

INTRODUCTION

1.1. General Introduction

Oxygen is an essential element for life to perform biological functions such as catabolism of fats, proteins and carbohydrates in order to generate energy for growth and other activities. However, a parallel role of oxygen as a toxic agent for living tissues has also been discovered. Oxygen, though not dangerous by itself, is involved in the generation of various kinds of “reactive oxygen species” (ROS). ROS, formed during metabolism or through the action of ionizing radiation, can interact with bio-molecules and ultimately lead to an onset of degenerative diseases such as cancers, cardiovascular diseases (CVD) and other illnesses. To protect against the destructive action of free radicals, nature has created an antioxidant defense system composed of a group of compounds and enzymes potent enough to remove free radicals before they cause tissue damage. Some antioxidants are produced in the body, while others must be sequestered from the diet or through supplementation. Most citrus and dried fruits, cruciferous vegetables, garlic, onions, carrots, tomatoes, sweet potatoes, sesame and olive oil are rich sources of antioxidants. There are thousands of naturally occurring and synthetic antioxidants known. These antioxidants belong to different classes of compounds, such as carotenoids, polyphenolics, polyamines, gallic acid derivatives, tannins and catechins. Examples include phytic acid, lipoic acid, bilirubin, melatonin, quercetin, carnosol, carnosic acid, hydroxytyrosol, rutin, butylated hydroxyanisole, and butylated hydroxy toluene. Vitamins E and C are among the most effective antioxidants with preventive effects against heart diseases and cancers.

1.2. Reactive Oxygen Species (ROS)

Primarily ROS play an important role in the host defence mechanism against microorganisms, but the increased production of ROS is associated with the onset of a variety of diseases including cancers (Wiseman and Halliwell, 1996), inflammation (Sco *et al.*, 1995), neurodegeneration (Leboritz *et al.*, 1996), Parkinson's disease (Jenner, 1994), atherosclerosis (Witztum, 1994) and pre-mature aging (Orr and Sohal, 1994). A number of different free radicals and non-radicals are produced during normal aerobic metabolism (Halliwell, 1995; Greenwald, 1991). A collective term, "reactive oxygen species" (ROS), is used for oxygen-derived species including oxygen bearing free radicals, as well as certain non-radicals. Some non-oxygenated radicals are also generated in biological systems, such as carbon-centered free radicals (e.g. alkyl radical, $R-H_2C^*$) and sulfur-centered radicals (e.g. thiyl radical, $R-S^*$), which are produced by the attack of free radicals on hydrocarbons and the oxidation of glutathione, respectively (Ivanova and Ivanov, 2000; Cheeseman and Slater, 1993).

1.2.1. Free Radicals -Highly Reactive Species

A free radical is a chemical species, which is capable of independent existence and possesses one or more unpaired electrons that bestow it with immense reactivity. This reactivity is inversely related to their stability (Aitken and Fisher, 1994).

1.2.2. Types and Sources of ROS

A number of ROS are continuously generated in living system as a consequence of normal metabolic processes. These can be produced in two ways: by enzymatic

reactions involving xanthine oxidase (XO), NADPH oxidases and lipoxygenases; by non-enzymatic sequence of reactions such as the catalytic action of free transition metals (for example iron and copper), by the toxic action of certain chemicals such as doxorubicin, by the attack of electrons leaked from the mitochondrial electron transport chain and by the effect of radiation including UV light and radon (Ra) gas. Different types of ROS generated include both free radical and non-radical species (Bakonyi and Radak, 2004; Kerr *et al.*, 1996; Ahmad, 1996; McCord, 1993). The major ROS have been listed in Table 1.1.

Table 1.1: Types of ROS

ROS	Types	Symbol
Radicals	Superoxide	$O_2^{\bullet -}$
	Hydroxyl	$\bullet OH$
	Alkoxy	$LO^{\bullet} / RO^{\bullet}$
	Peroxy	$LOO^{\bullet} / ROO^{\bullet}$
	Nitric oxide	NO^{\bullet}
	Thiyl radical	$R-S^{\bullet}$
Non-radicals	Hydrogen peroxide	H_2O_2
	Hypochlorous acid	$HOCl$
	Ozone	O_3
	Singlet oxygen	1O_2
	Peroxynitrite	$ONOO^-$
	Lipid peroxide	$LOOH$

Alkyl, alkoxy, alkperoxy radicals and lipid peroxide (R^{\bullet} , RO^{\bullet} , ROO^{\bullet} , and $LOOH$) are the products of attack of free radicals in lipid and fatty acid molecules. These biomolecules are more susceptible towards free radical attack due to the presence of unsaturation.

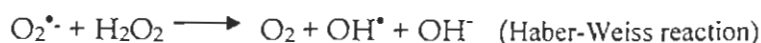
Superoxide anions ($O_2^{\bullet-}$) are the reduced form of oxygen. Though $O_2^{\bullet-}$ itself is not damaging, it plays an important role in the formation of ROS like H_2O_2 and nitric oxide radicals, and also acts as a reductant for transition metals (Cheeseman and Slater, 1993).

There are three main sites of $O_2^{\bullet-}$ production in cells: the mitochondria, endoplasmic reticulum, and cell cytoplasm membranes. In mitochondria, besides other enzymes of the electron transfer chain, two enzymatic sites have been clearly identified as major sources for one-electron reduction of oxygen: ubiquinone-cytochrome C reductase, which involves autooxidation of the ubisemiquinone (Sun and Trumpower, 2003; Han *et al.*, 2001; Nohi and Jordan, 1986) and NADH dehydrogenase, which involves autooxidation of semi-flavin cofactor (Kalinowski and Malinski, 2004; Aust *et al.*, 1972). In the membrane of the endoplasmic reticulum, $O_2^{\bullet-}$ is produced by the oxy complex of cytochrome P-450 and the action of NADH-cytochrome P-450 reductase (Henderson and Chappell, 1996). In plasma, NADPH oxidase is involved in the generation of $O_2^{\bullet-}$ radicals by transferring one electron to molecular oxygen by the enzyme electron transfer chain reaction (Curi *et al.*, 2002). Xanthine oxidase has also been proposed to be an important source of $O_2^{\bullet-}$ generation in reperfused tissues. It uses molecular oxygen as its acceptor, producing superoxide anion (Prigmore *et al.*, 1995, McCord and Omar, 1993).

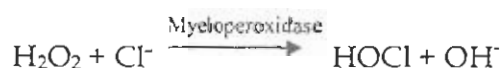
Hydroxyl radical (OH^{\bullet}) is an extremely reactive radical species that can react to every bio-molecule. A number of sources generate these radicals. Hydrogen peroxide, $O_2^{\bullet-}$, and transition metals (Fe^{+2} and Cu^+) are generally involved in the

generation of OH^\bullet . Hypochlorous acid is another source of hydroxyl radicals (McCormick *et al.*, 1998; Cheeseman and Slater, 1993).

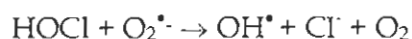
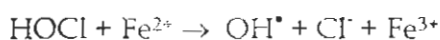
Hydrogen peroxide (H_2O_2) is an oxidising agent and a main source of hydroxyl radicals as mentioned earlier. It produces OH^\bullet through Haber-Weiss and Fenton reactions in the presence of $\text{O}_2^{\bullet-}$ and Fe^{2+} , respectively.



H_2O_2 also produces another oxidizing agent, hypochlorous acid (Lin and Kao, 1998; Halliwell and Gutteridge, 1992). Hypochlorous acid (HOCl) is a potent antibactericidal agent produced by the activated polymorphonuclear cells. It is generated by the action of myeloperoxidase on chloride ions in the presence of H_2O_2 :



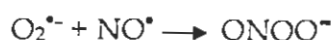
It can cross cell membranes and, in the presence of transitional metal ions, it can generate hydroxyl radicals (OH^\bullet) by reacting with superoxide anion radicals and ferrous iron (Pullar *et al.*, 2001; Aruoma, 1994):



Nitric oxide (NO^\bullet) is a common gaseous free radical. It plays an important role in vascular physiology and is also known as endothelium derived relaxing factor.

Vascular endothelium produces nitric oxide, like neutrophils and macrophages, from arginine, using the enzyme nitric oxide synthetase (Mazzetti *et al.*, 2001; Beckman and Crow, 1993; Moncada *et al.*, 1991).

Peroxynitrite (ONOO^-) is produced due to the release of significant quantities of NO^\bullet and $\text{O}_2^{\bullet-}$ from activated macrophages and neutrophils during the inflammatory response (Grace *et al.*, 1998).



Singlet oxygen ($^1\text{O}_2$) is an electronically excited and mutagenic form of oxygen. It is generated during exercise, by radiations, by the action of peroxidases or lipoxygenases, and by the reaction of hydrogen peroxide with hypochlorite or peroxynitrite, as well as during the respiratory burst of phagocytes (Mascio *et al.*, 1994; Steinbeck *et al.*, 1993). In mammalian cells, singlet oxygen can be generated during oxidative stress and it is able to attack many cellular molecules such as amino acids, nucleic acid bases and membrane lipids. Sunlight contains high energy short-wavelength ultraviolet photons (comprising the UVB spectra, 290–320 nm) which are potentially detrimental because of their destructive interactions with many cellular biomolecules. Chronic exposure to sunlight is thus a significant causative factor in the development of skin cancer (Estevam *et al.*, 2004).

1.2.3. Adverse Effects of Free Radicals

Because of the immense reactivity of free radicals, they can easily react with several bio-molecules including DNA, lipids, proteins and carbohydrates. ROS react with

the bio-molecules, leading to local injury and eventual organ dysfunction. They also accelerate the aging and related degenerative processes. Moreover, ROS are also involved in the promotion of heart diseases, chronic inflammation, and cancers (Ivanova and Ivanov, 2000).

The susceptibility of biological membranes to peroxidation is due to the presence of polyunsaturated fatty acids (PUFA). The presence of double bonds in PUFA weakens the C-H bond of the adjacent carbon atom (allylic carbons) and facilitates the hydrogen abstraction step, which initiates the peroxidation reactions (Aitken and Fisher, 1994). Cell membranes contain a variety of PUFA such as linoleic, linolenic and arachidonic acids, mainly in the form of esters with phospholipids, triglycerides, or with cholesterol. Overall attack of one reactive free radical on PUFA molecule can convert multiple fatty acid side chains into lipid peroxides, damage membrane proteins, make the membrane leaky and eventually cause breakdown of the membrane (Schafer *et al.*, 2000; Cheeseman, 1993).

Protein and nucleic acids are generally less susceptible to free radical attack than PUFAs and hence have less possibility to take part in the progression of chain reactions. This can happen only if radicals are allowed to accumulate, or if the damage is focused on a particular site of the protein (Leeuwenburgh *et al.*, 1998; Stadtman and Oliver, 1991).

Deoxyribonucleic acid (DNA) is a sensitive target for free radicals-mediated damage in a living system. A free radical can damage the specific site of DNA, leading to breaking of strands, or it might delay the repair before replication occurs, leading to mutations (Cheesman and Slater, 1993). It has been reported that free radicals may

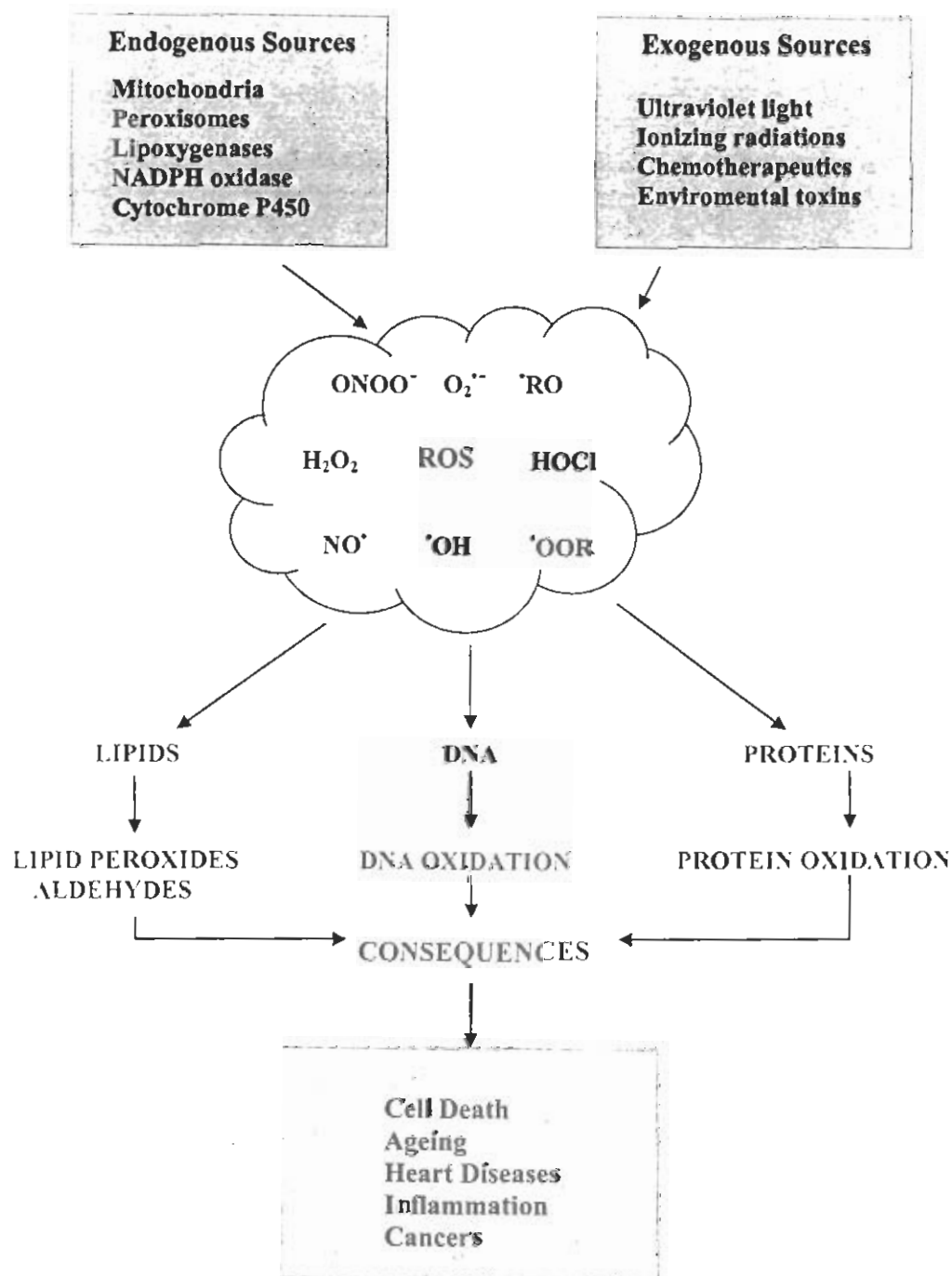


Figure 1.1: Sources and consequences of reactive oxygen species (ROS): Oxidants are continuously generated as a result of normal intracellular metabolism in mitochondria and peroxisomes as well as from a variety of cytosolic enzyme systems. In addition, number of exogenous sources can generate ROS. The ROS are harmful to cells by damaging biomolecules which results in acceleration in ageing and of certain other age-related diseases.

be involved in cell death or sub-lethal injuries such as mutations, chromosomal aberrations or carcinogenesis by damaging DNA and the DNA repair processes (Aust and Eveleigh, 1999; VanRensburg *et al.*, 1992).

1.2.4. Mechanism of Action of Free radicals

There are three main stages in free radical mediated reactions: initiation, propagation and termination. Initiation starts with the abstraction of a hydrogen atom from the bio-molecule. For example fatty acid (LH) can be converted into radicals (L^{\bullet}). The hydroxyl (OH^{\bullet}), alkperoxyl (ROO^{\bullet}), and alkoxy (RO^{\bullet}) radicals are all capable of oxidizing PUFAs. Extremely rapid addition of oxygen to the fatty acid radicals then generates peroxy radicals (LOO^{\bullet}) that propagate the reaction by initiating a new chain of oxidation with the formation of lipid hydroperoxide ($LOOH$). This chain reaction continues till an antioxidant interrupts it through scavenging the radicals: the termination step (Schafer *et al.*, 2000; Aitken and Fisher, 1994). The mechanism of peroxidation of linoleic acid as a model is presented in Figure 1.2.

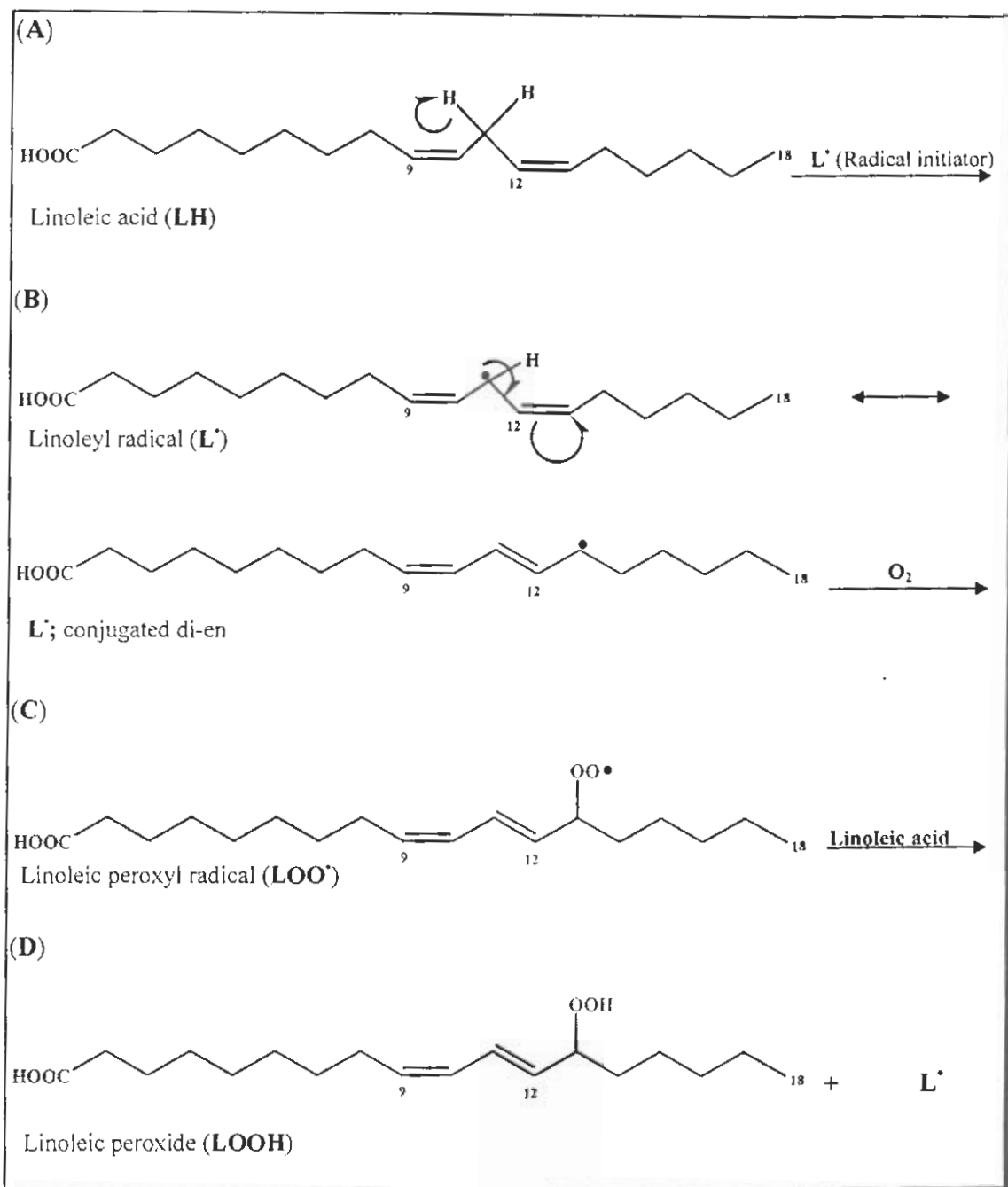


Figure 1.2: Mechanism of lipid (Linoleic acid) peroxidation: (A) Hydrogen abstraction from the allylic position of linoleic acid (LH) by an initiator leads to the formation of L[•]. (B) Delocalization of the odd electron yields a conjugated diene. (C) Formation of LOO[•] as a result of reaction between oxygen and L[•]. (D) Generation of new L[•] with the formation of LOOH as a result of attack of LOO[•] to a new LH.

1.3. Antioxidants

To protect cells and organs from the oxidative stress induced by ROS, living organisms have evolved with an extremely efficient and highly sophisticated protective system, the so-called "antioxidant defensive system". It involves a variety of components, both endogenous and exogenous in origin. These components function interactively and synergistically to neutralize free radicals (Percival, 1998). A broader definition of an antioxidant is "any substance which, when present at low concentrations compared to those of oxidizable substrates, significantly delays or prevents oxidation of those substrates". The term oxidizable substrates includes DNA, lipids, proteins and carbohydrates, which are the essential building blocks of a biological system (Halliwell *et al.*, 1995). Oxidative stress occurs as a result of an increase in oxidative metabolism, which produces a number of ROS. To avoid oxidative stress, antioxidants can play an important role conferring beneficial healthy effects (Vaya and Aviram, 2001). High dietary intake of proven antioxidants can significantly lower the risk of several chronic diseases such as heart diseases, cancers and cataracts.

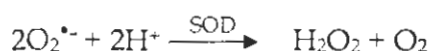
1.3.1. Classification of Antioxidants

A variety of antioxidants are collectively required for the removal of free radicals to protect the body from adverse effects of ROS. Certain enzymes as well as non-enzymatic cellular molecules are involved in the detoxification of ROS. Based on the nature of antioxidants, the human antioxidant system can be categorized into two broader classes: enzymatic and non-enzymatic (Jakus, 2000).

1.3.1.1. Enzymatic Antioxidants

The major primary intracellular endogenous antioxidant defences are the enzyme system. This antioxidant enzymatic system includes superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidase (GSHPx) (Yang *et al.*, 1999; Halliwell and Gutteridge, 1990).

Superoxide dismutases (SODs) have been found in three isoforms. Manganese containing SOD (MnSOD) is a tetrameric protein that is localized in the mitochondrial matrix. It plays a key role in scavenging $O_2^{\bullet-}$ generated from the electron transport chain. Copper and zinc containing SOD (CuZnSOD) is a dimer protein that is localized in the cell cytoplasm. It is thought to remove $O_2^{\bullet-}$ generated by endoplasmic reticulum and cytosolic oxidases. The extracellular SOD is a tetrameric protein found in the extracellular space (Noor *et al.*, 2002). SODs catalyze the dismutation of superoxide into H_2O_2 (Ivanova and Ivanov, 2000).



Catalase (CAT) is located in peroxisomes and mitochondria. It is a large tetrameric protein which removes H_2O_2 by catalyzing its conversion into water (Krinsky, 1992).



Glutathione peroxidases (GSHPx) are a group of selenium-dependent enzymes. Four isoforms of GSHPx have been described: cytosolic GSHPx1, plasma GSHPx, phospholipid-hydroperoxide PHGSHPx and gastrointestinal GSHPx-GI. All GSHPx

require glutathione (GSH) as a cofactor and secondary enzymes, such as glutathione reductase and glucose-6-phosphate dehydrogenase (G-6-PDH), to function. G-6-PDH generates NADPH to recycle the GSH (Takebe *et al.*, 2002; Ivanova and Ivanov, 2000).



1.3.1.2 Non-Enzymatic Antioxidants

Non-enzymatic antioxidants may be further classified into two groups: endogenous and exogenous antioxidants. The major extracellular endogenous antioxidants found in human plasma are transition metal binding proteins. These include ceruloplasmin, transferrin, hepatoglobin and albumin. They bind with transition metals and hence control the production of metal-catalyzed free radicals. Albumin and ceruloplasmin are the copper ions sequesters. Hepatoglobin binds with haemoglobin while ferritin and transferrin bind with free iron (Ivanova and Ivanov, 2000; Halliwell and Gutteridge, 1990). Lipoic and uric acids, bilirubin, ubiquinone and glutathione are non-protein endogenous antioxidants which inhibit the oxidation processes by scavenging free radicals (Shahidi, 1997).

Many effective exogenous antioxidants are generally of dietary origin. The best known are vitamins such as ascorbic acid, vitamin E, carotenoids, quinones, and polyphenols. These molecules can inhibit oxidative reactions by scavenging free radicals, while certain compounds may chelate redox active metals or inhibit particular oxidative enzymes. Vitamin E is a lipid soluble, chain breaking antioxidant which reacts with lipid peroxy radicals to yield a relatively stable lipid

hydroperoxide and thus protects against membrane lipid peroxidation (Maguire *et al.*, 1989). On the other hand, vitamin C has multiple antioxidant properties, including the ability to regenerate α -tocopherol by reducing α -tocopheroyl radicals at the membrane surfaces of membranes. It also scavenges others free radicals and certain non-radicals such as HOCl (Packer and Cadenas, 2002).

1.3.2. Mechanism of Action of Antioxidants

Antioxidants can remove both free radical and non-radical species through different modes of action depending upon what ROS is required for neutralization. On the basis of the nature of ROS, two basic mechanisms have been proposed for the action of antioxidants: mechanism of removal of ROS initiators and a chain breaking mechanism.

1.3.2.1 Mechanism of Removal of Initiators of ROS

This process is mainly based on the inhibition of the enzymes involved in the production of ROS. Xanthine oxidase (XO) is one of the major sources of superoxide anions production (Borges *et al.*, 2002). Lipoxygenase, during arachidonic metabolic pathway, produces lipid peroxides (Dailey and Imming, 1999). Antioxidants inhibit these enzymes so that they are not available to harm the cellular system. The other main sources of free radicals are free transition metal ions which, by virtue of their attachment with carrier proteins, act as pro-oxidants and are therefore toxic to the body. There are several metal binding proteins that chelate transition metals, which are capable of reacting with hydroperoxides to produce free radicals:



The proteins which bind with the transition metal ions include transferrin, lactoferrin and ferritin. These proteins function to keep iron-induced oxidant stress in control. Ceruloplasmin and albumin proteins are the copper and iron sequestrants, respectively (Vaya and Aviram, 2001; Krinsky, 1992).

1.3.2.2. Chain Breaking Mechanism of Action

Antioxidants scavenge free radicals by donating an electron to them while being oxidized themselves during the process. This is known as a “chain breaking antioxidation” (CBA). The chain reactions of free radicals can be stopped with the help of antioxidants, which can neutralize free radicals formed during the overall reaction. Most of the polyphenolics work through the chain breaking (CB) mechanism of action. Vitamins C and E, carotenes, flavonoids and coumarins are examples of chain breaking antioxidants (CBA).

Vitamin E (tocopherol, TH₂) is the most widely distributed lipid soluble antioxidant in nature. Its main function is to prevent the peroxidation of membrane phospholipids and to avoid cell membrane damage (Maguire *et al.*, 1989). A detailed study of the effects of benzene ring substituents on the rate of radical scavenging reaction showed that the reaction is accelerated by the presence of a 4-methoxy and C-2 and C-6 methyl groups. The presence of C-1 hydroxyl group contributes to donating H[•], whereas functional groups at C-4, C-2, and C-6 stabilize the resulting tocopheroyl radical (Shahidi, 1997; Burton and Ingold, 1981). The reaction of vitamin E (TH₂) with peroxy radical (LOO[•]) is summarized in Figure 1.3.

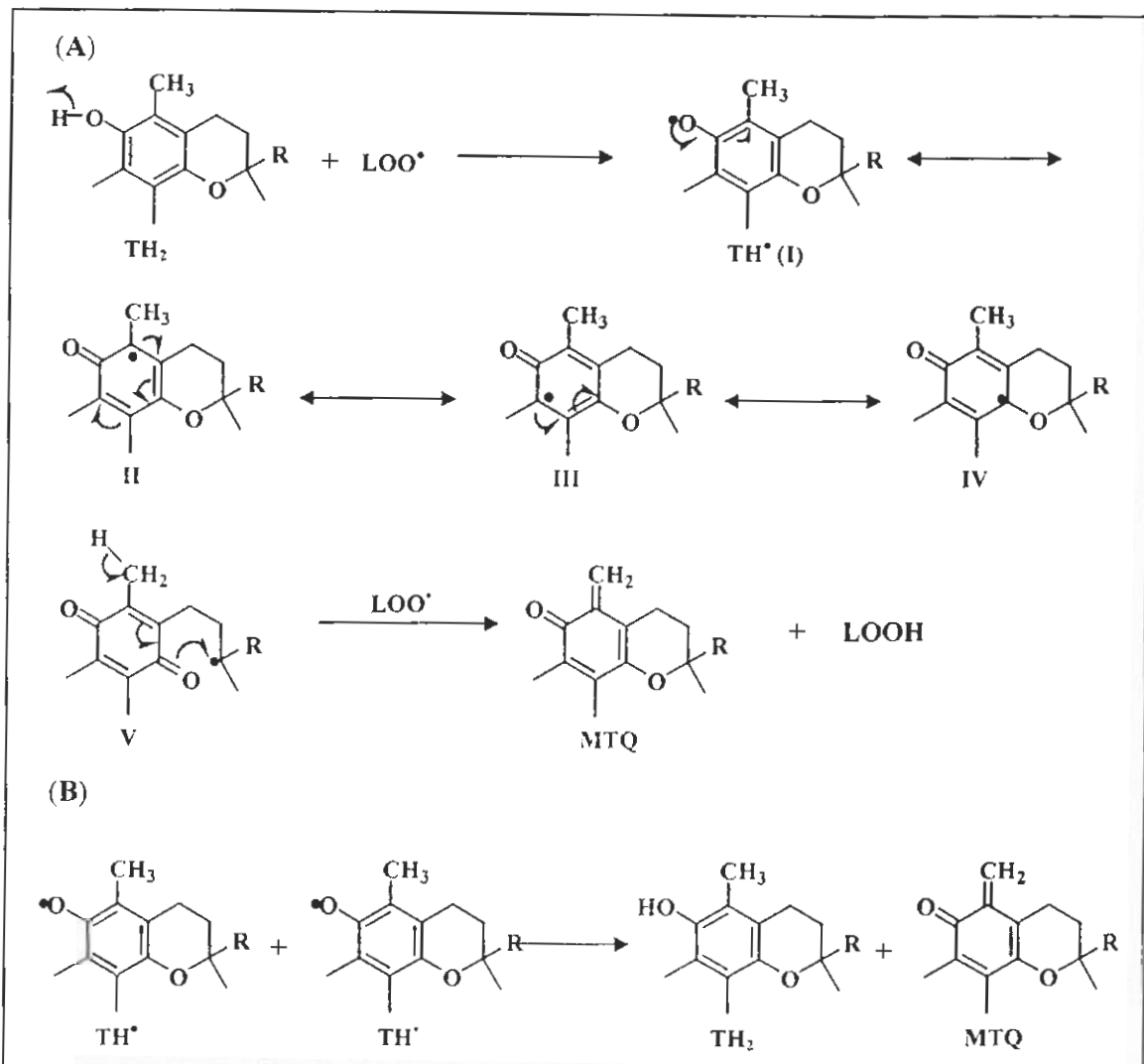


Figure 1.3: Chain breaking mechanism of action of vitamin E: (A) Generation of tocopherol radical (TH[•]) by the donation of H[•] from TH₂ to LOO[•]. The TH[•] is stabilized by the delocalization of unpaired electron over the benzene ring through five resonance structures (I-V). TH[•] reacts with another LOO[•] to yield methyl tocopherylquinone (MTQ) and hydroperoxide (LOOH). **(B)** TH[•] may react with another TH[•] radical to give methyl tocopherylquinone and a regenerated molecule of tocopherol, TH₂.

1.3.3. Protective Role of Antioxidants on Biological Functions

Substantial research work had been carried out to investigate the preventive role of antioxidants in different diseases. Every antioxidant has some significance and the best protection against oxidative stress comes from the presence of a wide assortment of interrelated antioxidants and their cofactors. The function of each particular antioxidant depends on what type of oxidative stress is imposed (Percival, 1998).

Lipid peroxidation can damage low-density lipoprotein (LDL) particles in several ways. *In vitro* studies have demonstrated that lipoxygenase, superoxide anion, peroxynitrite and myeloperoxidase can oxidize LDL (Yla-Herttuala *et al.*, 2000) which can lead to heart diseases. Studies showed that antioxidants may protect against coronary heart diseases (CHD) (Chow, 2001; Parthasarathy *et al.*, 1999; Diaz *et al.*, 1997). Vitamins have been shown to reduce the susceptibility of LDL to oxidation and are also known to be involved in elevating the levels of protection factors like HDL-cholesterol. Studies suggest that vitamin C may reduce the risk of hypertension (Noguchi and Niki, 2000). In addition, a high intake of vitamin C appears to protect against gastric cancer, probably through scavenging ROS formed in the gastric mucosa (Woodford and Whitehead, 1998). Further investigations on vitamin C have proved its preventive effects on inhibition of tumor promotion (Lee *et al.*, 2002).

Dietary antioxidants are important to maintain good health. Vegetables and fruits contain a variety of important nutrients that are essential for the normal functioning of the body. Tomato intake, the main source of lycopene, has been found to be

associated with a lower risk of a variety of cancers in several epidemiological studies (Agarwal and Rau, 2000). Epidemiological investigations on vitamin E suggested that it may be protect against the occurrence of Parkinson's disease. Some researchers have also shown that vitamin E intake can slow down the progression of Alzheimer's disease (Vatassery *et al.*, 1999). It was reported that supplementations with vitamins C and E, mixed with other antioxidants, can reduce symptoms of oxidative stress during exercise (Clarson and Thompson, 2000).

Flavonoids are polyphenols and they are abundantly found in fruits, vegetables, grains, bark, roots, stems, flowers, and tea. They possess anti-inflammatory, antiallergic, antiviral and anticarcinogenic properties due to their antioxidant potential (Nijveldt *et al.*, 2001; Hollman *et al.*, 1996).

Tea is found to be particularly rich in antioxidants. It exerts beneficial effects on the regulation of blood cholesterol levels. Catechin, the major constituent of tea, has been found to regulate blood pressure and help to lower the blood sugar levels (Ramarathanam *et al.*, 1995).

1.3.4. Some Common Antioxidants

There are hundreds of antioxidants of natural and synthetic origin. Increasing interest in such compounds is due to their effective role against the destructive actions of free radicals. Several studies have been focused on identifying different classes of antioxidants. Some important antioxidants have been discussed in this section.

Vitamin E is the most common naturally occurring antioxidant. Its structure is closely related to phenolic benzochroman derivatives. It has a phytyl side chain

attached to its chromanol nucleus. The ability of donating two electrons confers for radical scavenging activity (Packer and Cadenas, 2002).

Ascorbic acid (vitamin C) is a water soluble electron donor vitamin. To act as an antioxidant, it donates two electrons from the C-2 and C-3 double bond carbons, which results in the formation of an intermediate free radical, semidehydroascorbic acid E. The resulting ascorbate free radicals readily reduce to a neutral ascorbate molecule (Feri *et al.*, 1989; Packer and Cadenas, 2002).

Carotenoids are a large group of compounds with various structural features. The basic skeleton of carotenoids consists of a polyisoprenoid C₄₀ carbon chain with a number of conjugated double bonds. Due to the presence of high conjugation, effective delocalization of electrons can occur along the entire length of the polyene chain. This distinctive character of carotenoids makes them effective as singlet oxygen quenchers. β -Carotene is bicyclic in nature with β -ionone rings at both ends of the molecule (Packer and Cadenas, 2002).

Phenolic phytochemicals can be categorized into three major classes: non-flavonoid polyphenols, flavonoids, and phenolic acids (Figure-1.4). Phenolic compounds have aromatic rings containing one or more hydroxyl groups such as caffeic acid E. Polyphenols contain multiple phenol rings within their structures; examples include catechin and ellagic acids. Flavonols and catechins are major components of tea (*Camellia sinensis*) and are responsible for its antioxidant properties (Wiseman *et al.*, 1997). Tannins are high molecular weight phenolic mixtures. All classes of phenolics possess antioxidant activities, depending on the number of hydroxyl groups present on benzene rings (Packer and Cadenas, 2002). Flavonoids have been shown to have

antioxidant activities both by acting as hydrogen donors or chelating with metal ions. Their structures consist of two rings, A and C, fused with the phenyl ring B through its C-1' to C-2 of ring C (Evans *et al.*, 1997). The structures of some antioxidants are given in Table 1.2.

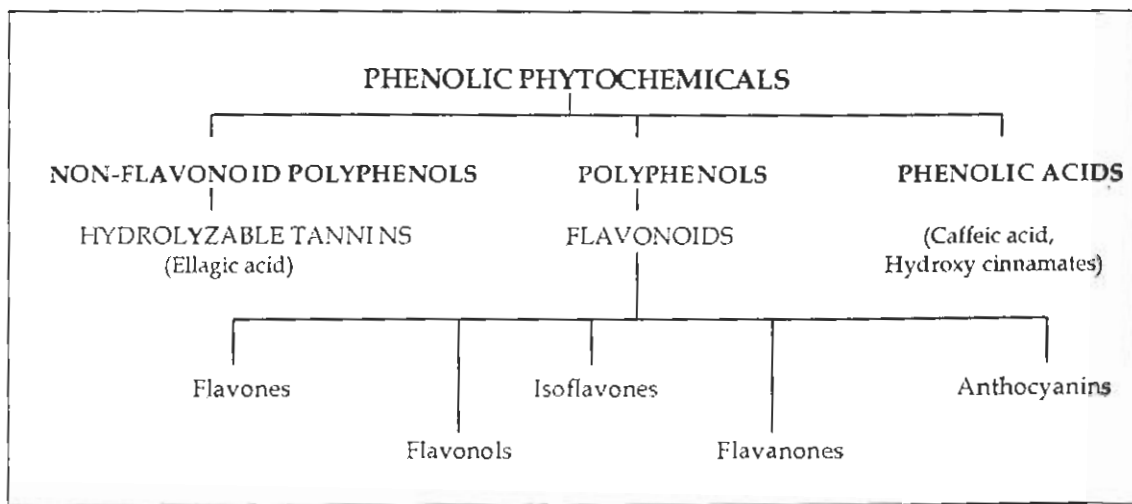


Figure 1.4: Classification of phenolic phytochemicals: Ellagic and caffeic acids are examples of non-flavonoid polyphenols and phenolic acids, respectively.

Table 1.2: Some antioxidants of natural and synthetic origin.

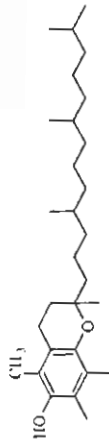
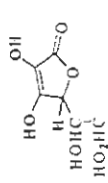
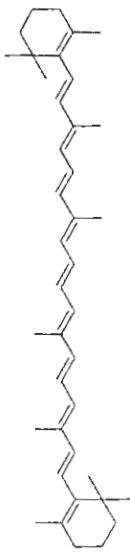
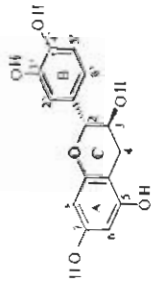
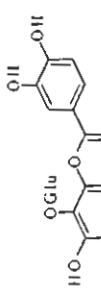
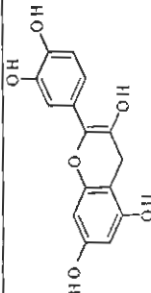
Sr. No	IUPAC/ Common Name	Structure	Source	Mechanism of Action	Reference
1	α -Tocopherol		Vegetables, fruits, seeds, fish oils etc.	Chain breaking	Packer and Cadenas, 2002
2	Ascorbic acid		Citrus fruits, vegetables etc.	Chain breaking	Shahidi, 1997
3	β -Carotene		Fruits, vegetables	Singlet oxygen quencher Chain breaking	Polyakov <i>et al.</i> , 2001
4	(+)-Catechin		<i>Camellia sinensis</i> L.	Chain breaking	Wiseman <i>et al.</i> , 1997
5	Hypolaetin-8-glucoside		<i>Sideritis javanlambrensis</i> Pau.	Chain breaking	Rios <i>et al.</i> , 1992
6	Quercetin		<i>Polygonum hydropiper</i> L. <i>Bridelia ferruginea</i> Benth	Chain breaking Inhibits Xanthine oxidase	Haraguchi <i>et al.</i> , 1992 Cimanga <i>et al.</i> , 2001

Table 1.2: Some antioxidants of natural and synthetic origin.

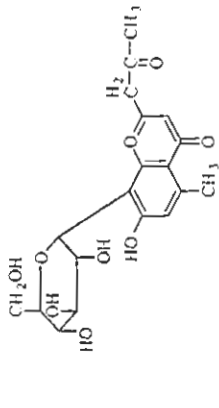
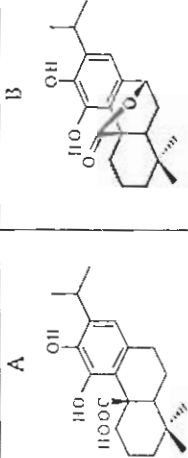
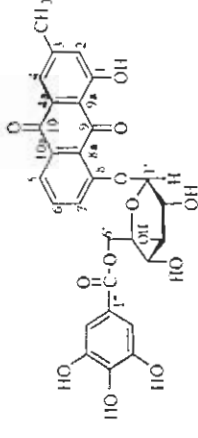
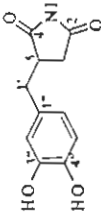
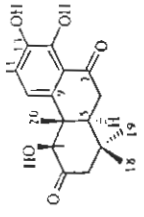
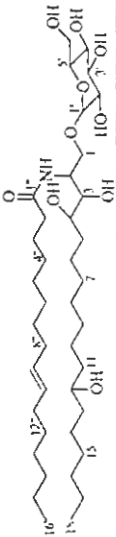
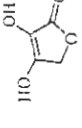
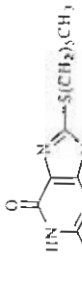
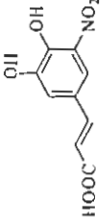
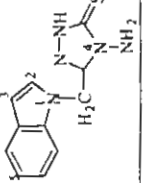
Sr. No	IUPAC/Common Name	Structure	Source	Mechanism of Action	Reference
7	Aloesin		<i>Aloe vera</i>	Chain breaking	Yag <i>et al.</i> , 2002
8	A) Carnosic acid B) Carnosol		<i>Rosmarinus officinalis</i> L.	Chain breaking	Haraguchi <i>et al.</i> , 1995
9	Chrysohanol 8-O-β-D-(6'-galloyl)-glucopyranoside		<i>Rhizum undulatum</i> L.	Chain breaking	Matsuda <i>et al.</i> , 2001
10	5-(3,4-Dihydroxyphenylmethyl)oxazolidine-2,4-dione		<i>Perille frutescens</i> var. <i>japonica</i> Hara	Chain breaking	Nagatsu <i>et al.</i> , 1995

Table 1.2: Some antioxidants of natural and synthetic origin.

Sr. No	IUPAC/ Common Name	Structure	Source	Mechanism of Action	Reference
11	1 β ,13,14-Trihydroxy-8,11,13-podocarpatriene-2,7-dione		<i>Taiwania cryptomerioides</i>	Chain breaking	Kuo <i>et al.</i> , 2002
12	1,3,4,12-Tetrahydroxy-2-(9-hexadecenyl amino)-n-octadecane-1-O-glucopyranoside		<i>Conyza bonariensis</i> L.	Inhibits Xanthine oxidase	Kong <i>et al.</i> , 2001
13	Reductive acid/ 2,3-dihydroxy-2-cyclopentenone		Synthetic	Chain breaking Inhibits Xanthine oxidase	Mashino <i>et al.</i> , 2000
14	8-11-oxylthio-xanthine		Synthetic	Inhibits Xanthine oxidase	Biagi <i>et al.</i> , 2001
15	5-Nitro-3,4-dihydroxycinnamic acid		Synthetic	Chain breaking Inhibits Xanthine oxidase	Grenier <i>et al.</i> , 1996
16	3-[(5-Hydroxy-1H-indolyl)methyl-4-amino-4,5-dihydro-1H-1,2,4-triazole-5-thione		Synthetic	Chain breaking	Andreadou <i>et al.</i> , 2002

1.3.5. Measurement of Antioxidant Activity

Free radicals have been implicated in a number of disorders in biological systems and certain other fields, i.e. deterioration of food, degradation of polymers, rubbers, plastics and cosmetics etc. To prevent the damaging effects of free radicals, a number of investigations have been made to discover antioxidants using various methodologies. A number of experimental models have been developed for the determination of antioxidant activities of different samples. These methods can be divided into two major categories (Vaya and Aviram, 2001):

- 1) Measuring the potential of a sample to donate an electron or α hydrogen atom to a specific reactive oxygen species or to any electron acceptor.
- 2) Measuring the ability to remove any source of oxidative initiation, e.g. inhibition of enzymes, chelation of transition metal ions and absorption of UV radiation.

Several factors influence the efficiency of an antioxidant and require consideration of its bioavailability, the site of action, its stability, its toxicity, and the type of reactive oxygen species that the antioxidant must react with. It is therefore necessary to select an appropriate method for evaluation of antioxidant potential. The following assays are commonly used for measurement of antioxidant activity:

1.3.5.1. Radicals Scavenging Assays

Stable free radical species are commonly used to determine the antiradical activity of compounds. These radicals include 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Lee *et al.*, 1998) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Re *et al.*, 1999). Other radicals scavenging assays include superoxide anion scavenging assay (Gaulejac *et al.*, 1999) and hydroxyl

radical scavenging assay (Yoshiki *et al.*, 1995). In most studies, either the phenazine methosulfate-NADH system (Gaulejac *et al.*, 1999) or the xanthine/XO system is used to produce superoxide anion radicals (Cos *et al.*, 1998).

In many methods of detecting antioxidant activity, the ability of an antioxidant to stop the oxidation of poly-unsaturated fatty acids (PUFA), such as linoleic acid is determined by exposing the PUFA to oxygen, light or free radical generators. The frequently used methods for measuring lipid peroxidation/peroxy radical scavenging ability include:

Conjugated diene assay: This assay is used for quantification of conjugated diene formed as a result of initial PUFA oxidation by measuring UV absorbance at 234 nm (Esterbauer *et al.*, 1989).

Lipid peroxide assay: In this system, oxidation of PUFA initiated by oxygen at allylic position of lipid results in an unstable mixture of lipid peroxides. The total amount of lipid peroxides can be detected iodometrically (El-Saadani *et al.*, 1989).

Thiobarbituric acid reactive substances (TBARS) assay: In this assay model, lipid peroxides formation is measured by the detection of a stable product formed as a result of reaction of thiobarbituric acid with aldehydes, a decomposed product of lipidperoxides (Armstrong, 1998).

1.3.5.2. Enzyme Inhibition Assays

There are certain enzymes which produce reactive oxygen species during reactions. These enzymes include lipoxygenases, cyclooxygenases and xanthine oxidase etc. Xanthine oxidase catalyzes the production of uric acid and superoxide anion using xanthine/hypoxanthine as substrate (Richardson and Finley, 1997), whereas

lipoxygenases and cyclooxygenases produce lipid hydroperoxides as a result of arachidonic acid metabolism (Dailey and Imming, 1999). The inhibitors of these enzymes may decrease reactive oxygen species in biological systems (Vaya and Aviram, 2001).

1.3.5.3. Chelation of Transition Metals

The transition metals iron and copper are essential cofactors of several enzymes which are involved in oxygen metabolism. In biological systems, these metals are found with proteins and enzymes but when these are present in free state, they can catalyze free radical reactions. The relative chelating capacity of samples can be studied spectrophotometrically by measuring the ability to release iron ions from an iron-EDTA complex and to chelate iron ions (Acker *et al.*, 1996).

1.3.5.4. Chromatographic Procedures

Lipid hydroperoxides are the major end products obtained during oxidation of PUFA. Many methods developed to date measure either primary hydroperoxides or secondary aldehydic products of lipid oxidation. High performance liquid chromatography (HPLC) is one of the useful methods in the quantitative measurement of lipid hydroperoxides. These HPLC methods employ chemiluminescence detection for lipid hydroperoxides and thiobarbituric acid (TBA) assay (Lunec and Griffiths, 2000).

1.4. Aims of the Present Study

Antioxidants have attracted immense interest of researchers because of their implied role in the protection of biological systems. Though all living organisms possess their own antioxidant defense systems, no one can disregard the importance of dietary or exogenous antioxidants as these have an important role in the prevention of a variety of diseases. There is, therefore, a need to discover new and effective radical scavengers from natural sources. Natural antioxidant lead molecules can lead to the synthesis of a variety of more effective derivatives in laboratory in order to investigate their biological activities and carry out SAR studies.

The primary goal of the work presented in this thesis was to characterize the antioxidant properties of a variety of chemical constituents isolated from several medicinal plants along with a variety of synthetic molecules. The specific objectives are as follows:

- To evaluate the antioxidant potential of test samples using three complimentary *in vitro* bioassays, DPPH (1,1-Diphenyl-2-picrylhydrazyl radical) scavenging assay, NADH-PMS superoxide scavenging assay and xanthine oxidase inhibition assay.
- To carry out a structure-activity relationship study of active compounds in order to discover the functionalities responsible for enhanced activity.
- To study the *in vivo* antioxidant activities of selected compounds by using hepatoprotection as an indication of antioxidant effects, through the analysis of

the changes in concentrations of bilirubin and estimating the serum enzymes, ALT/GPT and AST/GOT after carbon tetrachloride-induction.

- To study the cytotoxic nature of hepatoprotective compounds on human neutrophils in order to investigate their suitability as potential drugs.