Review of Literature
CHAPTER - 2
REVIEW OF LITERATURE

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2.1 CARCINOGENESIS
2.1.1 Introduction

Carcinogenesis is a complex and dynamic interaction of host and environment. Important host factors include genetic constitutions and health status (Friend, 1993). Among environmental factors, diet, environmental pollutants, occupation and life style factors such as smoking have implicated. Furthermore, cancer is believed to be preventable (Doll and Peto, 1981).
Theories of carcinogenesis can be dated to the 4th century when Hippocrates proposed that cancer was a disease of excess of black bile. Pott in 1775 first described the association of occupational exposure of chimney sweeps to soot and scrotal cancer.

In 1895, a German clinician Rehn provided further support for the concept of chemical carcinogenesis when he linked cancer of bladder with occupational exposure to the chemicals in the aniline dye industry.

Kennaway (1925 and 1955) and Kennaway and Hieger (1930) performed studies with benzo (a) pyrene [B(a)P] that provided the impetus for experimental carcinogenesis.

2.1.2 Carcinogens

A carcinogen is an agent whose administration to animals leads to a statistically significant increased incidence of neoplasms of one or more histogenetic types as compared with the incidences in appropriate untreated animals. Carcinogens may be either drugs, industrial and other chemicals, food additives, hormonal and metabolic agents, infectious microorganisms and viruses, physical agents and environmental factors (IARC, 1981). They occur relatively as simple and defined forms or may remain more complex and ill defined. Carcinogenicity may
thus be defined as a clinical experience or experimental induction of tumors due to exposure to carcinogens (IARC, 1973; Yagi et al., 1977).

2.1.3 Classification of carcinogens

Carcinogens are classified as genetic carcinogens that undergo covalent reaction with DNA and epigenetic carcinogens that do not undergo covalent reaction with DNA. Most of the genotoxic carcinogens require metabolic activation before it interacts with DNA. The metabolism of most carcinogen involves mixed function monooxygenase enzyme systems. Polycyclic aromatic hydrocarbons, nitrosamines, aromatic amines, azodyes, nitrofurans, urethane, aflatoxin are examples of genotoxic agents that need enzyme activation to convert them into an ultimate carcinogenic forms. However beta propeolactone, propane sulfone, epoxides, imines, nitrogen mustard do not require metabolic activation for their genotoxic activity (Harris, 1985).

Carcinogens have been placed into three categories depending upon their mechanism of action that is

a. Indirect acting or procarcinogens
b. Secondary or precarcinogens
c. Cocarcinogens or promoters
The direct acting carcinogens act often at the point of application and sometimes in remote tissues. They do not require metabolic activation but are subject to detoxification and excretion. The secondary or precarcinogens usually do not act at the point of application (exception do exists) but often affect specific tissues. They must undergo metabolic activation and ultimately detoxification. CC14 is an example for nongenotoxic procarcinogen and B(a)P is a well established genotoxic carcinogen in human and in animals which requires metabolic activation. Promoters work only at the sustained and comparatively higher doses, while the initiators may need only one hit at a relatively lower dose to produce cancer (Gupta et al., 1991; Natarajan and Obe, 1986).

2.1.4 Methods of Testing Mutagenicity/Carcinogenicity

There are several short-term methods to detect mutagenicity and carcinogenicity. The following are the review of specific methods utilized in the present study.

2.1.4.1 Cytogenetic test system

With the cytogenetic test system morphological evidence of damage to the genetic material may be detected. Some of the obvious advantages are a large number of species, including humans, can be examined by this method; it can be performed on both in vivo and in vitro systems, the genetic materials can be observed directly; and the tests can be
accomplished in a relatively rapid manner. Studies can be conducted with either acute, subacute or chronic doses of the potential mutagens, and both the parent compound and its metabolic products can be tested. Procedures have been worked out for direct bone marrow preparations as well as spermatogonial analysis (Legator and Mailing, 1971).

The basic prerequisite for cytogenetic investigations include - the availability of cell in active division (either natural cell division as in cultured cells, or by use of a mitogenic stimulating agent such as in phytohaemagglutinin in leukocyte culture), accumulation of sufficient metaphase plates usually through the use of a mitotic arresting agent; hypotonic swelling to further disperse the chromosomes within the cell; preparation of hypotonic swelling to further disperse the chromosomes within the cell; preparation of one optical plane and photomicroscopy to obtain proper plates for analysis.

a. Micronucleus test

This test was initially developed by Schmid (1975) and Bedell (1983) independently and is being increasingly used in the field of chemical carcinogenesis. General procedure is that the animals are exposed either acutely or chronically to a test substance. After the exposure the

1. Rupture of the swollen cells to spread the chromosomes in one optical plane and photomicroscopy to obtain proper plates for analysis.
animals are sacrificed and the bone marrow is extracted, spread on slides and stained. The frequency of micronucleated cells among the newly formed (RNA containing) erythrocytes is determined and the frequency is compared among the various treatment groups. The newly formed erythrocytes are identified by staining the residual RNA which remains in the newly formed cells for 2 days after the inoculation. Cells stain uniformly positive for RNA are referred to as polychromatic or polychromatophilic erythrocytes (PCEs); cells which do not stain positively for RNA are referred to as normochromatic erythrocytes (NCEs). Increase in the frequency of micronucleated PCEs relative to the vehicle control group indicates that the test substances induced chromosomal damage or lagging chromosomes in the nucleated erythrocytic cells. Document of MacGregor et al. (1983) provides recommended guidelines for performing the mammalian in vivo bone marrow micronucleus assay. Under appropriate test conditions measurement of the frequency of newly formed micronucleated erythrocytes in bone marrow provides a convenient index of chromosomal damage in nucleated erythrocyte precursor cells.

b. Chromosomal aberrations (CA)

Chromosomal aberrations can be studied in any cycling cell population or in any non cycling cell population, that can be stimulated by a mitogenic agent to enter the cell
cycle. In animals there are several cell types that fit this criteria, but for human studies, there are only two cell type that are practically suitable. These are the bone marrow cells, which are a cycling population and the peripheral blood lymphocytes, which are normally non-cycling but can be stimulated to enter the cell cycle by in vitro culturing with a mitogen such as a phytohaemagglutinin-M (PHA-M). However, because of the ease of obtaining blood sample, in contrast to bone marrow sample, lymphocyte assays have been used in the majority of studies on the induction of chromosomal aberrations in human beings.

Genotoxic effects of mutagen and carcinogen was assessed by chromosomal aberration system.

Oya et al. (1986) investigated the cytogenetic effect of NaF and H$_2$O$_2$ respectively in the human embryonic fibroblasts. Kulkarni et al. (1982) used human leukocyte culture.

c. Sister chromatid exchanges (SCEs)

The induction of cytogenetic effects has proven to be one of the most sensitive methods for determining if an agent can damage DNA and consequently be biologically hazardous. The phenomenon of SCEs can be used to gather a considerable amount of basic biological information regarding how various chemical agents interact with DNA and
produce genetic damage (Kulkarni et al., 1982; Kato, 1983; Buckton and Evans, 1979).

2.1.4.2 The in vivo and in vitro assay for assessing DNA repair and DNA replication.

An assay for quantifying DNA repair and DNA replication in rat hepatocytes following chemical treatment in vivo as a predictive for hepato-carcinogenicity has been developed and studied extensively (Ashby et al., 1985; and Mirsalis et al., 1982). Carcinogenesis is a complex multistage process which appears to have both genetic and epigenetic components in its aetiology. Thus, in order to assess fully the carcinogenic potential of a chemical compound, it is necessary to measure the chemical ability to induce both genetic and epigenetic event that may lead to carcinogenesis. In the in vivo-in vitro hepatocyte assay, the potential of a chemical to induce hepatic tumors by a genotoxic mechanism is assessed by measuring its ability to induce DNA repair. The measurement of chemically induced DNA repair has been shown to be a valuable tool for assessing a compound's organ specific genotoxic potential (Doolittle et al., 1984; Doolittle and Butterworth, 1984; Mirsalis et al., 1982; and Working and Butterworth, 1984) and has been correlated with carcinogenic potential (Mirsalis and Butterworth, 1982).
In the most of the above mentioned in vivo studies incorporation of labelled thymidine was examined in one organ only. However, an in vivo technique of Hellman et al. (1985) indicated that measurement of $^3$H-thymidine ($\gamma$-ray) incorporation into DNA in various tissues of experimental animals is a useful short term bioassay to evaluate the potential tissue specific carcinogenicity of the carcinogens using a modified Schmidt-Tennenhaus procedure (Munro and Fleck, 1966).

2.1.5 Food and Environmental Carcinogens
2.1.5.1 Polynuclear aromatic hydrocarbon

This widespread group of substance is synthesised mainly in the combustion of organic materials; they are thus present in all kinds of soot and smoke. They occur in tobacco smoke, in smoked fish and meal, in coal and coal tar pitch and in the atmosphere of all urban areas, as well as in increasing quantities in rural areas, mainly as a result of the discharge of the exhaust gases from gasoline and diesel engines. Man is exposed to complex mixtures of these substances and exposure occurs under a variety of circumstances and through different routes. One of the most abundant widespread or the most potent polynuclear carcinogen is benzo (a) pyrene [B(a)P] (Wattenberg, 1983, 1985; Harris, 1985). It has been found in the air in concentrations ranging from 0.01 to 100 $\mu$g/1000 $m^3$. 

In the most of the above mentioned in vivo studies incorporation of labelled thymidine was examined in one
corresponding to an annual intake of 0.05 - 500 ug, on the basis of 5000 m$^3$ of air inhaled per year per individual. B(a)P is a component of all kinds of soot and smoke, including wood smoke. It also occurs in coal tar and processed rubber. B(a)P has been found in smoked fish (1.0 to 6.9 ppb) and shell fish (0.8 to 9.85 ppb), charcoal broiled steaks (8.0 ppb). B(a)P has also been found in other food stuffs, e.g., spinach (3.3 ppb), seaweed (31.1 ppb), carrots (0.7-1.9 ppb), tea leaves (6.8-7.6 ppb), roasted coffee (0.2-0.6 ppb), bread (0.18-0.26 ppb), rice (0.03-0.08 ppb) and potato (2.4-3.0 ppb) (Bornoff et al., 1968; and Hikke, 1981). Other carcinogenic polycyclic aromatic hydrocarbons present in food are anthracene, benz (a) anthracene, benz (a, b) flouroanthracene, dibenzo (a) pyrene, anthracene and chrysine. Most snuff manufactured in the United States contains ppb quantities of B(a)P (Hoffmann et al., 1986).

2.1.5.2 Chlorinated hydrocarbons

Some of the compounds belonging to this group are widely used as industrial solvents, reactive intermediates, fumigants or pesticides and are therefore widely distributed in the environment. Among them, carbon tetrachloride, chloroform and pesticides DDT, aldrin, dieldrin, heptachlor and HCH are of special consideration. Carbon tetrachloride may be found in industrial environments and as contaminants
in food, e.g., carbon tetrachloride in bread (WHO, 1972). CCl₄ is referred to as potent environmental human carcinogen. It induces cancer in liver when administrated orally to mice. Types of human contact was found to be as solvent, degreasing agent, dry cleaning agent, fire extinguishing agent, production of freon, grain fumigant and extractive of oils (Environmental Pollution and Chronic Diseases, 1992).

2.1.6 Lung and forestomach model system for carcinogenesis

Forestomach tumor was studied using a forestomach tumor model developed in Swiss mice or A/J mice by Wattenberg (1972 and 1981) and Wattenberg et al. (1980 and 1985) by the oral administration of 1 mg/3 mg of benzo (a) pyrene [B(a)P] in sesame oil and 20 mg/kg body weight of N-nitrosodiethylamine (DEN) in 0.2 ml water.

2.1.7 Carcinogenesis

Dietary factors are known to play an important role in the development of cancer (Miller et al., 1979). This observation is supported by epidemiological studies on immigrant populations in different parts of the world, biochemical and epidemiological studies by Perera et al. and Sun (1985), Harris (1984) and Hoel et al. (1985).
2.1.8 Mechanism of Carcinogenesis

Early experimental models supporting the stepwise development of tumors, were based on Berenblum and Shubik's (1947) studies of cancer formation in mouse skin. They stated that there are at least two stages - initiation and promotion in chemical carcinogenesis - progression was first used by Foulds (1957 and 1958) to describe his observations of changes during advancement of neoplasms.

Majority of chemical carcinogens are precarcinogens/procarcinogens, which are converted in vivo to ultimate carcinogens or the final reactive forms of these agents. The active metabolites of these procarcinogens generally react covalently with nucleic acids and proteins in vivo in non-enzymatic nucleophilic substitutions. These reactions are catalysed by enzymes and may include the formation of one or more intermediate metabolites of proximate carcinogens. The common denominator of these ultimate reactive metabolites of carcinogens is their electrophilicity (Electron deficient reactants). They are compounds which react with electron rich sites in cellular nucleic acids and proteins causing mutagenic effects frequently paralleled by the onset of DNA repair processes.

Miller and Miller (1976 and 1979) proposed a generalized scheme of the metabolic activation of chemical
carcinogens. Possible mechanisms of action of these agents and steps involved in the carcinogenic process:

**Procarcinogen**
- Indirect acting carcinogen
  - Metabolic activation

**Ultimate carcinogen**
- (Electrophile, electron deficient species \( R' \))
- Reactive site
  - (Nucleophile, electron rich donors \( R' \))
  - \( O, N, S \) and \( C \) atoms in cellular macromolecules

**DNA**
- Modified DNA
- Mutant DNA (initiation)
- Genotoxicity
  - Promotion
    - (expression of altered information-increased proliferation)

**RNA**
- Repair
- Protein, enzyme

**Nucleophilic traps**
- Endogenous (GSH, GST)
- Exogenous
  - (Plant phenols, porphyrins, tetrapyrroles)
  - Inactivation elimination (detoxification)

**Neoplasm**
- Growth of clones
- Gross tumor

*Miller and Miller (1976 and 1979)*
Scheme represents the chemical carcinogenesis illustrating the formation of ultimate carcinogens electrophile (R), the competitive chemical sites for its reaction with cellular nucleophiles and the alternative pathways to detoxification, repair, cytotoxicity or promotion to neoplasia (Miller and Miller, 1976 and 1979).

Initiation - the first irreversible step requires a single application of a carcinogen and thus is a rapid process. However, it does not produce any remarkable morphological alterations and the event can remain unexpressed for a period ranging from few days to even lifetime of the animals unless it is subjected to large number of exposures to the promoters. Initiation is thus a change in a large tissue or organ induced by exposure to a carcinogen that can be promoted or selected to develop focal proliferations, one or more of which can act as sites of origin for the ultimate development of malignant neoplasia (Farber, 1982).

Nowell (1986) described tumor progression as developing in a stepwise fashion through qualitatively different stages. He also suggested that the genetic instability was the fundamental mechanism responsible. These sequential changes include cellular morphology and behaviour, increase in growth rate, escape of local growth control, continual
proliferation in place of terminal, differentiation or death, metabolic alterations, decrease in antigenicity and acquisition of drug resistance.

Ling et al. (1986), studied the genetics of tumor progression and their findings support the concept of genetic instability as a cause in cancer development. The instabilities they observed were characterized among other genetic alterations, as chromosomal breaks, non-dysfunctions and ploidy changes.

Farber's studies of hepatocarcinogenesis in the rat (Farber and Cameron, 1980; and Farber and Sharma, 1987) have provided the most detailed information available concerning the stepwise development of tumors in animals. They characterized the development of neoplasms in stages and indicated that there are steps in biological events within the stages of initiation, promotion and progression.

Studies of cellular and molecular events using mammalian cells in culture also provide evidence for the stepwise changes (McCormick and Maher, 1989). These stages/steps are linked to the experimental design for exploring the mechanism of carcinogenesis.

Cancer development in humans and animals normally spans a substantial fraction of their life span; this period of
time or latency must occur before the disease is expressed. The developing pathology is accompanied by progressive molecular, cellular and tissue changes (Farber, 1982; and Pitot, 1986b). The biological events or phenotypic cellular changes observed are described as the stages of carcinogenesis.

These stages are further described as initiation, promotion and progressions. The term initiation is used to describe early changes in cancer development associated with damages to DNA. Promotion as a stage is less well understood, but is usually used to describe events subsequent to DNA damage and is associated with cell proliferation activities. Progression is used to describe the last stage of cancer development and is characterised by increase in chromosomal/karyotype changes and progressive ability of the cell to escape normal control growth and differentiation.

This stepwise development of cancer is generally referred to as the multistage concept of carcinogenesis with the major stages characterised as initiation, promotion and progression (Pitot, 1986b; and Farber and Sharma, 1987).

2.1.9 Environmental Factors Contributing to Carcinogenesis

The identification of factors contributing to cancer relies heavily on epidemiology studies and animal toxicities
testing. The belief that cancers are avoidable led to intense efforts to search for environmental factors that contribute to the development.

Chemicals only represent one of the many environmental factors that can affect cancer development. The most intensive effort has been devoted to the identification of industrial chemicals that can induce cancer. IARC (1987a) evaluated some 628 agents, industrial processes, occupational exposures and cultural habits for human carcinogenic risk and identified 50 of these as human carcinogens.

The recognition that not all exposed individuals develop cancer indicates that genetic determinants may play an important role in cancer development.

Knudson (1986) stated that the majority of human cancer probably result from interactions of genetics and environmental factors; some cancers may be induced by environmental factors alone. Examples of such environmental factors may include the human carcinogens (Vinyl chloride) identified by IARC (1987a).

2.1.9.1 Mechanism of action of benzo (a) pyrene [B(a)P]

Benzo (a) pyrene is a potent animal carcinogen and a pervasive environmental contaminant in cigarette smoke, work
place, tar roofing operations and ambient air, food and water (NAS, 1972; IARC, 1973; Gelboin and Ts'O, 1978; Jerina and Dally, 1974; Jerina et al. 1977; Conney et al. 1977; and Sims, 1977). Examination of the metabolites of DNA in vitro established that B(a)P 7, 8 - dihydrodiol was the substrate which was most effectively bound. Subsequently evidence was provided which indicated that a B(a)P 7, 8 - diol - 9, 10 - epoxide was responsible for this binding and the exact structures of some of the nucleic acid adducts were identified (Weinstein et al., 1976; and Huberman and Sachs, 1966). Tumor studies indicated that B(a)P 7, 8 - oxide was the only one of four arenë oxides of B(a)P tested which had high carcinogenic activity. B(a)P 7, 8 - dihydrodiol a metabolic product of B(a)P 7, 8 - oxide, was found to be even more carcinogenic. Reduction of the 9, 10 - double bond in either B(a)P 7, 8 - oxide or B(a)P 7, 8 dihydrodiol resulted in complete loss of carcinogenic activity for these compounds (Levin et al., 1977). Tumor studies established that the enantiomer of the diastereomeric B(a)P 7, 8 - diol - 9, 10 - epoxides were much more tumorigenic than the parent hydrocarbon or other isomers or the diol epoxide (Yang et al., 1977).

Diol epoxides in which epoxide group formed part of a bay-region of the hydrocarbon would be the most active diol
epoxide and not K-region epoxides establishing the carcinogenic activity (Dipple et al., 1984).

\[
\text{ER (P}_{450}\text{)} \quad \text{NADPH + O}_2 \quad \rightarrow \quad \text{B(a)P 7, 8 - oxide}
\]

\[
\begin{align*}
\text{B(a)P-SG conjugate} & \quad \text{B(a)P 7, 8 - dihydrodiol} \\
\text{ER (P}_{450}\text{)} & \quad \text{detoxification} \\
\text{GSH transferase} + \text{GSH} & \quad \text{by glucuronyl transferase} \\
\text{Bay region} & \quad \text{B(a)P - 7, 8 - dihydrodiol glucuronide} \\
\text{Cellular DNA} & \quad \text{B(a)P - 7, 8-diol - 9, 10 epoxide}
\end{align*}
\]

Metabolic activation and GSH conjugation of B(a)P

2.1.9.2 Mechanism of action of carbontetrachloride (CCl\textsubscript{4})

Carbon tetrachloride is an example of a compound that is metabolically activated in the liver. CCl\textsubscript{4} is metabolized by the cytochrome P\textsubscript{450} electron transport chain in the endoplasmic reticulum to a primary radical species the
A summary chart for the activation of \( \text{CCl}_4 \) in rat liver endoplasmic reticulum (Slater, 1984 and 1987; and Connor et al., 1986)
trichloromethyl radical \( \text{CCl}_3^* \) and this can undergo a variety of secondary reactions. \( \text{CCl}_3^* \) radical is a very reactive species that initiates liver damage (Slater, 1984; and 1987; Connor et al., 1986; and Patel et al., 1993). Studies with spin traps demonstrated that \( \text{CCl}_4 \) is metabolised in vitro and in vivo to \( \text{CCl}_3^* \). With oxygen it reacts at diffusion controlled rates to yield \( \text{CCl}_3^\text{OO}^* \). This derived radical is much more reactive chemically than \( \text{CCl}_3^* \) by several orders of magnitude (Packer et al., 1981).

\[
\begin{align*}
R - C^* \text{ carbon centered (e.g., fatty acid radicals)} \\
R - C - \text{O}^*\text{CCl}_3 \text{ peroxyl (CCl}_3^\text{OO'})
\end{align*}
\]

This peroxyl radical is much more reactive chemically than \( \text{CCl}_3^* \) and is probably the main initiating species for lipid peroxidation, destroy the cellular membranes leading to degeneration of liver cells (Teufel et al., 1990; and Singh et al., 1993).

Carbon tetrachloride and nitrofurantoin generates free radicals in redox cycling that is chemicals undergo an electron reduction to form radical species which can then react with oxygen forming superoxide and regenerating the parent molecule.
2.1.9.3 Mechanism of lipid peroxidation

Lipid peroxidation proceeds by a classic chain reaction that includes three discrete phases of initiation, propagation and termination.

Initiation

Initiation triggers lipid peroxidation, when an unspecified oxidant gives rise to an initiating lipid peroxyl radical (LiOO\(^{•}\)) by reaction with either a lipid (LH) or a pre-existing lipid hydroperoxide (LOOH). Central mechanistic concentration on lipid peroxidation both in vitro and in vivo is whether LOOH are participating. On the basis of this type of initiation, phases of lipid peroxidation have been classified as LOOH independent and LOOH-dependent (Minotti, 1993). The former results from the introduction of an initiator with a lipid acyl chain, whereas latter from the interaction of an initiator with a pre-existing LOOH (Slater, 1987).

Propagation

LiOO\(^{•}\)'s enter the propagation phase by abstraction of the comparatively labile bis methylene hydrogen atom of a polyunsaturated fatty acyl chain to yield a resonance stabilised penta dienyl radical. Diffusion controlled addition of oxygen generates a lipid peroxyl radical, the chain carrying species of lipid peroxidation. Propagation is
cycled through rounds of LOO* abstraction of bis allylic hydrogen atoms to generate new LOO's which results in net conversion of lipids to LOOHs. Many propagation reactions can occur for each initiation.

Termination

Lipid peroxidation termination involves the reaction of two LOO's to form non-radical products or reaction of one LOO* with another terminating radical to generate non-propagating radical species which then self quench by various pathways. Quenching of LOO's by antioxidants must be the primary mechanism of termination.
Chemical transformation of three phases of lipid peroxidation

Pathological basis of lipid peroxidation

The net result of lipid peroxidation is the conversion of di and polyunsaturated lipid acyl chains to LOOH and destabilization of hydrophobic membrane lipid bilayer integrity and function by the need to accommodate polar hydroperoxide groups (Jaya et al., 1993; and Richter, 1987).
In addition a number of toxic compounds are generated during lipid peroxidation and propagating LOO's can react with protein, nucleic acids and endogenous or exogenous molecule and thus lipid peroxidation products are known to interact with biological material and cause cellular damage (Dhuley et al., 1993).

The complete reduction of oxygen by the univalent pathway results in the formation of superoxide anion \( \cdot O_2^- \), \( H_2O_2 \) and \( \cdot OH \) radicals as the intermediates. These intermediates are too reactive and hence are not tolerated by living tissues. Free radicals can be generated mainly by two ways (a) radiation (b) redox reactions mostly catalysed by transition metals or by enzyme catalysis (Slater, 1987).

Compounds can undergo one electron reduction to form radical species which then can react with oxygen forming superoxide \( (\cdot O_2^-) \) and regenerating the parent molecule. This process is called redox cycling (Cohen and Doerty, 1987).

Paraquat, \( CCl_4 \), adriamycin, B(a)P are helpful in more generation of free radicals (Das et al., 1993).

Hydroxy radical (OH') attacks occur at the other locations along the acyl chain of bis unsaturated fatty acyl chain. Although OH' radicals are almost detectable in metal dependent peroxidation system, addition of OH' scavengers or
catalase rarely inhibits the peroxidation observed (Boivin et al., 1990).

Peroxy radicals initiate lipid peroxidation by bis allylic hydrogen atom and are formed in a number of metal catalysed processes and in the metabolic activation of CCl₄ which was an important link between free radicals and pathology (Poli et al., 1987).

2.2 ANTICARCINOGENESIS

2.2.1. Anticarcinogens

Plant extracts with anti mutagenic activities are often anticarcinogenic as well. Antimutagens may be of two types.

a. Desmutagens - that deactivate the chemical carcinogens by acting directly on them

b. Bioantimutagens - that suppress cellular mutagenesis by increasing the cellular defenses by promoting cell repair mechanisms. Hence both the terms are used for any agent suppressing mutagenicity (Kada et al., 1978).

In the hope of defining an ideal anticarcinogenic agent which could be useful in humans several studies were carried out by Wang et al. (1992), Agarwal and Chauhan (1991), Chen et al. (1993) and Abraham et al. (1986). These studies revealed the usefulness and mechanism of action of anticarcinogens
(polyphenols) present in fruits, vegetables and plants as chemopreventive agents.

2.2.2 Plant foods as anticarcinogens

Plant foods are rich in phenols, indoles, aromatic thiocyanates, coumarines, protease inhibitors and sterols which inhibit carcinogenesis. Extracts of a number of plants have been shown to have anticancer properties. The relationship of cancer with higher plants was known many centuries ago. Both cancer inducing and cancer reducing properties have been attributed to a large number of plants in folklore and in systems of traditional medicine all over the world. Even about 3500 years ago, in the Ebers papyrus obtained from Egypt, symptoms of cancer have been described, together with the use of Allium sativum extracts for its cure (Sharma, 1990). Wattenberg (1983) reported that foods contain large number of antimutagens and anticarcinogens which counteract the effect of carcinogens.

2.2.2.1 Glutathione (GSH)

Glutathione is present in food and is one of the major antioxidants in the soluble fraction of cells. Dietary glutathione has been shown to be an effective anticarcinogen against aflatoxin. Glutathione functions in many cellular processes, including catalysis, transport and reductive phenomena.
Glutathione with its reactive free thiol group is a highly potent blocking agent (Chasseaud, 1976). It functions to protect cells against toxic compounds of endogenous and exogenous origin. The glutathione transferases are major defense against oxidative and alkylating carcinogens (Warholm, et al., 1981). GSH together with glutathione S-transferase catalyses the conjugation of a whole range of xenobiotics and hence plays a critical function on detoxification and cellular protection in both in vivo and in vitro experiments (Shallom and Chitinis, 1990).

2.2.2.2 Pyrrole pigments

Chlorophyll and copper chlorophyllin have also been shown to be effective in inhibiting mutagenesis induced by the carcinogens 3-methylcholanthrene and benzo (a) pyrene using Ames salmonella/microsome activating system (Lai, 1979; and Lai et al., 1980).

Pyrrole pigments were found to inhibit mutagenesis induced by certain classes of carcinogens. Arimoto and coworkers (1980 a and b) showed that hemin, biliverdin, chlorophyllin and protoporphyrin were effective in inhibiting the activity of a series of mutagens in experiments using the Ames salmonella/microsome assay.
2.2.2.3 Beta carotene and vitamin A

Vitamin A and its synthetic analogues have been shown to prevent the development of carcinomas in animals exposed to chemical carcinogens. Other epidemiological studies also support the finding that low vitamin A or provitamin A intake significantly increases the risk of cancer, especially respiratory tract and lung (Sporn et al., 1976; and Sporn, 1978). Vitamin A and its analogues are highly potent physiological agents, playing important role in the control of cancer formation (Sporn and Newton, 1981; Sporn and Robert, 1983; Ames, 1983; and Alfred, 1985; 1p and 1p, 1981).

Beta carotene reduces clastogenicity and genotoxicity in vivo (Raj et al., 1983). Carotenoids (in green and yellow vegetables) have been implicated to be anticarcinogens in human (Peto et al., 1981; and Tuyns, 1982). Carotenoids and retinols are reported to have a pronounced antimutagenic action (Renner, 1990).

Vitamin A may interact with the initiation of a cancer either by direct interaction with the initiation itself (for example, beta carotene quenches singlet oxygen generated by uv light) or by enhanced susceptibility to carcinogenic influences due to vitamin A deficiency (Nettesheim et al., 1976; Nettesheim and Williams, 1976; and Krinsky and Deneke, 1982).

2.2.2.4 Ascorbic acids

Ascorbic acid has been used in the treatment of cancer since the vitamin became available. The effect of ascorbic acid on cancer has been studied in vitro in animals and in patients (Hanck, 1985).

2.2.2.5 Tocopherols

Among the antimutagens present in the food materials, vitamin E possesses strong antioxidative property and acts as free radical scavenger during chemical and biochemical processes. Among other tocopherols, alpha tocopherol is the most active antioxidant which inhibits nitrosamine formation and the carcinogenicity of nitrosamines (Lathia and Blum, 1989). Protective effect of tocopherol against radiation-induced damage and mutation and dimethyl hydrazine-induced carcinogenesis have also have been observed. Vitamin E destroys nitrite, an essential component in the food chain associated with the production of cancer. Vitamin E
especially together with ascorbic acid is an excellent nitrite trapping agent (Mirvish, 1981).

2.2.3 Anticarcinogenesis

Anticarcinogens acts at different levels of cell division and may detoxify or eliminate those agents which may lead to the formation of cancer at any of the stages of initiation, promotion and progression. In understanding the inhibition of cancers, both the mode of action and of detoxification of the known carcinogen and mutagens have to be taken into account.

2.2.4 Mechanism of anticarcinogenesis

The plant products may act at different stages of carcinogenesis and through various interactions. Extracellular protection can be given by the inhibitor by acting on the carcinogen during the first stages of carcinogenesis. The penetration of the carcinogen into the cell may be hindered, as done by putrescine, fatty acids and aromatic amino acids. Toxicants or carcinogens already present may be removed by fibrous plant products through (i) binding by fibres of initiators and promoters (ii) modification of their mechanism and (iii) increase in faecal bulk, for example, refined corn bran is seen to bind mutagenic nitropyrenes irreversibly (Sharma, 1990; Sharma et al., 1994).
Schematic presentation of protective mechanism/anticarcinogenesis

(Polasa, 1989)

Carcinogenic precursors

Carcinogens → Deactivation by desmutagens (from diet) enzyme induced by them

Activation

Reactive carcinogens (electrophilic) → Formation of nontoxic products

Detoxification by desmutagens/enzymes induced by them

Formation of nontoxic products

Attack target site → Protection of cellular DNA by bioantimutagens

Formation of neoplasia → Neoplasia not formed
The endogenous formation of carcinogens can also be hampered. The reaction between nitrate and nitroso amines or amides in the acidic gastric environment gives rise to N-nitroso compounds, a majority of which has been found to be carcinogenic. This reaction has been inhibited by complex mixtures including tea, coffee, vegetables and fruit juices and may be attributed to the presence of ascorbic acid and vitamin E and natural phenolics in these plant products.

Deactivation of mutagens in the alimentary tract is mediated by enzymes with peroxidase and NADPH-oxidase activities present in vegetables like cabbage and broccoli and also by plant extracts containing thiols and various antioxidants.

In the stage two of cancer formation, anticancer activity has been shown by several plant products, acting inside the cell. These agents may modulate the metabolism by (i) inhibiting the cell replication (ii) sequestering mutagens in non-target cells so as to render them inactive as well as by (iii) inhibiting activation of promutagens and (iv) inducing detoxification.

The principal reactive groups are retinoids, thiols and phenolic compounds. The reactive molecules of carcinogens
can be blocked from acting with the nucleophilic sites of DNA by reacting with the active electrophiles, (sulphur compounds) or by scavenging reactive oxygen (antioxidants) or through directly binding with these sites (ellagic acid and retinoids). Some plant products (vanillin, thiols, cinnamaldehyde and coumarins) may increase the repair of DNA damage, thus reducing the chances of carcinogenesis. Protease inhibitors may inhibit error-prone DNA repair (found in beans). After the formation of neoplastic cells, promotion of tumor can be prevented in addition by scavenging free radicals (antioxidants), inhibiting cell proliferation and inducing cell differentiation (retinoids, and glucocorticoids, vitamin D3). Progression of tumors may further be hampered by plant hormones and protease inhibitors acting on growth factors and immuno regulators, like retinoids, acting on the immune system (Roberts and Sporn, 1984).

The oxidative reactions of phase I result in the formation of metabolites, which subsequently undergo conjugation at phase II by enzymes like epoxide hydrolase or glutathione transferases to form conjugates (Sharma, 1990).
2.2.4.1 Modulation of xenobiotic enzyme systems

Induction of detoxifying enzyme systems/inhibition of monoxygenase activity (De Flora & Ramal, 1988)

<table>
<thead>
<tr>
<th>Enzyme systems present in mammalian cells</th>
<th>Modulation</th>
<th>Leads to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 isoenzymes</td>
<td>Inhibition of monoxygenase enzyme activity</td>
<td>Decreased formation of reactive metabolites (of ultimate mutagenic/carcinogenic species)</td>
</tr>
<tr>
<td>GSH</td>
<td>Induction of detoxifying enzyme activity</td>
<td>Increased destruction of reactive compounds (of ultimate mutagenic/carcinogenic species)</td>
</tr>
<tr>
<td>SOD</td>
<td>Catalase</td>
<td></td>
</tr>
</tbody>
</table>

GSH - Glutathione
SOD - Superoxide dismutase

Xenobiotics are metabolized in two stages

(i) Biotransformation or phase I metabolism followed by
(ii) Conjugation or phase II metabolism.

During detoxification of toxic agents, the oxidative reactions of phase I (Adams, 1985; and Adesnik, 1986) result in the formation of metabolites, which subsequently undergo conjugation at phase II by enzymes like epoxide hydrolase or glutathione transferase to form conjugates. The conjugates are eliminated from the cell and finally from the organism.
In contrast, activation of the chemical by oxidation leads to the formation of proximate carcinogens or reactive intermediates. The latter were poor substrates for the conjugating enzymes. Therefore these reactive intermediates undergo a non-enzymatic interaction with intra cellular constituents like proteins, RNA and DNA. Such covalent binding leads to the formation of neo-antigens, mutations, cancer induction and subsequent cell death. These two possible alternative routes of oxidative biotransformation resulting in detoxification or activation indicate two different modes of oxygenation. Thus two families of enzymes may be responsible for the alternative pathways (Sharma, 1990) and the same enzyme system whose primary function is detoxification can also bring about the reverse effect namely, activation and transformation of a biologically inert compound into a potentially toxic entity.

Among the predominantly detoxicating phase II enzymes, human GST plays a major role in the detoxification of many reactive electrophilic compounds by conjugation with glutathione but also by noncovalent binding of many xenobiotics (Ditwach et al., 1971). Human cytosolic GSTs can be classified by their isoelectric points into atleast four classes. The basic class alpha (GST 2; GST A), the near neutral class (GST1, GSTM1), the acidic class (GST 3; GST P) and the class 0. Within most of these classes, multiple
isoenzymes are known, GSTM 1, activity was first identified in human liver as the B(a)P oxide conjugating enzyme (Warholm et al., 1981).

2.2.4.2 Modulation of antioxidant enzymes

Antioxidant enzymes can remove or prevent the formation of reactive oxygen species in a catalytic fashion. Free radical mediated toxicity moderated by enzyme such as superoxide dismutase, catalase, peroxidase and selenium dependent glutathione peroxidase. Catalases and peroxidases catalytically decompose hydrogen peroxide. Catalase also scavenges electrons besides hydroxy radicals (Das, 1991).

Superoxide radicals in biological system damage the cells by the oxidation of SH groups to thiol radicals.

\[ \text{RSH} + \text{O}_2^- \rightarrow \text{RS} + \text{H}_2\text{O}_2 \]

This progress of radical damage in cells may involve lipid peroxidation which in turn is inhibited by superoxide dismutase (Petkau, 1987; and Nandhi and Chatterjee, 1988).

This suppression of superoxide radicals is done mainly by certain scavengers, probably non-enzymatic antioxidant and enzymatic antioxidant (Simic et al., 1992). Especially vitamins and enzymes mainly superoxide dismutase is involved in this process (Palozza and Krinskyni, 1992).
2.2.4.3 Mechanism by which plant foods can lead to the inhibition of the action of mutagens and carcinogens

a. Prevention of mutagen/carcinogen formation or limitation of uptake

The formation of nitrosamines was inhibited by natural food components such as ascorbic acid, tocopherol, sulfur compounds, melanoidins and phenolic compounds (Bartsch et al., 1988) through competition with the amine due to their antioxidant properties.

Several nutrients are known to inhibit nitrosation reactions. Among vitamins, vitamin C and E are known for their capacity to inhibit formation of nitroso compounds. Low vitamin E levels have been identified as risk factor in oesophageal cancers (NIN, 1994).
b. Deactivation of reactive species produced by mutagens/carcinogens by non enzymic antioxidants

In the normally metabolizing aerobic cell oxygen undergoes a tetravalent reduction with acceptance of four electrons resulting in formation of $H_2O$. During this process, several free radical intermediates or highly reactive substances are formed. With the first electron transfer, the superoxide anion radical ($O_2^-$) is formed. Acceptance of second electron results in the generation of hydrogen peroxide, a reactive compound that is not a free radical but can participate in reactions resulting in free radical generation. With the third electron transfer, the very reactive hydroxyl radical ($\cdot OH$) is formed. The hydroxyl radical is widely believed to account for much of the cell damage caused by free radicals. Normally, cytochrome oxidase system of the mitochondria can detoxify the free radicals generated and thus prevent damage to the cells. It is also known that mitochondria generate a steady flux of free radicals which can be controlled by the scavenging mechanisms available. A decrease in the intracellular scavenging mechanisms, an increase in the flux of free radicals and/or a combination there of can lead to free radical induced injury to macromolecules and membranes (Halliwell and Gutteridge, 1990).
Direct scavenging capacity of food products inhibit the free radical induced injury/mutagenicity/carcinogenicity.

\[
\begin{align*}
\text{Food borne} & \quad \text{direct} & \quad H_2^* \\
\text{Antioxidants} & \quad \text{scavenging} & \quad O_2^- \\
\text{(Vitamin C)} & \quad \text{} & \quad OH^- \\
\text{Beta carotene} & \quad \text{} & \quad H_2O_2 \\
\text{\(\alpha\)-tocopherol} & \quad \text{} & \quad \\
\end{align*}
\]

Antioxidant compounds, compounds of non protein nature, e.g., ascorbic acid, tocopherols, carotenoids, glutathione and a large number of other chemicals which can remove or prevent the formation of reactive oxygen species non enzymatically and have to be regenerated enzymatically or non enzymatically in order to function in a catalytic fashion. Singlet oxygen has the capability of generating superoxide free radical (Foote, 1982). Additionally, vitamin C has been shown to protect the antioxidant capacity of vitamin E, the fat-soluble antioxidant found in cell membrane (Packer et al., 1981).

2.3 MEASURES TO PREVENT AND CURE CANCER

Except for a few spontaneous neoplasms a majority of human cancer are shown to be due to the environmental factors and hence potentially preventable (IARC, 1978).
The knowledge gained on the molecular and cellular basis of carcinogenesis coupled with new technologies, may provide new avenues of therapeutic approaches. These include identification of the agents that cause cancer and eliminating them and intervening carcinogenesis through chemoprevention or therapy.

In protecting against cancer the plant products may act at different stages of carcinogenesis and through various interactions. Extracellular protection can be given by the inhibitors by acting on the carcinogen during the first stage of carcinogenesis. In the stage 2 of cancer formation, anticancer activity has been shown by several plant products acting inside the cell. These agents may modulate the metabolism by inhibiting cell replication, sequestering mutagens in cells and make them inactive, inhibiting activation of promutagens and inducing detoxification. The reactive groups involved are retinoids, thiols, phenolic compounds, mustard seeds (Ames, 1986; NIN, 1992)

2.3.1 Cytotoxic effect of plant extracts

Cytotoxic agents are those which inhibit the multiplication and development of cancer cells or cell lines. The extracts of a large number of plants were found to have cytotoxic effect on various cell lines. Various plant extracts have been successfully screened for a wide
range of biological activities. *Withania somnifera* and *Plumbago rosea* have reported to possess antibacterial and antitumor activities (Si arada *et al.*, 1993). Gintona, *Asparagus racemosus*, *Osmium sanctum*, *Panax Ginseng*, *Withania somnifera* and *Tinospora cordifolia* showed anticancer and immunomodulatory activities (Seena *et al.*, 1993). *Centella asiatica* showed cytotoxicity to EA, DLA and L929 cells (Babu *et al.*, 1992; Ferns and Jose Padikkala, 1993). Chalcones are distributed throughout the plant kingdom and were found to be cytotoxic, tumor reducing, antioxidant and anti-inflammatory (Sukumaran and Kuttan, 1991; and Ruby *et al.*, 1993). Nagabhushan and Bhide (1986), Nagabhushan *et al.* (1987), Huang *et al.* (1991), Srinivas and Shalini (1991) and Shylaja *et al.* (1992) exposed the anticancer activity of turmeric extract and curcumin against environmental mutagens/carcinogens (Srivastava and Srimal, 1985).

### 2.3.2 Conclusion

The interest in plants as a source of raw material for developing new pharmaceutical products is increasing. Large pharmaceutical companies are speeding up their efforts to screen developing countries plant wealth for new medical compounds, while these countries are establishing a plant export industry or commercial extraction facilities. However, new techniques are being developed in
industrialized countries that facilitate the production of plant derived drugs in bioreactors.

In 1989, the Rural Advancement Fund International (RAFI) reported plant material collecting activities of Merck and company, New Jersey, USA, in Latin America, while Upjohn, (Michigan, USA) has been said to study compounds from ancient Chinese herbal medicines with the goal of developing new drugs to combat cancer, cardiovascular disease and disorders of central nervous system. The British Biotics Limited has carried out phytochemical screening programmes, since 1986(44, the screening of developing countries flora by industrial and other specialized research organisations). In 1988, the British Technology Group (BTG) and in 1989, the screening of Ghanaian flora by UK pharmaceutical company Glaxo; Imperial Chemical Industries (ICI). Thus several international agencies are involved in screening of medicinal plants.

In India search for natural/plant extracts and synthetic compounds that may revert or inhibit the process of chemical mutagenesis or carcinogenesis has begun since early 1970s. This area is gaining prominence in view of its potential role in preventing environmental cancer.

There are new technologies, however, which bring unknown risks leading to harmful consequences. Moreover, the
treatments are very expensive and not accessible to the common man. Hence, there is a need to find alternative drugs, which are effective without harmful side effects, inexpensive, physically and financially accessible to the common man.