Chapter 1

INTRODUCTION
1. INTRODUCTION

In modern society, herbal medicine continues to flourish and play a pivotal and indispensable role in public healthcare. Exploring the bioactive constituents represents a promising approach towards discovery of new drugs. Medicinal plants used in traditional medicine would be a good source for this area of research (Mbwambo et al., 1996). In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. There has always been research on plants as medicines. A wide variety of the traditional herbal remedies are used by liver diseases patients, especially in the third world countries and may therefore represent new avenues in the search for alternative drugs.

Medicinal plants and their derivatives have found important position in modern medicine due to the presence of several curative chemicals. Their secondary metabolites represent a large reservoir of structural moieties which work together exhibiting a wide range of biological activities. Microorganisms have the genetic ability to transmit and acquire resistance to antibiotics and have become a major global healthcare problem in the 21st century. Over 75% of the antibacterial drugs in clinical use are of natural origin (Newmann et al., 2003). Plant derived antimicrobial compounds might inhibit bacteria through different mechanisms and provide clinical value in the treatment of infections caused by resistant microbes. There is a need to evaluate the herbs scientifically for their antimicrobial activity against the antibiotic-resistant microorganism, in order to develop complementary phytochemical strategies.
1.1. ALTERNATIVE MEDICINES AGAINST LIVER DISEASES

Medicinal plants have been widely used to treat a variety of infectious and non-infectious ailments. The use of natural remedies for the treatment of liver diseases has a long history, starting with the Ayurvedic treatment, and extending to the Chinese, European and other systems of traditional medicines. The 21st century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver diseases by carefully synergizing the strengths of the traditional systems of medicine with that of the modern concept of evidence-based medicinal evaluation, standardization of herbal products and randomized placebo controlled clinical trials to support clinical efficacy. In spite of the availability of more than 300 preparations for the treatment of jaundice and chronic liver diseases in Indian systems of medicine using more than 87 Indian medicinal plants, only four terrestrial plants have been scientifically elucidated (Thyagarajan et al., 2002).

Mankind has been continuously using the plants in one or the other way in the treatment of various ailments. The traditional systems of medicines-Ayurveda, Siddha and Unani are based on the experience in the use of plant products in amelioration of many diseases. Nearly 80% of the world population depends upon traditional system of healthcare. Allopathic drugs have brought a revolution throughout the world but the plant based medicines have their own status. The local use of plants as a cure are common particularly in those areas that have little or no access to modern health services such as the innumerable villages and hamlets in India. Currently available toxicity drugs play an important role in the treatment of liver diseases. However, one thing that toxicity drugs have in common is that they are expensive with many adverse effects. Therefore there is a need for population-based, cost-effective; adverse-effect free liver diseases control strategies to be developed.
People with viral hepatitis are increasingly investigating and using non-traditional treatments, especially herbal supplements, to help combat hepatitis-related liver disease. Parents of children with viral hepatitis infections are no exception. E-mail message boards and online discussions reflect a lively interest in herbal supplements that may help protect children’s livers and bolster their immune systems. But to date, little research has been conducted on the safety and efficacy of herbal supplements in children and adults with viral hepatitis, nor in the healthy adult population in general. Sometimes, experts warn, these supplements can interfere with and lessen the effectiveness of conventional drugs prescribed by doctors. Physicians and alternative medical practitioners agree it is important that patients and parents talk to physicians ahead of time when considering alternative herbal supplements and, more critically, when taking them.

1.2. INDIAN SCENARIO

In India, knowledge and wisdom have been passed on from one generation to the next by oral means through verbatim, songs and poems, which scholars and physicians had to learn by heart and recite. The history of health care goes back to 5000 years B.C., when health care needs and diseases were noted in ancient literatures like ‘Rig-Veda’ and ‘Atharva-Veda’. Later, the texts like ‘Charak Samhita’ and ‘Sushruta Samhita’ were documented in about 1000 years B.C., where use of plants and polyherbal formulations was highlighted for health care. Evolution of Ayurveda and plant based remedies for health care through day-to-day life experiences is a part of cultural heritage of India. In almost all the traditional systems of medicine, the medicinal plants play a major role and constitute their backbone. Indian Materia
Medica includes about 2000 drugs of natural origin almost all of which are derived from different traditional systems and folklore practices (Narayana et al., 1998).

In this traditional system of medicines, a single drug or combinations in raw form are preferred over compound formulations. The system offers excellent remedies for gastrointestinal, cardiovascular and nervous disorders. Though the origins and development periods of these systems of medicine are of different there is a common thread running through these systems in their fundamental principles and practices by using the plants and plant-based formulations in the health care (Ramakrishnappa, 2002).

1.3. LIVER

Liver (Figure 1) is the largest mass of glandular tissue and the second largest organ after skin in the body (Moore and Dalley, 2002). It represents about 2-5% of the body weight in man and other mammals such as the rat and mouse (human liver: 2%; rat liver 4%; mouse liver 5%) (Junquiera, 2003). It is located in the upper right hand portion of the abdominal cavity, beneath the diaphragm and on top of the stomach, right kidney and intestines. Shaped like a cone, reddish brown in colour because of the rich supply of blood flowing through it. The liver divided into left and right anatomical lobes by the falciform ligament, an anterior extension of the peritoneal folds that connects the liver to the diaphragm and anterior abdominal wall. Two smaller lobes are found on the posterior surface (caudate lobe) and the inferior surface (quadrate lobe) of the right lobe. Riedel’s lobe is an anatomical extension of the right lobe of the liver and consists of a projection that may feel like a mobile tumor in the right abdomen. It has a dual blood supply. The hepatic portal vein from the gastrointestinal tract which accounts for 75% of the liver's blood supply and the hepatic artery, which supplies 25%
of the liver's blood. The liver consists of several lobes and each lobe contains thousands of six-sided units called a lobule, which is a structural and functional unit of the liver (Dekant, 1992). Liver has a number of essential roles in the body including glycogen storage, plasma protein synthesis and it plays an important role in metabolism of protein and lipid (Junqueira, 2003). The liver also has an important role in detoxification and breaking down substances such as xenobiotic chemicals and metabolic waste; there are two types of metabolism, phase I, where by the liver is using cytochrome P450 mixed function oxidase enzyme pathway to oxidize the potential toxicant and phase II, the conjugation pathway, where by the hepatocytes add another substance (e.g. glutathione or a sugar) to a toxic chemical or drug to make it water soluble, so it can then be excreted from the body via watery fluids such as bile or urine (Hayes, 2001).

**Types of liver cells**

The liver is the first site of passage for venous blood arriving from the intestines via vena porta. The areas around the influx blood vessels are named periportal. The areas surrounding efflux blood vessels are the perivenous. The periportal area is highly complex and consists of a dense matrix containing collagen where afferent blood vessels are found, together with bile ducts, nerves and lymph. Spaces within the matrix contain a variable cell population, such as fibroblasts, hematopoietic cells and inflammatory cells. Also found here are epithelial cells of the bile ducts, endothelial cells of the blood vessels, and smooth muscle of arteries and veins (Grisham, 1983). The liver lobule consists mainly of plates of hepatocytes and sinusoids, with a light matrix of collagen to form a network between the two. Kupffer cells, as well as fat-storing stellate cells are found here. These types of cells reside mainly in the tissue space between the hepatocyte and the sinusoids. Terminal bile ductules connect here to the bile cannaliculi between hepatocytic plates. The walls of the hepatic sinusoid are
Figure 1. Structure of liver

Biliary System

Right Hepatic Duct
Liver
Gallbladder
Cystic Duct
Common Bile Duct
Duodenum
Common Hepatic Duct
Pancreas
Stomach
Left Hepatic Duct
Pancreatic Duct
lined by three different cell types: the sinusoidal endothelial cell, Kupffer cells, and stellate cells. Additionally, pit cells, the liver specific NK T cells are often present in the sinusoidal lumen. The main parenchymal mass is normally that of hepatocytes. In rat, the hepatocytes make up about 60% of liver cell count, but 70% of its mass. The remaining 40% non-parenchymal cells only make up for about 6-7% of the liver volume, while the remaining volume of approximately 23% is formed by extracellular space (Hendriks Brouwer et al., 1990).

**Liver as a detoxifying organ**

The adult liver is the main organ responsible for detoxifying (Scheme 1) and metabolizing a variety of exogenous as well as endogenous compounds, rendering them more hydrophilic, which often affects their potency and activity. The enzymes responsible for these actions are primarily expressed in hepatocytes and mainly divided into two groups: Phase I and Phase II.

The phase I enzymes are predominantly from the P450 family of genes, whose general function is to add polar groups, such as hydroxyl groups, to lipophilic molecules thus rendering them more hydrophilic (Park Pirmohamed et al., 1995). The main function of the phase II enzymes is to covalently attach a water soluble moiety to the polar group added by the phase I enzymes. Usually such molecules are sugars or peptides, such as glucuronic acid or glutathione. This usually renders the compound less reactive. Examples of phase II enzymes are glutathione S-transferase and UDP-glucuronosyl transferase. If the phase II reaction is impaired for some reason, or the phase I reaction is induced, this may leave the organism with an excess of reactive molecules from the phase I reaction, which can be detrimental. This can occur in the case of drug induced hepatotoxicity, when reactive metabolites of the parent compound
Scheme 1. Detoxification of liver
are formed, which subsequently negatively affect cellular functions (Liu and Kaplowitz, 2002).

1.4. HEPATOTOXICITY

Hepatotoxicity is a general term for liver damage. It is a major cause of compound (drug) withdrawal during the discovery and development (Rialland et al., 2000). There are several specific conditions that all fall within the general category of hepatotoxicity.

These conditions include hepatitis, inflammation of the liver, hepatitis necrosis - death of liver cells and hepatic steatosis - too much fat in the liver, may be associated with a life threatening condition called lactic acidosis.

Hepatitis

Hepatitis is a common liver disease in the world especially in the developing countries. Despite, considerable progress in the treatment of liver disease by oral hepatoprotective agents, search for newer drugs continues because the existing synthetic drugs have several limitations. Hence, there are many researches of traditional medicines attempting to develop new drugs for hepatitis (Liu, 1989). The symptoms of chemically-induced (drug induced) hepatitis are similar to that of infectious hepatitis.

Symptoms of Hepatotoxicity

The first sign of damage to the liver is an increase in liver enzyme, levels in the blood. When the liver is damaged, its enzymes are released into the bloodstream, where the levels can be measured by blood tests. Enzyme levels that are routinely checked as part of liver function tests (LIFTs) include: Alanine aminotransferase
(ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP) and Gamma glutamyl transpeptidase (GGT).

**Symptoms of Liver damage**

Jaundice characterised by yellowing of the skin, whites of the eyes, and mucous membranes (moist areas around eyes and mouth) due to high levels of bilirubin in the extracellular fluid. Other symptoms includes nausea, vomiting, abdominal pain, loss of appetite, diarrhoea, feeling tired or weak, generalized itching, hepatomegaly (liver enlargement) and any unusual swelling in the feet and legs, or weight gain of greater than 3 to 5 pounds in one week.

**1.5. DRUG INDUCED HEPATOTOXICITY**

Drug induced liver injury is a health problem, and is expected to increase as the number of drugs being consumed increases, both prescription and non-prescription, as well as due to the current trend of usage of pharmacologically active substances in complementary and alternative medicine (Schoepfer Engel *et al.*, 2007). Drug induced hepatotoxicity is the most common reason cited for withdrawal of already approved drugs from the market. It also accounts for more than 50 percent of cases of acute liver failure in the United States. The exact incidence of drug induced liver injury is difficult to estimate, and in general, studies aiming at measuring its incidence suffer from drawbacks such as under reporting and that data in general come from retrospective studies. Often, there is also a lack of information about self medication and usage of herbal products that may interact with prescription and non-prescription drugs (De Abajo Montero *et al.*, 2004). However there is one prospective community-based study performed in France where, over a 3 year period, the annual incidence of hepatic drug reaction was found to be 14 cases per 100,000 inhabitants (Sgro Clinard *et al.*, 2002).
Isoniazid (INH) and rifampicin (RIF), the most important first line antitubercular drugs (ATD) have been used for the treatment of TB. These drugs are also used in combination with other medicines to treat co-infections (Yossepowitch et al., 2007). However, a variety of adverse reactions have been reported. One of the well-known toxic effects is hepatotoxicity (Fernandez et al., 2004). Antitubercular drugs (ATD) are the most common cause of drug-induced acute liver failure in India (Agal et al., 2005). Most of the potent ATD, particularly INH-RIF and pyrazinamide are hepatotoxic (Yew et al., 2006). The frequency of hepatotoxicity is increased when these drugs are used in combination (Tahagolu et al., 2001). RIF is a potent inducer of several cytochrome P450 isoenzymes (e.g. CYP3A4) (Lee, 1995). RIF increases INH toxicity, most probably by increasing the formation of its toxic metabolite hydrazine (HYD) (Sarma et al., 1986).

The drug directly impairs the structural and functional integrity of the liver by the metabolism of the drug produces a metabolite, usually an oxidizing or alkylating species, which alters hepatocellular structure and function. In this case, injury occurs when cellular protective mechanisms have been overwhelmed, a drug metabolite binds to hepatic proteins to produce new antigenic determinants that become the target of a specific immune response and the drug initiates a systemic hypersensitivity response (drug allergy), as part of which the liver is damaged.

Hepatic cellular dysfunction and death also have the ability to initiate immunological reactions, including both innate and adaptive immune responses. Hepatocyte stress and/or damage could result in the release of signals that stimulate activation of other cells, particularly those of the innate immune system, including Kupffer cells (KC), natural killer (NK) cells, and natural killer T (NKT) cells. These
cells contribute to the progression of liver injury by producing proinflammatory mediators and secreting chemokines to further recruit inflammatory cells to the liver (Blazka et al., 1996).

DILI (Drug Induced Liver Injury) can affect both parenchymal and nonparenchymal cells of the liver, leading to a wide variety of pathological conditions, including acute and chronic hepatocellular hepatitis, fibrosis/cirrhosis, cholestasis, steatosis, as well as sinusoidal and hepatic artery/vein damage (Larrey, 2000). The predominant forms of DILI include acute hepatitis, cholestasis, and a mixed pattern (Gunawan and Kaplowitz, 2004). Acute hepatitis is defined as a marked increase in aminotransferases coinciding with hepatocellular necrosis. Cholestasis is characterized by jaundice with a concurrent elevation in alkaline phosphatase, conjugated bilirubin, and \( \gamma \)-glutamyl transpeptidase. Mixed-pattern of DILI includes clinical manifestations of both hepatocellular and cholestatic injury.

ISONIAZID AND RIFAMPICIN (INH-RIF)

Isoniazid (INH) and Rifampicin (RIF), (Figure 2 and 3) two front-line drugs used in antituberculosis therapy, have been known to be potentially hepatotoxic and may lead to drug-induced liver injury (Hwang et al., 1997). A meta analysis of studies involving the use of a multiplicity of antituberculosis drug regimens predominantly in adults has shown an incidence of toxic hepatitis of 1.6% in patients with isoniazid alone, 1.1% in patients with RPF alone and 2.55% in patients with isoniazid plus rifampicin (Steele et al., 1991).

Isoniazid is thought to be initiated by cytochrome P450 mediated metabolism of isoniazid to acetylhydrazine and hydrazine that is hepatotoxic (Yue et al., 2004; Bhaduria et al., 2007; Preziosi, 2007). Rifampicin, which is generally co-administered
Figure 2. Structure of Isoniazid

Figure 3. Structure of Rifampicin
with isoniazid in treatment of tuberculosis, is toxic to hepatocytes (Christiane and Peter, 2006). In addition, as a powerful inducer of drug metabolizing enzymes in man and rats, rifampicin aggravates isoniazid-induced hepatotoxicity by enhancing the production of toxic metabolites (Tasdug et al., 2007).

It is generally accepted that rifamycins can induce many drug metabolizing enzymes including cytochrome P450 (CYP) 3A4 (Jamis-Dow, et al., 1997). Thus, drug–drug interactions sometimes occur with the combination of rifampicin and drugs metabolized by the same enzymes. For example, patients taking rifampicin had low area under the curve (AUC) ratio of fluconazole (Lazar, et al., 1990), which are both metabolized by CYP3A4. In addition, rifampicin has been reported to cause toxic injury to hepatocytes (Yew et al., 2006). However, the involvement of rifampicin in hepatic injury remains unclear because rifampicin are frequently used in combination with other antitubercular drugs such as isoniazid and pyrazinamide (Sarma et al., 1986). Rifampicin is also used for the treatment of leprosy, some types of osteomyelitis and encocarditis.

**Silymarin**

Silymarin, a known standardized extract obtained from seeds of Silybum marianum (Family: Compositae) is widely used in treatment of liver diseases of varying origin (El-Samaligy et al., 2006). Silymarin is a purified extract from milk thistle (Silybum marianum) composed of a mixture of four isomeric flavonolignans: silibinin (its main, active component), isosilibinin, silydianin and silychristin. Silymarin has been used medicinally to treat liver disorder, including acute and chronic viral hepatitis, toxin/drug-induced hepatitis, and cirrhosis and alcoholic liver disease. Silymarin also provides hepatoprotection against poisoning by ethanol,
thiocetamide, halothane, acetaminophen, and carbon tetrachloride (Fraschini et al., 2002) hence silymarin used as a reference drug in this study.

1.6. FREE RADICALS

Free radicals (Scheme 2) are simply defined as any species capable of independent existence that contain one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. Examples of free radicals are superoxide ($O_2^-$) and hydroxyl (OH$^+$), both oxygen centered radicals, thiyl (RS$^+$ a sulphur-centered radical), and nitric oxide. The dot designates the presence of unpaired electrons (Halliwell and Gutteridge, 1989).

Generation of free radicals

The hydrogen atom has one proton and a single electron, thus qualifying as a radical. Hence removal of a hydrogen atom from a biological molecule leaves behind an unpaired electron on the atom, producing free radicals. Highly reactive radicals such as OH$^+$ frequently attack biological molecules by abstracting hydrogen. This is one of the mechanisms of starting of lipid peroxidation.

$$L - H + \text{OH}^+ \rightarrow \text{H}_2\text{O} + L^+$$

Free radicals and other reactive oxygen species are continuously produced in vivo and OH$^+$ radical is produced in living organisms by at least two mechanisms.

i) Reactions of transition metal ions with $\text{H}_2\text{O}_2$

$$\text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \text{O}_2^-$$

Superoxide anion
Scheme 2. Free radicals may be initiated within cells in several ways

A. FREE RADICAL GENERATION

B. CELL INJURY BY FREE RADICALS

C. NEUTRALIZATION OF FREE RADICALS – NO CELL INJURY
Fe^{2+} + O_2^{-} + 2H^+ \rightarrow Fe^{3+} + H_2O_2

Hydrogen peroxide

Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + \cdot OH

Hydroxyl radical (Fenton Reaction)

ii) Homolytic fission of water due to background exposure to ionizing radiation.

H_2O \xrightarrow{\text{\gamma-rays}} H^* + OH^*

Some of the O_2^{-} production that occur in vivo appear to be a chemical accident due to auto-oxidation reactions and the 'leakage' of electrons from electron transport chains to oxygen (Fridovich, 1989).

In addition, some superoxides (O_2^{-}) are made deliberately. For instance, activated phagocytes generate large amounts of superoxide as part of the mechanism by which foreign organisms are killed. During chronic inflammation this normal protective mechanism may become damaged. NADPH-oxidase in leukocytes generates ROS.

NADPH + H + O_2 \rightarrow NADP + O_2^{-} + 2H

Increased generation of O_2^{-} and H_2O_2 within the cell often leads to DNA damage, but neither of these species reacts directly with DNA or with membrane lipids. These two reactive species generate more dangerous radicals, such as highly reactive OH radicals (or) the protonated form of O_2^{-}, the radical HO_2^{-} (Halliwell and Aruoma, 1991).
Haber-Weiss reaction (superoxide driven Fenton reaction)

\[
\begin{align*}
\text{O}_2^* + \text{H}_2\text{O}_2 & \rightarrow \text{O}_2 + \text{OH} + \cdot\text{OH} \\
\text{Singlet oxygen}
\end{align*}
\]

The \( \text{O}_2^* \) rapidly dismutases to form \( \text{H}_2\text{O}_2 \)

\[
\begin{align*}
2\text{O}_2^* + 2\text{H} & \overset{\text{SOD}}{\rightarrow} \text{H}_2\text{O}_2 + \text{O}_2
\end{align*}
\]

1.7. OXIDATIVE STRESS

A free radical is any chemical species that contains one or more unpaired electrons. Unpaired electrons act as electron acceptors from other molecules, leading to their oxidation. The most common cellular free radicals are hydroxyl radical, superoxide radical, and nitric oxide (NO). Free radicals and related molecules are classified as reactive oxygen species (ROS) for their ability to lead to oxidative changes within the cell. A wide variety of ROS are produced in the course of the normal metabolism in biological systems and they have several important physiological functions, but their accumulation beyond the needs of the cell can potentially damage lipids, proteins, and nucleic acids. The cells possess an intricate network of defence mechanisms to neutralize excessive ROS accumulation, including antioxidant compounds (e.g., glutathione (GSH), arginine, citrulline, taurine, creatine, selenium, zinc, vitamin E, vitamin C, vitamin A) and antioxidant enzymes (e.g., superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidases); therefore, under physiological conditions, cells are able to cope with the flux of ROS. Oxidative stress describes a condition in which cellular antioxidant defences are insufficient to keep the levels of ROS below a toxic threshold (Schulz et al., 2000).
Oxidative cell injury

Many hepatotoxic agents exert their toxicity only after their biotransformation to reactive intermediates as a consequence of oxidative metabolism catalysed by microsomal cytochrome P450 (Pake and Ioannidis, 1984). The elevated production of reactive oxygen intermediates may contribute to the hepatotoxic action of acetaminophen. Microsomes from RIF-INH treated rats have been shown to generate reactive oxygen species such as

1. Superoxide anions (O$_2^{-}$)
2. Hydroxyl radicals (OH$^*$)
3. Hydrogen peroxide and
4. Lipid hydroperoxides (Barry and Gutteridge, 1989).

The radicals have been implicated in the pathogenesis of tissue damage particularly in liver.

The steps in oxidative stress

1. Oxidation of thiols
2. Peroxidation of lipids
3. Damage of biomolecules

Oxidation of Thiol Status

Thiol groups are known to be important for normal protein functions and for cell viability during cytotoxic events (Reed, 1996). Reduced GSH maintains cell membrane sulphhydril groups and other structural proteins in stable form (Gaetani et al., 1989). Both free radical generation and lipid peroxidation are increased by Acetaminophen intoxication (Itokazu et al., 1999). Since the reduction of lipid
peroxides by GSH utilize NADPH for the regeneration of GSH from GSSH, the rate of NADPH production can be limiting during oxidative stress (Reed, 1986). Therefore, GSSH may be transported from the liver when reduction is limited by low levels of NADPH (Akerboom et al., 1982). Decreased availability of NADPH and GSH can impair GSH-dependent detoxification pathway for hydrogen peroxide (Jones et al., 1981) and free radicals (Burk, 1983) can decrease protection of thiols in proteins. Thus the process involving NADPH, GSH and thiols in proteins, appear to be critically involved in cellular homeostasis during drug-induced toxicity (Reed, 1996).

The Lipid Peroxidation Process

Oxidative stress that occurs in the cells, because an imbalance between the prooxidant/antioxidant systems, causes injury to biomolecules such as nucleic acids, proteins, structural carbohydrates, and lipids. Among these targets, the peroxidation of lipids is basically damaging because the formation of lipid peroxidation products leads to spread of free radical reactions. The general process of lipid peroxidation consists of three stages: initiation, propagation, and termination (Catala, 2006). The initiation phase of lipid peroxidation includes hydrogen atom abstraction. Several species can abstract the first hydrogen atom and include the radicals: hydroxyl (·OH), alkoxyl (RO'), peroxyl (ROO'), and possibly HO2· but not H2O2 or O2· (Gutteridge, 1988).

The membrane lipids, mainly phospholipids, containing polyunsaturated fatty acids are predominantly susceptible to peroxidation because abstraction from amethylene (–CH2–) group of a hydrogen atom, which contains only one electron, leaves at the back an unpaired electron on the carbon, –CH–. The presence of a
double bond in the fatty acid weakens the C–H bonds on the carbon atom nearby to the double bond and thus facilitates H' subtraction. The initial reaction of 'OH with polyunsaturated fatty acids produces a lipid radical (L'), which in turn reacts with molecular oxygen to form a lipid peroxy radical (LOO'). The LOO' can abstract hydrogen from a adjacent fatty acid to produce a lipid hydroperoxide (LOOH) and a second lipid radical (Catala, 2006). The LOOH formed can suffer reductive cleavage by reduced metals, such as Fe^{2+}, producing lipid alkoxyl radical (LO'). Both alkoxyl and peroxy radicals stimulate the chain reaction of lipid peroxidation by abstracting additional hydrogen atoms (Buettner, 1993). Peroxidation of lipids can disturb the assembly of the membrane, causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes (Nigam and Schewe, 2000). Injure to mitochondria induced by lipid peroxidation can direct to further ROS generation. In addition, LOOH can break down, frequently in the presence of reduced metals or ascorbate, to reactive aldehyde products, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal (4-HHE) and acrolein (Lee et al., 2001).

These unstable intermediates then under peroxidative degradation with loss of membrane permeability, changes in membrane fluidity, altered enzyme function, cellular death and necrosis (Ferber, 1984, Rieter, 1995).

1.8. DAMAGE TO BIOMOLECULES

Protein Damage

Proteins are an important target for oxidative challenge. ROS modify amino acid side chains of proteins such as arginine, lysine, threonine and proline residues to form protein carboxyls (Chevion et al., 2000). Oxidation cystine residues may lead to
the reversible formation of mixed disulphides between protein thiol groups (-SH) and low molecular weight thiols, in particular GSH (S-glutathiolation) (Dalle-Donne et al., 2005). A random attack of reactive oxygen species on proteins is unlikely to be very damaging. In recent years, apart from the well documented inhibition of protein synthesis, it has been suggested that reactive oxygen species produced by activated macrophages might be the primary cause in acetaminophen-induced liver damage (Hu and Chen, 1992).

**DNA Damage**

DNA damage is one of the most sensitive biological markers for evaluating the oxidative stress (Anderson, 1996). The importance of reactive oxygen species (ROS) in inducing genetic toxicity was widely accepted and subsequently was extensively studied in the past decade (Simic, 1994). Direct breakage of DNA strands (Scheme 3) occurs when ROS interacts with DNA. Superoxide radicals can directly or indirectly damage DNA whereas hydrogen peroxide mediates DNA damage by the production of hydroxyl radical via events such as the Fenton reaction (Imlay et al., 1988).

The cumulative and inevitable effect of free radical attack on mitochondrial DNA is an increased frequency of mutations, which is likely to result in the production of proteins with impaired function (Michikawa et al., 1999). An accumulation of errors or damage of primary genetic material has already been proposed as initiators during cellular senescence and death of cell (Bohr and Anson, 1995). Recent research has suggested an intriguing possibility that ethanol may exert its cell toxicity via DNA damage (Singh and Khan, 1995)
Scheme 3. Mechanism of oxidative damage to DNA

ROS

Increased intracellular Ca²⁺

Activation of endonucleases (DNase)

Addition of OH⁺ to C=C of base

Abstraction of H atom from sugar moiety

Chemical modification of bases

DNA strand break

Hydroxy methyl uracil
Thiamine fragments
Adenine ring open products
γ-oxoguanine & 5-hydroxyctosine
Altered cellular calcium homeostasis is an important event in hepatocellular degradation. Body cells are bathed by an extracellular fluid containing very high concentration of ionized calcium relative to its intracellular concentration (Ferber, 1984). This concentration is maintained by specific plasma membrane ATPase that transport calcium out of the cell or by calcium sequestration within mitochondria and endoplasmic reticulum.

Hepatotoxins can cause sustained elevation of cytosolic Ca\(^{2+}\). Increase in cytosolic Ca\(^{2+}\) can result from lack of energy as required for ATP-dependent calcium pumps, plasma membrane damage permitting excessive influx of Ca\(^{2+}\) from the extracellular fluid, oxidative stress leading to the oxidation of GSH and protein thiols followed by inhibition of microsomal and plasma membrane Ca\(^{2+}\) pumps and direct toxin-induced lesion of mitochondria or endoplasmic reticulum causing an intracellular redistribution of calcium (Moore, 1985, Tsai et al, 1997).

Increased Ca\(^{2+}\) in turn activates a number of enzymes, with potential deleterious cellular effects. The enzymes known to be activated by calcium include Phospholipase (thus promoting membrane damage), Proteases (which break down membrane and cytoskeletal proteins), ATPase (thereby causing ATP depletion) Endonucleases (which are associated with chromatin fragmentation) . Thus an increase in the intracellular calcium is considered to be responsible for cell death (Tsai et al, 1997).

1.9. ANTIOXIDANTS

Antioxidants are the compounds of exogenous or endogenous in nature which either prevent the generation of toxic oxidants or intercept any that are generated and inactivate them and thereby block the propagation of chain reaction produced by these
oxidants (Rangan and Bulkley, 1993). These can be classified as enzymatic antioxidants, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, non-enzymatic antioxidants like (nutrient antioxidants) beta-carotene, alpha-tocopherol, ascorbic acid, bioflavonoids and metabolic antioxidants like glutathione, ceruloplasmin, albumin, bilirubin, ferritin, transferrin, uric acid and lactoferrin (Pillai and Pillai, 2002).

**Preventive Antioxidants**

Preventive antioxidants reduce the rate of chain initiation. e.g. catalase and glutathione peroxidase.

**Chain Breaking Antioxidants**

Chain breaking antioxidants trap directly thereby shortening the chain-length. Examples: Dismutase, Vitamin E.

**ENZYMATIC ANTIOXIDANT**

**Superoxide Dismutase (SOD)**

SOD is one of the most important enzymes in the antioxidant defense system of the bodies. The major function of SOD is to catalyse the conversion of superoxide anion radicals to $\text{H}_2\text{O}_2$ and hence reduces the toxic effects due to this radical or other free radicals derived from secondary reactions (Sen and Hanninen, 1994).

$$2\text{O}_2^* + 2\text{H} \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2$$

There are 3 SOD isoenzymes in the mammalian body (Alschier et al, 2002), Cu-Zn SOD, present in the cytosol, Mn-SOD, present in mitochondrial matrix and extra cellular SOD.
Catalase (CAT)

Catalase, a heme protein enzyme, catalyzes dismutation of H$_2$O$_2$ into H$_2$O and oxygen (Nogueira et al., 2005).

\[ 2\text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2\text{H}_2\text{O} + \text{O}_2 \]

The enzyme plays a central role in controlling the hydrogen peroxide concentration in human cells and more than 98% of blood catalase is localized in erythrocytes. These cells with their high catalase content provide a general protection against the toxic concentration of this small H$_2$O$_2$ molecules (Chance et al, 1952).

Glutathione Peroxidase (GPx)

Glutathione peroxidase is a selenium containing enzyme, present in the cytosol. It catalyzes peroxide reduction utilizing GSH as the substrate and converting it to GSSG (Freeman and Crapo, 1982).

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

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

Lipid alcohol hydroperoxide

Non-Enzymatic Antioxidants

Reduced Glutathione (GSH)

GSH is one of the most important endogenous antioxidants; it plays the role of a sulphydryl group provider for direct scavenging reaction. GSH acts both as a substrate in the scavenging reaction catalysed by GPx and as a scavenger of vitamin C and E radicals (Comporti, 1987).
Vitamin C

Vitamin C is considered as the most important water soluble antioxidant in extracellular fluids. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. It also has ability to regenerate \( \alpha \)-tocopherol by reducing \( \alpha \)-tocopheryl radicals present on the surface of membranes (Frie et al., 1989).

\[
\text{Ascorbic acid} + \text{Vit-EO}^* \rightarrow \text{Vit-E-OH} + \text{Dehydroascorbic acid}
\]

\( \alpha \)-tocopherol radical

Vitamin E

Vitamin E, a major lipid-soluble antioxidant is the most effective chain breaking antioxidant within the cell membrane. Vitamin E quenches the singlet oxygen and also reacts with lipid peroxide radical to form vitamin E radical but it does not abstract \( H^* \) from the membrane lipids. Thus it act as a chain termination and protects cellular structure against damage (Niki, 1995).

\[
\text{Vit E-OH} + \text{ROO}^* \rightarrow \text{ROOH} + \text{Vit E-O}^*
\]

\begin{align*}
\text{Vitamin E} & \quad \text{lipid hydroperoxide}
\end{align*}