Review of literature
2. Review of literature

Ethnomedicine is a part of either general ethnobotany or classical ethnobotany. When it corresponds to general ethnobotany, it has the pharmacological prospective, which seeks the potential efficacy of the tribal or indigenous herbs in biological terms. When it corresponds to classical ethnobotany, it has the symbolic prospective which views plants as part of particular cultural system of beliefs and practices surrounding health and healings (Montellano and Browner, 1985). Along with material culture like food, medicine and shelter, plants have been closely associated with many social customs and mythological rituals of man. Many flowers, fruits or whole plants have been used for offering in worship and some plants themselves are worshipped or considered sacred (e.g. Ocimum sanctum and Ficus religiosa). It is the fact that primitive or ethnic populations have their own medical lore, which is being practiced from time immemorial and some of their therapeutic practices have been found significantly important for modern medical knowledge.

The huge diversity of plant species leads to the expectation that many therapeutic worthwhile compounds remain undiscovered. Random screening of these compounds is not an effective approach as the National Cancer Institute failed to find a compound with clinical anti-cancer activity among 114,000 plant extracts from 35,000 species (Reynold, 1991). The use of folk beliefs and traditional healers as a short cut to the discovery and isolation of pharmacologically active compounds has been a productive approach. Virtually, almost all the currently used drugs derived from plants including reserpine, quinine, digoxin, digitoxin, tubocurarine, morphine and codeine were discovered through scientific investigation of folklore claims.

There is an extremely large and readily accessible body of traditional medicine describing a wide range of plants and other substances, that has not recently been investigated systematically. These plants are rapidly being destroyed and with them the potential of discovering new drugs based on phytochemicals (Holland, 1994). So, an attempt has to be made to scientifically validate the medicinal properties of such plants and their active principles, which precisely is the objective of the present study.
2.1 Carcinogenesis

Neoplasm is a mass of abnormal cells that possesses uncontrolled proliferative activity. In general, neoplasms are irreversible, and their growth is relatively autonomous. In contrast to benign neoplasms, malignant neoplasms of either epithelial origin (carcinoma) or non-epithelial origin (sarcoma) have the added property of invasiveness and metastasis. Such lesions emerge through a multistep process which includes initiation, promotion, and progression. The whole process involves multiple genetic as well as epigenetic alterations. This process of carcinogenesis has been illustrated in Figure 1.

It is now established that cancer is the result of mutational events. Genotoxic carcinogens undergo host mediated biochemical activation and are thus converted to reactive electrophilic metabolites which interact with nucleophilic centers in DNA and also RNA and protein. The carcinogen modified DNA template forms the basis for the production of an altered DNA. Cells bearing such changed DNA are considered initiated cells. This set of reactions is also carried out by specific viruses or radiation. In addition to altering DNA through activation of prot-oncogenes by retroviral insertion, mutation, gene amplification and chromosomal translocation can also affect tumor suppression genes. Initiated cells are known to exhibit altered responsiveness to microenvironment and selective growth advantage over normal cells in response to tumor promoting stimuli. These properties help in clonal expansion of initiated cells as compared to the surrounding normal cells. The initiated cells are also found to be less responsive to the inducers of terminal differentiation, negative growth factors and/or programmed cell death (Morse et al., 1990).

In a later promotion sequence, the growth and development of initiated cells are subject to a different set of endogenous and exogenous growth controlling elements that operate through distinct mechanisms. This results in the proliferation of initiated cells leading to the development of a microcolony of cells with altered phenotype. Tumor promoters bring about membrane changes like increase in phospholipid synthesis, an increase in protease activity, cyclic AMP dependent protein kinase activity, induction of cell proliferation, ornithine decarboxylase and subsequently polyamines. They are also known to produce pro-oxidant conditions such as a decrease in epidermal superoxide dismutase and catalase activities. Altered differentiation plays a critical role in tumor
Figure I. Sequential major events in chemical carcinogenesis and potential chemopreventive strategies.
promotion. In contrast to the initiator, promoter induced changes are generally reversible and appear to be epigenetic. Basically, promoters are not directly mutagenic, but they can change the expression of genes and cell proliferation.

Progression of a benign neoplasm to malignancy is characterized by an increased autonomy from both environment and the host. This stage is irreversible and is associated with an increased frequency of genetic alterations and morphologically identifiable changes in the cell (Pitot, 1986). Progression leads to the increased growth rate, invasiveness and metastatic capability, changes in response to hormones and drugs, and alterations in the karyotype.

2.2 Cancer control - a chemopreventive approach

Chemoprevention is the use of pharmacologic or natural agents that inhibit the development of invasive cancer either by blocking the DNA damage that initiate carcinogenesis, or by arresting or reversing the progression of premalignant cells in which the damage has occurred. The goals of chemoprevention are 1) inhibition of carcinogenesis, 2) logical intervention for persons at genetic risk for cancer, 3) treatment of precancerous lesions and 4) confirmation and translation of leads from dietary epidemiology into intervention strategies (Kelloff et al., 1997; Wattenberg, 1997).

Dietary intake of nutrients and non-nutrients are specific to individuals, population and countries. It is therefore, necessary to identify dietary and other naturally occurring agents which may form a part of the lifestyle in prevention of cancer. Most of the cancers appear due to environmental load of chemicals from water, air or food. Considerable effect is being directed to identify the agents of plant origin especially associated with food which over a period of time can help in mitigating the effects of carcinogens or act as blocking agents that can decrease the risk of cancer (Steinmetz and Potter, 1991).

2.2.1 Mechanisms of chemoprevention

There are several mechanisms of chemoprevention ranging from inhibition of activation of carcinogen to scavenging the active electrophiles and activating antimetastasis genes employing various potential chemopreventive agents.
A plethora of possible mechanisms for chemoprevention have been suggested (Table II) (Kelloff et al., 1996). However, the classification scheme proposed by Wattenberg (1985) based essentially upon the time period at which these agents appear to have their activity, recognizes three major types of chemopreventive agents.

1. Inhibitors of carcinogen formation.
2. Blocking agents.

The majority of compounds that inhibit the formation of carcinogens, prevent the formation of nitrosamines from secondary amines and nitrite in an acidic environment. Blocking agents are inhibitors of tumor initiation (Table III), while suppressing agents are inhibitors of tumor promotion/progression (Table IV). Many well-characterized chemopreventive agents act at one or more steps in both tumor initiation and promotion/progression. Based on the mechanisms of action of the chemopreventive agents, many chemoprevention strategies have been developed to reduce the risk and incidence of cancer.

2.2.2 Chemoprevention strategies

The most desirable way of eliminating cancer in humans is by prevention. The first set of strategies for achieving this objective is to remove the causative agents. At present, causes of most cancers in human are not known and even in future it is likely to remain incomplete. Thus, the development of a second line of prevention based on chemoprevention assumes considerable importance. Interestingly, compounds belonging to over 20 different classes of chemicals have been shown to have chemopreventive capacities. These compounds have been classified into 1) anti-initiation compounds - prevent the formation of carcinogens from precursor substances, for example ascorbic acid, α-tocopherols and phenols have the capacity to inhibit the formation of nitroso-compounds, 2) blocking agents - prevent carcinogenic compounds from reaching or reacting with critical target sites in the tissue, for example phenols, indoles, coumarins, flavones, diterpenes, etc., and 3) suppressing agents - suppress the expression of neoplasia
<table>
<thead>
<tr>
<th>Mechanisms</th>
<th>Examples of Potential Chemopreventor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carcinogen blocking activities:</strong></td>
<td></td>
</tr>
<tr>
<td>1. Inhibition of uptake of carcinogen in target cells.</td>
<td>Calcium binds to excess bile and free fatty acids that are known to promote tumorigenesis in colon.</td>
</tr>
<tr>
<td>2. Inhibition of carcinogen formation from its precursors.</td>
<td>Ascorbic acid, α-tocopherol, caffeic acid, ferulic acid and gallic acid inhibit formation of N-nitroso compounds from secondary amines and nitrites.</td>
</tr>
<tr>
<td>3. Inhibition of activation of carcinogens.</td>
<td>Arylalkyl isothiocyanates, NSAIDS, polyphenols, DHEA.</td>
</tr>
<tr>
<td>4. Detoxification/deactivation of carcinogens.</td>
<td>Indole-3-carbinol, oltipraz; GST and GSH-enhancing agents.</td>
</tr>
<tr>
<td>5. Prevention of carcinogen binding to DNA.</td>
<td>Oltipraz, polyphenols.</td>
</tr>
<tr>
<td>6. Enhancement of level or fidelity of DNA repair.</td>
<td>Protease inhibitors, N-acetyl-l-cysteine (NAC)</td>
</tr>
<tr>
<td>a. increase in overall level of DNA repair;</td>
<td></td>
</tr>
<tr>
<td>b. stabilization of poly (ADP-ribosyl) transferase - a DNA-damage modulator;</td>
<td></td>
</tr>
<tr>
<td>c. inhibition of error prone repair system.</td>
<td></td>
</tr>
<tr>
<td><strong>Antioxidant/antiinflammatory activities:</strong></td>
<td></td>
</tr>
<tr>
<td>7. Scavenging of reactive electrophiles.</td>
<td>GSH-enhancing agents.</td>
</tr>
<tr>
<td>8. Scavenging of oxygen radicals.</td>
<td>β-carotene; tocopherol; polyphenols.</td>
</tr>
<tr>
<td>9. Inhibition of arachidonic acid metabolism.</td>
<td>NAC; NSAIDS, polyphenols, tamoxifen.</td>
</tr>
<tr>
<td>Antiproliferation and antiprogression activities:</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>13. Inhibition of polyamine metabolism.</td>
<td>DFMO, retinoids, tamoxifen.</td>
</tr>
<tr>
<td>15. Restoration of immune response.</td>
<td>Selenium, α-tocopherol, NSAID</td>
</tr>
<tr>
<td>16. Enhancement of intercellular communication.</td>
<td>Retinoids, carotenoids</td>
</tr>
<tr>
<td>17. Restoration of tumor suppressor gene function.</td>
<td>?</td>
</tr>
<tr>
<td>19. Inhibition of telomerase.</td>
<td>?</td>
</tr>
<tr>
<td>20. Correction of DNA methylation imbalances.</td>
<td>Folic acid</td>
</tr>
<tr>
<td>21. Inhibition of angiogenesis.</td>
<td>Retinoids, genistein, tamoxifen.</td>
</tr>
<tr>
<td>22. Inhibition of basement membrane degradation.</td>
<td>Protease inhibitors</td>
</tr>
<tr>
<td>23. Activation of antimetastasis genes.</td>
<td>?</td>
</tr>
</tbody>
</table>
### Table III. Categories of blocking (anti-initiation) agents.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of cytochrome P450</td>
<td>dithiocarbamates, isothiocyanates, diallyl sulfide, ellagic acid</td>
</tr>
<tr>
<td>Induction of cytochrome P450</td>
<td>indol-3-cabinol, beta-naphthoflavone</td>
</tr>
<tr>
<td>Induction of phase II enzymes</td>
<td>isothiocyanates, polyphenols, dithiolethiones</td>
</tr>
<tr>
<td>Scavenging electrophiles</td>
<td>ellagic acid, N- acetyl cysteine, sodium thiosulfate</td>
</tr>
<tr>
<td>Induction of DNA repair</td>
<td>vanillin</td>
</tr>
</tbody>
</table>

### Table IV. Categories of suppressing (anti-promotion/anti-progression) agents.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of polyamine metabolism</td>
<td>alpha-difluoromethyl ornithine, substituted putrescines</td>
</tr>
<tr>
<td>Inhibition of arachidonic acid metabolism</td>
<td>piroxicam, indomethacin, aspirin, quercitin</td>
</tr>
<tr>
<td>Protease inhibition</td>
<td>tosyl phenylalanine, chloromethyl ketone, antipain, Bowman-Birk protease inhibitor</td>
</tr>
<tr>
<td>Induction of differentiation</td>
<td>retinoids, calcium and vitamin D</td>
</tr>
<tr>
<td>Inhibition of oncogene expression</td>
<td>lovastatin, limonene, antisense oligonucleotides</td>
</tr>
<tr>
<td>*Inhibition of product post-translational modification</td>
<td>staurosporin, threo-dihydro sphingosine</td>
</tr>
<tr>
<td>*Inhibition of transcription or translation</td>
<td>sarcophytol A, epigallocatechin gallate, selenium</td>
</tr>
<tr>
<td>*Inhibition of protein kinase C</td>
<td></td>
</tr>
<tr>
<td>*Inhibition of oxidative DNA damage</td>
<td></td>
</tr>
</tbody>
</table>
in cells previously exposed to carcinogenic agents, for example retinoids, carotenoids and selenium (Figure II). (Wattenberg, 1985, Kelloff et al., 1997).

A large number of diverse group of compounds (both naturally occurring and synthetic) fall under the category of blocking agents which induce the activity of enzyme systems having the capacity to enhance carcinogen detoxification. The inhibitors of this group are of particular interest because they have the capacity to inhibit a wide range of carcinogens. Some blocking agents act by scavenging the reactive forms of carcinogens, for example, GSH and ellagic acid. Since large number of these blocking agents are naturally occurring substances, it may be possible eventually to assess the role that these compounds play in inhibiting the occurrence of neoplasia in specific population groups (Stoner et al., 1997).

The chemical diversity of the inhibitors indicates that inhibition of carcinogenesis is not a highly selective phenomenon and that multiple strategies exist for bringing about this desired effect. It also makes it probable that additional compounds with chemopreventive properties will be identified in the future, again adding to the choices that would be available.

2.3 Chemopreventive agents

2.3.1 Macronutrients, micronutrients and bioactive compounds

Epidemiologic and experimental studies indicate that the risk of certain cancers in humans is influenced by a variety of dietary factors, including macronutrients such as fat and fibres; micronutrients such as vitamins and minerals; and hundreds of natural and bioactive chemicals present in vegetables, fruits and other natural products. The discovery of such relationship provides the basis for cancer prevention research as demonstrated by the strong evidence that dietary and hormonal factors affect breast cancer risk.

Macronutrients, micronutrients, natural and synthetic bioactive chemicals (spices, herbs, condiments, food additives and drugs) are being studied in depth for their cancer chemopreventive efficacy.

Among macronutrients, fibres (complex carbohydrates) in food are known to inhibit carcinogenesis in several organs especially colon (Jacobs, 1986; Weisburger et al.,
**Category of inhibitors**

**Inhibitors preventing formation of carcinogens**
(e.g. ascorbic acid, tocopherols, phenols like caffeic acid, ferulic acid)

**Blocking agents**
(e.g. indoles, flavones, coumarins, phenols like BHA and BH 7)

**Suppressing agents**
(e.g. retinoids, carotenoids, plant sterols, selenium salts, protease inhibitors)

**Sequence leading to neoplasia**

Precursor compounds

Carcinogenic species

Reactions with cellular targets

Neoplastic manifestations

Figure II. Three categories of chemopreventive agents and their time of action.
bile acid concentration, physical dilution of faecal contents, decreased fermentation, production of short chain fatty acid and butyrate, lower pH, lower ammonia levels, decrease in mutagenecity of intestinal contents, alterations in mucosal kinetics, decrease in ornithine decarboxylase or aryl hydrocarbon hydroxylase, reduced gut hormones and other peptide growth factors. Both obesity and high-fat/low-fibre diet in women may be associated with increased estrogen levels which are widely assumed to increase the risk of developing breast cancer (Stoll, 1997).

Fat is one of the most extensively studied dietary factors in cancer prevention research. A strong positive relationship between colon, breast and prostate cancer risk and total fat intake has been suggested. In addition to the total amount of fat, the type of fat consumed appears to be more important in cancer development. For example, diets high in polyunsaturated omega-6 fatty acids have a strong positive association with breast cancer whereas polyunsaturated omega-3 fatty acids (from fish oil) or olive oil (high in monounsaturated oleic acid) may protect against breast cancer (Kaizer, et al., 1989, Franceschi, et al., 1996, Knight, 1999).

The concept of chemoprevention of cancer by micronutrients is based upon evidences from human epidemiology, from studies of animal carcinogenesis models and cell culture studies for cancer inhibiting potential of certain minerals and vitamins. These micronutrients are diverse with respect to chemical structure and physiological effects and include vitamins A, B, C, D and E; and calcium, selenium, magnesiu, etc. The dietary intake of various micronutrients has been observed to alter the incidence and mortality of various women cancers. Micronutrients have been found to modulate the formation and bioactivation of carcinogens, modify the promotion and progression of carcinogenesis, alter cellular and host defense and affect cellular differentiation ultimately leading to variation in tumor incidences (Reddy, 1996; Blot, 1997; Shklar, 1998).

Vitamin A (retinol, retinal-retinyl esters or retinoic acid) deficiency could increase the susceptibility of cancer development (Tanaka, et al., 1983). Also, several epidemiological studies indicated an inverse relationship between vitamin A and cancer development in respiratory organs, urinary bladder, breast and skin (Rao, et al., 1990; Weisburger, 1991). The biological functions of retinoids which are considered to be responsible for their inhibitory effects on carcinogenesis are: suppression of malignant
transformation, counteracting the tumor promoters, inhibition of cell proliferation that is influenced by growth factors, maintenance of intercellular cell communication through gap junction and alteration of humoral and cell mediated immunity. However, all retinoids are associated with hypervitaminosis as their side effect.

β-carotene is a naturally occurring pigment and is most efficiently converted into provitamin A carotenoids. There is an inverse association between β-carotene intake and the risk of cancer. β-carotene supplemented with vitamin E and selenium has been found to lower cancer mortality rate in animal model systems (Blot, 1997).

Vitamin C (ascorbic acid) is an essential nutrient for humans and is present in fruits and vegetables. It is recognized as an agent with broad biological function and importance for synthesis of hormones and neurotransmitters, cytochrome P450 activity and detoxification of exogenous compounds, antioxidant functions with protective action that may operate against carcinogenesis (Das et al., 1993). It has been shown to be an effective chemopreventive agent against mammary carcinogenesis in rat (Ramesha et al., 1990). Wattenberg (1985) categorized vitamin C as a preventing agent in the formation of certain carcinogens from precursor compounds.

Vitamin E (α-tocopherol) is physiologically important in the activation of enzymes associated with hematopoiesis, drug metabolism and pollutant detoxification. α-tocopherol at low pH could inhibit endogenous nitrosation reactions that lead to the formation of carcinogenic nitrosoamines. Epidemiological data have suggested that diet rich in vitamin E is associated with lowered risk of cancer (Franceschi, 1997; Peng et al., 1998).

A significant amount of data suggest that a deficiency of either vitamin B12 or folic acid enhances the activity of various chemical carcinogens in various organs suggesting a co-carcinogenic effect of inadequate intake of this vitamins. Deficiency of folic acid increases the expression of certain chromosomal fragile sites, which are associated with oncogenes and breakpoints thought to be relevant for specific cancers.

Among minerals, the best defined function of selenium is its role as a cofactor for glutathione peroxidase, an enzyme that protects against oxidative tissue damage. It also suppresses cell proliferation, enhances immunoreponses and/or alters the metabolism of carcinogens towards production of less toxic compounds via its role in mixed function oxidase system in the liver. Sufficient amount of data clearly indicate a protective role of
oxidase system in the liver. Sufficient amount of data clearly indicate a protective role of selenium against certain type of tumors (skin, liver, lung, breast, cervix and colon cancers) in animal models, although some experiments showed enhancing effects of selenium on liver, skin and pancreatic carcinogenesis (Birt, 1986; Rao et al., 1990; Ramesha et al., 1990; Hussain et al., 1992).

Increase in the dietary level of calcium has been found effective in reducing the ornithine decarboxylase activity in the human and rodent colonic mucosa. Chemopreventive trials have shown that diet rich in calcium may be protective against breast cancer (Negri et al., 1996). Several studies have indicated the role of magnesium as antitumorigenic agent (Tanaka, et al., 1989; Ramesha et al., 1990; Mori, et al., 1992). Iron, zinc, copper and molybdenum which are essential for many enzyme systems are also implicated in modulating carcinogenesis, but sufficient evidences are not available to link such associations.

2.3.2 Plants/plant products as chemopreventive agents

_Curcuma longa_ (rhizome) has been shown to possess chemopreventive potential against stomach cancer and skin cancer induced by chemical carcinogens B(a)P and DMBA, respectively. Its neoplastic action was supported by increase in hepatic GSH level and GST activity and decrease in hepatic cytochrome b5 and cytochrome P450 levels (Magnus et al., 1992). Curcumin (diferuloylmethane), an active component of turmeric, is a strong antioxidant, free radical scavenger and a potent inhibitor of nitrosation reaction (Nagabhushan et al., 1988; Krishnasamy and Raghuramulu, 1998; Choudhary et al., 1999).

Mustard oil (_Brassica_ sps.) exerts its chemopreventive effect by inducing the enzymes of drug detoxification and also changing the profile of the antioxidant defense system (Kumari, 1990, Hashim et al., 1998). Garlic (_Allium sativum_) is a popular spice and is added to several edible preparations all over the world. It has been shown to have chemopreventive action on skin carcinogenesis induced by B(a)P and DMBA in animal model system (Sadhana et al., 1988; Rao et al., 1990). Garlic contains several organo-sulphur compounds like allyl methyl trisulphide, diallyl sulphide and diallyl disulphide which have been shown to inhibit the carcinogen induced neoplasia in mice by inhibiting the activation of carcinogen (Wattenberg et al., 1989; Guyonnet et al., 1999). Rao (1984)
tumorigenesis and DMBA induced mammary tumor incidence in rats. Betel leaf is rich in ascorbic acid, phenols, asparagine, glycine, proline and tryptophan which are known to act as good antioxidants, may contribute to anticarcinogenic action of betel leaf. The aril of *Myristica fragrans*, commonly known as mace, is consumed as spice and is also used in traditional medicine. It has been shown to enhance the hepatic GST activity and acid soluble GSH level, hence might be effective in detoxification of xenobiotics including chemical carcinogens (Kumari and Rao, 1989). Chemopreventive action of mace has also been reported on methylcholanthrene-induced carcinogenesis in the uterine cervix of mouse (Hussain and Rao, 1991). Epigallocatechin gallate (EGCG), a tea tannin, structurally a polyphenolic compound from *Camellia sinensis* has been reported to inhibit the skin cancer promoted by okadaic acid and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Fujiki *et al.*, 1996). Hydroalcoholic extract of *Ocimum* leaf has been shown to be effective in inducing drug metabolizing enzymes and antioxidant system and also in reducing the incidence of cancer in animal model system (Prashar *et al.*, 1994; Banerjee *et al.*, 1996).

Our laboratory has also investigated the influence of essential oils from naturally occurring plant dietary items such as cardamom, celery seed, cumin seed, coriander, ginger, nutmeg and xanthoxylem on hepatic carcinogen metabolizing enzymes. The observations suggest that the intake of these essential oils favourably affects the enzymes associated with activation and detoxification of xenobiotic compounds and also suppresses the formation of DNA adducts with carcinogens *in vitro* (Banerjee *et al.*, 1994; Hashim *et al.*, 1994). The oil from sandal wood (*Santalum album*) has been shown to increase the hepatic GST activity and GSH level, indicating its possible chemopreventive action on carcinogenesis through a blocking mechanism (Banerjee *et al.*, 1993).

The bioactive compounds which have been investigated for their plausible or putative cancer preventing properties include allium compounds, coumarins, dithiolthiones, flavones, glucosinolates, indoles, isothiocyanates, isoflavones, phenols, protease inhibitors and plant sterols (Wattenberg, 1992; Raj *et al.*, 1996; Cline *et al.*, 1998; Manson *et al.*, 1998; Hetch, 1999; Lahiri-Chatterjee *et al.*, 1999; Rice-Evans, 1999).

Natural products also play a dominant role in pharmaceutical care. This is especially obvious in the case of antitumor drugs. Currently, there are about 50 anticancer
drugs available. Many of these are either natural products or derivatives of plant products which are capable of inhibiting the process of carcinogenesis. Some of the widely used products are (Pezzuto, 1997):

1) Paclitaxel/taxol - *Taxus braevifolia* (Taxaceae)
2) Vincristine/oncovin - *Catheranthus roseus* (Apocyanaceae)
3) Podophyllotoxin - *Podophyllum peltatum* (Beriberidaceae)
4) Camptothecin - *Camptotheca acuminata* (Nyssaceae)

### 2.4 Xenobiotic metabolism

Most xenobiotics and endobiotics undergo extensive biotransformation in mammals in which lipophilic substances are converted into hydrophilic substances and then excreted out of the body. Two classes of enzymes have been proposed by Williams in 1971 for the biotransformation of xenobiotics, namely, phase I and phase II enzymes. Phase I enzymes introduce polar groups to the substrate compounds making them more water soluble which undergo conjugation reactions catalyzed by phase II enzymes (Table V). The reactions detoxifying foreign compounds are exceedingly important in preventing toxicity from a wide variety of xenobiotic compounds including chemical carcinogens. With the vast variety of reactions catalyzed by phase I and phase II enzymes, it is anticipated that some adverse reactions might occur. But overall, this complex system is highly protective. Presumably the overall inductive effects on both phase I and phase II systems in aggregate result in enhanced detoxification of xenobiotics, including carcinogens.

#### 2.4.1 Phase I metabolism

Phase I metabolism generates reactive electrophilic species which can interact with nucleophilic sites on target molecules (including DNA) of the cell. This task is accomplished by some of the monooxygenases of phase I machinery - cytochrome P450 system. Any modulation in the levels/activities of these enzymes will affect the xenobiotic metabolism as well as the process of carcinogenesis. So, these enzymes can serve as biochemical indices for assessing the chemopreventive ability of a compound.
Table V. Reactions classified as Phase I and Phase II metabolism.

**Phase I**
1. Oxidation involving cytochrome P450 and others
2. Reduction
3. Hydrolysis
4. Hydration
5. Dethioacetylation
6. Isomerization

**Phase II**
1. Glucuronidation/glucosidation
2. Sulfation
3. Methylation
4. Acetylation
5. Amino acid conjugation
6. Glutathione conjugation
7. Fatty acid conjugation
8. Condensation
2.4.1.1 Cytochrome P450 system

The cytochrome P450 system consists of mainly NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase, cytochrome P450 and cytochrome b5. It is located in membranes of endoplasmic reticulum and mitochondria (Ahmad et al., 1996). This enzyme system is present in all living organisms (Kargel, 1996).

Cytochrome P450

Cytochrome P450 is the terminal electron acceptor of an electron transfer system and is the site where interactions among electrons, drugs and/or xenobiotic compounds take place (Paine, 1981). Liver shows highest concentration of cytochrome P450 (Eugene et al., 1992). It is suggested that cytochrome P450 is selected during evolution to detoxify the atmospheric oxygen (Nebert, 1994). Cytochrome P450 is tightly bound to membrane and is a highly amphiphilic protein (Dean and Gray, 1982; Eugene et al., 1992). Although cytochrome P450 catalyzes the oxidation of a variety of xenobiotic chemicals through monoxygenase reaction, it is also found to have peroxidase activity in which cytochrome P450 catalyzes substrate hydroxylation using various hydroperoxides as well as H₂O₂ as co-substrate (Hrycay and O’Brien, 1972; Renneberg et al., 1978; O’Brien and Rahimtula, 1980; Aust and Svingen, 1982; Cadenas and Sies, 1982; Cavallini et al., 1983; Kappeli, 1986; Hollenberg, 1992; Thompson et al., 1995; Anari et al., 1995; Segura-Aguilar, 1996). It contains iron protoporphyrin IX as the prosthetic group which is non-covalently bound to the apoprotein. Cytochrome P450 exhibits a spectral absorbance maximum at 450 nm when reduced and complexed with carbon monoxide only when it is intact and catalytically functional. The heme-iron in conjunction with associated NADPH-cytochrome P450 reductase undergoes in a cyclic oxidation/reduction process.

There are multiple forms of cytochrome P450 which have been classified in different families and subfamilies. A number of polymorphisms have been recognized in P450 which exert a dramatic effect on its catalytic activity (Goldstein and Morais, 1994).
NADPH-cytochrome P450 reductase

Sometimes it is also referred as NADPH-cytochrome C reductase because of its ability to reduce exogenous cytochrome C (a mitochondrial heme protein that is not present in endoplasmic reticulum) in presence of NADPH+H+. This is an unique flavoprotein enzyme which contains both FAD and FMN (one mole each/mole of protein) as its prosthetic group. FAD acts as electron acceptor from NADPH+H+ and FMN as donor to cytochrome P450 (Iyanagi and Mason, 1973). A recently discovered enzyme nitric oxide synthase (Bredt et al., 1991) has regions that are homologous to NADPH-cytochrome P450 reductase.

\[
\text{NADPH-Cyt P450 reductase} \\
\text{NADPH + H}^+ \rightarrow \text{FAD} \rightarrow \text{FMN} \rightarrow \text{Cyt P450}
\]

NADPH-cytochrome P450 reductase catalyzes electron transfer from NADPH to cytochrome P450 (Alvares and Pratt, 1990), cytochrome b₅ (Enoch and Strittmatter, 1979; Ilan et al., 1981), heme oxygenase (Schacter et al., 1972), fatty acid elongase as well as nonphysiological electron acceptors (Williams and Kamin, 1962). It has been widely used in subcellular distribution studies as a marker enzyme for the membranes derived from the endoplasmic reticulum (ER) (Kargel et al., 1996).

Cytochrome b5

Cytochrome b5 is mainly present in the endoplasmic reticulum in the liver. It is also found in many other tissues (Mangum, 1970; Tamura et al., 1988). Cytochrome b5 is shown either to stimulate or to inhibit or to have no effect or even be an obligatory component of cytochrome P450 dependent oxidations, depending on the isozyme of cytochrome P450 involved and the nature of xenobiotic metabolism. Cytochrome b5 is linked with NADH and NADPH dependent electron transfer pathways through NADH-cytochrome b5 reductase and NADPH-cytochrome P450 reductase, respectively (Omura, 1978; Tamura et al., 1990; Yamazaki et al., 1996). Cytochrome b5 has been considered to be an electron donor in cytochrome P450-mediated drug and lipid metabolism (Keyes and Cinti, 1980; Tamura et al.; 1988; Yamazaki 1996). The ratio of hepatic microsomal P450
to NADPH-cytochrome P450 reductase to cytochrome b5 may be about 20:1:10 (Estabrook et al., 1971).

It is now well established that reduced cytochrome b5 can interact with the stearyl-CoA desaturase of microsomes, thereby participating in oxidative metabolism of fatty acids. Its ubiquitous distribution in various organs, coupled with its versatility in catalyzing the oxidation of structurally different xenobiotics makes it one of the most important enzymes of biotransformation.

NADH-cytochrome b5 reductase

NADH-cytochrome b5 reductase, a FAD containing flavoprotein is membrane bound enzyme and is found in the liver in highest concentration. It is also found in all tissues and organs (Tamura et al., 1987). NADH-cytochrome b5 reductase transfers the electron from NADH to cytochrome b5 which provides electron in several kinds of cytochrome P450 mediated drug metabolism and lipid metabolism (Keyes and Cinti, 1980; Lee and Kariyar, 1986; Tamura et al., 1988; Yamazaki, 1996).

2.4.1.2 Synergism between NADH and NADPH dependent electron transport systems

It is well established that NADH-dependent system and NADPH-dependent system interact with each other (Cohen and Estabrook, 1971; Yamazaki et al., 1996). It is proposed that one of the two electrons required by cytochrome P450 for hydroxylation reaction is supplied by NADH via NADH-cytochrome b5 reductase and cytochrome b5 (Noshiro and Omura, 1978; Tamura et al., 1990; Holmans et al., 1994; Yamazaki et al., 1996). Cytochrome b5 can also receive electron from NADPH via NADPH-cytochrome P450 reductase (Lu et al., 1974; Kargel et al., 1996). Electron flow in NADH and NADPH dependent system is shown in scheme 1.
2.4.1.3 Reaction Cycle of cytochrome P450

Reduction and oxygenation of cytochrome P450, while interacting with substrate (RH), electron from donor molecules and molecular oxygen ($O_2$) is shown in scheme 2.

- The cytochrome P450 ($Fe^{3+}$) interacts with substrate leading to formation of their complex.
- The substrate-cytochrome ($Fe^{3+}$) complex undergoes one electron reduction donated by NADPH via NADPH-cytochrome P450 reductase to form ferrous hemoprotein-substrate complex (Blanck et al., 1989).
- The reduced cytochrome P450 binds with oxygen, and oxy complex is formed. This complex has the ability to release superoxide anion (Rein et al., 1986).
- The second electron is introduced in the cycle is provided either by cytochrome b5 or by NADPH-cytochrome P450 reductase (Bonfils et al., 1981; Schenkman, 1993). The interaction of electron with oxy complex leads to formation of peroxy complex. From this peroxy complex hydrogen peroxide can split off.
- From the peroxy complex, terminal oxygen is removed in the form of $H_2O$ and iron-oxo intermediate is formed (Rein and Jung, 1993; Rein et al., 1986).
- The hydrogen atom is abstracted from the iron-oxo intermediate (a radical). The resulting reactive species immediately recombines to produce stable product. This process is designated as "oxygen rebound" (Groves and McClusky, 1976). Finally the hydroxylated product dissociates and the cycle starts again.
- Interestingly, in the shunt reaction (denoted by dotted line inside reaction cycle), the substrate can be hydroxylated immediately by peroxides such as hydrogen peroxide without interacting with an electron donating system.
Scheme 2. Schematic outline of the cytochrome P450 system. Fe$^{3+}$ (ferric-cytochrome P450) Fe$^{2+}$ (ferrous-cytochrome P450), P450-R (NADPH-cytochrome P450 reductase) b$_5$-R (NADH-cytochrome b$_5$ reductase), b$_5$ (cytochrome b$_5$), e (electron from donor molecule).
It is well established that the physical state of the membrane (fluidity) during irradiation plays an important role in determining the levels of injury and repair following the exposure (Yatvin et al., 1984). Numerous studies support the idea that the lipid conformation or association in membranes can influence the process of lipid peroxidation (Patterson and Redpath, 1977; Edwards and Quinn, 1982; Raleigh, 1988). Lipid peroxidation is also known to be interlinked with cytochrome P450 (Varshney and Kale, 1990).

Cytochrome P450 system plays a very significant role in biological functions. It is essential for disposition of vast number of drugs and foreign compounds. This system is also important in metabolism of endogenous compounds such as steroids, fatty acids and prostaglandins (Guengerich and Shimada, 1991; Gonzalez, 1992; Wrighton and Stevens, 1992). The cytochrome P450 system frequently prevents the lethal dose of ingested compounds being accumulated within an organism. Several factors including age, sex, radiation, etc, influence the activity of cytochrome P450. As mentioned earlier, cytochrome P450 has been shown to have peroxidase activity (Renneberg et al., 1978; O'Brien and Rahimtula, 1980; Aust and Svingen, 1982; Cadenas and Sies, 1982; Cavallini et al., 1983; Kappeli, 1986; Hollenberg, 1992; Thompson et al., 1995; Anari et al., 1995; Segura-Aguilar, 1996) and partially replaces catalase in protecting the cells against oxidative stress (Morichetti et al., 1989). Therefore, an attempt has been made to examine the effect of plant extracts on the activity of cytochrome P450 system and its link with the antioxidant potential of animals by studying antioxidative parameters GST, DTD, GSH, GPX, GR, SOD and CAT.

2.4.2 Phase II metabolism

The xenobiotic compounds which are acted upon by the phase I enzymes, become the substrates for phase II enzymes. This process of conjugation is biosynthetic which leads to the water soluble non-toxic products that are easily excretable through bile or urine. The two most important phase II enzymes investigated are glutathione S-transferase and DT-diaphorase.
2.4.2.1 Glutathione S-transferases

GSTs are predominantly present in the cytosolic fractions in most of the tissues of various organisms (Jakoby and Habig, 1980). The enzymes are suggested to have a crucial role in bringing the two substrates in close juxtaposition and in increasing the nucleophilicity of GSH probably by ionizing GSH in thiolate ion (Ketterer, 1986). In addition to catalysis of detoxification reactions, GSTs have the ability to bind various endogenous compounds which are not substrates for their enzymatic reactions. GSTs also play a profound role in binding proteins that participate in the transport or storage of exogenous and endogenous toxic compounds like bilirubin in liver. However, selenium independent GSTs are shown to exhibit glutathione peroxidase like activity with a variety of organic hydroperoxides including free fatty acid hydroperoxides (Prohaska and Ganthar, 1977; Prohaska, 1980). Thus, GSTs along with glutathione peroxidase are believed to be involved in augmenting GSH dependent defense against lipid peroxidation (Horton and Fairhurst, 1987). GSTs catalyze the nucleophilic addition of tripeptide glutathione to the substrates having functional group, with the primary role of detoxification of endogenous and exogenous compounds including carcinogens. The enzyme is found in most of the aerobic microorganisms, plants and animals (Armstrong, 1991).

Cytosolic GSTs are dimeric proteins existing as homodimer or heterodimer. GSTs represent a multigene family. In higher organisms there are evidences of three gene classes of cytosolic enzymes designated as α, μ and π. Each gene class consists of two or more genes that encode different subunit types. GST isoenzymes exhibit tissue, age and sex dependent expression (Mannervik and Danielson, 1988). GST π is a characteristic marker of tumors (Coles and Ketterer, 1990).

Glutathione conjugate is converted to corresponding cysteine conjugate following sequential removal of glutamate and glycine. Cysteine conjugate is either metabolized to a mercapturate by acetylation or cleaved to a mercaptan by β-lyase. In addition to mercapturic acid pathway (Habig et al., 1974; Pickett and Lu, 1989), methylation of thiol to form methylthio containing metabolite and glucuronidation of mercaptan to form thioglucuronide represents important metabolic steps for biotransformation of cysteine conjugate. They may be excreted via bile or urine. Thiols act as protective agents against
electrophiles, radical damage and oxidative stress. GST is one of the enzymes which catalyzes the antioxidant processes of thiols (Choudhary et al., 1997).

2.4.2.2 DT-diaphorase [NAD(P)H: Quinone oxidoreductase]

Ernster first characterized and termed this enzyme as DT-diaphorase (DTD) for its catalyzing property of the oxidation of NADH and NADPH (earlier known as DPNH and TPNH) at equal rates. This flavoprotein is predominantly present in the cytosolic fraction (95% of the total activity) of liver. DT-diaphorase [NAD(P)H:quinone oxidoreductase], is a FAD containing flavoprotein and consists of two identical subunits (Lind et al., 1990). It is widely distributed in animal kingdom except pigeons. Almost all tissues have DT-diaphorase, however, the richest source is liver (Benson et al., 1980; Schlager and Powis, 1990; Belensky and Jaiswal, 1993). It has shown to have a broad specificity for a variety of hydrophobic quinones including benzoquinones, naphthoquinones, ubiquinones and vitamin K derivatives (Ernster et al., 1962) which serve as substrates during xenobiotic metabolism.

There are two alternative pathways in the quinone reduction. A one electron reduction (catalyzed cytochrome P450 system) yields a semiquinone radical in presence of oxygen. Most semiquinones rapidly autooxidize to form the superoxide anion radical (O$_2^-$) and have been invoked to explain the cytotoxic and antitumor properties of quinoid drugs via binding to nucleic acids (Benson et al., 1980). In the other pathway, quinone is reduced by two electrons catalyzed by DTD into non-toxic hydroquinones. These are subsequently converted into sulfate or other conjugates in the presence of other phase II enzymes. Sulfotransferases, methyl transferases and N-acetyl transferases are the other phase II conjugation enzymes which bring about sulfation, methylation and acetylation of various functional groups. All these conjugates are primarily eliminated in urine (Jakoby and Habig, 1980).

DT-diaphorase is unique as it displays non-specific reactivity towards NADH and NADPH and shows a broad electron acceptor specificity, catalyzing the reduction of quinones, quinone epoxides, quinoneimines, certain aromatic nitro compounds, aromatic C-nitroso compounds, azo dyes, hexavalent chromium etc. (Lind et al., 1990; Cadenas et al., 1992).
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The most striking feature of DTD is its ability to catalyze two electron transfer (Iyanagi and Yamazaki, 1970) leading to the formation of hydroquinones from quinones.

\[ Q + \text{NAD(P)H} + H^+ \xrightarrow{\text{DTD}} \text{QH}_2 + \text{NAD(P)}^+ \]

Although, some metabolites generated from DTD catalyzed reaction could be cytotoxic. This enzyme is known for its antioxidant property. Indeed, DTD belongs to a family of phase II detoxification enzymes which includes GST and glutathione peroxidase with other transferases and reductases (Nebert, 1994) which divert potentially active electrophiles from damaging interactions with nucleophilic group of DNA and ultimately protect tissues against carcinogenic and mutagenic compounds (Talalay and Benson, 1982; Riley and Workman, 1992; Ross et al., 1993).

DTD prevents the formation of semiquinones by one electron reduction and in turn the generation of free radicals from the autooxidation of semiquinones (Lind et al., 1982). DTD also decreases the electrophilic characters of quinones by aromatization of quinonoid ring, and restricting its participation in arylation reaction, thereby avoiding cytotoxic effects (O'Brien, 1991).

DTD activity is reported to increase with the activity of other antioxidant enzymes such as SOD, catalase and glutathione peroxidase (Whitney and Frank, 1993; Prestera et al., 1993). Recently, DTD has been shown to protect biological membrane against oxidative damage (Landi et al., 1997). The antioxidant functions of DTD are attributed to its ability to maintain membrane bound enzyme, coenzyme Q (CoQ) in reduced antioxidant state and to provide protection against free radical damage. It is suggested that DTD had been selected during evolution to act as CoQ reductase to protect cellular membrane components from free radical damage (Beyer et al., 1996).

2.4.3 Phase III Metabolism

Glutathione conjugates (cysteine conjugates and mercapturic acid) are further metabolized by c-s lyase and glucuronidase in the intestinal microflora transfering the -SH group from glutathione to the substrate. A subsequent methylation and reabsorption takes
place, while S-methylate compound is oxidized in the liver to a methylthio-derivative and then excreted (Gustaffson et al., 1981).

2.4.4 Antioxidant defense mechanism

To cope up with the damaging effect of superoxide radical \( \left( O_2^- \right) \), \( H_2O_2 \) and other reactive species which are produced by the exposure to radiation or xenobiotics, cells exhibit various inherent protective features (Figure III), which are collectively termed as 'antioxidant defense mechanisms' (Puglia and Powell, 1984). Superoxide dismutase and catalase are the most important antioxidant enzymes which protect the cells from reactive oxygen species.

\[
\text{SOD} \quad O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2
\]

\[
\text{CAT} \quad 2H_2O_2 \rightarrow 2H_2O + O_2
\]

Catalase is localized exclusively in peroxisomes and deals with the large amount of \( H_2O_2 \) present in them. Some iron binding macromolecules like transferrin and ferritin are prevented from forming free radicals by the system. Superoxide dismutase catalyzes the dismutation of \( O_2^- \) (formed by the monovalent pathway of biological oxidation) to \( H_2O_2 \) and \( O_2 \). The SOD-catalase system forms a first line of defense against oxygen toxicity. Glutathione cycle (involving GSH, GPX and GR) also plays an important role in eliminating reactive oxygen species (Figure IV). Oxidative stress has profound effect on carcinogen activation, gene expression and enzyme activity which are closely linked to the process of carcinogenesis (Figure V).

2.4.4.1 Glutathione (GSH)

Glutathione (\( \gamma \)-glutamylcysteinyl glycine) is one of the most abundant, small organic and unique molecule in which glutamyl moiety is bound via the \( \gamma \)-carboxyl group. Most of its biological functions depend on thiol group of its cysteinyl residue (Chasseaud, 1979). \( \gamma \)-glutamyl bond renders it less susceptible to autooxidation and resistant to
Free Radicals in Chemical Pathogenesis

Figure III. Diagrammatic representation of several hypothetical pathways which may mediate (radical) oxidant-induced cellular damage.
Figure IV. Reactive oxygen species production and disruption of cellular homeostasis. ROS can be produced by both endogenous and exogenous sources. An overload of the normal antioxidant defense system by these reactive oxygen molecules will result in oxidative stress and eventual oxidative damage to critical cellular macromolecules. Abbreviations: CAT, catalase; GSH, reduced glutathione; GPX, glutathione peroxidase; SOD, superoxide dismutase; Vit C, vitamin C; Vit E, vitamin E.
Oxidative stress interacts with all three stages of the cancer process. During the initiation stage oxidative DNA damage may produce gene mutations and structural alterations of the DNA, resulting in a heritable mutation. During the promotion stage ROS and oxidative stress can contribute to abnormal gene expression, blocking of cell-to-cell communication, and modification of second messenger systems, resulting in an increase in cell proliferation or a decrease in apoptosis in the initiated cell population. This results in the clonal expansion of the initiated cells to pre-neoplastic focal lesions. Oxidative stress may also participate in the progression stage of the cancer process by imparting further DNA alterations to the initiated cell population. These changes may result in changes in enzyme activity and make the lesions resistant to normal growth control. Abbreviation: GJIC, gap junctional intercellular communication.
peptidases. The main functional form of glutathione is the reduced form (GSH) which is dynamically interchangeable with its oxidized disulfide form (GSSG).

Glutathione performs numerous diverse functions like synthesis of protein and DNA, transport of acids, ions and/or sugars, regulation of enzyme activity, protection of cells against reactive oxygen compounds, maintenance of membrane integrity, etc. (Meister, 1994). It participates in spontaneous scavenging of electrophiles or free radicals and in reactions catalyzed by enzymes like GST and GPX. Glutathione also functions as redox buffer by reacting reversibly with other thiol groups, thus affecting redox state of protein-thiol functions. Glutathione can also serve as storage form of cysteine. GSH depletion may occur in response to starvation, excess electrophilic burden on the body, oxidative stress, hormonal imbalance, growth and development (Kretzschmar and Klinger, 1990).

2.4.4.2 Glutathione peroxidase

Mills first demonstrated glutathione (GSH) dependent activity of glutathione peroxidase (GPX) in 1957, in bovine red cell lysate. Two major types of GPX have been found. One type is known as selenoenzyme, in which selenium is covalently bound as selenocysteine, in its active site. This selenium dependent enzyme is active with both organic hydroperoxides and H₂O₂. It is homotetrameric protein of about 80 kd, each subunit containing one selenium atom. Its catalytic site forms a flat depression on the surface of the molecule (Mannervik, 1985).

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

GPX activity has been detected in all mammalian tissues and it mostly resides in the cytosolic fraction. Selenium independent GPXs show similarity with the properties of some of the GSTs and have substrate specificity and kinetic characteristics that are different from those of selenium dependent activity. They have low activity towards organic hydroperoxides and none at all for H₂O₂ (Mannervik, 1985). The biological significance of non-selenium dependent GPX activity has not been determined.
On the basis of sequence similarity, six members of the selenium-dependent GPX have been recognized, which are summarized in the following table;

<table>
<thead>
<tr>
<th>Systematic designation</th>
<th>Other designations</th>
<th>Year recognized as distinct form</th>
<th>Selenocysteine present</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPX-1</td>
<td>Cellular or classical GPX</td>
<td>1957</td>
<td>Yes</td>
</tr>
<tr>
<td>GPX-2</td>
<td>GPX G1</td>
<td>1990</td>
<td>Yes</td>
</tr>
<tr>
<td>GPX-3</td>
<td>Plasma or extracellular GPX</td>
<td>1986</td>
<td>Yes</td>
</tr>
<tr>
<td>GPX-4</td>
<td>Phospholipid hydroperoxide GPX</td>
<td>1985</td>
<td>Yes</td>
</tr>
<tr>
<td>GPX-5</td>
<td>Epididymis GPX like protein</td>
<td>1990</td>
<td>No</td>
</tr>
<tr>
<td>GPX-6</td>
<td>Olfactory GPX like protein</td>
<td>1991</td>
<td>No</td>
</tr>
</tbody>
</table>

GPX-1 is the most abundant selenoprotein which contains almost one-third of the total body selenium. Liver GPX-1 is more sensitive to selenium supply and because of this, its activity in liver serves as an excellent index of selenium nutritional status. It is restricted to use free hydroperoxides as substrates.

The physiologic function of GPX-2 may be to use large amount of hydroperoxides that arise during oxidative stress. The substrate specificity of GPX-2 was similar to that of GPX-1 except that the activity of GPX-2 was relatively higher towards organic hydroperoxides when normalized against H$_2$O$_2$ metabolizing activity (Chu et al., 1993).

GPX-3 and GPX-5 are extracellular in location. GPX-3 activity has been measured in plasma, liver, kidney and breast (Yoshimura et al., 1991). GPX-5 is expressed in epididymis, secreted into the lumen of seminiferous tubules and is associated with the head of the sperm (Jimenez et al., 1992). It has been suggested that it prevents the acrosomal reaction. GPX-4 is a monomeric protein and is first purified from the pig heart in 1985. It has been postulated to protect against lipid peroxidation as it is capable of reducing fatty hydroperoxides that are esterified in phospholipids (Ursini et al., 1985). It has a
characteristic tissue distribution and its activity is low in rat liver but very high in testis (Roveri et al., 1992). GPX-6 appears to be expressed only in the Bowman's glands of the olfactory system and thus has been postulated to metabolize odorants (Dear et al., 1991).

Enhanced GPX expression is known to protect cells from hydroperoxides but not from radiation and doxorubicin (Liebmann et al., 1995). Activity of the GPX varies among tissues and between species and is also affected by environmental factors, including selenium nutritional status and oxygen tension. GPX proteins are regulated at transcriptional, post-transcriptional and translational levels. The details and precise mechanisms of the regulation are still not known (Roveri et al., 1994). Selenium deficiency is likely to be related to mRNA stabilization (pre-translational effect) because transcription does not appear to be involved (Sunde, 1990). The informations available, indicate that the regulation of these enzymes is complex and multifactorial.

General mechanism of selenium dependent GPXs is as follows; (Flohe, 1988)

![Mechanism of selenium dependent GPXs](image)

The mechanism has two components: oxidation of the enzyme and reduction of it. These processes are distinct from one another and thus the mechanism is ping-pong. The
reduced selenolate form of the enzyme reacts rapidly with a hydroperoxide (ROOH) to
yield the detoxified alcohol (ROH) (water in case of H₂O₂) and the selenic acid form of the
enzyme. GSH (or other thiols) reduces the enzyme in two steps, back to the selenolate with
the generation of GSSG. The rapidity of the reaction varies among the enzymes.

2.4.4.3 Glutathione reductase

Glutathione reductase (GR) is a dimeric protein of 120 kDa, containing two FAD
molecules at its active site (Sun, 1990). It is distributed in cytosol and mitochondria of
almost all the organs of the body. GR catalyzes the NADPH dependent reduction of
 glutathione disulfide to glutathione. This reaction is essential for maintaining glutathione
level which is important for several cellular functions including detoxification and
protection against free radical damage (Le et al., 1995). It is known as secondary
antioxidant as it helps in regeneration of reduced glutathione which is consumed by GPX
to protect cells from H₂O₂ and hydroperoxide mediated damage.

\[
\text{GR} \quad \text{GSSG} + 2 \text{NADH} + \text{H}^+ \underset{\text{SOD}}{\longrightarrow} 2 \text{GSH} + 2 \text{NADP}^+
\]

2.4.4.4 Superoxide Dismutase

Superoxide dismutase (SOD) was isolated by Mann and Keilin in 1939, as a copper
containing protein from bovine erythrocytes and called as hemocuprein. Its antioxidant
function of catalyzing the dismutation of superoxide radicals was identified by McCord
and Fridovich (1969). SOD catalyzes the conversion of O₂⁻ to H₂O₂ in the presence of any
substrate that provides protons and the overall rate of dismutation at pH 7.0 is about 5 \times
10⁵ M⁻¹S⁻¹ (Harris, 1992; Baez et al., 1994).

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \underset{\text{SOD}}{\longrightarrow} \text{H}_2\text{O}_2 + \text{O}_2
\]

There are two families of genes encoding SODs. First family encodes Cu, Zn-SOD,
which is ubiquitously distributed among eukaryotes as a cytosolic enzyme. These are also
secreted from cells as glycosylated extracellular SODs (EC-SODs) (Marklund et al., 1982). The second family contains SODs that utilize either manganese or iron as the redox-active metal cofactor.

2.4.4.5 Catalase

Catalase (hydrogen peroxide oxidoreductase), a heme protein with a single substrate-H$_2$O$_2$, is ubiquitously distributed in tissues of all species, which was first isolated from ox liver and later from blood and other sources (Aebi, 1984; Harris, 1992). Its maximum activity has been found in liver and erythrocytes. It serves two functions (a) decomposition of hydrogen peroxide and (b) oxidation of hydrogen donor e.g. methanol, ethanol, formic acid, phenols, etc. with the consumption of one molecule of peroxide.

Catalase degrades H$_2$O$_2$ to oxygen and water with a turnover number of the order of $10^8$. It is one of the few protein based enzymes in mammalian cells that does not follow classical Michaelis-Menten kinetics (Chuang et al., 1989). Besides degrading H$_2$O$_2$, catalase also mediates the peroxidation of alcohol to aldehyde. Thus, catalase decomposes H$_2$O$_2$ into different reaction modes, catalytic (equation 1 and 2) and peroxidatic (equation 1 and 3).

\[
\begin{align*}
\text{catalase} + \text{H}_2\text{O}_2 \rightarrow & \quad \text{catalase-H}_2\text{O}_2 \quad & \text{... 1} \\
\text{catalase-H}_2\text{O}_2 + \text{H}_2\text{O}_2 \rightarrow & \quad \text{catalase} + 2\text{H}_2\text{O} + \text{O}_2 \quad & \text{... 2} \\
\text{catalase- H}_2\text{O}_2 + 2\text{R-OH} \rightarrow & \quad \text{catalase} + 2\text{R}=\text{O} + 2 \text{H}_2\text{O} \quad & \text{... 3}
\end{align*}
\]

It has been demonstrated that the supply of H$_2$O$_2$, and not enzyme activity, is in fact the predominant rate limiting factor for ethanol metabolism via catalase-H$_2$O$_2$.

\[
\begin{align*}
\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O}_2 \rightarrow & \quad \text{CH}_3\text{CHO} + 2 \text{H}_2\text{O} \quad & \text{... 4} \\
\text{ROOH} + \text{AH}_2 \rightarrow & \quad \text{H}_2\text{O} + \text{ROH} + \text{A} \quad & \text{... 5}
\end{align*}
\]

The absolute rate of peroxidation depends upon the relative concentrations of catalase heme, H$_2$O$_2$ and peroxidative substrates.
2.4.5 Lipid Peroxidation

The possible role of free radicals in the initiation, promotion and progression of carcinogenesis is getting well established. Free radicals are generated in many ways which can attack the polyunsaturated fatty acids (PUFA) of membrane lipids. This oxidative deterioration of membrane lipids is known as 'lipid peroxidation'. Lipid fluidity confers several properties on membranes which are essential to the correct functioning of the cell (for example, operation of membrane-associated receptors, enzymes etc., and efficient diffusion of water). In eukaryotes, fluidity is maintained by incorporated PUFA chains into membrane lipids. In radiolytic systems, free radicals generated from water can attack fatty acids and result in homolytic dissociation of C-H bond which may lead to the initiation of lipid peroxidation. Lipid peroxidation is a chain reaction (reactions 1-7) which involves three distinct stages: (a) initiation, (b) propogation and (c) termination.

Initiation

\[
\begin{align*}
H_2O & \rightarrow OH_\cdot, H_\cdot, e^-_\text{aq}, O^-_2 & \quad \ldots 1 \\
LH + OH_\cdot & \rightarrow L' + H_2O & \quad \ldots 2
\end{align*}
\]

Propagation

\[
\begin{align*}
L' + O_2 & \rightarrow LOO' & \quad \ldots 3 \\
LH + LOO' & \rightarrow LOOH + L' & \quad \ldots 4
\end{align*}
\]

Termination

\[
\begin{align*}
L' + L' & \rightarrow LL & \quad \ldots 5 \\
LOO' + LOO' & \rightarrow LOOL + O_2 & \quad \ldots 6 \\
LOO' + L' & \rightarrow LOOL & \quad \ldots 7
\end{align*}
\]

A free radical that has sufficient energy to abstract an allylic hydrogen from methylene carbon of PUFA, can initiate lipid peroxidation process. OH\textsuperscript{•} (hydroxyl radical) free radical is considered to be responsible for initiation (reaction 2). The presence of double bond in fatty acids weakens the C-H bond adjacent to the double bond and makes hydrogen removal easier. The carbon centered radical (L\textsuperscript{•}) reacts rapidly with molecular
oxygen to form LOO' (reaction 3). In subsequent much slower reaction, LOO' attacks another lipid molecule (LH) forming non-radical LOOH while generating new lipid radical L' (reaction 4). The latter can be converted to LOO' on encounter with oxygen, closing the self propagating cycle (reaction 3). Thus, once initiated, lipid peroxidation proceeds to establish a chain reaction with a low energy requirement. In termination, two free radicals combine to yield a non-radical product to end the chain reaction (reaction 5-7) (Patterson and Redpath, 1977; Edwards and Quinn, 1982; Kale and Sitawad, 1990).

The major detrimental effects of lipid peroxidation are imparted on cellular membranes. Free radicals derived from PUFA can form cross linkages of proteins, thereby inactivating their enzymatic/receptor functions. Products of lipid peroxidation like MDA can interact with DNA to form toxic products. In addition, destruction of cytochrome P450, loss of activity of glucose-6-phosphotase and UDPGT has also been reported. In mitochondria the peroxidation causes membrane swelling, deterioration of electron transport and organelle lysis. The mutagenic and carcinogenic effects of barriers fatty acid hydroperoxides and MDA have been demonstrated by the Ames test (Horton and Fairhurst, 1987).

2.4.6 Lactate dehydrogenase

Lactate dehydroganase (LDH) is an oligomeric enzyme and is reported to be present in all tissues. LDH catalyzes the reversible conversion of pyruvate to lactate in the presence of coenzyme NADH (Bergmeyer and Bernt, 1974).

\[
\text{LDH} \quad \text{Pyruvate} + \text{NAD}^+ + \text{H}^+ \rightleftharpoons \text{Lactate} + \text{NAD}^+
\]

LDH is a cytoplasmic marker enzyme which is well known indicator of damage induced by several factors including xenobiotic compounds and radiation (Reddy and Lokesh, 1996; Deters et al., 1998).
2.4.7 Review of work on plants investigated

Recent upsurge in identifying non-dietary natural products associated with high degree of safety margin as cancer chemopreventive agents, has been hailed by many investigators to be practically beneficial, especially when the carcinogenic insult is mild to moderate. Our present knowledge on chemoprevention of cancer has revealed the presence of a diverse array of naturally occurring bioactive compounds which inhibit carcinogenesis at almost every site (Tanaka, 1994; Morse and Stoner, 1996; Pezzuto, 1997). The present investigation deals with the cancer chemopreventive potential of extracts of *Tinospora cordifolia*, *Andrographis paniculata*, *Adhatoda vesica*, *Aloe vera*, *Aegle marmelos*, *Clerodendrum inerme*, *Lawsonia alba*, *Prosopis juliflora* and *Decalepis hamiltonii* in the murine model system. The chemopreventive potential is assessed by the evaluation of the levels/activities of phase I and phase II drug metabolizing enzymes and antioxidant status in mice.

2.4.7.1 *Tinospora cordifolia*

*Tinospora cordifolia*, a glabrous, climbing shrub of family Menispermaceae has been known in India, to be beneficial in treating a wide variety of diseases. The aerial roots and the stem of the plant are the sources of drug preparation. A number of different principles including, alkaloids (berberine), bitter compounds (tinosporin, tinosporic acid and tinosporol), essential oil and a mixture of fatty acids have been identified as contributing to the observed medicinal effects (Wealth of India, 1989). In Indian system of medicine, *Tinospora* has been known to promote longevity and to increase body resistance against diseases (Bhatt and Bhatt, 1996). Animal investigations have revealed that *Tinospora* affords protection against xenobiotic induced liver damage including fibrosis, and stimulates liver regeneration (Rege et al., 1984). Furthermore, *Tinospora* has also been accredited to alleviate immunomodulatory response and Kupffer cell functioning in human beings, and to exhibit insulin-like action under experimental conditions (Wadood et al., 1992; Nagarkatti et al., 1994; Sohni and Bhatt, 1996; Kapil and Sharma, 1997).
2.4.7.2 Andrographis paniculata

Andrographis paniculata (Acanthaceae), traditionally employed as folklore remedy for a wide spectrum of ailments, is now a days incorporated into a number of herbal medicinal preparations. It is a shrub with considerable reputation as potent adjunct for various ailments including jaundice, cholestasis, inflammatory conditions, and ameliorating toxicity by hepatotoxins (Deng et al., 1982; Handa and Sharma, 1990; Shukla et al., 1992; Visen et al., 1991, 1993). In addition, its effectiveness as immunostimulant (Puri et al., 1993) and anti-HIV (Chang and Yeung et al., 1988) has been reported. Furthermore, its efficacy in treating common cold and pharyngotonsillitis has also been described (Thamlikitkul et al., 1991; Haneke et al., 1995). No acute or subchronic toxic effects of A. paniculata are known (>17 gm/kg, LD₅₀) (Chang and But, 1987). However, reports on induction of infertility in male rats by subchronic doses of this plant have been contradicted by Burgos et al., (1997) who found no subchronic testicular toxic effect in male rats.

The active principle in Andrographis, andrographolide (a diterpenoid) has been accredited with significant protective activity against liver disorders induced by a number of hepatotoxics, as judged by significant reduction in serum enzymes-SGOT, SGPT and alkaline phosphatase and by assessing the degree of liver damage histologically (Handa and Sharma, 1990; Bhatt and Bhatt, 1996).

2.4.7.3 Adhatoda vesica (Justicia adhatoda)

Adhatoda vesica, an evergreen, gregarious, stiff and perennial shrub of family Acanthaceae has been used in treating a wide variety of diseases by folk and Ayurvedic medicine practitioners. Leaves of the plant are the main source of drug preparation. A number of different principles including, alkaloids (vesicine, vesicinone, vesinol), essential oil (betane), vitamins (vitamin C, β-carotene), a non-crystalline steroid (vasakin) and a mixture of fatty acids have been identified as contributing to the observed medicinal effects of the plant (wealth of India, 1989). The shrub is the source of the drug ‘vasaka’, well known in the indigenous system of medicine for its beneficial effects, particularly in bronchitis. The leaves as well as flowers, fruits and roots are extensively used for treating
cold, whooping cough, asthma and as antihelmintic. The leaf juice is stated to cure diarrhea, dysentry and glandular tumor. As a corollary to this, in vivo investigations have revealed that Adhatoda possesses a promising teratologic effect in rats (Nath et al., 1992), and has growth inhibitory effect on Mycobacterium tuberculosis (Grange and Snc!!., 1996). Furthermore, Adhatoda has also been accredited to afford protection against allergen-induced bronchial obstruction in guinea pigs (Dorsch and Wagner, 1991).

In present study, an attempt to investigate cancer chemopreventive potential in hydroalcoholic extract of Adhotoda vesica has been undertaken by evaluating its role, if any, in intervening carcinogenesis process by appreciably inducing detoxification (glutathione S-transferase, DT-diaphorase) and/or blocking activation enzymes viz., microsomal hemeproteins and cytochrome P450 dependent mixed function oxidases. Extrapolating the results obtained would highlight mechanistic insight to antihepatotoxic and hepatotherapeutic activity reportedly associated with this plant, since previous investigators concluded the hepatoprotective ability of this plant extract based on serum marker enzymes and barbiturate induced sleeping time as a measure of enzyme induction.

2.4.7.4 Aloe vera

Aloe vera belonging to family Liliaceae is predominantly found in dry localities in most parts of India from dry westward vallies of the Himalayas upto Cape Comorin. Aloe leaves reportedly have immense medicinal value. Its juice is commonly used on burns and minor cuts for enhancing healing of dermal wounds (Chitra et al., 1998). In addition, it is also used as an emollient, purgative, laxative, antibacterial, anaesthetic and as antiseptic. Washing eyes with Aloe is suggestive of protecting the eyes from ultraviolet rays. Furthermore, recently angiogenic activity of Aloe vera gel has been documented in vitro (Lee et al., 1998). Aloe vera gel along with vitamin C supplementation were found to be effective to reduce the severity of chemical carcinogenesis in rat (Shamaan et al., 1998). Another independent study by Lissoni et al., (1998) found additive protective effect of Aloe along with melatonin in terms of stabilization of disease and survival, in patients with advanced solid tumors, for whom no other standard effective therapy is available.

A major sugar present in Aloe gel which is associated with wound healing activity and anti-inflammatory property of the leaf extract has been identified as a growth
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substance mannose-6 phosphate (Davis et al., 1991, 1994; Vazquez et al., 1996). Hypoglycemic effect of Aloe gel plays a role in accelerated healing of dermal wound in diabetic rat (Chitra et al., 1998). Anti-inflammatory potency of Aloe has also been evaluated experimentally against wide spectrum of irritants like kaolin, carrageenan, albumin, dextran, gelatin and mustard.

2.4.7.5 Aegle marmelos

Aegle marmelos is a spinous tree belonging to family Rutaceae. It is extensively planted near Hindu temples for its leaves and wood which are used for worship, and also for its edible fruits which are valued in indigenous medicine. Besides fruits, the leaf, root, bark and seed of Aegle are also valued in Ayurvedic system of medicine. The root is an ingredient of the 'dasmula' (ten roots), a medicine commonly used by the Ayurvedic practitioners. The leaves are bitter and used as fabricule. It is also used as a remedy for ophthalmia, ulcers, dropsy, cholera and beriberi associated with weakness of heart.

Fresh leaf extract of Aegle is reported to have cardiotonic effect and also found to decrease the requirement of circulatory stimulant. Its aqueous and alcoholic extracts possess cardiotonic effect like digitalis. It contains alkaloids (aegeline, aegelenine) and essential oil. The alkaloid aegeline is efficacious in asthma and other respiratory problems. Essential oil contains cineol, p-cymene, citronellol, citral, cumminaldehyde, d-limonene and eugenol. The essential oil has shown a broad spectrum of antibacterial and antifungal activity. (Pattnaik et al., 1996; Rana et al., 1997). The aqueous decoction of Aegle leaf has shown significant hypoglycemic effect (Karunanayake et al., 1984). Aegle leaf extract also helps in the regeneration of damaged pancreas (β-cells) in diabetic rats (Das et al., 1996) and is found to be as effective as insulin in restoration of blood glucose and body weight to normal levels (Seema et al., 1996).

2.4.7.6 Clerodendrum inerme

Clerodendrum inerme is a green shrub belonging to family Verbanaceae. It is cultivated as hedge plant. It contains iridoid glycosides (inerminosides A, A1, B, C and D) (Calis et al., 1994) and sterols (ethyl sterols- α and β forms) (Akihisa et al., 1989). Two
proteins, CIP 29 and CIP 34 isolated from Clerodendrum leaves, shown to induce systemic resistance against viruses and are also found to have ribosome inactivating properties (Olivieri et al., 1996).

2.4.7.7 Lawsonia alba

Lawsonia is a glabrous, much branched shrub that belongs to family Lathyraceae. It is commonly called as Henna which is widely used in India and other countries for colouring palms and feet and dyeing silk and wool. The principle components identified in Henna leaves include lawsone, gallic acid, glucose, mannitol, fat, resin and traces of alkaloid. Henna leaves are also used against skin diseases, boils, burns, bruises and skin inflammation.

The crude ethanolic extract of Lawsonia has been reported to have significant and dose dependent anti-inflammatory, analgesic and antipyretic effects in rats. The extract also increases the pentobarbitone-induced sleeping time (Ali et al., 1995). Hydroalcoholic extract of Lawsonia has been shown to possess hepatoprotective activity against CCl₄-induced liver toxicity (Anand et al., 1992). Tuberculostatic activity of henna has also been shown in vitro as well as in vivo in guinea pigs and mice (Sharma, 1990). Bark extract of Lawsonia has shown broad fungitoxic spectrum against ringworm fungi (Singh and Pandey, 1989).

2.4.7.8 Prosopis juliflora

Prosopis is a xerophytic shrub or small tree belonging to family Mimoseae. It is commonly known as mesquite bean. Mesquite pollen can cause serious pollinosis in the area where it is localized. Peptidases Imess and peptidase II mess isolated from Prosopis pollen play an important role in inflammatory responses in allergic asthma (Matheson and Travis, 1998).

Many antigenic glycoproteins isolated from Prosopis pollen have been shown to exhibit allergic activity (Thakur, 1991). Julifloricine, an alkaloid isolated from Prosopis is associated with antimicrobial property (Aqeel et al., 1989). Based on ethnobotanical survey of medicinal plants in Israel, it was found that Prosopis is used for treating diabetes
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(Yaniv et al., 1987). *Prosopis* contains flavanoids and alkaloids, (prosopin and prosopinine). Oleanolic acid, one of the constituents of *Prosopis* possesses anti-HIV activity which has been shown to inhibit HIV-I replication *in vitro* (Kashiwada et al., 1998).

2.4.7.9 *Decalepis hamiltonii*

*Decalepis hamiltonii* is a climbing shrub belonging to family Asclepiadaceae. Its root is markedly fleshy and cylindrical and has a strong aromatic odour. It has a sweet sarasaparilla like taste (accompanied by a tingling sensation on the tongue) and is considered to be an appetizer and blood purifier.

*Decalepis* root contains 92% fleshy matter and 8% woody core. Volatile principle responsible for the aroma and taste of *Decalepis* root is 4-O-methylresorcyaldehyde which is present in a concentration of 0.8% in the air-dried material that can be isolated with alcoholic extraction of powdered root. The root also contains inositols, saponins, tannins, a crystalline resin acid, an amorphous acid, a ketonic substance and sterols (α- and β-amyrins and lupeol, both free and as esters). The root can be stored for long periods and is unaffected by microorganisms and insects; this is apparently due to the presence of the volatile principle which possesses bacteriostatic and toxic properties (Wealth of India, 1989).