CHAPTER 1

Introduction and Review of Literature
World Health Organization in its 1990 report stated that cancer occupied the second position in the list of killer diseases in the industrially advanced countries, while in the developing world it ranked as the fifth (WHO, 1990). Cancer is a disease of misguided cells, which have high potential of excessive proliferation without apparent relation to the physiological demand of the organ involved (Prescott et al., 1982).

Liver is the major tissue in which ingested chemicals are largely metabolized. Hence, disease of the liver is one of the common medical problems. Liver injury poises a serious problem in the metabolism and disposition of a large number of foreign chemicals to which vertebrates are continuously exposed. Liver damage commonly results by viral and protozoan infections, toxicity due to drugs, food additives and fungal toxins. Chronic hepatitis B virus infection and carcinogenic insult from aflatoxin B₁ and nitrosamines lead to liver cancer. Plants and plant products have been shown to play an important role in the management of various liver disorders (Subramaniam et al., 1998).

Hepatocarcinoma is a major disabling disease that effects a major part of the world population, and there are only a very few drugs that can reduce the onset and course of the disease. Hepatocellular carcinoma accounts for 90% of all primary hepatic malignancies (Okuda., 1992) and causes over 5% of the deaths from cancer throughout the world (WHO., 1997). The geographic
differences in the incidence of HCC suggest that environmental factors frequently contribute to its development (Thung and Gerber., 1997). Its epidemiological frequency and pathogenesis are due to chronic viral infection with hepatitis B and hepatitis C, consumption of aflatoxin contaminated foods, alcohol abuse (Di Bisceghe et al., 1988), industrial chemicals, air and water pollutants (Peers and Linsell., 1973). N-nitrosodiethyl amine is a potent carcinogenic dialkyl nitrosamine present in tobacco smoke, water, cheddar cheese, cured and fried meals and in a number of alcoholic beverages and has been considered to be a potent hepatocarcinogen producing reproducible hepatocellular carcinoma after repeated administration in experimental animals.

Pharmacological, endocrinal and nutritional interventions to inhibit or delay the process of cancer promotion can have an effective role in cancer prevention. A basic concept in cancer prevention is that there is a relation between dietary habits and cancer risk (Rogers and Longnecker., 1988). During the later part of the 20th century herbalism has become mainstream worldwide. This is due in part to the recognition of the value of traditional and indigenous pharmacopoeias, the incorporation of some derived from these sources into pharmaceuticals (DeSmet., 1997, Winslow and Kroll., 1998), the need to make health care affordable for all, and the perception that natural remedies are somehow safer and more efficacious than remedies that are pharmaceutically derived (Bateman
et al., 1998; Murphy., 1999). For a variety of reasons more individuals are nowadays preferring to personal control over their health, not only in the prevention of diseases but also to treat them. This is particularly true for a wide variety of chronic and incurable diseases such as cancer, diabetes, arthritis or acute illnesses that are readily treated at home (common cold etc.) (Kincheloe., 1997). In fact thousands of plants have been studied for their pharmacological properties (Dahanukar et al., 2000; Rates., 2001; Rajesh and Latha., 2001). A detailed investigation and documentation of plants used in local health traditions and ethno pharmacological evaluation to verify their efficacy and safety can lead to the development of invaluable herbal drugs or isolation of compounds of great therapeutical value including effective cure for dreadful diseases like cancer and AIDS.

A variety of bioactive compounds and their derivatives have been shown to inhibit carcinogenesis in a number of experimental systems involving initiation, promotion and progression (Ho et al., 1992, Huang et al., 1992). Plants contain abundant quantities of substances that have consistently been shown to be associated with a lower risk of cancers at almost every site (Steinmetz and Potter, 1991). Efforts, therefore, are being made to identify naturally occurring anti-carcinogens, which would prevent, slow and/or reverse the cancer induction and its subsequent development (Chuang et al., 2000).

Since ancient times, plants of the genus *Phyllanthus* (Family- Euphorbiaceae) have commonly been used in treatments for liver
diseases (Thyagarajan and Jayaram., 1992). Jaundice and other liver abnormalities are being treated regularly with *Phyllanthus* administration in India, China, Burma, Pakistan, Philippines, Guam, West Indies, South America, East and West Africa and elsewhere in tropic and sub tropic areas (Blumberg et al., 1989). *P. amarus* is also used in Ayurvedic medicine to combat many stomach disorders and diseases of genito-urinary system, liver and kidney. In clinical trials it has been effective against infective hepatitis (Bratati et al., 1990).

Without accompanying biological data, the discovery of new medicinal plant constituents is nothing more than pure phytochemistry. So the search for new pharmacological activities is important in drug discovery (Suffness and Douros., 1982). This thesis analyzes the pharmacological properties of *Phyllanthus amarus* in a most scientific manner and possible mechanism of action has been explained.
REVIEW OF LITERATURE

Oxidative Stress

Chemical compounds and reactions capable of generating potential toxic oxygen species/ free radicals are referred to as pro-oxidants. On the other hand, compounds and reactions disposing off these species, scavenging them, suppressing their formation or opposing their actions are called anti-oxidants. In a normal cell, there is an appropriate pro-oxidant - anti-oxidant balance. However, this balance can be shifted towards the pro-oxidant, when production of oxygen species is increased or when levels of anti-oxidants are diminished. This state is called oxidative stress and can result in serious cell damage if the stress is massive or prolonged. Oxidative stress is implicated in the etiopathogenesis of a variety of human diseases (Frei., 1994, Peterhans., 1997, Domenico et al., 1998).

During the last two decades, there has been a growing interest in studies that concern with the prevention of uncontrolled oxidative processes leading to various diseases in the living system. Several studies have shown the role of oxidative stress in the causation and progression of different diseases including atherosclerosis, carcinogenesis, neurodegenerative diseases, chronic inflammatory diseases, radiation damages, ageing and various other pathobiological effects (Treitinger et al., 2000, Beck., 2000).
Free radicals are species with very short half-life, high reactivity and damaging activity towards macromolecules like proteins, lipids and DNA. These species may be either oxygen derived (Reactive Oxygen Species, ROS) or nitrogen derived (Reactive Nitrogen Species, RNS). The oxygen-derived species include $O_2^-$ (superoxide), $\cdot$HO (Hydroxyl), $\cdot$HO$_2$ (Hydroperoxyl), $\cdot$ROO (Peroxyl), $\cdot$RO (alkoxyl) as free radicals and H$_2$O$_2$ (Hydrogen peroxide), HOCl (Hypochlorous acid), O$_3$ (Ozone), and $^1$O$_2$ (singlet oxygen) as non-radicals. Nitrogen derived oxidant species are mainly NO (Nitric oxide), ONOO (Peroxynitrite), NO$_2$ (Nitrogen dioxide) and N$_2$O$_3$ (Dinitrogen trioxide).

**Oxygen free radicals**

Free radical can be defined as chemical species possessing an unpaired electron, which is formed by homolytic cleavage of a covalent bond of a molecule by the loss of a single electron from a normal molecule or by the addition of a single electron to a normal molecule. Most of the molecular oxygen consumed by aerobic cells during metabolism is reduced to water by using cytochrome oxidase in mitochondria. However, when oxygen is partially reduced it becomes "activated" and reacts readily with a variety of bio molecules. This partial reduction occurs in one electron step, by the addition of one, two or four electron to O$_2$, which leads to successive formation of reactive oxygen metabolites (ROMS). These are five possible species: superoxides ($O_2^\cdot$), hydroperoxyl radical (HO$_2^\cdot$), peroxide ion (HO$_2^-$),
hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($\cdot OH$) (Frei., 1994; Peterhans., 1997)

$$e^- \rightarrow O_2^-$$

$$2e^- \rightarrow H_2O_2$$

$$H_2O_2 \rightarrow OH + OH$$

The $O_2^-$ and $H_2O_2$ so formed, in presence of metal catalysts such as $Fe^{2+}$ or $Cu^+$ may lead to the formation of the most reactive, $\cdot OH$. $O_2^-$ is reduced to $H_2O_2$ by the catalytic activity of superoxide dismutase (SOD).

**Oxidants**

**Superoxide anion ($O_2^-$)**

Superoxide anion is the first reduction product of $O_2$. $O_2^-$ can be produced either by the univalent reduction of $O^2$ or by the univalent oxidation of $H_2O_2$. It is a base with the equilibrium with its conjugate acid, the hydroperoxyl radical $H_2O_2$, whose $pKa$ is 4.8. In aqueous solution, at neutral or slightly acid pH, $O_2^-$ is a relatively non-reactive species and dismutates to $H_2O_2$. This reaction either occurs spontaneously or is catalyzed by intracellular enzyme, SOD. It has been proposed that $O_2^-$, owing to its unreactivity, can diffuse through a long way from its site. At low pH in the cell, it becomes protonated ($HO_2^*$) and hence reactive. The lifetime of $O_2^-$ in the water cellular environment is approximately $10^{-6}$s (Pryor., 1986).

The most important source of $O_2^-$ is oxidative enzymes, among which Xanthine oxidase (XO) and NADPH/NADH oxidase are the most
effective sources (Cross and Jones., 1991). These enzymes possess flavin or transition metal such as Zn, Cu, Fe, which serve as electron donors (Mohazzab and Wolin., 1994). Several oxidative enzymes such as aldehyde oxidase and dihydrotic dehydrogenase have been shown to produce substantial amounts of $O_2^*$ (Mc Cord and Frodovich., 1969). $O_2^*$ itself directly affects the activity of catalase and peroxidase. Experimental studies showed that $O_2^*$ directly effects some intracellular enzymes, changing their activities such as epinephrine and creatine phosphokinase (McCord and Russell., 1988), lactate dehydrogenase bound NADH (Bielski and Chan, 1973), aconitase and 6- phosphogluconate dehydratase (Gardner and Fridovich., 1992). Researches have demonstrated an increased production of $O_2^*$ during the proliferation of endothelial cells (Arnal et al., 1996) and involvement of this species in proliferation of B-lymphocytes (Morikawa and Moridawa., 1996). It is capable of initiating the peroxidation of unsaturated lipids (Pederson and Aust., 1973) and can cause the oxidation of thiols. Photochemical or enzymatic generation of $O_2^*$ resulted in an increase in chromosome breakage, rearrangement, and sister chromatid exchanges (SCEs) (Emerit et al., 1982). Thus $O_2^*$ may be one of the possible factors for increased risk of carcinogenesis.

**Hydrogen peroxide ($H_2O_2$)**

Hydrogen peroxide is the most stable ROS. This is to say that it is the least reactive and the most readily detected. $H_2O_2$ may be
generated directly by divalent reduction of O2 or indirectly by univalent reduction of O2·-. H2O2 is the primary product of the reduction of O2 by numerous oxidases, such as XO, uricase, D-aminoacid oxidase and α-hydroxy acid oxidase localized in peroxisome (Oshino et al., 1973). In any system producing O2·-, substantial amount of H2O2 is formed. The H2O2 is decomposed, although not readily, to H2O and O2. H2O2 like most peroxides is very sensitive to decomposition by the species that it reacts with. The reaction is catalyzed by the redox-active metal complexes, of which catalase and peroxidase is the most effective exponent. Metal ions have a strong effect on the chemistry of O2 and its reduction product. Experiments with antioxidant enzymes show that H2O2 rather than O2·- is the more essential species to induce cell injury (Junod., 1987). Other researches also indicated H2O2 as the most effective species to induce cellular injury (Rao et al., 1996). It has been demonstrated that H2O2 stimulates proliferation of smooth muscle cells (Nishio and Watanabe., 1997). The well-known Fenton reaction is initiated when Fe2+ comes in contact with H2O2. Ions of Cu and Co and Ni can also participate in a similar reaction.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-
\]

H2O2 also react with O2·- to initiate Haber-Weiss reaction producing OH in presence of Fe2+.2.

\[
\text{O}_2^{\cdot-} + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \cdot\text{OH} + \text{OH}^-
\]
H$_2$O$_2$ has been found to be effective in activating DNA binding with NF-kB \textit{in vivo} but not \textit{in vitro} (Sen and Packer, 1996). Certain estrogen metabolites such as catecholestrogens are involved in carcinogenesis, where H$_2$O$_2$ plays an important role. The most active catecholestrogens are the 4-hydroxy derivatives that produce about 2.5 times more DNA-double stranded breaks than the 2-hydroxyderivatives, meanwhile estradiol and 16α-hydroxyestrone are inactive. In addition, results show that 4-hydroxyestradiol (4-OHE$_2$) at physiological concentration is capable of exhibiting DNA cleaving activity. The formation of these catecholestrogens-induced DNA strands have been associated with the utilization of O$_2$ and the generation of H$_2$O$_2$ (Thibodeau and Paquette, 1999). H$_2$O$_2$ exposed to cultured MCF-7 cells have been shown to inhibit binding of estrogen receptor to DNA (Lu et al., 1998).

H$_2$O$_2$ has been associated with induction of cancer in animals and has been found to induce molecular damage that leads to the formation of transformed cells \textit{in vitro} (Shamberger, 1972). It has been shown to be mutagenic and carcinogenic (Pryor, 1986). Studies have also demonstrated that H$_2$O$_2$ stimulated the proliferation of smooth muscle cells (Nishio and Watanabe, 1997). H$_2$O$_2$ is believed to be involved in the initiation and promotion of carcinogenesis (Birnboim, 1986). Many reports have suggested that H$_2$O$_2$ could induce DNA breaks in an intact cell and also purified DNA (Imlay et al., 1988). H$_2$O$_2$ has been known to cause DNA damage in the form of
single stranded breaks and double stranded breaks (Thibodeau and Paquette., 1999), chromosomal aberrations (Sofni and Ishidate., 1984) and single chromatid exchanges (Mac Rac and Stich., 1979). The induction of chromosomal aberrations by H\textsubscript{2}O\textsubscript{2} was also reported in a retrospective study (Tsuda., 1981). Significantly higher H\textsubscript{2}O\textsubscript{2} concentration and SCEs (Ray et al., 2001) have been reported in breast cancer patients. An experimental study suggested that H\textsubscript{2}O\textsubscript{2} could induce high frequency of SCEs when applied at low concentrations (Schoneich., 1967). Studies by Ray et al suggested that there might be an optimum concentration of H\textsubscript{2}O\textsubscript{2} that could induce DNA damage and of SCEs in breast cancer.

**Hydroxyl radical (\textbullet OH)**

Hydroxyl radical is highly reactive. It can react with practically any molecule present in cells. For this reason it is short lived. This insufficient stability does not allow it to diffuse through the cells. Therefore, it reacts with an organic substrate or near the sites of its formation. The life span of \textbullet OH at 37\textdegree C is 10\textsuperscript{-9}s. It does not survive beyond a few collisions, after its formation. The reactions of \textbullet OH are thus site-specific. This \textbullet OH is produced following the reaction of O\textsubscript{2}\textsuperscript{*} and H\textsubscript{2}O\textsubscript{2} in the presence of metallic ions such as Fe\textsuperscript{2+} or Cu\textsuperscript{+}. Lipids are very susceptible to \textbullet OH attack and initiate LPO. As a result of interaction of \textbullet OH with DNA, formation of many types of oxidized nucleosides have been reported. 8-OHdG is one of the most commonly occurring products of these DNA modifications (Kasai., 1997). \textbullet OH is
the most potent among ROS, reacting with a wide range of macromolecules at a high rate constant (Hutchinson., 1985). •OH is known to induce conformational changes in DNA including strand breaks, base modifications, damage to tumor suppressor genes and enhanced expression of protooncogenes, (Halliwel and Aruoma., 1991). •OH is responsible for DNA damage and high frequency of SCEs (Tsuda., 1981) and LPOs (Chessman and Slater., 1993).

Singlet oxygen (¹O₂)

Singlet oxygen can be generated in biological systems by two different routes namely, 'light reaction' due to photo excitation and 'dark reaction' due to chemiexcitation.

Many cellular constituents such as flavins, porphyrins, cytochromes, 4-thiouridine etc as well as biologically active drugs like tetracycline, chlorpromazine, merbromin, thiazides, poralens, etc have the ability to generate ¹O₂ under illumination. The dark reaction that generates ¹O₂ includes enzymatic reaction catalyzed by dioxygenases, lactoperoxidases, myeloperoxidases, pyrrolase, and lipoxygenases. The formation of ¹O₂ by this mechanism is likely to be increased under the influence of certain xenobiotics capable of inducing oxidative stress. In human plasma, which is rich in antioxidants, the lifetime of ¹O₂ is calculated to be one microsecond. ¹O₂ causes damage to biomolecules like lipids, proteins and DNA.
Lipid peroxidation

Lipid peroxidation is a consecutive process of oxidative degradation of polyunsaturated fatty acids in the phospholipids present in the biological membranes and produces a great variety of secondary products including several aldehydes such as malonaldehyde (MDA). Lipid peroxidation in vivo is increased during lung damage by oxygen toxicity and liver damage by various drugs, herbicides and pesticides, particularly those, which deplete intracellular GSH levels. The metals Fe, Cu or Pb also enhance lipid peroxidation in vivo.

Malondialdehyde (MDA)

Malondialdehyde is the major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acid (PUFA) (Vaca et al., 1988). MDA, a secondary product of LPO, is used as an indicator of tissue damage by a series of chain reactions (Okhawa et al., 1979). MDA is also a byproduct of prostaglandin biosynthesis (Hayaishi and Shumizu, 1982). It reacts with thiobarbituric acid and produce red coloured products. It is said to be a product of normal metabolism, and is present in a variety of fat containing food items (IARC., 1985). It has also been proposed that the effect of dietary fat on cancer occurs through the activation of procarcinogens to ultimate carcinogens by fat oxidation products such as lipid hydro peroxides (Vaca et al., 1988). Peroxides and hydro peroxides have also demonstrated tumour-promoting activity in vivo.
It can result in the formation of cyclic DNA adducts (Bartsch., 1996). MDA forms adducts with DNA adenine and cytosine, which contributes to the carcinogenesity and mutagenecity in mammalian cells (Marnett and Tuttle., 1980). MDA induced mutations include frame shift mutation and base pair substitution. Higher plasma MDA level has also been reported in solid tumour and human cell lines (Diplock et al., 1993) and in breast cancer patients (Ray et al., 2000).

**Reactive nitrogen species**

Nitric oxide is an inorganic free radical gas, containing odd number of electrons and forming a covalent link with other molecules by sharing a pair of electrons. It is synthesized by a family of isoenzymes called nitric oxide synthetase, located in various tissues, and plays an active role in free radical and tumour biology (Felley-Bosco., 1998). There are three isoenzymes of NOS. NO plays a vital role as a signaling molecule in vascular, nervous and immune systems (Moncada et al., 1991). It regulates numerous physiological processes, including neurotransmission, smooth muscle contractibility, platelet reactivity and cytotoxic activities of immune cells. Prolonged exposure of NO inhibits the activity of number of enzymes such as aconitase, complexes I and II, and cytochrome c oxidase (Clementi et al., 1998). On the other hand, excessive and unregulated NO synthesis has been implicated as causal or
contributing to path physiological conditions, including many lethal and debilitating diseases (Gross and Wolin., 1995).

NO and its derivatives produced in inflamed tissues contribute to carcinogenesis. It is believed that NO plays a dual role in cancer. It is a cytotoxic agent, which causes cell killing when generated at higher concentrations by cytokine activated macrophages and endothelial cells, and at low concentrations, it promotes tumor growth and metastasis (Jenkins et al., 1995).

NO plays an active role in free radical and tumor biology (Tamir and Tannenbaum., 1996). Moreover it may have a role in carcinogenesis by inducing DNA strand breaks (Yoshie and Oshimima., 1998). NO is known to be a potential mutagen (Arroyo et al., 1992). It can bind to nonheme iron of ribonucleotide reductase to inhibit DNA synthesis (Lepoivre et al., 1991). NO may have a role in carcinogenesis by impairing the tumor suppressor function of p53 (Wang and Liehr., 1995). It has been suggested that NO can stimulate $O_2^-$ /$H_2O_2$ /$^*OH$- and induce LPO (Rubbo et al., 1994).

**Sources of oxygen free radicals**

Free radicals are constantly produced in the body during the normal cellular metabolism and oxidative phosphorylation. About 1-4% of oxygen taken up in the body is converted as free radicals. There are two important pathways for the production of radicals in living systems: (i) enzymatically controlled one electron reduction of oxygen. For e.g., formation of superoxide anion radicals by xanthine oxidase,
aldehyde oxidase, dihydro orotate dehydrogenase and peroxidases. (ii) reactions initiated by xenobiotics. Xenobiotics can lead to the production of radicals by several distinctive mechanisms. (a) Substances can trigger the production of $H_2O_2$ and $O_2^-$ from phagocytic cells. Unreactive materials (polystyrene beads, asbestos) as well as reactive substances (a cigarette smoke) can cause this effect. (b) Xenobiotics can be metabolized by radical mediated paths; for e.g., $CCl_4$ (reduced by cytochrome P450 to $Cl^-$ and $CCl_3$). (c) A few toxins are themselves radicals; e.g. are NO, NO$_2$ and organic combustion products such as tobacco smoke and automobile exhaust. These minerals can react with biomolecules to produce radicals without the intervention of enzymes. (d) A small group of toxins, while not radicals themselves, can react to form radicals or radical precursor compounds by non-enzymatic paths. This group includes very reactive species such as ozone and singlet oxygen.

**Role of oxidative stress in human body**

When overall generation of ROS and RNS exceeds the total antioxidant activity in the body, the resulting condition is called oxidative stress. This oxidative stress may be mild or severe. Based on various reports (Ghosh and Myers.,1998., Lee et al., 1998), the causes and aftereffects of severe oxidative stresses are shown in flow chart.
Responses and signals during oxidative stress

Excess ROS/RNS & Low antioxidant defense

Damage to biomolecules (Lipid, DNA, Protein)

- Lipid peroxidation (Damage to membrane ion channel, ion transporters).
- DNA damage (Strand breakage Base Modification).
- Protein damage (Damage to receptor, enzyme, ion channel).

- Raised intracellular Ca$^{2+}$

Cellular damage with release of more radicals

Cell death and tissue damage

Carcinogenesis, atherosclerosis, ageing etc.

Cardiovascular diseases

Epidemiological studies have indicated that vitamin C and E exert protective effect against cardiovascular diseases. Low plasma level or low dietary intakes of vitamin C are associated with high blood pressure and unstable coronary syndrome. Taddei et al., 1998, concluded that in essential hypertension, vitamin C improves the endothelium dependent vasodilatation by increasing type III NOS (eNOS) expression and ensuring NO production. Similarly, Riemersma et al., 1991, showed that low plasma levels of vitamin E were
associated with risk of angina. Vitamin E supplementation reduced non-fatal heart attack, risk of coronary diseases and atherosclerosis by inhibiting oxidation of LDL by free radicals (Stephens et al., 1996; Evans et al., 1998).

**Cancer**

The incidence of most cancers rises with the fourth or fifth power of age in animals (including humans), e.g. about 35% of humans have cancer by age 85. At first sight this seems high, but the enormous number of mutations that occur over a lifetime caused by polymerase errors, spontaneous deamination and depurination of DNA, plus the actions of ROS, RNS and exogenous carcinogens makes one admire the high efficiency of protective mechanisms in ensuring that the majority of animals not developing cancer. (Loft and Poulsen., 1996)

As early as 1984 it was shown that exposure of mouse fibroblasts to ROS leads to malignant transformation. (Zimmerman and Cerutti., 1984) Incubation of plasmids bearing proto-oncogenes with ROS followed by their transfection into cells has been reported to lead to all transformation (Jackson., 1994). Similarly, incorporation of 8-OHdG into the first or second position of codon 12 in K-ras causes it to gain transforming ability. Several organic peroxides (e.g. benzyl peroxide) are tumour promoters in mouse skin; their conversion into free radicals is thought to be involved in their tumour-promoting effect. (Kensler and Taffe., 1986) Increased steady-state levels of
multiple oxidative DNA base damage products have been found in DNA from cancerous breast tissue compared with DNA from surrounding areas. Similar elevations are found in lung, colon, stomach, ovary and brain cancers (Malins., 1996) Whether these rises are due to increased oxidative DNA damage or decreased repair is as yet unclear.

**Diabetes**

Diabetic patients are often stated to be under an oxidative stress. Indeed, the link between diabetes and oxidative stress has been extensively discussed for years, but rigorous experiments to elucidate its importance are still awaited (Hunt., 1995). Plasma lipid peroxides appear higher than normal in diabetics.

There is disagreement as to whether plasma α-tocopherol levels in diabetic patients are sub-normal, but general agreement is that vitamin C levels are lower than normal in plasma (Hunt., 1995), despite the fact that elevated blood glucose has been reported to inhibit the uptake of ascorbate and dehydroascorbate into cells. (Washko and Levine., 1992). Erythrocyte GSH levels are also slightly sub-normal. The significance of oxidative stress in the disease pathology is uncertain, but it is frequently proposed to be related to the hyperglycemia. Other possible sources include elevated plasma lipids leading to increased lipid oxidation and decreased levels of the antioxidant defense systems. Maternal diabetes increases the
incidence of fetal abnormalities, and oxidative stress has been suggested to contribute.

**Inflammatory diseases**

It is well established that there is increased oxidative damage to DNA, lipids and proteins. In chronic inflammatory condition as in rheumatoid arthritis as phagocyte derived ROS have been implicated in inflammation related injury. Intracellular protection of cytoplasmic components against phagocyte derived oxidative injury is mediated predominantly by antioxidant enzymes like SOD, catalase and glutathione peroxidase. Synovial fluid from RA patients shows an accumulation of stable prostaglandin, TBA reactive materials and fluorescent products of lipid peroxidation. The development of mutations in p53 tumor suppressor gene and other key regulatory genes promotes inflammation into chronic disease in rheumatoid arthritis and other inflammatory disorders (Tak et al., 2000., Taysi et al., 2002; Kumar et al., 2002).

**Ageing**

Ageing in humans is associated with changes in physical characteristics and the decline of many physiological functions. Increased accumulation of free radicals heightens the vulnerability of older individuals to a variety of oxidative insults. These radicals are capable of causing apoptosis, necrosis and cell death (Niki., 2000; Orr and Sohal., 1994).
Liver diseases

The involvement of free radicals in the pathogenesis of liver injury has been investigated for many years in a well defined experimental system (Poli., 1993). Several studies were conducted to find out the role of oxidant stress and in alcohol induced cirrhosis (Farzach and Moore., 2001; Lieber., 2000), which is considered as the terminal irreversible stage of liver diseases. These studies have shown increased lipid peroxidation by \( \cdot \text{HO} \) radical and hydroperoxides in experimental acute and chronic alcoholic liver diseases. Also it has been suggested that ROS and lipid peroxidation may play role in pathogenesis of hepatic fibrosis with loss of normal liver architecture. In another study (Floreani et al., 2000) it is found that plasma levels of antioxidant vitamins are low in patients with chronic cholestatic liver diseases.

Hepatitis viruses are taxonomically quite diverse and include Picorna, Flavia, Hepadna and Calciviruses as well as delta agent associated with hepatitis B virus. Viral hepatitis is caused by both RNA (Hepatitis-B, C, D, E) viruses and DNA virus (HBV). In these infections, there is an associated enhanced production of ROS/RNS (Peterhans., 1997) via long term oxidant stress. In another study (Boya et al., 1999) oxidative stress was observed in peripheral blood mononuclear cells from chronic hepatitis C patients.
Alcohol and Oxidative Stress

Prolonged intake of excessive amounts of ethanol by humans causes severe damage to many tissues, especially the liver, which may become cirrhotic. Hepatic iron overload is common in patients with alcoholic liver diseases. Alcohol abuse is also a major risk factor for pancreatitis, and inflammatory disease causing intense pain and extensive destruction of pancreatic tissue, and for brain damage. It may cause fetal damage in pregnant women.

Both large doses of ethanol, and smaller doses given repeatedly, have been shown to increase lipid peroxidation in the livers of rats and baboons, as followed by accumulation of conjugated dienes or the production of ethane or F₂-isoprostanes. Large doses of ethanol have also been observed to decrease the levels of GSH in liver, brain and kidney cells of animals. A fall in GSH might lead to increased lipid peroxidation. Much ethanol toxicity (including mitochondrial damage) in liver may be due to acetaldehyde. This is mainly metabolized by aldehyde dehydrogenase in liver, but it is possible that some of it could be acted upon by the molybdenum containing enzyme aldehyde oxidase, which is known to produce O₂⁻. Xanthine oxidase can oxidize acetaldehyde, producing O₂⁻ and H₂O₂, and the xanthine oxidase/dehydrogenase ratio has been claimed to increase in the liver of ethanol-treated rodents. However, the affinity of xanthine oxidase for acetaldehyde is low, so that xanthine and hypoxanthine may out-compete acetaldehyde as a substrate in vitro.
Acetaldehyde undergoes reversible reactions with GSH and also bind with protein –SH and -NH₂ groups. Acetaldehyde could react with dopamine and serotonin in the brain to form neurotoxic products, such as tetrahydroisoquinolines and tetra hydro-β-carbolines (Han and Dryhurst., 1996). Other potentially toxic products generated from ethanol include fatty acid ethyl esters which have been suggested to be neurotoxic. Withdrawal of ethanol from neurons ‘adapted’ to its presence in culture can also, paradoxically, cause damage.

Heavy drinkers are often also cigarette smokers, compounding the pathology of two different toxic agents. Iron overload in the cirrhotic liver may represent an additional mechanism of oxidative injury, depending on the ability of the extra iron to catalyse free-radical damage. The inflammation that can result from exposure of the liver and stomach to large intakes of ethanol may also involve damage by ROS/RNS generated by activated phagocytes.

Alcohol is an important cause of hepatic cirrhosis, which is sometimes complicated by hepatocellular carcinoma, although alcohol may also have an independent effect on the risk of this cancer. The role of alcohol in other cancers remains uncertain. Rectal cancer in men has shown positive geographic correlations with beer consumption, but the findings from the analytical studies have been inconsistent. Recent interest has centered around the possible relationship of alcohol with breast cancer, with a series of prospective
studies showing an excess risk and dose response gradient (Schatzkin et al., 1987).

**Stomach cancer**

The stomach cancer rate in the United States and the number of deaths from this disease have got dramatically over the past 60 years. Stomach cancer is more common in some parts of the world—such as Korea, parts of Eastern Europe and Latin America—than in the United States. People in these areas are preserving the food by drying, smoking, salting or pickling. Exposure to certain dusts and fumes in the workplace has been linked to a higher than average risk for cancer. Also some scientists believe smoking may increase stomach cancer risk.

Environmental factors are more important than genetic factors in the development of gastric cancer. The ingestion of meat and salted fish, especially the processed or smoked varieties, correlates positively with the risk of gastric cancer. A high starch and animal fat intake may be linked to stomach cancer risk but vegetable fats appear too protective.

Nitrates are a common constituent of our diet and primarily found in vegetables, cured meat and drinking water. Nitrates are converted to nitrites which then can be nitrosated i.e. combined with amines and amides to form nitrosamines and nitrosamides (NOC). Humans are exposed to preformed NOC in addition to a wide variety of nitrogen containing compounds and nitrosating agents that can
react in gastric lumen to form NOC. Such compounds can also be synthesized endogenously by bacteria and macrophages within inflamed or infected organs. Nitrates and amines ingested as part of normal dietary intake react directly in the acid medium of the normal stomach to form NOC. Gastritis, once it becomes atrophic, is a risk factor for gastric cancer. The loss of normal acidity is followed by bacterial colonization and the reduction of intragastric nitrate to nitrite. Nitrosation of protein substrate then result in the formation of carcinogenic NOC.

**Antioxidants**

An antioxidant or free radical scavenger is defined as a substance, which at low concentrations, can prevent or delay the oxidation of an oxidisable substrate. Such substrates include proteins, carbohydrates, lipids, DNA, and other cell constituents. The major intracellular antioxidants are SOD, catalase, glutathione peroxidase, and the biological membrane containing antioxidants, alpha-tocopherol and ascorbic acid etc. The major extracellular antioxidants include uric acid, bilirubin, caeruloplasmin, albumin, tocopherol, ascorbic acid etc.

Enzymatic antioxidants

**Superoxide dismutase (SOD)**

SODs are a family of metalloenzymes that convert $O_2^+$ to $H_2O_2$ according to the following reaction.

$$2H^+ + O_2^+ + O_2^\rightarrow H_2O_2 + O_2$$

SOD
SOD is the most important enzyme because it is found virtually in all aerobic organisms. There are four families of SOD. Each type of SOD has its own peculiarities; however, all types of the enzymes have similar properties (Oberley and Oberley., 1984). Moreover SOD is considered to be a stress protein, which is synthesized in response to oxidative stress (Mc Cord., 1990). SOD has been detected in a large number of tissues and organisms, and is thought that it is present to protect the cell from damage caused by O$_2^*$ (Fridovich., 1972). The inactivation could be prevented by xanthine, urate and formate (Fridovich., 1995). SOD inhibits nuclear transcription factor AP-I and NF-κB in human breast cancer cell (Li et al., 1998). During oxidative stress, cell respond to ROMs with SOD (Allen., 1991). SOD can act as anti-carcinogens, and inhibitor at initiation and promotion/ transformation stage in carcinogenesis. Mutation caused by potassium superoxide in mammalian cells can be blocked by SOD (Cunningham and Lokesh., 1983). From the results of experiments with ROMs and antioxidant enzymes, Simon et al., 1981, concluded that elevation of intracellular SOD increased the cell damage allowing more H$_2$O$_2$ to generate.

Elevated SOD activity in various diseases including breast cancer had been reported (Abdel-Aziz and El- Naggar., 1997; Ray et al., 2000). The exact cause of this elevation in SOD activity in these studies is not clear. Production of ROMs has been found to be higher in various pathological conditions. To counter the deleterious action of
ROMs, antioxidants enzymes are also synthesized in response to the higher production of ROMs (Mc Cord., 1990).

**Glutathione peroxidase (GPx)**

Glutathione peroxidase enzyme is a well-known first line of defense against oxidative stress, which in turn requires glutathione as a cofactor. Among the many functions of glutathione, it is involved in the generation of the nucleotide precursors of DNA via the reduction of ribonucleotides to deoxyribonucleotides (Meister., 1991). GPx catalyses the oxidation of GSH to GSSG at the expense of H$_2$O$_2$. By its selenium (Se)-dependency, GPx can be divided into two forms; Se dependent GPx and Se- independent GPx. The former is a tetramer (MW 84000) with very activity toward both H$_2$O$_2$ and hydro peroxides. It is found in both cytosol (70%) and mitochondria (30%) of various tissues. Iodoacetate, cyanide and O$_2$•- are considered as inhibitors of this enzyme (Blum and Fridovich., 1985).

GPx catalyses the oxidation of GSH to GSSG. This oxidation reaction occurs at the expense of H$_2$O$_2$. Plasma GPX activity was found significantly elevated with respect to the control in the breast cancer patients regardless of clinical stages and menopausal status (Ray et al., 2000). Dillio et al., 1985, reported higher GPx activity in human breast tumor tissue than non-tumor tissue. Therefore higher GPx may be an indicator of malignancy. There are also many contrasting reports suggesting its less effectiveness with regard to antioxidant activity.
Catalase (CAT)

Catalase is an enzyme, which is, present in most cells, and catalyses the decomposition of hydrogen peroxide to water and oxygen. CAT is a heme- containing protein. The mechanism of action is:

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

CAT is found to act 10^4 times faster than peroxidase. It is localized mainly in mitochondria and sub cellular respiratory organelles (Pryor., 1986). CAT is present in peroxisomes (80%) and cytosol (20%). It has 240,000 molecular weight and consists of four protein subunits, each containing a heme Fe (III)- protoporphyrin group bound to active site. The encodes of human CAT is found on chromosome 11p13(Mc Alpine et al., 1988).

Catalase catalyses the decomposition of \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) and \( \text{O}_2 \). Most of the \textit{in vitro} studies suggested that this antioxidant functions as promotion/ transformation inhibitor in carcinogenesis. CAT is found to reduce SCE levels resulting treatments with \( \text{H}_2\text{O}_2 \) (Mac Rac and Stich., 1979). GPx and CAT were found to be important in the inactivation of many environmental mutagens (Nagao et al., 1986). There are reports that many tumors appear to have a decreasing effect in the expression of antioxidant enzymes. In breast cancer tissue CAT activity was found decreased, while the activities of SOD and GPx were elevated (Punnonen et al., 1994).
**Anti-oxidative vitamins**

Anti-oxidative vitamins have a number of biological activities such as immune stimulisation, inhibition of nitrosamine formation and an alteration of metabolic activation of carcinogens (Van and Van., 1997). They can prevent genetic changes by inhibiting DNA damage induced by the ROMs (Sun., 1990). Lupulescu., 1996, indicated that cancer cells synthesized an increased amount of DNA, and RNA as when compared to normal cells, which may be controlled by the administration of vitamins. Another case control study suggested inverse association of vitamins A, C and E and the risk of breast cancer (Bohlke et al., 1999). Several studies have also observed inverse association between β-carotene, tocopherol, vitamin C, and breast cancer risk (Ambrosone et al., 1995). The major protective function of vitamins against cancer is the scavenging of ROMs (Torn et al., 1995).

**Vitamin A**

Vitamin A is a fat-soluble vitamin, which is essential for growth, maintenance of visual function, reproduction and differentiation of epithelial tissue. The naturally occurring preformed vitamins include the compounds retinol and its esters, retinylaldehyde and retinoic acid. Vitamin A occurs mainly as the alcohol (retinal) in plasma and circulates as a 1:1:1 complex with two hepatically synthesized proteins, retinol binding protein (RBP) and transthyretin. The amount in the circulation remains constant as the body stores decline during
a period of deficiency, until the liver stores become too low to maintain this normal circulating level in the plasma (Bates., 1997).

Vitamin A is reported to play a crucial role in suppressing carcinogenesis by increasing immunity to tumors through several mechanisms. Vitamin A deficiency has been associated with a higher incidence of cancer and increased carcinogenesis (Tachibana et al., 1984). A number of epidemiological studies have shown that low dietary intake of vitamin A or carotenoid was correlated with the increased incidence of mortality from lung or breast cancer (Basu et al., 1988). Vitamin A deficiency promote carcinogenesis, but paradoxically, an excess of vitamin A may have a similar effect. De Carli et al., 1987, noted that foods providing large amount of retinol increase the risk of cancer of the esophagus.

Both natural and synthetic analogues of vitamin A have been shown to be effective in suppressing micro nucleated cells, reversing oral leukoplakia, and preventing new and recurrent lesions in subjects with oral leukoplakia, as well as in reducing the occurrence of head and neck cancer (Sankaranarayanan and Mathew., 1996).

Vitamin E

Vitamin E is another lipid soluble vitamin. Vitamin E occurs in plasma as a variety of tocopherols, of which the alpha and gamma isomers are usually the major ones. All vitamins in the human body is derived from the diet. Major dietary sources of vitamin E are vegetable oils, margarine, nuts, seeds, whole grains, and wheat sprout.
Different foods vary in the tocopherol isomers. \( \alpha \)-tocopherol is biologically the most active isomer in mammals. Because of antioxidant properties vitamin E neutralizes ROMs and reduces oxidative DNA damage and genetic mutation (Frei, 1994). Vitamin E is thought to be an important chain breaking antioxidant, which plays an important role in various stages of carcinogenesis through its contribution to immuno competence, membrane and DNA repair, and decreasing oxidative DNA damage (Kimmick et al., 1997). Vitamin E can directly scavenge ROMs. It is the major lipid soluble antioxidant present in all cellular membranes which protects against LPO (Machlin and Bendich, 1998). Vitamin E can directly act with a variety of oxyradicals including the peroxo radicals (ROO\(^\bullet\)), CCl\(_3\)\(^\bullet\), \( \cdot \) OH, \( \cdot \) O\(_2\)\(^\bullet\) (Fukuzawa and Gebicki, 1983) and singlet oxygen (Littarru et al., 1984). The major function of vitamin E is its role as a physiological membrane bound antioxidant, protecting cell membrane lipids from oxidative damage initiated by ROMs. In vitro studies showed that vitamin E can prevent oxidation of DNA by inhibiting activated neutrophils (Van Staden, et al. 1993). The lipophilic antioxidants, \( \alpha \)-tocopherol has been shown to protect LDL from oxidation (Traber, 1997).

**Vitamin C**

Vitamin C (Ascorbic acid) is an important water soluble antioxidant in biological fluids and an essential micronutrient required for normal metabolic functioning of the body (Jaffe, 1984). It
readily oxidizes to dehydroascorbic acid. Human beings have no ability to synthesise vitamin C due to mutation in the gene coding for L-gulonolactone oxidase, an enzyme required for the biosynthesis of vitamin, via the glucuronic acid pathway (Woodal and Ames., 1997). Thus, vitamin C is obtained through the diet. The vitamin is plentiful in fresh fruits, especially, citrus fruits and vegetables (Bendich., 1997). The molecular mechanisms of the antiscorbutic effect of vitamin C are not completely known. Vitamin C is a cofactor for several enzymes involved in the biosynthesis of collagen, carnitine and neurotransmitters (Burri and Jacob., 1997).

Vitamin C has been implicated for steroid metabolism in adrenal gland. Hydroxylation of aromatic drug and carcinogens by hepatic cytochrome P 450 is also enhanced by reducing agents such as vitamin C (Tsao.,1997). The temporal order of antioxidant consumption in human beings with plasma exposed to a constant flux of aqueous peroxyl radicals in vitamin C > bilurubin> uric acid>vitamin E. Plasma is devoid of vitamin C, but no other endogenous antioxidant, is extremely vulnerable to oxidant stress and susceptible to peroxidative damage to lipids (Frei et al., 1989). Vitamin C readily scavenges ROMs, ozone, ONOO, NO2, NO and hypochlorous acid (Noroozi et al., 1998). Vitamin C neutralizes ROMs and reduces oxidative DNA damage and genetic mutations (Frie., 1994). It has also been reported that vitamin C may enhance host immunological functions (Kelley and Bendich., 1996). Epidemiological
studies have indicated an inverse association between vitamin C intake and the risk of cancers (Hecht., 1997). It can prevent carcinogenic nitrosamine formations in cancer, which is yet another protective function of vitamin C (Tannenbaum and Wishnok., 1991). Vitamin C can act as a co-antioxidant by regenerating α-tocopheroxyl radicals produced during scavenging of ROMs (Packer., 1997).

Vitamin C can protect host cells against harmful oxidants released into the extracellular medium. Serum vitamin C has been shown to decrease the risk of colon, breast and stomach cancers in many studies (Block., 1991).

β-carotene

Basic research has demonstrated that beta carotene can trap organic free radicals and/or deactivate excited oxygen molecules, which may prevent the onset of cancer (Buring and Hennekens., 1995). Beta-carotene also may prevent genetic changes by preventing DNA damage directly induced by free radicals. Enhanced cell-to-cell communication has been described with the use of beta-carotene. This would restrict clonal expansion of initiated cell (Wolf., 1994).

β-carotene that acts as antioxidants under normal physiological conditions (low oxygen tension), can also act as pro-oxidants at high concentrations and more oxidizing conditions (such as lungs of smokers) (Hennekens et al., 1986).
**Chemo prevention**

Chemo prevention (CP) is the use of pharmacological or natural agents that inhibit the development of invasive cancer either by blocking the DNA damage that initiates carcinogenesis or by arresting or reversing the progression of premalignant cells in which such damage has already occurred. CP is an important defense strategy against human cancer since it is highly unlikely for one to avoid all carcinogenic insults. The human body is composed of sixty-three trillion cells, plus or minus a few hundred billion. Each day a typical human cell undergoes five thousand mutations (Hong and Sporn., 1997). Each mutation of course can lead to the initiation of cancer, but it usually does not because a particular mutation does not necessarily occur in both strands of the DNA helix and therefore, is repairable. The objective of CP is to administer one or more chemical agents naturally occurring or synthetic, which may have multiple biological mechanisms to inhibit various stages of carcinogenesis (Arnold et al., 1995) The multistage nature of the process of cancer development is a cyclical process of DNA damage, proliferation, colonal selection and progression. This process could potentially be modulated by chemicals that effect cellular enzyme systems, gene expression, signal transduction pathways, differentiation or interactions with surrounding cells and extracellular matrices. Many chemical compounds have the ability to inhibit, retard or reverse one or more stages of carcinogenesis and thus affect the overall cancer
incidence. The steps of carcinogenesis and possible points of inhibition of chemo preventive agents are shown below (Ho et al., 1992).

**Pre-carcinogen**

Metabolic Activation

**Ultimate carcinogen**

Reaction with DNA

**DNA carcinogen adducts**

One or two cycles of DNA replication to initiated cell

**Initiated cell**

Conversion of initiated cell to pre-neoplastic cell

**Preneoplastic cell**

Conversion of preneoplastic cell to neoplastic (Cancer cell)

**Chemo preventive activity**

Inhibits formation of carcinogens by
- Blocking activation
- Diverting to less genotoxic metabolites.

Prevent ultimate carcinogen reacting with DNA by
- Intercepting Ultimate carcinogen
- Enhancing carcinogen metabolism/excretion.

Stimulation of DNA repair

Slow down promotion by
- Blocking promoting agents
- Antioxidant activities
- Anti-inflammatory activities
Mechanism of action of chemo preventers

Mechanism of action of chemopreventer is complex. It can be categorized according to the site of action or by specific type of action. The activity could be the result of simultaneous action of several factors on the same event. It appears that most chemopreventers act primarily as antioxidants. Broadly the mechanism of action of chemopreventers can be categorized, depending on whether they act extracellularly or intracellularly.

Some important bioactive compounds and their roles in cancer prevention (Table 1) are discussed below (Huang et al., 1992).

**Table 1**

**INHIBITORY EFFECTS OF SOME PHYTOCHEMICALS IN FRUITS AND VEGETABLES ON CHEMICALLY INDUCED CARCINOGENESIS IN ANIMAL MODELS**

<table>
<thead>
<tr>
<th>Group</th>
<th>Phytochemicals</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allylic compounds</td>
<td>Allyl mercaptan</td>
<td>Allium sp. vegetable (garlic and onion)</td>
</tr>
<tr>
<td></td>
<td>Allyl methyl disulfide</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>Allyl methyl trisulfide</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>Diallylsulfide</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>Diallyl disulfide</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>Diallyl trisulfide</td>
<td>&quot;</td>
</tr>
<tr>
<td>Isothiocyanates</td>
<td>Benzyl isothiocyanate</td>
<td>Cruciferous vegetables</td>
</tr>
<tr>
<td></td>
<td>Phenethyl isothiocyanate</td>
<td>Cruciferous vegetables</td>
</tr>
<tr>
<td>Indoles</td>
<td>Indole-3-cabinol</td>
<td>Cruciferous vegetables</td>
</tr>
<tr>
<td></td>
<td>Indole-3-acetonitrile</td>
<td>Cruciferous vegetables</td>
</tr>
<tr>
<td>Monoterprenes</td>
<td>D-Limonen</td>
<td>Citrus fruit oils</td>
</tr>
<tr>
<td></td>
<td>D-Carvone</td>
<td>Caraway seed oil</td>
</tr>
<tr>
<td>Vitamins</td>
<td>Ascorbic acid</td>
<td>Fruits and vegetables</td>
</tr>
</tbody>
</table>

36
<table>
<thead>
<tr>
<th>Carotenoids</th>
<th>Tocopherol</th>
<th>Vegetable oils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitamin a</td>
<td></td>
</tr>
<tr>
<td>β-Carotene</td>
<td>ORange-yellow vegetables</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>Chlorophyll</td>
<td>Green vegetables</td>
</tr>
<tr>
<td></td>
<td>Chlorophyllin</td>
<td>Green vegetables</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Quercetin</td>
<td>Vegetables and fruits</td>
</tr>
<tr>
<td></td>
<td>Rutin</td>
<td>Citrus</td>
</tr>
<tr>
<td></td>
<td>Tangeretin</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>Nobiletin</td>
<td>&quot;</td>
</tr>
<tr>
<td>Cinnamic acids</td>
<td>Caffeic acid</td>
<td>Fruits, coffee bean and soybean</td>
</tr>
<tr>
<td></td>
<td>Ferulic acid</td>
<td>Fruits and soybean</td>
</tr>
<tr>
<td></td>
<td>Chlorogenic acid</td>
<td>Fruits, coffee bean and soybean</td>
</tr>
<tr>
<td></td>
<td>Curcumin</td>
<td>Curcuma longa</td>
</tr>
<tr>
<td></td>
<td>Saponin</td>
<td>Soya bean</td>
</tr>
</tbody>
</table>

**Synthetic antioxidants**

Chain breaking antioxidants, especially butylated hydroxyanisole (BHA) and butylated hydroxy toluene (BHT) are widely used in the food industry to prevent rancidity. Because of alleged toxicity problems of BHA and BHT there are attempts to replace them as preservatives in certain foodstuffs by natural phenolic compounds.

**Mercaptopropionylglycine and other thiols:** They have been found to protect against reperfusion injury and clinically effective as antioxidants in a variety of diseases.

**Ebselen:** It is a glutathione peroxidase congener with anti-inflammatory action, which may also inhibit PGs and LTs.

**Xanthine oxidase inhibitors:** Xanthine oxidase is the prime source of reactive oxygen species during reperfusion or injury. They are the inhibitors of the enzyme, allopurinol and oxypurinol, have been
shown to protect against myocardial, cerebral and gastro-intestinal reoxygenation injury. In addition, these drugs may be effective in organ transplantation and have been shown to attenuate duodenal ulcer relapse.

**Disferrioxamine:** This drug used as an iron chelator, can inhibit iron-dependent lipid peroxidation and generation of oxidative free radicals. It has been found to be useful in Alzheimer’s disease and reperfusion injury. Disferrioxamine has antimalarial activity, suggesting that reactive oxygen species are involved in pathogenesis of malaria. It is also effective in rheumatoid arthritis.

**Probucol and chain breaking antioxidants:** Phenolic chain breaking antioxidants, like probucol, have anti-inflammatory effect and antiatherosclerotic effects. They inhibit lipid peroxidation and interfere with synthesis of eicosanoids (PGs and LTs). Bioflavanoids of plant origin have similar action.

**Herbal formulations as pharmacotherapeutic agents**

Herbal medicines have been used since the dawn of civilization to maintain health and to treat diseases. The World Health Organisation estimates that about three quarters of the world’s population currently use herbs and other forms of traditional medicines to treat their diseases. Even as we commence the new century with its exciting prospects of gene therapy, herbal medicines remains one of the common forms of therapy available to much of worlds population. Ayurveda, Siddha, Unani systems of medicine are
widely used in India. Pharmacoepidemiological survey carried out by Karandikar et al in adults over 60 years of age revealed that about 47% of the elderly population uses herbal drugs (Karandikar et al., 1997). China is another country where traditional medicines are widely used. Faith in these traditional systems of medicine is related to cultural practices and beliefs. Several publications are available on medicinal plants of India. Authors like Chopra, Nadkarini have published reviews on medicinal plants. Indian Council of Medical Research (ICMR) has published several booklets on the same subject and formed a scientific advisory group for traditional medicines. In spite of the wide spread interest, knowledge regarding the scientific basis of the use of herbal medicines is not known to large majority of physicians and users of these preparations.

Focus on plant research has been going on for many years in India. Large numbers of plants have been tested for their pharmacological effects. These have been presented in various reviews (Vaidya., 1997; Aswal et al., 1996; Gupta., 1994; Vaidya and Antarkar., 1994). Herbal preparations which have achieved wide spread acceptability as therapeutic agents include noortropics, anti-hypertensives, hepato-protective, anti-inflammatory agents, anti-diabetics and lipid- lowering agents. Some of these have undergone detailed experimental and clinical studies, which are highlighted below.
**Hepatoprotective agents**

Liv52, a polyherbal formulation to improve ethanol metabolism in a rat model of chronic alcohol administration (Chauhan et al., 1994). It also prevented lipid peroxidation in \( \text{CCl}_4 \) induced liver damages as seen by significant decrease in malondialdehyde content (Pandey et al., 1994; Kataria and Singh., 1997). Liv100 improvised herbal formulation of Liv52, as been reported to reduce the peroxidation effect to hydrogen peroxide in rat liver homogenate (Saraswathy et al., 1998). Jigrine, a unani polypharmaceutical herbal formulation containing fourteen medicinal plants was evaluated in three models of hepatic damage induced by alcohol, carbon tetrachloride or paracetamol in rats. Jigrine significantly reduced liver enzymes and improved histopathological findings (Kapur et al., 1994). Thyagarajan et al., 1998; Jayaram and Thyagarajan., 1996) have studied the effects of *Phyllanthus amarus* on hepatitisB virus. Significant hepatoprotective activity was reported in several individual plant extracts (Asha., 2001; Emmamul., 2001; Subramoniam et al., 1998; Kumar and Kuttan., 2000; Jose and Kuttan., 2000; Babu et al., 2001; Ajith and Janardhanan., 2002).

**Centrally acting drugs**

Mentat, a polyherbal formulation has been shown to augment acquisition and retention of learning in experimental studies (Bhattacharya., 1994; Handa and Bhargava., 1997; Bhardwaj and Srivastava., 1995). Trasina (Bhattacharya and Kumar., 1997) a herbal
formulation is known to exert nootropic effects. Ginkgolic acid conjugates isolated from leaves of Indian *Ginkgo biloba* Linn is known to have anxiolytic activity (Satyan et al., 1998; Manocha et al., 1997). *Withania sominifera* is another plant which has been studied extensively for neuropharmacological effects (Kulkarni and Ninan., 1997).

**Herbal formulation for cardiovascular disorders**

Abana, a polyherbal formulation is marketed as an antihypertensive agent. This preparation has been tested in hypertensive animals (Bhatt et al., 1998). Bark extract of *Terminalia arjuna* has been tested in clinical trials in patients with refractory congestive cardiac failure and was reported to show improvement in symptoms and signs of heart failure (Bharani et al., 1995; Dwived and Jauhari., 1997). Rutin, a flavonoid from the plant *Sophora japonica*, is reported to have beneficial effects in ischaemic heart diseases (Chopra and Singh., 1994). Guggu lipid (an active principle of *Commiphora mukul*) is an agent that has been widely investigated for its hypolipidaemic activity (Dalviss et al., 1994). Preparations containing garlic and onion are known to have antiatherogenic effect. The active ingredient is S-allyl cysteine sulphoxide which has antiatherogenic effect (Angusti., 1996).

**Antidiabetic agent**

Numerous plants and natural products have been studied for antidiabetic activity in different laboratories (Manickam et al., 1997;
Dubey et al., 1994; Dhawan et al., 1996). Detailed studies have been undertaken on *Pterocarpus marsupium* for its effect in experimentally induced diabetes in animals as well as in patients. D-400, a polyherbal formulation has been studied for its effects on alloxan-induced diabetes in rabbits (Dubey et al., 1994; Dhawan et al., 1996). The list of agents which have been tested for hypoglycemic activity includes wide variety of plants, Ayurvedic and Unani preparations (Tripathi and Chaturvedi., 1995; Gomes et al., 1995; Saxena et al., 1996; Agarwal et al., 1996). Many indigenous Indian Medicinal plants have been found to be successfully used to manage diabetes (Nagarajan et al., 1987, Jain and Sharma., 1967, Anjali and Manoj., 1995) and some of them have been tested and active principle isolated. Several drugs have shown antidiabetic activity when assessed using presently available experimental techniques (Saifi et al., 1971, Mukherjee et al., 1972, Coimbra et al., 1992, Ajith Kar et al., 1999, Jafri et al., 2000)

**Miscellaneous**

Antiinflammatory activity has been studied in various models of inflammation in experimental animals. The plants, which are reported to have anti-inflammatory property, include *Ocimum sanctum*, *Pongamia pinnata*, *Gmelina asiatica*, *Nelumbo nucifera*, and *Gymnema sylvestre*. Polyherbal formulations Ease and Jegrine and Ayurvedic drugs like Sandhika are known to have antiarthritis effect (Chaurasia et al., 1995; Chatterjee and Das., 1996; Karunakar et al., 1997). Plant
products have been studied for various therapeutic effects such as anti and profertility action, cytoprotective effects, pro- and antikinetic effects in gastro-intestinal system, ulcerative colitis, antimicrobial, antiviral and antifungal actions, antihelmintic and antiprotozoal action (Dahanukar et al., 2000). Many researchers have focused their attention also on anticancer plants and immunomodulatory plants and antimutagenic plants (Upadhyay., 1997., De et al., 1998).

*Phyllanthus amarus*

*Phyllanthus amarus* Schum & Thonn., is one of the most important medicinal plants used as a traditional medicine in India and elsewhere. It is used for the treatment of jaundice and other diseases (Chopra et al., 1956). It is a Euphorbiaceous herb, found easily during rainy season. In clinical trials diuretic (Srividya and Periwal., 1995), urolytic (Campos and Schor., 1999), and hypertensive effects have been documented. Decoctions are used in diarrhoea (Odetola and Akojenu., 2000), diabetes (Moshi et al., 1997), jaundice (Dixit and Achar., 1983), and genitourinary diseases and to treat kidney and bladder calculi.

Extracts of *Phyllanthus* sp had been reported to have pharmacological effects such as antibacterial (Macrae et al., 1988), antihyperglycaemic (Moshi et al., 1997), antihepatotoxic, (Syamasundar et al., 1985; Thabrew and Hughes, 1996), and antihepatitis B virus (Thyagarajan et al., 1982; Lee et al., 1996) activities. *Phyllanthus amarus* is widely used as an anti-viral agent
P. amarus extract has been shown to inhibit DNA polymerase of hepatitis B virus and related hepatitis viruses (Venkateswaran et al., 1987; Blumberg et al., 1990) and down regulate hepatitis B virus mRNA transcription and translation (Lee et al., 1996; Ott et al., 1997). Liver protective effect of Phyllanthus has been demonstrated in chemically induced liver toxicity models (Prakas et al., 1995). The extract reversibly inhibited cellular proliferation and suppressed HbsAg production in cultured hepatoma cell line HepA2 (Yeh et al., 1993) and inhibited the release of HbsAg in Alexander cell line, a human hepatocellular carcinoma derived cell line (Jayaram and Thyagarajan., 1996).

The P. amarus extract was found to have significant anti oxidant activity. P. amarus extract was found to scavenge the superoxides induced by photoreduction of riboflavin (Mc Cord and Fridovich 1969) (IC50-16 μg/ml), which was very close to ellagic acid (IC50-7.05 μg/ml) and curcumin (IC50-7.21 μg/ml) which have been reported to have significant anti oxidant activity. The P. amarus extract was also found to inhibit hydroxyl radicals (Okhawa et al 1979) (IC50-130 μg/ml) and inhibited the lipid peroxidation (IC50-125 μg/ml) (Joy and Kuttan., 1995).

P. amarus extract also produced significant reduction in solid tumour volume produced by DLA and EAC cells (Kumar et al., 2002). Simultaneous administration of P. amarus extract along with the carcinogen has been reported to inhibit hepatocellular carcinoma
development induced by NDEA (Joy and Kuttan., 1998) and also found to increase the life span of rats infected with it (Kumar and Kuttan., 2000). *P. amarus* extract could inhibit 20 methylcholanthrene induced sarcoma development and increased the survival of tumour harboring mice (Kumar et al., 2002).

Other than its activity in hepatitis B virus, the extract and ingredients isolated from the extract has been shown to inhibit the activity of HIV type 1 reverse transcriptase (Ogata et al., 1992). *P. amarus* extract has been shown to reverse the chromosomal alterations induced by genotoxic agents (Gowrishanker and Vivekanandan., 1994). *P. amarus* extract has been reported to inhibit aniline hydroxylase, a P-450 enzyme responsible for the activation of the carcinogen (Kumar et al., 2002). The extract was found to inhibit the activity of cdc25 tyrosine phosphatase, a key enzyme involved in cell cycle regulation (Jose et al., 1999). *P. amarus* inhibited the activity of cdc2 kinase only at a higher concentration (IC$_{50}$ was more than 1000µg/ml) (Kumar et al., 2002) The extract was also found to inhibit the activity of topoisomerase I and II in *Saccharomyces cerevisiae* mutant cell cultures (Kumar et al., 2002).

Several active compounds have been identified in *P. amarus* extract. Lignans like phyllanthin and hypophyllanthin (Somanabandhu et al., 1993) flavonoids like quercetin and atsragalin (Nara et al., 1997), ellagitannins like amarinic acid (Foo., 1995) and hydrolysable tannins like phyllanthisiin D (Foo and Wong., 1992) and
amarin (Foo., 1993) were reported from *P. amarus*. A variety of hydrolysable tannins purified from *P. amarus* were found to be potent inhibitors of wheat embryo Ca$^{2+}$- dependent protein kinase (CDPK), rat brain Ca$^{2+}$ protein kinase, phospholipid- dependent protein kinase C (PKC) and Ca$^{2+}$ calmodulin- dependent myosin light chain kinase (Polya et al., 1995).

Other ingredients are catechin, epicatechin, gallocatechin and epigallo catechin gallate, which are proven antioxidant polyphenolic compounds (Hara., 1990). Leaf oil contains limonene (4-5%). It also has repandusinic acid (Ogata et al., 1992), which has been indicated as one of the major antiviral agent present in this plant.

**Scope of the study**

In the present study we report several pharmacological properties of *Phyllanthus amarus*, which have not been reported earlier. The anti- mutagenicity of *P. amarus* extract was studied using several direct acting as well as mutagens leading microsomal activation. We have also studied the anti- carcinogenicity of *P. amarus* extract using MNNG induced stomach cancer model as well as dimethyl benzathracene induced skin cancer in small animals. A systemic study on the anti- diabetic activity of *P. amarus* was undertaken to study the hypoglycemic effect of the extract. The effect of the extract in reducing the alcohol induced gastric lesion as well carrageenan-induced inflammation has also been studied in animals. Preliminary study has been done on the anti- microbial activity of *P.*
amarus extract as well as its anti-fungal activity and inhibition of the production of aflatoxin by Aspergillus parasiticus. Anti-oxidant activity of P. amarus extract as well as its isolated fraction was also done in order to get an insight into the active fraction responsible for the biological activity of the extract. These studies produce a new insight into the pharmacological activity of P. amarus.