion, recurrent pregnancy loss and preeclampsia (Agarwal *et al*., 2012b).

### 2.5.8. Aging process

Aging is a complex phenomenon characterized by progressive decline in the physiological functions and subsequent increase in mortality, which is associated with many pathological diseases. According to Denham Harman who proposed the free radical theory of aging, the life span of an organism is determined by aging that results from the accumulation of deleterious effects caused by free radicals and by the ability of an organism to cope with cellular damage induced by ROS (Cui *et al*., 2012). Oxidative damage to mitochondria and mitochondrial DNA (mtDNA) contribute to apoptosis upon aging. Increased ROS production by mitochondria and increased 8-oxo-2'-deoxyguanosine content in the mtDNA are detected

in aged tissues. Oxidative damage also affects replication and transcription of mtDNA, thereby impairing the mitochondrial function, which, in turn, promotes enhanced ROS production and further mtDNA damage (Salminen *et al*., 2012). Increase in telomere shortening under mild oxidative stress was identified as the major cause of replicative senescence, a process of proliferation limit for metabolically viable cells (Xu *et al*., 2013).

## 2.6. ANTIOXIDANTS

Human body has developed a series of defence mechanisms to prevent the free radical mediated oxidative damage. One such mechanism is the antioxidant defences that monitor the generation of free radicals. An antioxidant is a stable molecule that neutralizes a reactive free radical by donating an electron, thereby inhibiting its ability to damage. Antioxidants either delay or inhibit free radical induced cellular damage through their free radical scavenging property (Mohammad *et al*., 2009).

### 2.6.1. Levels of antioxidant action

In the defence system, the antioxidants act at three different levels against free radical induced oxidative stress.

i) In the first line of defence, antioxidants prevent the formation of free radicals. For example, metal-induced decompositions of hydroperoxides and hydrogen peroxide are one of the important sources of free radicals *in vivo*. Preventive antioxidants such as catalase, glutathione S-transferase and glutathione peroxidase reduce such reactions by decomposing the hydroperoxides and hydrogen peroxide to alcohols and water, thus preventing the generation of free radicals and proteins that sequester metal ions.

ii) In the second line of defence, antioxidants scavenge the free radicals, especially peroxy radicals, in order to suppress the initiation or to break the propagation of chain reactions. Various endogenous radical-scavenging antioxidants (both hydrophilic and lipophilic) such as vitamins C, E, uric acid, bilirubin, albumin and thiols are involved.

iii) In the third line of defence, the repair enzymes recognize the oxidatively damaged molecules and either repair or decompose them. For example, the cytosolic and mitochondrial proteolytic enzymes such as proteinases, proteases and peptidases recognize and remove oxidatively modified proteins, thereby preventing their accumulation in the cells. Similarly, DNA repair enzymes such as glycosylases and nucleases repair the damaged DNA.

In addition to these three levels, another important function is involved in the defence system called the adaptation in which the antioxidant formation and its transport to the

reactive site is induced by the signal generated from the production and reactions of free radicals (Lobo *et al*., 2010).

Antioxidants are both endogenous and exogenous in origin. Endogenous antioxidants are predominantly enzymes that are produced inside the body whereas, exogenous antioxidants that include non-enzymatic, metabolic and nutrient antioxidants, are supplied through diet.

### 2.6.2. Classification of Antioxidants

Antioxidants have been classified based on their structure, solubility, mode of action and kinetics.

Antioxidants are classified into six categories based on their kinetics (Flora, 2009);

1. Chain breaking antioxidants, such as phenol, hydroquinone and aromatic amines that react with peroxyl radicals with weak O-H or N-H bonds.
2. Chain breaking antioxidants, such as quinones, nitrones, iminoquinones that react with alkyl radicals.
3. Antioxidants inducing the decomposition of hydroperoxide, such as sulphide, phosphide, thiophosphate.
4. Metal deactivating antioxidants, such as diamines, hydroxyl acids and bifunctional compounds.
5. Cyclic chain terminating antioxidants, such as aromatic amines and variable valence metal compounds.
6. Antioxidants that act synergistically, such as phenol sulphide in which the phenolic group reacts with peroxyl radical while the sulphide group reacts with hydroperoxide.

Antioxidants are also classified into two major categories namely enzymic and non-enzymic antioxidants. The enzymic antioxidants include endogenous enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and the non-enzymic antioxidants include metabolic and nutrient antioxidants such as vitamins E, C, A and reduced glutathione (GSH) (Gill and Tuteja, 2010).

### 2.6.3. Enzymic antioxidants

#### i) Superoxide dismutase (SOD)

Superoxide dismutase is an important intracellular enzymic antioxidant present in all aerobic organisms and subcellular compartments. It catalyzes the dismutation of superoxide

anion into hydrogen peroxide and oxygen (Kocot *et al*., 2013). Depending upon the metal cofactors, there are three major types of SOD namely copper/zinc SOD (Cu/Zn SOD), iron SOD (Fe SOD) and manganese SOD (Mn SOD) (Perry *et al*., 2010). The Cu/Zn SOD (SOD1) is the major form of SOD, which is localized in the cytoplasm and the Mn-SOD (SOD2) is localized in the mitochondria. SOD3 (EC-SOD) is primarily localized in the extracellular matrix (Maksimenko *et al*, 2010).

**ii) Catalase (CAT)**

Catalase is one of the most important endogenous enzymic antioxidants that catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is most abundant in peroxisomes (Goyal and Basak, 2012). High expression of catalase was found in tissues, where it protects the cells from ROS. For example, catalase prevents the accumulation of $H_2O_2$ in erythrocytes, which is formed during oxygen transport. Increasing evidences suggest that catalase also plays an important role in various other processes such as intergrin signaling (Glorieux *et al*., 2011).

**iii) Glutathione peroxidase (GPx)**

Glutathione peroxidase is a selenium-dependent enzyme with a selenium atom incorporated within the selenocysteine residue. It is an important indicator of the oxidative stress and plays a vital role against the oxidative damage caused by $H_2O_2$ or lipid peroxide that are produced in various cells of the body (Shim and Kim, 2013). Glutathione peroxidases (GPxs) catalyze the detoxification of hydrogen peroxide in the presence of reduced glutathione. Seven isoforms of GPx with different substrate specificities and tissue distribution have been identified. The plasma GPx (isoform GPx3), an extracellular enzyme is highly expressed in kidney and scavenges both organic and lipid hydroperoxides. GPx3 is also found in the liver, heart, lung, heart and white adipose tissue (Chung *et al*., 2009).

**iv) Glutathione S-transferase (GST)**

Glutathione S-transferase belongs to a large family of dimeric enzymes, which plays a vital role in cell defence system (Wu and Dong, 2012). GSTs reduce the reactivity of various endogenic and exogenic electrophilic compounds, such as carcinogens and antineoplastics, through conjugation with reduced glutathione, thereby making them more water soluble and enabling their elimination. GSTs also act as isomerases and peroxidases (Vasieva, 2011).

In humans, about 24 isoenzymes of GSTs, classified into 11 classes, have been identified. GST isoenzymes are involved in phase II of cellular xenobiotic metabolism (Tew and Townsend, 2012). In addition to detoxification, GSTs also catalyze glutathione-dependent

isomerization reactions that are involved in the synthesis of steroid hormones and prostaglandins, tyrosine catabolism and peroxide degradation (Board and Menon, 2013). GST isoenzymes are involved in many physiological processes such as regulation of cellular signaling, stress response control, cellular differentiation, proliferation, inflammation and apoptosis (Laborde, 2010).

**v) Glutathione reductase (GR)**

Glutathione reductase (GR) is a crucial enzyme in glutathione metabolism, which catalyzes the reduction of glutathione disulphide (GSSG) to its reduced form, GSH, in a NADPH dependent manner (Rousar *et al*., 2010). GR renders significant protection of the cell from the toxic effects of reactive oxygen species (Tandogan and Ulusu, 2010). GR is mainly involved in detoxification process, destruction of free radicals and metabolism of various exogenous and endogenous compounds (Tandogan *et al*., 2011a).

### 2.6.4. Non-enzymic antioxidants

**i) Vitamin C**

Vitamin C (ascorbic acid) is a potent water soluble antioxidant, which is supplied exogenously through diet. Ascorbic acid is a rapid electron donor and, thus, acts as a reducing agent to neutralize ROS such as hydrogen peroxide. It inhibits lipid peroxidation by scavenging aqueous reactive oxygen species (Telang, 2013). Influence of ascorbic acid in collagen biosynthesis, endothelial proliferation, gene expression, apoptosis and other cellular functions has been implicated (May and Harrison, 2013). Recent studies have confirmed the protective effect of ascorbic acid in lung and colorectal cancer. Ascorbic acid has been found to prevent oxidative DNA damage by decreasing lipid hydroperoxides formation or by preventing radical attack on proteins involved in DNA repair. It also prevents nitrosamine formation and subsequent formation of reactive nitrogen species (Pohanka *et al*., 2012).

**ii) Vitamin E**

Vitamin E (α-tocopherol) is a major lipophilic chain-breaking antioxidant present in biomembranes and mainly scavenges hydroperoxyl radicals (Joshi and Praticò, 2012). It is the main antioxidant present in human lipoproteins that inhibits radical chain formation as a strong pro-oxidant for LDL, thereby suppressing LDL lipid oxidation (Ozkanlar and Akcay, 2012). Vitamin E renders significant protection against oxidative-induced neuronal death in patients with Parkinson's disease (Sutachan *et al*., 2012). Diets enriched with α-tocopherol have been found to reduce disease-related oxidative stress in Rheumatoid arthritis (D'Orazio *et al*., 2012). Various *in vivo* and *in vitro* studies have confirmed its role in the prevention of

atherosclerosis (Riccioni *et al*., 2012).

**iii) Vitamin A and carotenoids**

Carotenoids are isoprenoid pigments synthesized in plants and microorganisms. Pure hydrocarbon carotenoids, such as β-carotene and lycopene are termed as carotenes. The ability of the carotenoids to eliminate excess energy, quench singlet oxygen and act as free radical scavengers make them inevitable in various physiological conditions (Amengual *et al*., 2010). Many *in vitro*, animal and human experiments have confirmed the antioxidant properties of β-carotene and lycopene. Provitamin-A carotenoids such as β-carotene are the precursor of retinoids (vitamin A and its derivatives) in the human diet (von Lintig, 2012). Evidence obtained from an *in vitro* study have shown that vitamin A deprivation increases oxidative stress, induces mitochondrial dysfunction and pleomorphic cell dysfunction, resulting in caspase-independent cell death (Chiu *et al*., 2008).

**iv) Reduced glutathione (GSH)**

Reduced glutathione is a tripeptide with potent electron donating capacity linked to its sulfhydryl (-SH) group. It is an important water soluble antioxidant that is mainly present in the cytosol of the cell (Abdalla, 2011). GSH is an essential cofactor for antioxidant enzymes and a major cellular redox buffer that directly scavenges hydroxyl radical and singlet oxygen. GSH eliminates hydrogen peroxide through the reaction catalyzed by glutathione peroxidase. GSH mediates the regeneration of both vitamin E and vitamin C to their active forms (Li *et al*., 2012a). The mitochondrial GSH is a crucial antioxidant involved in the maintenance of mitochondrial redox environment against ROS generated from electron transport chain (Vázquez *et al*., 2012).

**v) Phenols and flavonoids**

Polyphenols (phenolics and flavonoids) are the largest group of phytochemicals widely distributed in plants. Studies have shown that the plant polyphenols possess significant antioxidant properties and helps in the prevention of various oxidative stress associated diseases such as cancer, Parkinson's disease, cardiovascular disease and osteoporosis (Dai and Mumper, 2010). Polyphenols act synergistically with antioxidant vitamins and enzymes as a defence against oxidative stress. They also act as metal chelators. For example, polyphenols chelate $Fe^{2+}$ ions, thereby inhibiting the hydroxyl radical induced oxidation mediated through Fenton reaction (Perron and Brumaghim, 2009). The phenolic compounds render their antioxidant effects by scavenging ROS/RNS, reducing the ROS/RNS formation through

inhibition of enzymes or chelation of transition metal ions that are involved in free radical production and by upgrading the antioxidant defence (Tsao, 2010).

## 2.7. ALTERNATIVES TO ANIMAL TESTING METHODS

In recent years, the use of animals in research, teaching and testing has become an important ethical and political issue. Alternative scientific tests are being developed, which are more efficient and reliable than animal tests. Several non-animal tests have been developed, which are cost-effective, practical and expedient (Ranganatha and Kuppast, 2012).

These alternative methods are developed and validated based on the "3Rs approach" Reduction, Replacement and Refinement. At present, various methods are being used, such as cell and tissue cultures, computerized modeling, microarray technology, use of organ slices, physiology-based pharmacokinetic modeling, human clinical trials and use of omics technologies (genomics, transcriptomics, proteomics and metabolomics). Significant progress in the development of alternative methods has been made in various fields of research that includes toxicological, carcinogenic and pharmacological studies (Bhanushali *et al*., 2010).

### i) Precision-cut liver slices

The major advantage of using organ slices as *in vitro* model is that it represents the multicellular, structural and functional features of an *in vivo* tissue. Organ slices have been used extensively as a promising model for elucidating the mechanism of drug induced organ injury and for characterizing species susceptibilities (Vickers, 2009). Precision-cut liver slices is one such *in vitro* model, which is widely used to elucidate the pharmacological metabolism and to investigate the toxicology and efficacy of novel substances on primary material under standardized conditions (Zimmermann *et al*., 2009). Liver is an important organ that plays a pivotal role in the regulation of various physiological processes such as metabolism, secretion and storage. The heterogeneity and the cell-cell interactions within the original tissue matrix are maintained. The major advantage of using liver slices compared to cell culture models is that it mimics the *in vivo* situation of the liver due to the presence of its physiological extracellular matrix (de Graaf *et al*., 2010).

### ii) Cell culture

Cell culture is one of the major *in vitro* model systems used in cellular and molecular biology research to study metabolism, toxicology of drug compounds, mutagenesis and carcinogenesis. When compared to other *in vitro* systems, the use of cell culture has the major advantage of producing consistent and reproducible results (http://www.invitrogen.com /site/us/en/home/References/gibco-cell-culture-basics/introduction-to-cell-culture.html).

The use of mammalian cell culture has become inevitable for the production of new protein biopharmaceuticals, including monoclonal antibodies, cytokines and vaccines. Tissue engineering and therapies that are explored using stem cell culture is the major application of cell culture technology (Francis, 2010). Cell lines derived from human cancers are widely used to study the biology of cancer and to determine the efficacy of cancer treatment (Gillet *et al*., 2013). In recent years, three-dimensional (3D) cell culture using cancer cells that reproduce the natural tumour environment have been developed to improve the cell-based drug screening assays for anticancer drugs and to identify toxic and ineffective substances at an earlier stage of the cancer drug discovery, which reduces the cost and time of taking a drug to market (Lisa *et al*., 2010).

**iii) Yeast cells**

Many studies preferably use *Saccharomyces cerevisiae* as an *in vitro* model to explore the regulatory features of the oxidative stress response in human diseases. The budding yeast *S.cerevisiae* is a best studied unicellular eukaryotic organism whose genome has been completely sequenced. It is commonly known as baker's and brewer's yeast and is easily grown in the laboratory (Yáñez *et al*., 2009). The completely developed global "omic" analysis of yeast and the well conserved molecular mechanisms make it as an ideal *in vitro* model system (González-Siso and Cerdán, 2012). Yeast cell culture is an easily manipulated model system that is used to determine the preliminary cytotoxicity (Limberger *et al*., 2011). Recently, engineered yeast models have been developed to examine endogenous or heterologous proteins to unravel the molecular mechanisms involved in the pathogenesis of neurodegenerative diseases and cancer (Pereira *et al*., 2012). *S.cerevisiae* continues to be a premier organism to study the biology of telomerase and telomere in cancer research (Wellinger and Zakian, 2012).

**iv) Peripheral blood lymphocytes**

Peripheral blood lymphocyte culture is a simple, efficient and inexpensive *in vitro* culture system widely used for cytogenetic analysis. The abundance of mitotic cells in peripheral blood and the simplicity of the cell culture technique make the culture system the most convenient method to study human chromosome abnormalities for both clinical and research purposes (Benn and Delach, 2008). Chromosome instability and micronuclei formation induced by chemical or physical agents in short-term cultures of peripheral blood lymphocytes has been used as an indirect measure of individual susceptibility to cancer (Bonassi *et al*., 2011).

### v) Other model organisms and *in vitro* model systems

A number of non-mammalian model systems have been developed in recent years that help in studying the pathophysiology of various diseases and in accelerating the drug discovery process. *Drosophila melanogaster*, the common fruit fly, is a well-studied and highly tractable genetic model organism used in genetics and developmental biology (Pandey and Nichols, 2011). Other genetically tractable model organisms include *Arabidopsis thaliana* for molecular genetic studies (van Norman and Benfey, 2009), *Danio rerio* (zebrafish) to analyze malignant hematopoiesis (Jing and Zon, 2011), *Loligo pealei* (crayfish) to investigate apoptosis (Krumschnabel and Podrabsky, 2009), *Caenorhabditis elegans*, the round worm (Marsh *et al*., 2012) and *Xenopus laevi* (South African Clawed Frog) (Liu *et al*., 2013a) to study immunological diseases. Significant similarity between the chick embryo and human embryo at the molecular, cellular and anatomical levels have enabled the establishment of chick embryo fibroblasts as an ideal research model in the field of virology, cell biology, neuroscience, cancer biology and immunology (Vergara and Canto-Soler, 2012).

## 2.8. APOPTOSIS

In all living systems, a homeostatic balance between the proliferation of cells and their death is maintained for the development and maintenance of the biological system. Apoptosis, a physiological cell suicide program was first described by Kerr *et al*. (1972) and is characterized by distinct morphological and biochemical features such as cytoplasmic membrane blebbing, chromosomal condensation and aggregation around the nuclear periphery and formation of small apoptotic bodies (Cheung *et al*., 2012). Alterations in apoptosis play a pivotal role in the pathogenesis of many diseases like neurodegenerative diseases, autoimmune diseases and cancer (Sankari *et al*., 2012).

### 2.8.1. Apoptosis and oxidative stress

Reactive oxygen species (ROS) are products of normal metabolism and xenobiotic exposure. Apoptosis is initiated by extracellular and intracellular signals via two main pathways, namely the death receptor-mediated and mitochondria-mediated pathways. Increased ROS production, decreased antioxidant defences, disruption of intracellular redox homeostasis and irreversible oxidative modifications of lipid, protein and DNA due to oxidative stress can induce apoptosis (Circu and Aw, 2010).

### 2.8.2. Apoptosis and cancer

Carcinogenesis is caused due to the mutation or malfunction of genes that regulate cell division and growth. One of the most effective ways of inhibiting carcinogenesis is to induce

the cell death in cancer cells through apoptosis (Li *et al*., 2012b). Apoptosis reduction or resistance plays a key role in carcinogenesis, which occurs through various mechanisms such as disruption of the balance between pro-apoptotic and anti-apoptotic proteins, decreased caspase function and impairment of death receptor signaling (Wong, 2011). Apoptosis is closely related with anti-cancer therapy and many anticancer drugs exert their action by inducing apoptosis of cancer cells. The dysfunction of the highly regulated apoptosis is associated with drug resistance. Hence many preclinical and clinical studies are being carried out to identify the key regulatory factors of apoptosis that can be targeted by therapeutic strategies (Grimm *et al*., 2011). In addition to this, researches focusing on exploring natural products that can modulate apoptotic signaling pathways have been increased recently (Veeresham, 2012). For example, tocopheryl succinate, a vitamin E analog is reported to induce ROS generation and kill prostate cancer cells (Tomasetti *et al*., 2012). A recent study by Alwi *et al*. (2012) showed that β-phenylethyl isothiocyanate, a natural compound abundant in vegetables, selectively induces apoptosis in breast cancer cells.

## 2.9. PHYTOMEDICINE

Recent researches have shown that the antioxidants isolated from plants have gained importance as therapeutic agents in oxidative stress related diseases. Different plant extracts and their phytoconstituents have been identified as effective radical scavengers and inhibitors of oxidative damage (Ramchoun *et al*., 2009). The use of phytochemicals as drug therapy for oxidative stress related ailments has proved to be clinically effective and relatively less toxic than the existing drugs (Sen *et al*., 2010).

Plant-derived bioactive compounds known as phytochemicals, such as terpenoids, phenolic acids, tannins, flavonoids, alkaloids, and other metabolites, are rich in antioxidant and free radical scavenging properties (Ebrahimzadeh *et al*., 2010). Many research studies have been carried out to identify plants with significant antioxidant and anticancer potential by analyzing their cytotoxic, antiproliferative, apoptotic and radical scavenging activities using both *in vitro* and *in vivo* systems (Ghosh *et al*., 2010).

## 2.10 THE CANDIDATE PLANT - *Caesalpinia pulcherrima,* SWARTZ.

*Caesalpinia pulcherrima*, commonly known as peacock flower or "Barbados pride", is an ornamental plant which blooms in three different colours (orange, pink and yellow) with unique long stamens. The different parts of this plant have been used in traditional medicine as emmenagogue, abortifacient, purgative and stimulant (Pawar *et al*., 2008). It is also found to possess antipyretic, antimicrobial and antituberculur activities and is used to treat

bronchitis, asthma and malarial fever (Chichioco-Hernandez and Leonido, 2011).

The flowers have been found to exhibit antiviral (Chiang *et al*., 2003) and antioxidant properties (Padma *et al*., 2000). Earlier studies in our laboratory have shown that these flowers are a rich source of antioxidants, exhibit both antimutagenic and antioxidant properties, reduced cancer cell viability and protect DNA from oxidative damage (Aparna, 2000; Nirmala Priyadharshini, 2001; Yamuna, 2004).

The protective effect of the flowers against oxidative DNA damage, which is the main cause for the cancer development and their significant role in reducing cancer cell growth, has furthered the present study on the molecular mechanisms of the antioxidant and anticancer properties of the flowers. The study was carried out in four different phases and the methodology applied for each phase, with respective references quoted, is explained in detail in the following chapter.