A STUDY OF CANCER CHEMOPREVENTIVE POTENTIAL OF CERTAIN INDIAN MEDICINAL PLANTS

Dissertation Submitted to the
Jawaharlal Nehru University for the Degree of
MASTER OF PHILOSOPHY

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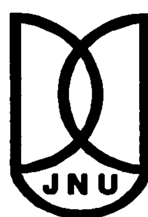

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2003

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CERTIFICATE

The research work embodied in the thesis entitled **"A STUDY OF CANCER CHEMOPREVENTIVE POTENTIAL OF CERTAIN INDIAN MEDICINAL PLANTS"** has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted in part or in full for any degree or diploma of any university.

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Acknowledgements

With due respect, I express my deep sense of gratitude to my supervisors, Prof. R.K. Kale and Prof. A.R. Rao, for their invaluable guidance, valuable suggestions, healthy criticisms, a positive attitude, and nonetheless for their constant support and encouragement which has led to the successful completion of this piece of work.

I express my heartfelt thanks to Prof. A. Bhattacharya, Dean and Prof. R.K. Saxena, former Dean, School of Life Sciences for ensuring the proper functioning of the School and the Central Instrumentation Facility.

I most heartily acknowledge the ever helping attitude of Dr. Ashu, Gagan, Anjali di, Rashmi, Dr. Trisha, Esther and Monisha.

Thanks are due to all my MSc classmates who have made my stay at the University campus, a memorable one.

My friends, Utsah, Amrita and Tanushree deserve special mention for sharing their friendship which shall always be cherished. A special word of thanks goes to Mr. Roshan Lal, Mr. Anwar and Mr. Bishen for their laboratory assistance.

I am thankful to Mr. R.N. Saini for his kind cooperation and expertise in photography.

I acknowledge the technical assistance rendered to me by the CIF staff members including Mr. A.C. Alexander, Mr. B.A. Khan, Mr. S.P. Sharma and Mr. S.K. Mishra. Thanks are due to the SLS office staff members for their patience and cooperation in dealing with the students.

I extend my thanks to the animal house staff members including Mr. Tyagi and Mr. Pandey.

I owe more than words to my family members for their love, care and constant moral support which has enabled me to face the tides of time.

I would like to acknowledge the CSIR for providing me financial assistance in the form of Junior Research Fellowship.

Bimala

ABBREVIATIONS

BSA	bovine serum albumin
B(a)P	benzo(a) pyrene
CDNB	1-chloro-2, 4-dinitrobenzene
DCPIP	2,6-dichlorophenolindophenol
DMBA	7-12, dimethylbenz(a)anthracene
DTD	DT-diaphorase
GSH	glutathione (reduced)
GST	glutathione-S-transferase
LP	lipid peroxidation
MDA	malondialdehyde
min	minute
ml	millilitre
mM	millimolar
µl	microlitre
µM	micromolar
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
nM	nanomolar
nm	nanometer
OD	optical density
s	second
SD	standard deviation
TBA	2-thiobarbituric acid
TCA	trichloroacetic acid
Tris	tris-(hydroxymethyl) aminomethane
v/v	volume/volume
w/v	weight/volume

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1. INTRODUCTION

1. INTRODUCTION

In the global scenario, cancer is one of the most devastating diseases, claiming more than 10 million lives every year. The aetiology and types of cancer vary in different geographical regions. The disease burden is immense, not only for the affected individual, but for the entire mankind. Cancer poses a major challenge for the health care system in both the developing and the developed countries alike. In a multicellular organism, the cell division and growth is meticulously regulated and is responsive to specific needs of the body. Cancer results from mutations that disrupt the control mechanism regulating normal cell division, resulting in the formation of an uncontrolled proliferative mass of tissue which is termed as a 'tumour' or neoplasm. The tumour is termed as benign, when the cells comprising the tumour remain clustered as a single mass. The benign tumour cells become malignant when they exhibit metastasis. Metastasis is a process whereby the benign tumour cells break loose, enter the blood stream or the lymphatic vessels and invade the normal tissue forming secondary tumours. Carcinogenesis, is a multifactorial, multiphasic and multigenic phenomenon. The process of carcinogenesis involves three stages namely initiation, promotion and progression. Initiation is an irreversible process whereby a carcinogen undergoes biochemical activation, converts into a reactive electrophilic metabolite and interacts with the nucleophilic centre of the DNA (Miller *et al.*,1981).

Promotion phase is generally reversible and is caused by repeated application at the proper dose level and frequency of a promoting substance. This phase is characterised by the clonal expansion of the initiated cells to preneoplastic lesions or benign neoplasm.

Progression phase is an irreversible phenomenon whereby the benign neoplasm develops into a malignant condition and is characterised by metastasis.

Based on the tissue of origin, there are four basic types of cancer in case of the malignant cells namely,

Carcinoma – originating from the epithelial tissue.

Sarcoma – originating from the mesenchymal tissue.

Leukemia – originating from the hemopoietic cells.

Lymphomas-originating from the lymphatic tissue.

The causative factor for cancer is mainly attributed to physical, chemical or viral agents. Physical agents include ionising radiations and ultra violet radiations. Chemicals constitute the predominant form of cancer causative agent and includes a plethora of environmental toxicants, dietary agents and hormones. Polycyclic aromatic hydrocarbon compounds are potent chemical carcinogens. Tumour causing (oncogenic) virus include both the DNA containing virus (example– Papova virus, Polyoma virus, Herpes virus) and RNA containing virus (example: Retrovirus, Human T cell leukemia virus).

The five most common types of cancer affecting mankind include those of the lung, stomach, breast, colon or rectum and uterine cervix. The increasing magnitude of cancer incidence and the failure of conventional chemotherapy of advanced invasive disease to reduce the mortality rates for the common forms of epithelial malignancy such as carcinoma of the lung, colon, breast, prostate and pancreas, indicate that new approaches to the control of cancer are critically needed. Furthermore, the misconception of cancer as a disease, whose most fundamental characteristic is excessive cell proliferation has led to an over emphasis of testing and development of cytotoxic drugs that kill cancer cells. Most cytotoxic drugs used in cancer chemotherapy are highly toxic to a wide spectrum of normal tissues, including those found in the gastrointestinal tract, bone marrow, heart, lungs, kidney and brain; iatrogenic failure of these organs is a frequent cause of death from cancer. Hence, as

an alternative approach, cancer can be looked upon as the 'end stage' of a chronic disease process and the need of the hour is to focus more effort on the control of the initial stage of carcinogenesis rather than attempting to cure the end stage disease (Sporn, and Suh, 2000). The logical way of dealing with any disease is by prevention. The majority of cancers are preventable. The goal of primary prevention is to avoid the development of cancer by reducing or eliminating exposure to cancer causing factors including environmental carcinogens and lifestyle factors such as nutrition and physical activity. The same holds true for cancer with all of its associated complexities. The term "Cancer chemoprevention" was coined by Sporn *et al* at the National Cancer Institute in the United States in the year 1976. Chemoprevention is a promising and relatively new approach to cancer prevention which involves the use of specific natural or synthetic substances with the object of reversing, suppressing or preventing the carcinogenic progression to invasive cancer.

Chemoprevention involves the administration of non-toxic agents to healthy individuals who may be at increased risk for cancer. Chemotherapy on the other hand, aims to kill cancer cells in the hope of preventing further cancer progression.

A large body of epidemiologic evidence alongwith data from animal and *in vitro* studies provide strong evidence that consumption of vegetables, fruits and whole grains in high amounts are associated with reduced cancer risk. Comprehensive reviews of case control studies and cohort studies revealed that the relationship between high intake of vegetables and fruits and reduced cancer risk appears to be strongest for cancers of the alimentary and respiratory tracts (cancer of the colon, lung, oesophagus, and oral cavity) and weakest for hormone-related cancers (cancers of the breast, ovary, cervix, endometrium and prostate) (Steinmetz and Potter, 1991; Block *et al.*, 1992; Negri *et al.*, 1991).

The first encouraging results of chemoprevention were reported by Hong *et al* (1991), whereby administration of isotretinoin proved to be highly effective in

preventing second primary cancers in patients, after treatment of primary cancer of the head and neck. In 1991, Thun *et al* reported that use of aspirin in both men and women, resulted in decreased death rates from colon cancer.

The concept of using chemopreventive agents to reduce cancer risk is firmly based on epidemiologic and experimental evidence from the last two decades, that indicates specific compounds may influence carcinogenesis at various sites, including the oral cavity, oesophagus, stomach, colon and rectum, lung, breast and prostate (Kelloff *et al.*,1994). Two fundamental concepts of carcinogenesis in humans namely "multistage carcinogenesis" and "field cancerization" have made it possible for clinicians to apply cancer chemoprevention to patients (Hong *et al* 1990). Since the initiation and the progression phases of carcinogenesis are characterised by irreversible changes, whereas promotion stage is a reversible process, therefore, inhibition of tumour promotion rather than initiation and progression seems to be the more practical approach to benefit humans (Wattenberg ,1992).

The credibility of chemoprevention as a serious and practical approach to the control of cancer has been greatly enhanced by the publication within the past few years, of the results of three major randomized clinical trials, in the field of breast cancer. Three different agents namely tamoxifen (Fisher *et al.*, 1998) raloxifene (Commings,1999) and 4, hydroxyphenylretinamide (fenretinide) (Varonesi *et al.*,1999) have exhibited to be effective chemopreventive agents for prevention of breast cancer in women at varying degrees of risk.

One of the major impediments in the validation of chemoprevention, is the required length of time, necessary for the determination of the efficacy and the inability to conclusively believe that at the time of administration of a particular chemopreventive compound, the concerned person does not have a premalignant lesion. Moreover, the chemopreventive agent must be administered to a homogenous cohort of individuals for a long time with specific end point (Kellen,1999).

Chemopreventive agents exhibit different strategies of chemoprevention amongst which the most promising one includes the induction of detoxification enzymes. Detoxification enzymes have been categorized under two broad groups based on their functional properties namely Phase I enzymes which metabolise and activate the xenobiotic compounds to generate highly reactive electrophilic product and Phase II enzymes which convert the electrophiles to inactive conjugates, rendering them more water soluble thus facilitating their easy excretion from the cell (Gibson and Skett,1994; Begleiter *et al.*, 1997; Wilkinson and Clapper,1997).

Hence induction of such xenobiotic metabolising enzymes can act as a reliable marker in assessing the chemopreventive efficacy of a given test compound against various carcinogen induced site specific carcinogenesis. Numerous naturally occurring agents namely garlic, betel leaf, *Acgle marmelos*, *Ocimum*, and non dietary agents namely magnesium, selenium, tocopherol, retinyl acetate, amino glutathemite, ergocryptin have been worked upon in our laboratory (Cancer Biology Laboratory–School of Life Sciences, Jawaharlal Nehru University, New Delhi, India) and their chemopreventive potential have been proved (Ramesha Rao *et al.*, 1980; Rao, 1984; Rao *et al.*, 1990a; Rao *et al.*, 1990b; Sadhana *et al.*,1998; Banerjee *et al.*, 1994; Banerjee *et al.*, 1996). Further, 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 zanthoxylem on hepatic carcinogen metabolising enzymes. The observations suggested that the intake of these essential oils favourably affects the enzyme associated with activation and detoxification of xenobiotic compounds and also suppresses the formation of DNA adducts with the carcinogen *in vitro* (Banerjee *et al.*, 1994; Mashin *et al.*, 1994).

Two modulators namely *Anacardium occidentale* (Cashew nut) kernel and *Trachyspermum ammi* (Bishop's weed or Ajowan) were selected for the present study to test their possible chemomodulatory potential. As earlies studies in this laboratory

have reported the tumour promoting action of cashew nut shell oil, there is need to assess the possible existence of this property in the edible kernel portion. *Trachyspermum ammi* is much valued for its antispasmodic, stimulant, tonic and carminative properties. The present study proposes to assess the chemomodulatory efficacy of *Anacardium occidentale* and *Trachsypermum ammi* by employing the following strategies:

1. To assess the possible tumour promoting effect, if any, of cashew nut kernel oil on 7,12 dimethylbenz (a) anthracene (DMBA) induced skin carcinogenesis in murine model system.

2. To assess the modulatory effect of *Trachyspermum ammi* at initiational and promotional levelson 7, 12 dimethylbenz (a) anthracene (DMBA) induced skin carcinogenesis in murine model system.

3. To evaluate the inducing potential of these two substances to modulate the specific activity of Glutathione-S-transferase (GST) and DT-diaphorase (DTD) in the liver.

4. To evaluate the lipid peroxidation status in the liver microsomes, in response to these substances.

2. REVIEW OF LITERATURE

2.1 THE NATURE OF CANCER

Cancer can be regarded as a disease of the cells whereby the exquisite control mechanism regulating the cell multiplication break down. Consequently, the cell begins to grow and divide in an unregulated manner ultimately resulting in an uncontrolled proliferating mass of tissue termed as a 'tumour' or 'neoplasm'. Tumours fall into two broad categories namely benign and malignant (Pitot,1986). Benign tumours are characterised by their relatively slow growth, encapsulation and non invasiveness alongwith microscopic similarity to the normal surrounding tissue. Cells from a benign tumour are generally of uniform size and shape and their nucleus appear normal and contain the usual number and arrangement of the chromosomes. In contrast malignant tumours usually exhibit rapid growth and invade adjacent normal tissue (metastasis). Metastasis involves the release of cancer cells into the blood or lymphatic circulation, from where they may colonise other normal organs and develop into secondary tumour. Cells from a malignant tumour are often large and exhibit an abnormal shape.

Cancer is a disease which has been known since ancient times (Pitot, 1986). Its occurrence is recorded in some of the ancient Indian (2000 BC) and Egyptian (1500 BC) writings. The term 'cancer' is derived from the Latin word meaning "crab" and appears to have been first described in a collection of works (500 BC to 200 AD) ascribed to Hippocrates and other Greek physicians. They recognized the clinical presentation of a variety of tumours and classified them as either carcinos or benign growth, which were circumscribed and did not spread; or as carcinomas or crab like growth, which invaded surrounding tissue causing the death of the patient. The term 'neoplasm' was coined later by Galen (200 AD).

The sequential molecular and cellular events involved in the development of cancer also termed as carcinogenesis, are believed to occur as a result of interplay of

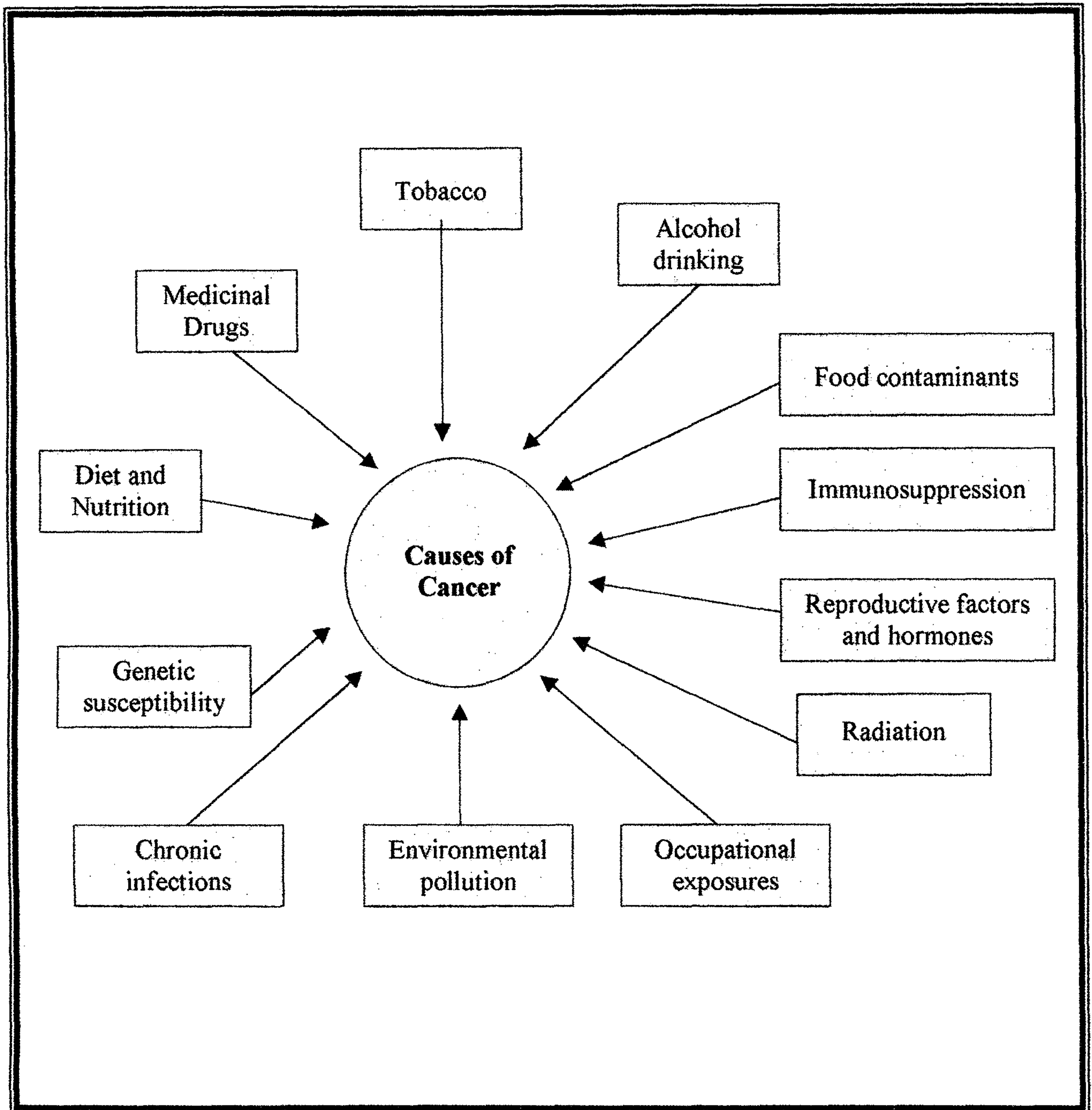
genetic and epigenetic aberrations. The process of carcinogenesis is multifactorial, multigenic and multistage in nature. It is multifactorial because multiple agents have been known to cause cancer and it is not attributable to only a single agent. Thus all ionising radiations, ultraviolet radiations, a plethora of chemicals and a variety of viruses are causative agents for cancer in both humans and animals. More than one gene is involved in the process of carcinogenesis and is hence said to be multigenic in nature. The manifestation of cancer is not an overnight phenomenon, but a multistage phenomenon. In order to have enough cells at risk for cancer, a substantial time and many generations of cell divisions of initiated cells must occur before multiple, rare genetic mutations can accumulate within an individual initiated cell. The complex process of carcinogenesis is divided into three stages namely intiation, promotion and progression (Cerutti,1988; Tanaka,1992).

Initiation is a critical event whereby an exogenous or endogenous carcinogen undergoes a biochemical activation, converts into a reactive electrophilic metabolite and interacts with the nucleophilic centre of the DNA of the target cell (Miller *et al.*, 1981). It is clearly established that initiation of carcinogenesis is a result of a mutational event (Weinberg, 1989). Such DNA mutation caused in a resting cell is repaired but during cell division, the modified DNA forms the template for the production of an altered DNA. Cells bearing such DNA are considered to be "initiated". This results in a genotypically altered cell and the alteration becomes irreversible once it is imprinted in the genome through cell division. Initiation is followed by promotion phase, which is characterised by the proliferation of the initiated cell, resulting in their clonal expansion to preneoplastic lesion or a benign neoplasm. Chemical substances of certain kinds, derived from endogenous or exogenous sources termed as promoters, can promote the events of this stage. Promotion is a reversible process, since removal of the promoter results in the reversal of the promotion phase.

Promotion stage is followed by the stage of progression, which is characterised by the clonal expansion of the precancerous cell population into malignant cancer. Progression is an irreversible process where DNA damage is widespread with loss, breakage and duplication of multiple chromosomes. Malignancy is associated with metastasis whereby the tumour cells break loose and migrate through the blood and lymphatic circulation to distant organs, forming secondary tumours. Malignant condition results in anaplasia (loss of tissue organisation and differentiation) drug resistance, escape from immunosurveillance and ectopic expression of genes which means expression of genes in cells or tissue not normally possessing the capability of such function; for example – lung cancer cells may usually secrete hormones. Numerous reports suggest the role of free radicals in tumour progression (Connell'O *et al.*, 1985; Sun, 1990).

2.2 CAUSES OF CANCER

Epidemiological studies have established associations between numerous environmental factors and the various types of cancer. According to Doll and Peto (1981) 35% of the cancer incidence in the United States of America maybe attributed to dietary factors and about 30% due to smoking. The fact that cancer arises from a single cell provides evidence that, once the abnormal behaviour arises the capacity of such behaviour is inherited by the daughter cells. This reveals that cancer is a disease that fundamentally involves the structure and function of DNA. DNA can be damaged by environmental agents including radiation and substances present in our food, water, air and workplace. Individuals exposed to constant high levels of DNA damaging agents for prolonged periods, such as cigarette smokers or those who have inherited a poor capacity to repair DNA damage are at high risk for cancer. The most important human carcinogens include tobacco, asbestos, aflatoxins and ultraviolet light. Almost 20% of cancers are associated with chronic infections. There is increasing recognition of the causative role of lifestyle factors, including diet, physical activity and alcohol consumption. Current concepts of the aetiology (pattern of causation) of human cancer have generally focused on three agents namely physical, chemicals and viruses.



Schematic illustration of the various causative factors of Cancer.

Physical carcinogens

Physical carcinogens include ultraviolet radiations and ionizing radiations. Ultraviolet radiation in the form of sunlight, has long been recognised as the major cause of human skin cancer. With the exception of malignant melanoma, skin cancers are seldom fatal and can easily be treated surgically. Ultraviolet radiation of the appropriate wavelength can be absorbed by the DNA bases, resulting in the production of dimers between adjacent pyrimidine residues in one of the DNA strands. Individuals possessing a non functional repair system (example: Xeroderma pigmentosum) are highly prone to skin carcinogenesis by exposure to ultraviolet rays in sunlight.

Exposure to ionising radiations (example: X rays, Gamma rays) is related to a variety of cancers particularly those involving the blood and the lymphatic systems, breast, thyroid and brain. It is estimated that only about 3% of all cancer deaths result from some form of radiation (Tomatis *et al.*, 1990). Although the population in Japan have developed neoplastic disease as a result of the use of nuclear weapons, recent human exposure to ionising radiation has been limited to medical X rays or radiation therapy, cosmic rays, radioactive air borne pollutants (including those from nuclear accidents and weapon testing) or to nuclear industry workers. The first observations of cancer after exposure to radiation was the appearance of skin tumours on the hands of early X ray workers in 1911. Since then, many experimental studies on animals have shown that an increase in the incidence of all types of naturally occurring cancers is attributed to radiation (Coggle,1985). The biological effect of radiation is caused by the absorption and distribution of the radiation energy in cells and tissues. The absorption of radiation is a random process. 70 to 90 percent of the mammalian tissue comprises of water. Hence most of the radiation absorbed is involved in the ionisation and excitation of the water molecules. The radiolysis of water generates

free radicals, which via a series of interactions, leads to the adverse biological effect of ionising radiation including chromosomal aberration.

Ionising radiation is a potent inducer of DNA damage, because of the ionisation of water and other biological molecules (Nikjoo *et al.*, 1994). Both single and double strand breakage of the DNA alongwith the modification of the bases is attributed to the ionising radiations.

Chemical carcinogens

The role of chemical carcinogens in human cancer was first suggested in the eighteenth and nineteenth centuries by clinical observations that prolonged contact with soot, coal, tar, pitch, shale and petroleum oils led to an increased occurence of cancers of the skin, lungs and other tissues. Dibenz [a, h] anthracene was the first pure chemical carcinogen to be synthesized. A chemical compound capable of inducing tumours on its own is termed as a complete carcinogen. An initiator refers to a chemical compound which only has the ability to initiate the process of carcinogenesis. Chemicals that have no appreciable carcinogenic activity on their own, but greatly enhance tumour development when given in conjunction with a carcinogen is termed as a promoter.

A majority of the chemical carcinogens, if not electrophilic by nature, undergo metabolic activation and convert themselves to a highly electrophilic form (Miller, 1988). It is these derivatives that are responsible for the biological effect of the parent compound. A few possible mechanisms by which different chemical carcinogens exert their effect are listed below:

1) Alkylating agents, acylating agents and epoxides exert their effect through direct interaction with the DNA.

2) Compounds belonging to the class of polycyclic aromatic hydrocarbons, N-nitrosoamines, aflatoxin, vinyl chloride undergo metabolic activation to form active metabolite of procarcinogen and then interacts with the DNA.

3) Some chemical carcinogens, interefere with DNA replication by reducing the fidelity, by increasing mismatching of the nucleotide (metal ions) by disturbing the nucleotide pool (Fluorouracil).

Among the various class of chemical carcinogens, the polycyclic aromatic hydrocarbons are examples of potent chemical carcinogens. Dimethylbenz (a) anthracene (DMBA – which is used as a carcinogen in the present animal tumour study) and benzo (a) pyrene, are good examples of polycyclic aromatic hydrocarbons. They are formed due to incomplete combustion of organic materials including fossil, fuels and vegetation. They are present in cigarette smoke, barbecued and smoked food. The microsomal monoxygenase system is responsible for the metabolism of the polycyclic aromatic hydrocarbons to arene oxides. According to a theory proposed by Jerina *et al* (1977), the "bay region" diol epoxides of the polycyclic aromatic hydrocarbon are important with respect to their carcinogenic activity. Bay region occurs when an angularly fused benzo ring is present.

Dimethylbenz (a) anthracene: It is one of the best known polycyclic aromatic hydrocarbon and is a potent chemical carcinogen. DMBA causes adrenal necrosis in rats (Huggins and Morii,1961). It yields derivatives which are mutagenic in bacteria and mammalian cells (Ames *et al.*, 1973; Huberman and Sachs, 1974). DMBA exhibits a complex metabolism (Figure I).

Hydroxylation of both methyl groups as well as oxidation at the 3, 4 -, 5, 6 – and the 8, 9 – ring positions has been reported. Hydroxylation of the 7- methyl group may be an early step in the activation of DMBA since it occurs extensively in the

microsomal systems (Yang and Dower, 1975). DMBA 3, 4 – diol 1, 2 – epoxide is known to be the ultimate carcinogen of the parent compound DMBA.

Three major adducts are formed by DMBA, which are identified as bay-region anti-dihydrodiol – epoxide: deoxyguanosine and: deoxyadenosine adducts and a bay region syn-dihydrodiol – epoxide: deoxyadenosine adduct (Dipple *et al.*, 1983). The ultimate carcinogenic form of DMBA is known to form DNA adducts by binding to the N^6 position of deoxyadenosine. This adduct causes A : T – T : A transversions (Lahiri *et al.*, 1999). Over 90% of tumours, including premalignant papillomas, initiated with DMBA have a characteristic A – T transversion at the second nucleotide of codon 61 of the Ha-ras gene (Quintanilla *et al.*, 1986).

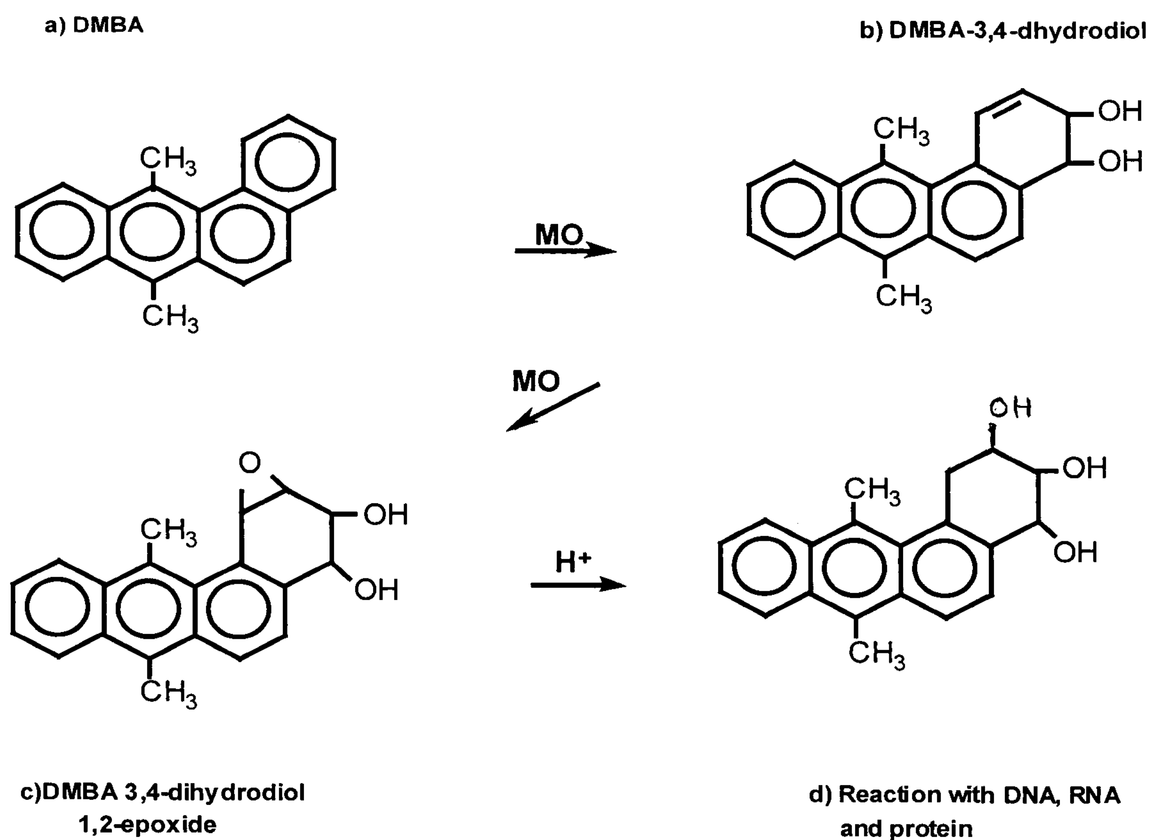


Figure I. Some of the major metabolites of DMBA and the metabolic pathway involved in the activation of DMBA.

a- 7,12-dimethylbenz (a) anthracene; b-Major metabolite of DMBA;

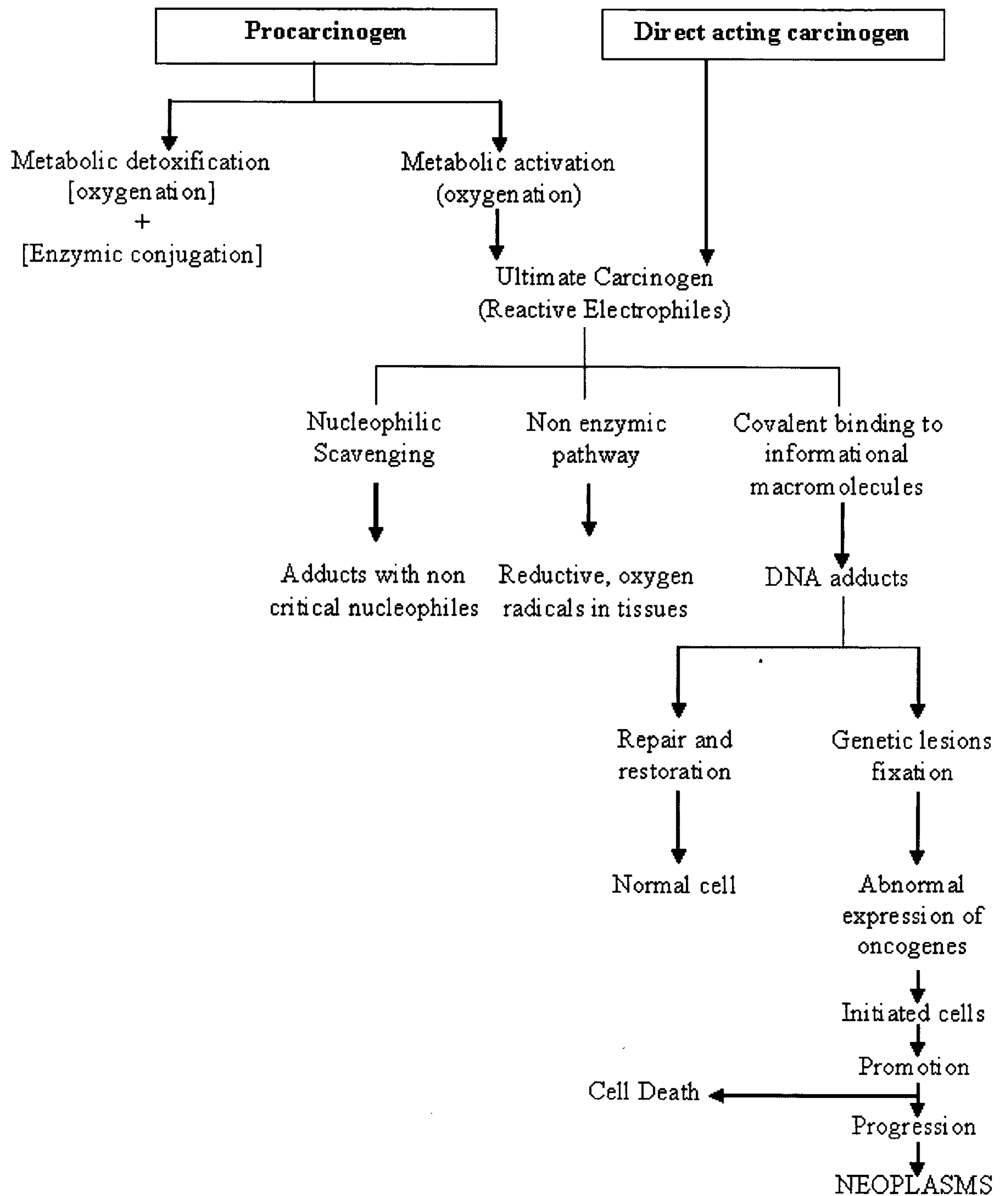
c- Ultimate carcinogenic from of DMBA; d-metabolite of DMBA that reacts with cellular macromolecules.

Viral carcinogens

The role of viruses in the spontaneous occurrence of cancer have been extensively studied in experimental animals and their mechanism of action have provided critical insights into the process of carcinogenesis. About 15% of human cancers have been attributed to viruses. 'Rous Sarcoma virus', identified in the chicken, was the first animal tumour virus to be discovered (Wyke 1991). There are two types of tumour causing (oncogenic) viruses including oncogenic DNA virus (contains DNA as their genetic element) and oncogenic RNA virus (contains RNA as their genetic element). The 'Papova virus associated with warts, Polyoma virus associated with carcinoma of the uterine cervix, Hepatitis B virus involved in liver cancer are some good examples of oncogenic DNA viruses. Some good examples of the oncogenic RNA viruses are the retrovirus family, namely Human T cell-leukemia virus 1 associated with adult T-cell leukemia; Human immunodeficiency virus I (HIV-I) involved in Kaposi's sarcoma. Epidemiological studies have implicated viruses as an aetiological agent only in certain cancer. These include the Epstein-Bar virus (responsible for Burkitt lymphoma and nasopharyngeal carcinoma), Hepatitis B and C virus (causes of liver cancer), Human Papilloma virus associated with cervical cancer and the human T cell leukemia virus.

Experimental carcinogenesis

Experimental carcinogenesis in mouse skin was pioneered by Mottram (1944) and Berenblum and Shubik (1947). It was extensively studied by Boutwell (1974). In the classical animal experiments, the initiation and promotion treatments are applied directly to the mouse skin epidermis. The first tumour appears after five to seven weeks in a benign condition and after 16–18 weeks become malignant. These time intervals represent about 5 and 12% of the life span of a mouse respectively.



Schematic representation of the summary of events in carcinogenesis

2.3 CHEMOPREVENTION

Chemoprevention is an attempt to use natural or synthetic compounds to intervene in the early stages of cancer, before the invasive disease begins. Although the concept is over forty years old, much credit for bringing this practical idea to the research scientists goes to two pioneers in the field of chemoprevention – Lee W. Wattenberg and Michael B. Sporn. Wattenberg initially called the concept of cancer prevention "Chemoprophylaxis of Carcinogenesis" (Watternberg, 1966) and in 1976 Sporn coined the term 'Chemoprevention' (Sporn *et al.*, 1976). Sporn extended the definition of cancer chemoprevention as follows – 'the use of non cytotoxic nutrients or pharmacological agents to enhance intrinsic physiological mechanisms that protect the organism against the development and progression of mutant clones of malignant cells' (Sporn,1993).

The original concept of chemoprevention was to inhibit or delay the occurence of cancer, by agents that could block one or more pathways in the induction of cancer. Currently, the concept of chemoprevention has been expanded and refined with continuous research and can be defined as the use of agents (often called drugs) to prevent the occurrence of precancerous lesions or markers, or to retard or reverse progression of pre malignancy to malignancy (Katiyar and Mukhtar, 1996; Lippman *et al.*, 1996). The major goals of chemoprevention include:

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).

Classification of chemopreventive agents

Chemopreventive agents can be placed under two broad categories. The first category includes compounds which are effective against complete carcinogen. The second includes compounds which are effective against the tumour promoters.

Inhibitors effective against complete carcinogens

Inhibitors of carcinogenesis can be divided into three categories based upon the time of the carcinogenic process at which they are effective. These include:

1. Compounds that prevent the formation of carcinogens from the precursor substances.

2. "Blocking agents" which inhibit carcinogenesis by preventing the carcinogenic compound from reaching or reacting with the critical target sites in the tissue.

3. "Suppressing agents" which act by the suppression of the expression of neoplasia in cells previously exposed to doses of a carcinogenic agent.

Compounds inhibiting the formation of carcinogens

A major focus of this group of inhibitors has been on the prevention of the formation of nitroso compounds from the reactions of the precursor amines or amides with nitrite. Ascorbic acid is effective in inhibiting formation of these carcinogens both *in vitro* and *in vivo* (Mirvish, 1981). α Tocopherols and phenols have the capacity to inhibit formation of nitroso compounds (Kuenzig *et al.*, 1984).

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Blocking Agents

Blocking agents prevent carcinogens from reaching or reacting with the critical target sites in the tissue. A large and diverse group of both naturally and synthetic occurring compounds falls into this category of inhibitors. There are three major mechanisms by which blocking agents act. One group acts by inhibiting the activation of a carcinogen to its ultimate carcinogenic form. An example of this type of inhibition is the prevention of symmetrical dimethylhydrazine induced neoplasia of the large bowel by disulfiram (L.W. Wattenberg, 1975).

A second group of blocking agents is effective by virtue of their capacity to enhance detoxification systems, whereby they increase the activity of enzyme systems having the capacity to enhance carcinogen detoxification. There are two general categories of blocking agents which enhance carcinogen detoxification system, namely type A and type B inhibitors. Both type A and type B inhibitors induce an increase in the activity of multiple enzymes, possibly by reacting with the receptors (Poland *et al.*, 1979; Wattenberg, 1981). The type A inhibitors induce an increase in the phase II enzymes (Conjugating enzymes) and some related systems (Wattenberg, 1983). A prominent feature of this enzymatic induction is a marked increase in the Glutathione–S transferase activity (Benson *et al.*, 1979), UDP glucuronosyltransferase activity, epoxide hydrolase and NAD(P)H quinone reductase activity (Cha *et al.*, 1979). The type B inhibitors characteristically induce a pronounced increase in the microsomal monooxygenase activity. A prototype of this class of inhibitors is β naphthoflavone. Type B inhibitors also enhance the activity of major conjugating systems such as Glutathione -S-transferase and UDP glucuronosyltransferase.

Blocking agents that enhance the activity of systems detoxifying carcinogenic chemicals are of special interest. These systems are inducible, and in many instances, induction of an increased activity has been shown to be protective against the occurrence of cancer. Phase II enzymes which are involved in conjugation and excretion reaction are particularly important in terms of their protective capacities.

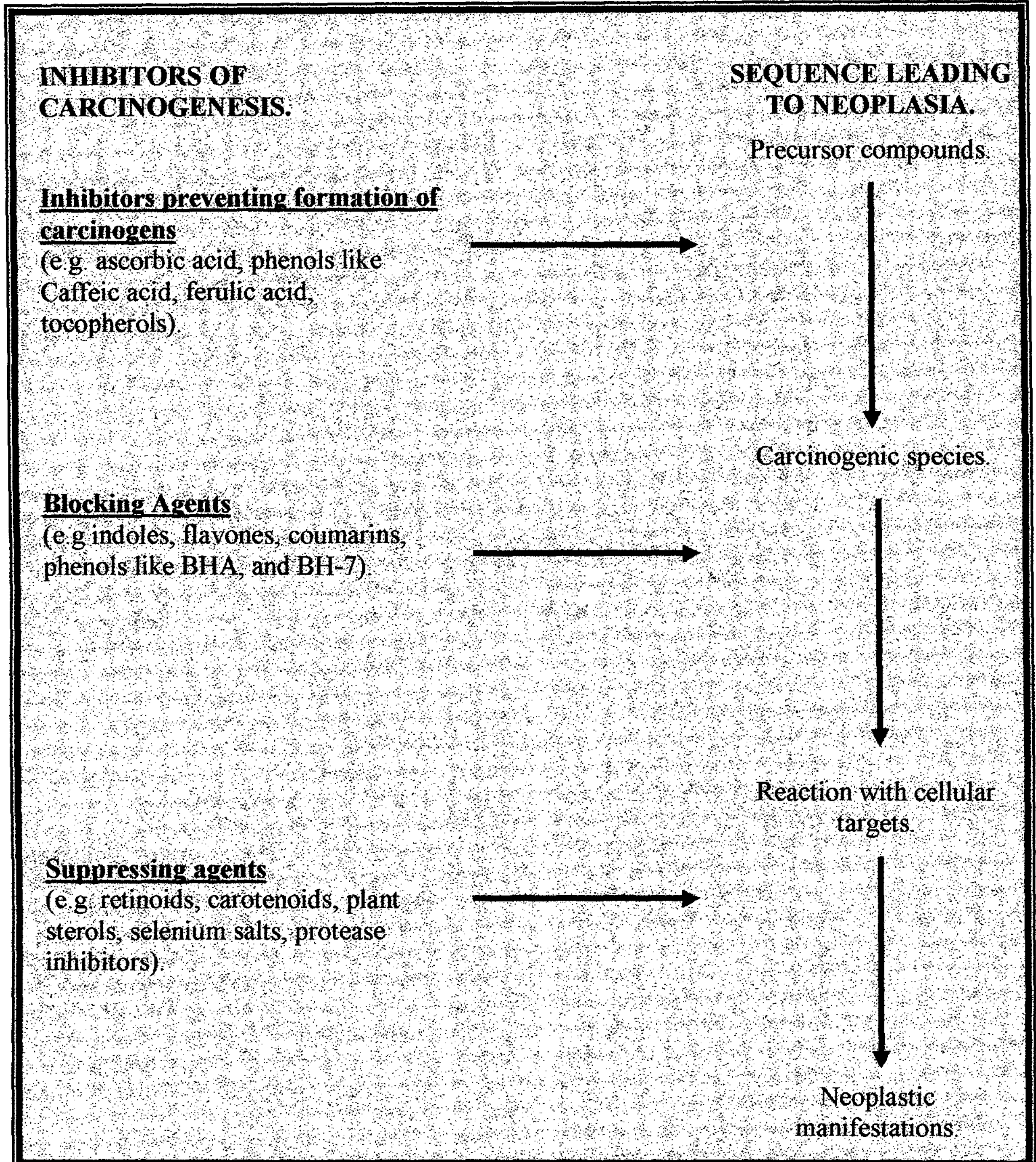
The single most critical system in this regard in Glutathione-S-transferase (Wattenberg, 1993). Many phase II inducing compounds occur as non nutrients in vegetables and fruits, and is possibly responsible for some of the protective effects imparted as a result of the consumption of the same.

A third group of blocking agents trap reactive carcinogenic species before they reach critical target sites (Hochalter *et al.*, 1988). Endogenous thiols, particularly glutathione, are important in this regard. N-Acetylcysteine is an efficient trapping agent of the reactive carcinogenic species and exhibits low toxicity.

Suppressing agents

Suppressing agents prevent the evolution of the neoplastic process in cells that otherwise would become malignant (Wattenberg, 1993). They are compounds that inhibit carcinogenesis when administered subsequent to a course of carcinogen administration ,which would result in the occurrence of cancer. Retinoids include the most extensively studied suppressing agents (Moon *et al.*, 1983; Sporn *et al.*, 1983). Retinoids control normal cell growth, differentiation, and apoptosis during embryonic development and within epithelial tissues in later life. Retinoids constitute a class of over 2,000 agents which include Vitamin A and its natural (example: retinyl esters) and synthetic (example: fenretinide, targretin) analogs. They have the potential to inhibit or reverse the process of carcinogenesis (Lotan, 1996; Lippman *et al*, 1994). Retinoids have proved to be effective in a wide range of *in vivo* experimental systems, including skin, bladder, lung, breast and oral carcinogenesis (Sporn, 1993; Lippman *et al.*, 1994). Numerous retinoids have been used alone or in combination for the treatment of basal cell carcinoma, squamous cell carcinoma of the skin, cervical cancer, melanoma, dysplastic nevus syndrome, cutaneous T cell lymphoma, acute promyelocytic leukemia, lung carcinoma, breast carcinoma, bladder carcinoma, renal cell carcinoma and squamous cell carcinoma of the head and neck (Lippman *et al.*, 1994; Davies *et al.*, 1996). β carotene, which is metabolised to retinols have been

reported to exhibit a suppressive effect on DMBA induced mammary neoplasia (Rettura *et al.*, 1983).



Schematic representation of the classification of chemopreventive agents, on the basis of the time at which they exert their protective effect (Wattenberg,1985).

Compounds which inhibit tumour promotion.

A major hypothesis concerning tumour promotion suggests that the oxygen radicals may play a role in its causation (Slaga *et al.*, 1983). Several groups of inhibitors which overall prevent the attack by oxygen radicals inhibit tumour promotion. Phenolic antioxidants inhibit tumour promotion by benzoyl peroxide (Slaga *et al*, 1983). Protease inhibitors prevent formation of oxygen radicals by TPA and inhibit tumour promotion (Troll, 1981).

Qualities of a chemopreventive agent.

A chemopreventive agent for human usage should ideally possess the following qualities:

a) Little or no adverse effects.
b) High efficacy against multiple sites.
c) Effectiveness at achievable dose levels.
d) Activity following oral consumption.
e) A known mechanism of action.
f) Low cost.
g) History of use by the human population; and
h) General human acceptance.

Possible mechanism of action of chemopreventive agents.

The growing body of information in the arena of mutagenesis and cell proliferation studies in relation to cancer biology, provides an insight into some of the possible mechanisms of chemoprevention. Table I depicts some of the proposed mechanism of action of current clinical chemopreventive agents. Some of the chemopreventive agents that have been or are being studied in specific disease site is depicted in Table II.

Table I: Proposed mechanism of Action of Current Clinical Chemopreventive Agents

Agent	Proposed Mechanism(s)
Vitamin C.	Water-soluble antioxidant, direct-acting reducing agent.
Selenium.	Water-soluble antioxidant functioning through selenium-dependent glutathione peroxidase enzyme system.
Vitamin E.	Non-water-soluble antioxidant of peroxidation.
Beta-carotene (carotenoids).	Non-water-soluble antioxidant, quencher of singlet oxygen and other free radical species; immunologic modulation.
Phenols.	Modulation of carcinogen catabolism.
Isothiocyanates.	Direct physical blockade of carcinogen damage.
Retinoids.	Regulation of differentiation, growth inhibition, and apoptosis; immunologic modulation.
Vitamin D.	Regulation of differentiation and growth inhibition.
Calcium.	Regulation of differentiation and growth inhibition.
Folate.	Immunologic modulation.
Progestin.	Antiestrogenic, antiproliferative effect on the endometrium.
Oral contraceptives.	Suppression of gonadotropin secretion and ovulation in the ovary; antiproliferative effect on the endometrium.
DHEA.	Antiproliferative effect through inhibition of oxygen free-radical generating pathways.
Oltipraz.	Elevation of reduced glutathione-related carcinogen deactivation.
N-acetylcysteine.	Elevation of reduced glutathione-related carcinogen deactivation.
Aspirin, sulindac.	Inhibition of prostaglandin synthesis.
Celecoxib, Vioxx.	Selective COX-2 inhibition
Finasteride.	Antiandrogenic effect on prostate (inhibition of 5α-reductase [type 2]).
SERMs (eg, tamoxifen, raloxifene, droloxifene).	Antiestrogenic effects on breast tissue.
Perillyl alcohol, limonene.	Ras inhibition.
Genistein.	Inhibition of receptor-associated tyrosine kinases.
DFMO.	Inhibition of ornithine decarboxylase.

COX 2= Cyclooxygenase 2; DFMO = Difluoromethylornithine;
DHEA = Dehydroepiandrosterone; SERMS = Selective Oestrogen Receptor Modulators.

[Source: Lippman *et al.*, 1998].

Table II :Chemopreventive Agents that have been or are being studied in specific disease sites.

Bladder	**Skin**	**Colon**
Retinoids Celecoxib DFMO Polyphenols Oltipraz N-acetylcysteine Multivitamins **Prostate** Selenium Vitamin E Finasteride 4-HPR DHEA Oltipraz DFMO Lycopene **Breast** SERMs (eg, tamoxifen, raloxifene, droloxifene) Retinoids DFMO Oltipraz N-acetylcysteine Perillyl alcohol Polyphenols DHEA LHRH agonists Sulindac sulfone Indole-3-carbinol Beta-carotene	Retinoids Selenium DFMO Oltipraz Curcumin Beta-carotene **Cervix** Retinoids DFMO Folate Beta-carotene **Ovary** Oral contraceptives Retinoids **Lung** Retinoids Vitamin E DFMO Olipraz N-acetylcysteine Curcumin Phenethyl isothiocyanate Beta-carotene **Upper Aerodigestive Tract** Retinoids Selenium Vitamin E N-acetylcysteine DFMO Polyphenols Curcumin Oltipraz Beta-carotene	Calcium NSAIDs Sullindac sulfone Celecoxib Selenium DMFO Perillyl alcohol Polyphenols N-acetylcysteine Antioxidant vitamins Beta-carotene Vitamin D_8 Folate Oltipraz Polyphenols Ursodiol Curcumin **Liver** Retinoids Oltipraz **Esophagus and Stomach** Antioxidant vitamins Beta-carotene Retinoids DFMO

DMFO = Difluoromethylortnithine; DHEA = Dehydroepiandrosterone; 4-HPR = N – 4, (hydroxyphenyl retinamide (Fenretinide); LHRH = Luteinising hormone-releasing hormone; NSAIDS = Non steroidal anti inflammatory drugs; SERMs = Selective estrogen receptor modulators.

[Source: Lippman, *et al.*, 1998]

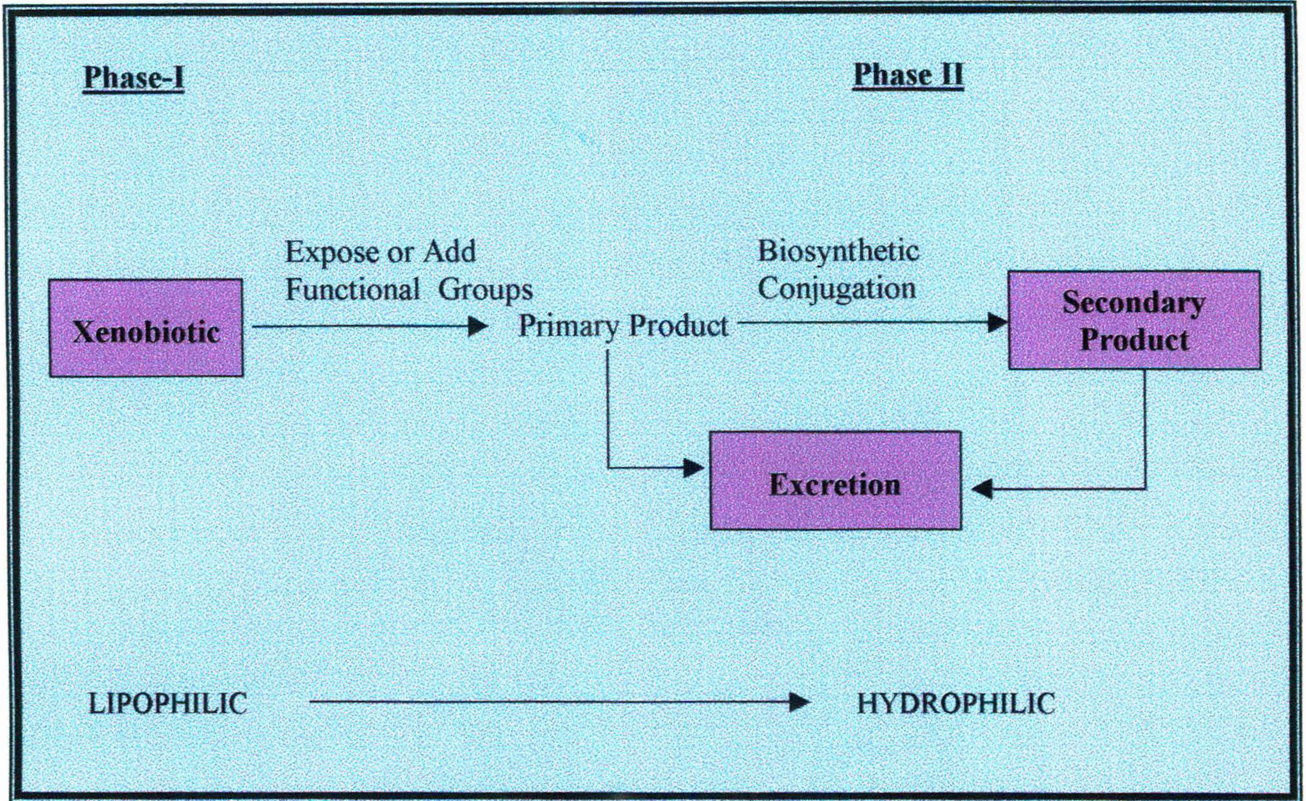
2.4 DIET AND CANCER

During the first half of the twentieth century two influential hypotheses on the environmental causes of cancer were developed. The first focussed on occupational cause, notably exposure of workers to carcinogenic agents (Hueper 1942). The second hypotheses focused on diet. Among the earliest formal epidemiological studies of diet and cancer were those by Orr (1933), who undertook an ecological study of oral cancer in India, and the other by Stocks (1933), who conducted a case control study of cancer in England and Wales; each identified distortions of dietary patterns, especially low intake. of vegetables and fruits as risk factors. According to a review by Wynder and Gori (1977) the preventive potential for all types of cancer, in case of both men and women was 80-90% and that diet accounted for 40% of all male cancers and 60% of all female cancers. Aspects of diet mentioned in Doll and Peto's report (1981) suggested that antioxidant vitamins, vegetables, such as carrots and leafy greens that are rich in these compounds, and bioactive microconstituents such as indoles and protease inhibitors, possibly exhibit a protective effect against cancer whereas possible dietary causes of cancer included over consumption (cancers of the uterus and gall bladder in women), fat (cancers of the breast, colon and rectum) and meat (cancers of the colon and rectum). The most important finding that has emerged strongly in recent years is that, a diet enriched with vegetables and fruits (therefore in fibre content, antioxidants and other bioactive microconstituents) are associated with reduced risk of most, if not all, epithelial cancers (Steinmetz and Potter, 1991, 1996). The nutrients showing modulatory effects in experimental cancer include: macronutrients (fats, carbohydrates, protein and fibre); vitamins (folic acid, riboflavin, β carotene, retinol, α-tocopherol and vitamin B_{12}); and minerals (selenium, zinc, magnesium and calcium).

2.5 XENOBIOTIC METABOLISM

Liver is regarded as the major organ exhibiting the function of detoxification, though other organs also possess the detoxification machinery. A majority of the potential carcinogens and mutagens are not active *per se*, and therefore, have to undergo metabolic activation to convert into reactive electrophilic forms, before exerting their biological effect (Wattenberg, 1985; Miller and Miller, 1986). A set of enzymes present in the microsome is responsible for such activation. These activated carcinogens are now free to act on cellular macromolecules causing cell damage. The integrated process of xenobiotic and endogenous compound metabolism involves two phases, which converts lipophilic compounds into excretable hydrophilic chemical species. Williams, 1979 proposed two classes of enzyme systems for the metabolism of xenobiotics. These are called Phase I and Phase II enzymes.

Phase I reactions (functionalisation) introduces polar groups (-OH, -SH, $-NH_2$, - COOH) into the xenobiotic compounds through the oxidative, reductive or hydrolytic reactions. The enzyme system involved in these reactions is the microsomal monoxygenase system. The presence of a polar group enables the compound to undergo subsequent conjugation reaction. Thus, the product of the Phase I reaction forms the substrate for the Phase II reactions, which are generally conjugating reactions. In Phase I reaction the procarcinogen compound is converted into ultimate carcinogen by the action of an enzyme termed as the cytochrome P450 system. These enzymes play a key role in the biotransformation of many endogenous compounds alongwith detoxification of numerous xenobiotics (Talalay, 1989; Guengerich and Shimada, 1991).



Schematic illustration of the Phase I and Phase II biotransformation reactions.

Table III: Reactions involved in Phase I and Phase II metabolism

Phase I

1. Oxidation involving cytochrome P 450 and others.
2. Reduction.
3. Hydrolysis.
4. Hydration.
5. Dethioacetylation,
6. Isomerisation.

Phase II

1. Glucouronidation /glucusidation.
2. Sulfation.
3. Methylation.
4. Acetylation.
5. Amino acid conjugation.
6. Glutathione conjugation.
7. Fatty acid conjugation.
8. Condensation.

Glutathione –S- transferases

Glutathione-S-tranferases (EC 2.5.1.18) comprises a group of highly versatile and inducible monofunctional proteins and is an important enzyme system of the Phase II metabolism. They are predominantly present in the cytosolic fractions in most of the tissues of various organisms (Jakoby and Habig, 1980). The enzymes are thought to play a physiological role in initiating the detoxification of potential alkylating agents (Booth, 1961; Boyland, 1969), including pharmacologically active compounds. The enzymes play a vital role in bringing the two substrates in close juxtaposition and in increasing the nucleophilicity of GSH, probably by ionising GSH in thiolate ion (Ketterer, 1986). These enzymes catalyse the reactions of compounds with the thiol (-SH) group of glutathione, thereby neutralising their electrophilic sites and rendering the products more water soluble. Glutathione conjugates are metabolised further by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue, to produce the final product, a mercapturic acid (Boyland, 1969). The mercapturic acid which is S-alkylated derivatives of N-acetylcysteine, are then excreted. In addition to mercapturic acid pathway (Pickett and Lu, 1989), methylation of thiol to form methylthio metabolite and glucuronidation of mercaptan to form thioglucuronide represent important metabolic steps for biotransformation of cysteine conjugate. These metabolites 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 that catalyses the antioxidant processes of thiols (Choudhary *et al.*, 1997).With the aid of phospholipase they are capable of inhibiting lipid peroxidation in cytomembranes (Tan *et al.*, 1984). These enzymes participate in the transport or storage of exogenous and endogenous compounds, by acting as intracellular binding proteins (Chasseaud, 1979; Mannervik, 1985). Cytosolic glutathione-S- transferases are dimeric proteins existing as homodimer or heterodimer and represent a multigene family. In higher organisms, there exists 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 tumours (Coles and Ketterer, 1990).

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

The enzyme DT- diaphorase (DTD) was first characterised and named by Ernster (1962) due to its ability to catalyse 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 exhibits 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 the xenobiotic metabolism.

DT- diaphorase is widely distributed in the animal kingdom except the pigeons. It is present in almost all the tissues, but the richest source is the liver (Benson *et al.*, 1980; Belensky and Jaiswal, 1993). There are two alternative pathways in the quinone reduction. One pathway involves the reduction of quinone by one electron (catalysed by cytochrome P450 system) yielding a semiquinone radical in presence of oxygen, most semiquinones rapidly autoxidise to form the superoxide anion radical ($O_2^{\cdot-}$) which explains the cytotoxic and antitumour properties of the quinoid drugs (Benson *et al.*, 1980). The other pathway involves catalysis by DT-diaphorase, whereby quinone in reduced by two electron into non toxic hydroquinones and is 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 excreted in the urine (Jakoby and Habig, 1980).

DT-diaphorase is unique since it exhibits a non specific reactivity towards NADH and NADPH and exhibits a broad electron acceptor specificity, catalysing 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).

The most striking feature of DT-diaphorase is its ability to catalyse the two electron transfer (Iyanagi and Yamazaki, 1970) leading to the formation of hydroquinones from quniones.

Q= Quinones

QH_2 = Hydroxyquinones

Although, some metabolites generated through DTD catalysed reactions are cytotoxic, the enzyme is mainly known for its antioxidant property. DTD belongs to the family of Phase II detoxification enzymes which also includes Glutathione-S-transferase and glucuronyl transferases (Nebert, 1994). These enzymes have the potential to prevent the active electrophiles from interacting with the nucleophilic group of the DNA and ultimately protect tissues against the carcinogenic and mutagenic compounds (Riley and Workman, 1992; Rose *et al.*, 1993). DTD prevents the formation of semiquinones by one electron reduction and in turn the generation of free radicals from the autoxidation of semiquinones (Lind *et al.*, 1982). DTD decreases the electrophilic characters of quinones by aromatisation of quinonoid ring, which restricts its participation in arylation reaction, thereby avoiding cytotoxic effects (O' Brien, 1991).

DT-diaphorase is reported to increase concomitantly with the increase in the activity of other antioxidant enzymes such as superoxide dismutase, catalase and

glutathione peroxidase (Whitney and Frank, 1993; Prestera *et al.*, 1993). DTD has been shown to protect biological membranes against oxidative damage. (Landi *et al.*, 1997). The antioxidant functions of DT-diaphorase is mainly attributed to its ability in maintaining membrane bound coenzyme Q (CoQ) in a reduced antioxidant state, which affords protection against free radical damage. Some of the colon carcinoma cell lines deficient in DT- diaphorase are more susceptible to quinone toxicity (Karczewski, 1999). It has been postulated that DTD was selected during evolution to act as coenzyme Q reductase, to protect cellular membrane components from free radical damage (Beyer *et al.*, 1996).

Lipid Peroxidation

Lipid peroxidation is a highly destructive free radical mediated process, inducing a plethora of pathological events in the biological system. In eukaryotes, the fluidity is maintained by incorporated polyunsaturated fatty acid (PUFA) chains into the membrane lipids. Free radicals generated through various ways, can bring about oxidation of the polyunsaturated fatty acids of the membranes. This process of oxidative deterioration of membrane lipids is termed as 'lipid peroxidation'. The toxicological significance of this process has been associated with spontaneous mutagenesis and carcinogenesis (Burcham, 1998). Malondialdehyde (MDA) is one of the major products of lipid peroxidation. MDA is mutagenic in bacterial and mammalian cells and carcinogenic is rats. In radiolytic system, free radicals generated from water can attack fatty acids resulting in the homolytic dissociation of its C- H bond which may lead to the initiation of lipid peroxidation. Lipid peroxidation is a chain reaction (reactions 1-7) depicted below, which involves three distinct stages namely (a) Initiation. (b) Propogation and (c) Termination

Initiation

$$H_2O \longrightarrow OH^{\cdot}, H^{\cdot}, e^{-}aq, O^{\cdot -2} \quad \text{---------1}$$

$$LH + OH^{\cdot} \longrightarrow L^{\cdot} + H_2O \quad \text{-------- 2}$$

Propogation

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \quad \text{--------3}$$

$$LH + LOO^{\cdot} \longrightarrow LOOH + L^{\cdot} \quad \text{--------4}$$

Termination

$$L^{\cdot} + L^{\cdot} \longrightarrow LL \quad \text{---------5}$$

$$LOO^{\cdot} + LOO^{\cdot} \longrightarrow LOOL + O_2 \quad \text{---------6}$$

$$LOO^{\cdot} + L^{\cdot} \longrightarrow LOOL \quad \text{----------7}$$

A free radical that has sufficient energy to abstract an allylic hydrogen from methylene carbon of PUFA, can initiate lipid peroxidation process. $OH^{\cdot}$ (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 ($L^{\cdot}$) then reacts rapidly with molecular oxygen to form $LOO^{\cdot}$ (reaction 3). Subsequently in a much slower reaction, $LOO^{\cdot}$ attacks another lipid molecule (LH) forming non-radical LOOH while generating new lipid radical $L^{\cdot}$ (reaction 4). $L^{\cdot}$ can again be converted to $LOO^{\cdot}$ on an encounter with oxygen, thus closing the self propogating 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(5-7), (Kale and Sitasawad, 1990).

The major detrimental effects of lipid peroxidation are imparted on cellular membranes. Free radicals derived from PUFA can form cross linkages with proteins,

thereby inactivating their enzymatic or receptor functions. Products of lipid peroxidation like malondialdehyde (MDA) can interact with DNA to form toxic products. In mitochondria, the peroxidation causes membrane swelling, deterioration of electron transport and organelle lysis.

The mutagenic and carcinogenic effects of fatty acid hydroperoxides and MDA have been demonstrated by the Ames Test (Horton and Fairhurst, 1987). An increasing attention has been focussed, on the potential role of oxygen radicals and lipid peroxidation in chemical carcinogenesis (Cerutti, 1985; Goldstein and Witz, 1990; Sun, 1990). Elevated levels of lipid peroxidation in tissues, have been associated with antioxidant depletion due to generation of reactive oxygen species. The basis of oxidative stress is the free radical reaction involving oxygen. Consequently, the prevention of lipid peroxidation becomes indispensable in all the aerobic organisms thus preventing DNA damage.

2.6 MODULATORS EMPLOYED IN THE PRESENT STUDY

Two plant modulators namely *Anacardium occidentale* (Cashew nut) Kernel and *Trachyspermum ammi* (Ajowan) were selected for the present study. The possible chemopreventive potential of these plants were examined by observing its modulatory effect in DMBA induced murine skin carcinogenesis model system and on the drug metabolising enzymes GST and DTD alongwith an estimation of the level of lipid peroxidation.

Anacardium occidentale

Common Name: Cashew, Caju, Cajueiro

Cashew nut (*Anacardium occidentale*) of family Anacardiaceae is consumed throughout the world with great relish and is highly nutritious. The cashew nut tree is an errect, spreading evergreen tree, growing upto a height of 40 inches with a rough

bark and a curious fruit. The fleshy portion, termed as the 'Cashew apple' is the swollen peduncle and disc, while the actual fruit includes the kidney shaped nut attached to the fruit. The cashew apple possesses a yellow or scarlet skin, and its flesh is soft and juicy. The drupaceous nut is greenish grey. They oleaginous shell or pericarp is hard, smooth and shining. It is thick and cellular and contains an acrid oil juice which has a powerful vesicant property. It encloses a slightly curved white kernel (about 30% of the nut) covered by a thin, reddish brown skin or testa.

The cashew nut tree is indigenous to South America. At present India is by far, the largest producer and meets about 95% of the world demand for kernel. The Indian Ayurveda system of medicine has accepted this rich nut as a potent remedy as well as a nutritive food. As for nuts in general, they have been popular with the people of the Indus valley: almonds were good for relieving eye problems, walnuts were regarded as brain tonics, and cashew nuts introduced in the Ayurveda texts in the sixteenth century was used as a stimulant, a rejuvenator and an appetizer medicine. The cashew nut constitutes of an internal kernel and a double layered outer shell which is thick and cellular.

The cashew nut kernel possess a pleasant taste and flavour. They are consumed either raw or fried. They constitute a highly nutritious food and are rich in vitamin A, D and K; minerals, proteins and fatty acids. Their composition is very similar to that of sweet almonds (*Prunus amygdalus* q.v.) and is depicted in Table IV.

The characteristics and composition of the fatty oil which constitutes 40-50% of the cashew nut kernel is depicted in Table V.

The oil consists mostly of glycerides of oleic (73.8%), linoleic (7.7%) stearic and palmitic acids (Patel, Sudborough and Watson, 1923). The cashew nut oil finds its application in the cosmetic industry. Its natural richness in Vitamin E protects it against oxidation and imparts a free radical scavenging activity to its formulated product.

Table IV: Composition of the cashew nut kernel

Water	5.9%
Proteins	21.2%
Fats	46.9%
Carbohydrates	22.3%
Mineral matter	2.4%
Calcium	0.05%
Phosphorous	0.45%
Iron	5.00mg
Calorie value	596/100g

(Reference: Health Bull. No. 23, 1941, 35).

Table V: Characteristics and composition of the fatty oil present in the cashew nut kernel

Specific Gravity/15°	0.9155-0.9180
n_D 40°	1.4623-1.4633
Acid value	2.2-8.2
Sap.value	2.2-8.2
Iod. Value	80.8-89.0
Unsap. Matter	0.41%

[Source: Wealth of India. Vol 1, 1948]

Experimental findings have reported the cashew nut shell oil to be a weak tumour promoting agent in a DMBA induced murine skin papillomagenesis model system (Banerjee and Rao, 1992). Furthermore, since the delicious edible kernels are the processed products, possible contamination of the kernel with the shell oil cannot be overlooked. Epidemic eczematous dermatitis, after consuming cashew nut contaminated with the shell oil constituents have been reported (Marks *et al.*, 1984; Rao, 1992). The present study was therefore done to assess any possible tumour promoting property, of the cashew nut kernel oil in a DMBA induced murine skin papillomagenesis model system. The effect of the cashew nut kernel oil on the drug metabolising enzymes, namely Glutathione-S-transferase (GST) and DT- diaphorase (DTD) along with the effect on the level of lipid peroxidation was also evaluated.

Trachyspermum ammi

Common Name: Ajowan, Omum, Bishop's Weed.

Trachyspermum ammi of family Apiaceae is much valued for its medicinal use and is extensively used for Indian culinary purposes. The plant is an errect, glabrous or minutely pubescent branched, annual upto 90 cm in height and is cultivated almost throughout India. The herb is said to be a native of Egypt. Although it is cultivated in the Mediterranean region and in South West Asian countries such as Iraq, Iran, Afghanistan and Pakistan, ajowan is chiefly produced in India.

Ajowan with its characteristic aromatic smell and pungent taste is widely used as a spice in curries. It is employed either alone or in mixture with other spices and condiments. It finds use in pickles, confectionary and beverages. But, the most important use of ajowan is medicinal and is much valued for its antispasmodic, stimulant, tonic and carminative properties. It is administered in flatulence, atonic dyspepsia and diarrhoea and is often recommended for cholera. It is used most frequently in conjunction with asafoetida, myrobalans and rocksalt. Ajowan is also

effective in relaxed sore throat and in bronchitis, and often constitutes an ingredient of cough mixture. Consumed with buttermilk, it is a common remedy for relieving difficult expectoration due to dried up phlegm. Externally, a paste of the crushed fruit is applied for relieving colic pains, and a hot and dry fomentation of the fruits on the chest is a common remedy for asthma. Ajowan is also used in the preparation of lotions and ointments, applied for checking chronic discharge. It has been shown to possess antibiotic activity against *Salmonella typhosa, Micrococcus pyogenes var. aureus and Escherichia coli.* The roots of the plant are reported to possess diuretic and carminative properties and are used in febrile conditions and in stomach disorders (Kirt and Basu, 1205; Krishna and Badhwar, 1953; George *et al.*, 1947; Bhatnagar *et al.*, 1947). The essential oil of *Trachyspermum ammi* exhibits fungitoxic properties (Singh *et al.*, 1986). An ethereal extract of *Trachyspermum ammi* exhibits antiaggregatory effects and alters arachidonic acid metabolism in human platelets (Srivastava, 1988).

Ajowan is included in the IPC. The fruit is a cremocarp. The drug occurs as entire cremocraps or separated mericarps. Cremocarps are ovoid- cordate to ovate, laterally compressed, 1.7-3.0mm thick, dirty yellow to yellowish brown in colour and half to two thirds apical portion has a slight purplish tinge. At the top of the cremocarp is a bifid stylopod surrounded by five minutes sepals. Each mericarp shows five light coloured ridges and is covered with light yellow protruberances. The drug has an agreeable odour and aromatic and warming taste like that of thymol. It does not contain more than two per cent of foreign organic matter (I.PC. 240, Quadry and Atal, loc. cit.) The composition of the fruit of *Trachyspermum ammi* is depicted in Table VI.

Table VI: Composition of the fruit of *Trachyspermum ammi*

Moisture	7.4
Protein	17.1
Fat	21.8
Fibre	21.2
Carbohydrates	24.6
Mineral matter	7.9
Calcium	1,525
Total phosphorus	443
Phytin phosphorus	296
Iron	27.7
Sodium	56.0
Potassium	1390
Thiamin	0.21
Riboflavin	0.28
Nicotinic acid	2.1 mg/100g
Carotene	71 μg/100g

[Source:Wealth of India. Vol 10, 1976]

Ajowan owes its characteristic odour and taste to the presence of an essential oil (2.4%). Other constituents in the fruits include sugars, tannins and glycosides. The alcoholic extract was found to contain a highly hygroscopic saponin, with a haemolytic index of 500. A yellow crystalline flavone (mp 291-94°) and a steroidal substance (m.p. 140-50°) have been isolated from the furits. (Nutritive value of Indian Foods 85, 120, 129; Roy Chowdhury 1963; Mukherjee, 1967; Rao, 1962; Chakraborti, 1956).

The essential oil obtained by steam distillation of the fruits is known as ajowan oil. It is used in medicine and is official in India pharmacopoeia. The oil, for a long time was the principle source of thymol. Since the introduction of synthetic thymol, production of the oil and of thymol from it, has considerably decreased.

The present study was carried out to evaluate any chemopreventive potential of ajowan, in a DMBA induced murine skin carcinogenesis model system, during the initiation and the promotion phases of skin carcinogenesis. A study was undertaken to test the effect of a test diet of ajowan on the specific activities of some hepatic drug metabolising enzymes namely Glutathione-S-transferase and DT-diaphorase alongwith the effect on the level of lipid peroxidation.

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 CHEMICALS

7, 12 – dimethylbenz (a) anthracene (DMBA), 1-chloro-2, 4 – dinitrobenzene (CDNB), reduced glutathione (GSH), 2, 6 – dichlorophenol indophenol (DCPIP), Triton X-100, ethylenediamine tetraacetic acid, bovine serum albumin (BSA), reduced nicotinamide adenine dinucleotide (NADH), thiobarbituric acid (TBA). All the above mentioned chemicals utilized were obtained from Sigma Chemical Co (St. Louis, MO, USA).

3.2 ANIMALS

Random bred, female Swiss albino mice (6 weeks old) were used for the experimental study. They were maintained in the air conditioned animal house facility (Jawaharlal Nehru University, New Delhi) with a 12hour light/dark cycle and was provided with standard food pellets (Golden Feeds - India) or test diet , accordingly, and tap water *ad libitum*. Animals were handled as per the rules and guidelines set by the animal ethics committee (IAEC).

3.3 PREPARATION OF TEST MATERIALS

Anacardium occidentale (cashew nut) kernel oil was obtained through acetone extraction. The required amount of cashew nut kernel was powdered and was mixed with double the volume of acetone. The mixture was stirred and left for 3-4 days after which the same was filtered. The acetone was allowed to naturally evaporate from the filtrate at room temperature for 3 days, followed by slight heating in the water bath (45^0C) such that a concentrated cashew nut kernel oil was obtained. For skin papilloma study, cashew nut kernel oil at 2.5% and 5% dilution with acetone was used. For assay of drug metabolising enzymes(GST and DTD)and the level of lipid

peroxidation, concentrated cashew nut kernel oil at two dose levels (50 µl & 100 µl) were used for oral feeding of the animals.

Trachsypermum ammi (Ajowan)

For skin tumour study 5% aqueous extract of ajowan was prepared. For assay of the drug metabolising enzymes (GST and DTD) and the level of lipid peroxidation; 2.5% and 5% test diet was prepared by mixing ajowan powder with powdered form of standard food pellets.

3.4 EXPERIMENTAL DESIGN

Experiment 1

The experimental procedure followed in the present study is essentially the same as explained in the work of Banerjee and Rao (1992).The hairs on the dorsal scapular region (2 cms diameter) of the mice were clipped off three days before the application of the carcinogen (DMBA – 50 µg/50 µl acetone per animal). The animals were assorted into control and experimental groups as schematically illustrated in the table below:

S.No.	Groups	Mode of treatment	Effective no. of animals
1.	Control		10
2.	DMBA (50 µg/50µl acetone)	Topical application	10
3.	Acetone (Vehicle)	Topical application	10
4.	DMBA+2% Croton Oil	Topical application	15
5.	DMBA+2.5% cashew nut kernel oil	Topical application	15
6.	DMBA+5% cashew nut kernel oil	Topical application	15
7.	5% cashew nut kernel oil	Topical application	15

DMBA (50 μg/50 μl acetone/animal) was topically applied on the shaven area of the 6 weeks old mice to achieve initiation. After a gap of one week, 2% croton oil (Promoter) was applied 3 times a week to achieve promotion, in the positive control group. In two separate experimental groups 2.5%, and 5% cashew nut kernel oil in acetone was topically applied. In the negative control group no DMBA was applied, only topical application of acetone was made three times a week. Number of papillomas, were noted down at weekly interval from the fifth week after initiation of croton oil treatment. The hairs on the dorsal scapular region was shaved off at regular intervals for tumour count and the body weight was noted at weekly intervals. The animals were sacrified at the end of four months. Skin papilloma of the size above 1 mm were counted and noted down.

Experiment 2

The experiment was designed to study the effect of cashew nut kernel oil on some drug metabolising enzymes (GST and DTD) and on the level of lipid peroxidation. Female Swiss albino mice (6 weeks old) were divided into the following groups:

Group I (n = 6): Animals were orally fed with normal drinking water (vehicle). This served as the control group.

Group II (n = 7): Animals were orally fed with 50 μl of concentrated cashew nut kernel oil.

Group III (n = 7): Animals were orally fed with 100 μl of cashew nut kernel oil.

(n refers to the total number of animals in each group)

Groups of mice were maintained in separate cages. Treatment with the modulator was initiated from day one and was continued upto the tenth day. At the end of the tenth day, the animals were starved overnight and sacrificed by cervical dislocation.

Experiment 3

This experiment was designed to study the effect of *Trachyspermum ammi* on the initiation and promotion phases of skin carcinogenesis in murine model system. The general experimental procedure followed was the same as that explained for experiment I. Animals were divided into the following groups as shown in the table below:

S.No.	Groups	Mode of treatment	Effective no. of animals
1.	Control	-	10
2.	DMBA (50 μg/50μl acetone)	Topical application	10
3.	DMBA+2% croton oil	Topical application	14
4.	Modulator+DMBA+2% croton oil	Oral feeding of the modulator (initiation phase)	15
5.	DMBA+2% croton oil+ modulator	Oral feeding of the modulator during the promotion phase	14
6.	DMBA+2% croton oil+ modulator	Topical application of the modulator throughout the experiment	15

Modulator = 5% and 10% aqueous extract of *Trachyspermum ammi* for oral feeding and topical application respectively.

Experiment 4

To study the effect of *Trachyspermum ammi* on the drug metabolising enzymes (GST and DTD) and on the levels of lipid peroxidation female Swiss albino mice (6 weeks old) were divided into the following groups:

Group 1 (n = 6): Animals were fed with normal diet. This served as the control group.

Group 2 (n = 6): Animals were fed with 2.5% test diet.

Group 3 (n = 6): Animals were fed with 5% test diet.

(n refers to the total number of animals in each group)

All the animals were given drinking water *ad libitum*. Groups of mice were maintained in separate cages. Treatment with the modulator was initiated from day one and was continued upto the tenth day. At the end of the tenth day, the animals were starved overnight and sacrificed by cervical dislocation.

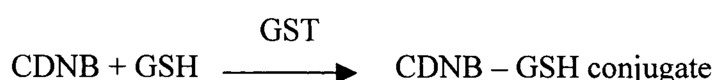
3.5 PREPARATION OF THE HOMOGENATE, CYTOSOL AND MICROSOME FRACTIONS OF THE LIVER FOR ENZYME ASSAY.

Animals were sacrificed by cervical dislocation and the entire liver was then perfused immediately with ice cold 0.9% sodium chloride (NaCl) and thereafter carefully removed, trimmed free of extraneous tissue, and rinsed in chilled 0.15 M Tris-KCl buffer (pH = 7.4). The liver was then blotted dry, weighed quickly and homogenized in ice cold 0.15M Tris-KCl buffer (pH=7.4) to yield a 10% (w/v) homogenate which was centrifuged at 10,000 rpm for 20 minutes. The resultant supernatant was transferred into pre cooled ultracentrifugation tubes and centrifuged

suspended in homogenizing buffer and was used for estimating the level of lipid peroxidation.

Determination of specific activity of cytosolic glutathione – S – transferase (GST)

Principle: The specific activity of glutathione – S – transferase was determined by the rate of formation of CDNB – GSH conjugate generated as a result of reaction between glutathione and CDNB catalysed by the enzyme glutathione – S – transferase. The conjugate absorbs at 340 nm wavelength.



Assay: The specific activity of GST was measured by using the method of Habig *et al* (1974). Three ml volume of the reaction mixture contained final concentration of 0.1 M sodium phosphate buffer (pH = 6.5), 1 mM CDNB in 95% ethanol, 1 mM GSH; and was incubated at 37^0C for five minutes. The reaction was initiated by the addition of the diluted cytosolic sample. The enzyme activity was followed for five minutes at 340 nm. The specific activity of GST was calculated using the following formula below, with 9.6 mM^{-1} cm^{-1} as the molar extinction coefficient and is expressed in terms of μ mole of CDNB – GSH conjugate formed/min/mg protein.

$$\text{Specific activity} = \frac{\Delta OD/\text{min} \times 3\text{x dilution}}{9.6 \times \text{mg protein in sample}}$$

Where 3 is the reaction volume.

ΔOD/min is the increase in the optical density per minute.

Determination of specific activity of DT-diaphorase

Principle: DT –diaphorase assay is based on the oxidation of NADH and reduction of the DCPIP, which is catalysed by DT-diaphroase.

Assay: The specific activity of DT-diaphorase was measured as described by Ernster *et al.* (1962). The reaction mixture contained 50mM Tris HCl buffer (pH= 7.5), 0.5 m M NADH, 40μM DCPIP and 0.08% Triton X-100 as an activator in a final volume of 1 ml. The reaction was initiated at 25°C by the addition of cytosolic fraction and the rate of reduction of DCPIP was measured at 600 nm. The specific activity was calculated using extinction co-efficient 21 mM^{-1} cm^{-1}. One unit of enzyme activity is defined as the amount of enzyme required to reduce one micromole of DCPIP per min.

$$\text{Specific activity} = \frac{\Delta OD/\text{min} \times 1 \times \text{dilution}}{21 \times \text{mg protein in sample}}$$

in which 1 is the reaction volume in ml.

Estimation of lipid peroxidation

Polyunsaturated fatty acids breakdown to simple aldehydes mainly malondialdehyde (MDA) as a result of peroxidative process. MDA serves as a convenient index for the estimation of peroxidative damage. It reacts with thiobarbituric acid (TBA) on heating under acidic condition to yield a pink coloured product which absorbs at 531.8 nm wavelength. Lipid peroxidation in microsomes prepared from liver was estimated spectrophotometrically by thiobarbituric acid – reactive substances (TBARS) method as described by Varshney and kale (1990) and is expressed in terms of malondialdehye (MDA) formed per mg protein.

In brief, 0.4 ml of microsomal sample was mixed with 1.6 ml of Tris KCl (0.15 M KCl + 10 mM Tris – HCl, pH = 7.4) buffer to which 0.5 ml of 30% TCA was added. Then 0.5 ml of 52mM TBA was added. The tubes were covered with aluminium foil and placed in a water bath at 80 degree Celsius temperature for 45 minutes, cooled in ice and centrifuged at room temperature for 10 minutes at 3000 rpm in REMI–T8 table top centrifuge. The absorbance of the clear supernatant was

measured against reference blank of distilled water at 538.1 nm in sepectrophotometer (Shimadzu UV-160).

The amount of MDA formed in the sample was estimated according to the equation:

$$\text{n moles of MDA} = \frac{V \times OD}{0.152 \times \text{mg protein in sample}}$$

where V = final volume of the test solution in ml.

OD = optical density

0.152 = Molar extinction coefficient.

Determination of the cytosolic and microsomal protein content

The protein content in the cytosolic and microsomal fraction was determined by using the method of Lowry *et al* (1951).

Principle: Protein reacts with the Folin-Ciocalteau Reagent to yield a coloured complex. The colour formed is due to the reaction of the phosphomolybdate with tyrosine and tryptophan residues of the protein.

Assay: Freshly prepared alkaline solution (50 ml of 2% sodium carbonate in 0.1 N sodium hydroxide and 1 ml of 0.5% copper sulphate ($CuSO_4$) in 1% sodium potassium tartarate) (5 ml) was added to 1 ml of test solution which contained BSA or protein sample. It was mixed thoroughly followed by incubation at room temperature for 15 minutes. Subsequently, 1 N Folin Ciocalteau Reagent (0.5 ml) was added and vortexed, followed by an incubation for 45 minutes at room temperature. The absorbance was measured at 660 nm against the reference blank. The protein content of each sample was evaluated from the standard curve made with BSA, and was expressed in mg/ml.

3.6 HISTOPATHOLOGICAL PREPARATIONS

After sacrificing the animals, the skin bearing the tumour were recovered and fixed in 10% formalin. The tissues were then processed, following standard techniques, for histopathological studies. Stained (Haematoxylin and Eosin) sections were observed under the microscope to ascertain whether the retrieved tissues had papillomas. Likewise the normal skin was also processed for comparison.

3.7 STATISTICAL ANALYSIS

The statistical significance of the difference in the enzyme activities between the control and the experimental groups was evaluated by analysis of variance (ANOVA) followed by Mann-Whitney Rank Sum Test. The difference in the mean number of tumours was analysed by ANOVA followed by students t-test.

4. RESULTS

4.1 CHEMOMODULATORY ACTION OF *Anacardium occidentale*

In DMBA induced skin carcinogenesis, the topical application of 2.5% and 5% cashew nut kernel oil did not produce any tumours. The tumour incidence (number of tumour bearing mice/effective number of mice) was 86% in the positive control group, with the mean number of tumours per tumour bearing mouse as 4.917 ± 1.084. (Table 1, Plate 1).

The study of the drug metabolising enzyme exhibited a significant increase in the specific activity of Glutathione-S- transferase at both the low dose (50μl) and high dose (100 μl) levels; the magnitude being 1.231 ($P<0.05$) and 1.4 77 ($P< 0.005$) respectively when compared to the control group (0.962). (Table 3, Figure 1).

There was no significant change in the specific activity of DT diaphorase at both the dose levels. (Table 3, Figure2).

The level of lipid peroxidation estimated as malondialdehyde formation in the microsomal homogenate fraction was significantly ($P<0.05$) decreased in the animal group treated with oral administration of 100 μl cashew nut kernel oil. The magnitude of lipid peroxidation in this group was 1. 291 ($P < 0.05$) when compared to the control group (1.872 ± 0.242) (Table 3, Figure 3).

4.2 CHEMOMODULATORY ACTION OF *Trachyspermum ammi*

In DMBA induced skin cancer, oral administration of the 5% aqueous extract of ajowan at the initiation and promotion phases inhibited the mean number of tumours per tumour bearing mouse to 3.100 ± 1.595 ($P < 0.006$) and 1. 875 ± 0.835 ($P < 0.001$) respectively. In the control group, the tumour incidence was 86% and the mean number of tumours per tumour bearing mouse was 5. 083 ± 1.165. The decrease in tumour multiplicity of the group of animals treated with the modulator at initiation phase and promotion phase was by 39% and 63.11% respectively. In case of

the group of mice with topical application of 10% aqueous extract of ajowan, the tumour incidence was reduced by 66.6% and the mean number of tumours per tumour bearing animals was 2.4 ± 1.075 ($P < 0.001$). The decrease in the tumour multiplicity was by 52.78% as compared to the control group (Table 4, Figure 4). (Plate 2-Plate 5)

The study of the drug metabolising enzymes, exhibited a significant increase ($P < 0.05$) in the specific activity of Glutathione-S- transferase at the high dose (5%) test diet, the magnitude being 1.120 ± 0.138 ($P< 0.05$) when compared to the control group (0.860 ± 0.106) (Table 5, Figure 5).

There was no significant change in the specific activity of DT diaphorase at both the dose levels of the test diet (Table5, Figure 6).

The level of lipid peroxidation estimated as malondialdehyde formed in the microsomal homogenate fraction was significantly increased by the high dose (5%) test diet, the magnitude being 0.500 ± 0.0027 ($P< 0.005$) when compared to the control group. (0. 543 ± 0. 0079). (Table5, Figure 7).

In case of histopathological preparations, the microtome sections of the skin papilloma and the normal skin were observed under the microscope (Plate 6, Plate 7).

Table 1: Effect of two different doses of cashew nut kernel oil in DMBA induced Swiss albino mice skin carcinogenesis model

S.No.	Groups	Mode of treatment	Effective no. of animals	Number of mice with skin tumours (%)	Mean number of tumours/ tumour bearing mouse
1.	Control		10	0	
2.	DMBA (50 µg/50µl acetone)	Topical application	10	0	
3.	Acetone (Vehicle)	Topical application	10	0	
4.	DMBA+2% Croton Oil	Topical application	15	12 (86)	4.917 ± 1.084
5.	DMBA+2.5% cashew nut kernel oil	Topical application	15	0	
6.	DMBA+5% cashew nut kernel oil	Topical application	15	0	
7.	5% Cashew nut kernel oil	Topical application	15	0	

Values are expressed as mean ± SD of 15 animals.

Table 2: Promoting effect of cashew nut shell oil in Swiss albino mice skin carcinogenesis model

Groups	Treatment	No. of mice (initial)	No. of mice (effective)	No. of mice with skin tumours (%)	Mean no. of tumours per effective mouse (a)
I.	Control	20	20	0	0
II.	DMBA	20	20	0	0
III.	DMBA+1% Croton Oil	20	19	18 (94.7)	6.6
IV.	DMBA+cashew nut shell oil (1%)	20	20	9 (45)[b]	1.1
V.	DMBA+cashew nut shell oil (2%)	20	18	10 (55)[c]	2.5
VI.	Cashew nut shell oil 2%	20	20	0 (0)	0

[a] no. of tumor bearing animals/ no. of survivors of the group.

[b] $p < 0.001$; group IV vs group II.

[c] $p < 0.001$; group V vs group II.

[Adapted from: Banerjee, S. and Rao, A.R., 1992. *Cancer Letters*, **62**, 149-152].

Row 1 Row 2 Row 3

Plate 1. Effect of topical application of two dose levels (2.5% and 5%) of cashew nut kernel oil in DMBA induced skin carcinogenesis of Swiss albino mice.

Row 1- DMBA + 2 % croton oil.(Tumour incidence=86%)

Row 2- DMBA + 2.5% cashew nut kernel oil. (Tumour incidence=0)

Row 3- DMBA + 5% cashew nut kernel oil (Tumour incidence =0)

Table 3: Effect of two different doses of cashew nut (*Anacardium occidentale)* kernel oil on the specific activity of mouse hepatic drug metabolising enzymes (GST and DTD) and on the level of lipid peroxidation.

Groups	GST ①	DTD ②	LP ③
Control (Drinking Water)	0.962 ± 0.197	0.0232 ± 0.0035	1.872 ± 0.242
Low Dose (50 μl of cashew nut kernel oil)	1.231 ± 0.100[a]	0.0207 ± 0.0045	1.603 ± 0.412
High Dose (100 μl of cashew nut kernel oil)	1.477 ± 0.180[c]	0.0277 ± 0.0079	1.291 ± 0.315[a]

Values are expressed as mean ± SD of 6-8 animals.
Values in parentheses represent relative change in parameters assessed (that is, levels of parameter assessed in liver of mice receiving test substance.

a ($p < 0.05$), c ($p < 0.005$) represent statistical significant difference between the experimental group and the control group.

① μ mole of CDNB – GSH conjugate formed/min/mg protein.

② μ mole of DCPIP reduced/min/mg protein.

③ n mole of malondialdehyde formed/mg protein.

Abbreviations: GST: Glutathione-S-transferase, DTD: DT diaphorase, LP: Lipid peroxidation.

Treatment duration = 10 days.

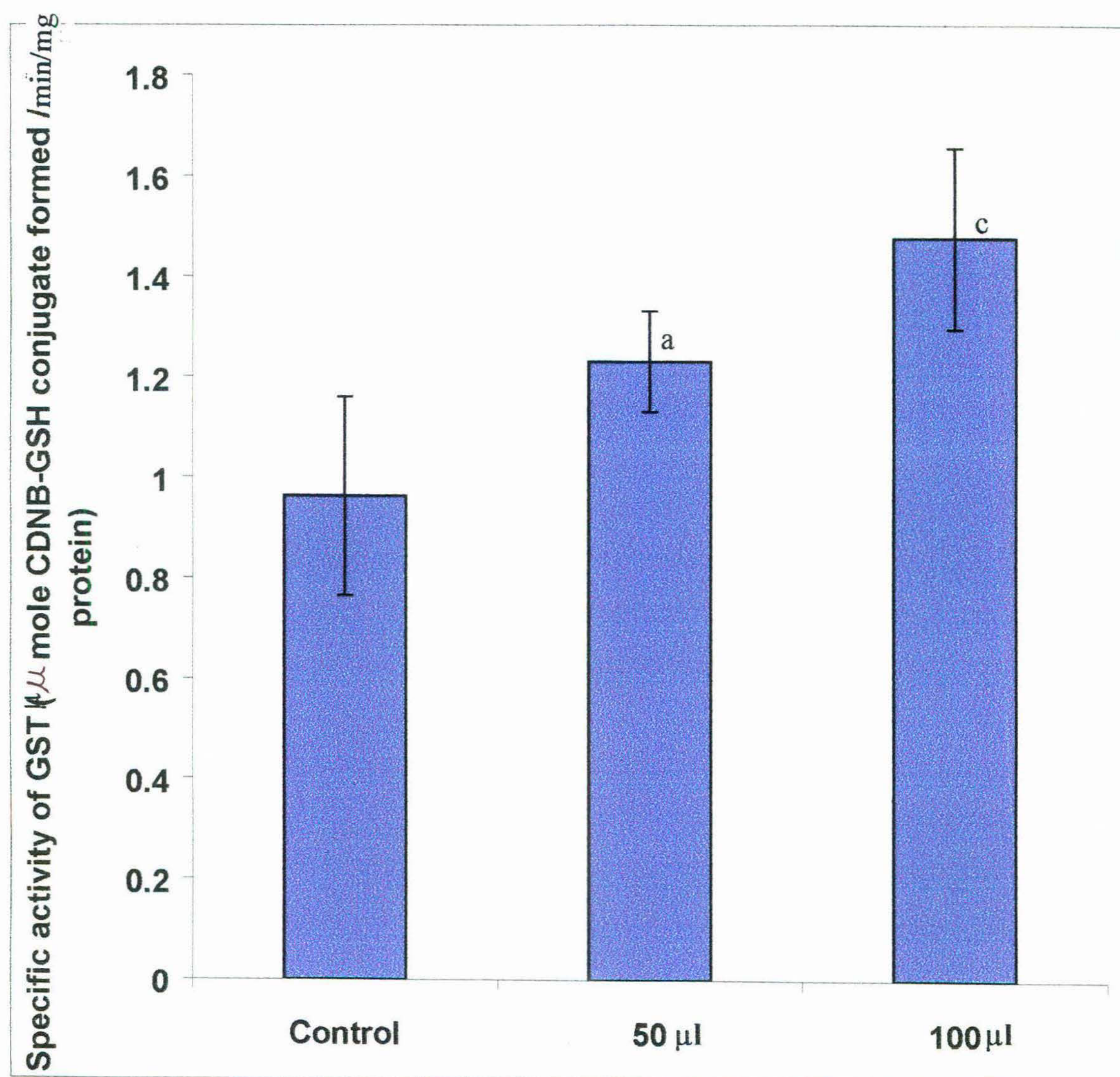


Figure 1. Effect of oral administration of two different doses (50µl and 100µl) of cashew nut (*Anacardium occidentale*) kernel oil on the specific activity of glutathione-S- transferase (GST) in the liver of female Swiss albino mice.

Error bars represent standard deviation.

a ($P<0.05$), c ($P<0.005$) represent the statistical significant difference between the experimental group and the control group.

Treatment duration = 10 days.

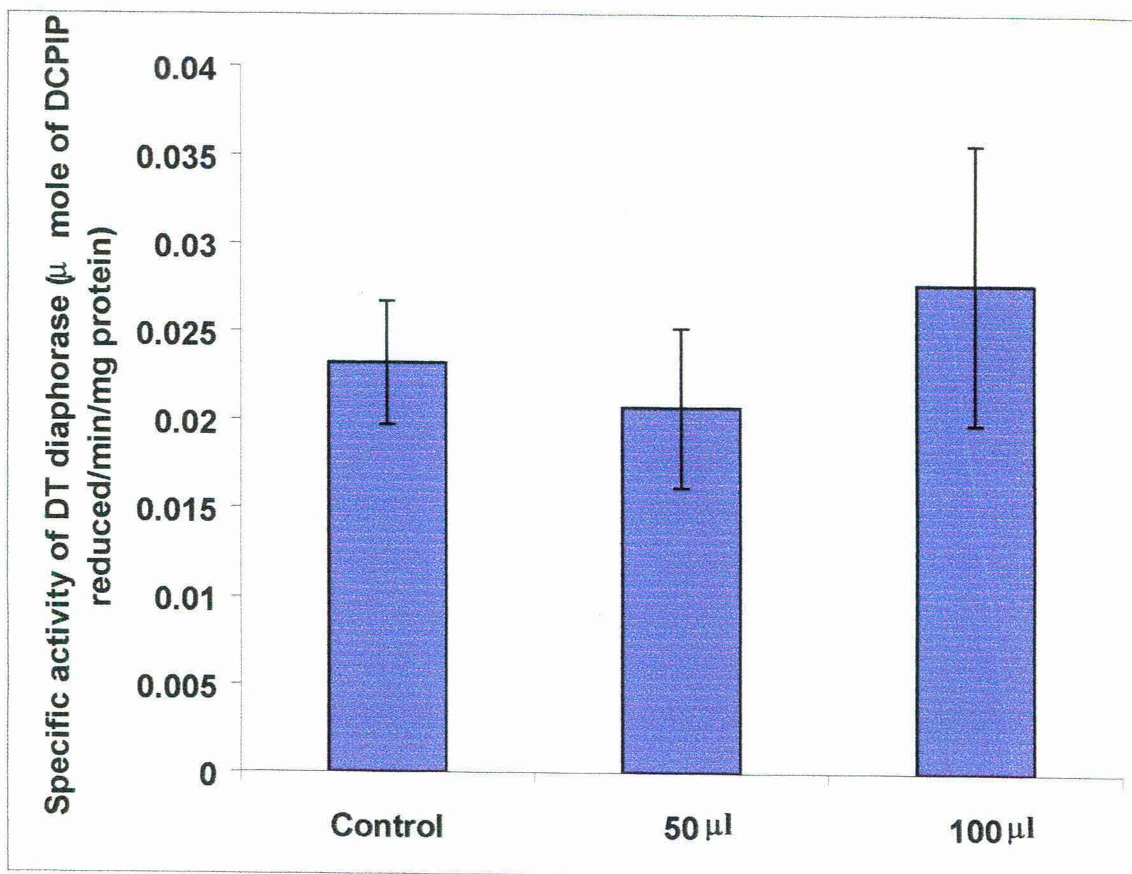


Figure 2. Effect of oral administration of two different doses (50μl and 100μl) of cashew nut (*Anacardium occidentale*) kernel oil on the specific activity of DT diaphorase (DTD) in the liver of female Swiss albino mice.
Error bars represent standard deviation.
Treatment duration = 10 days.

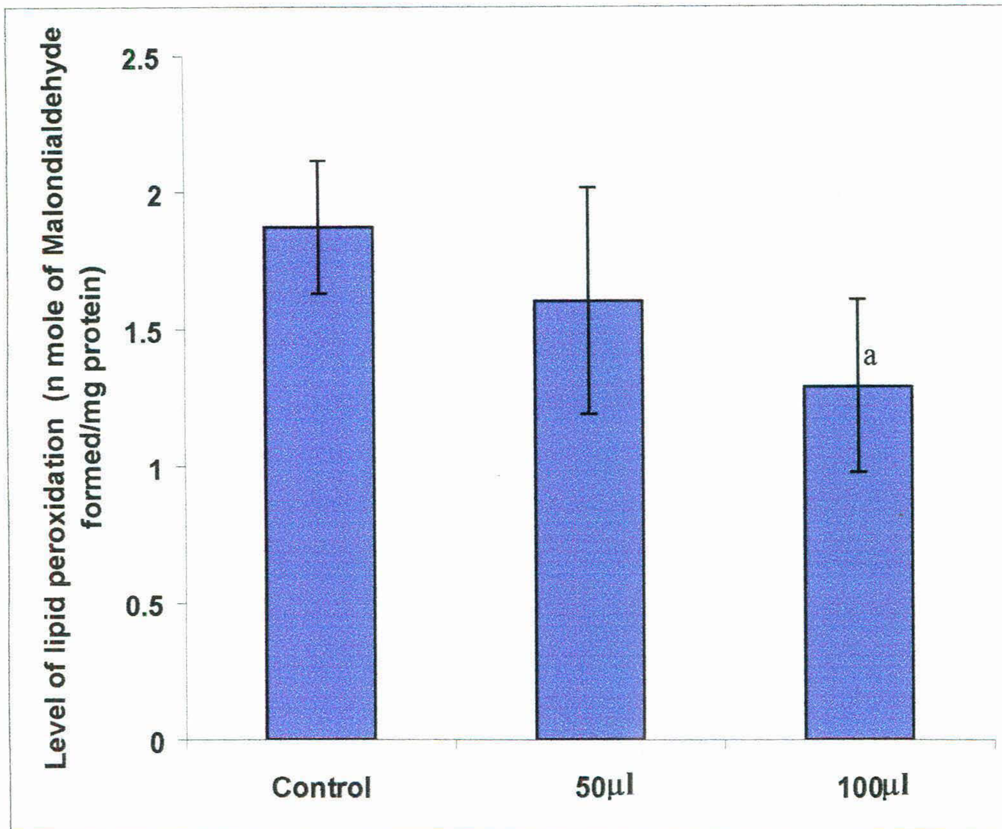


Figure 3. Effect of oral administration of two doses (50μl and 100μl) of cashew nut (*Anacardium occidentale*) kernel oil on the levels of lipid peroxidation in the liver of female Swiss albino mice.
Error bars represent standard deviation.
a ($P < 0.05$) represent statistical significant difference between the experimental group and the control group.
Treatment duration = 10 days.

Table 4: Modulatory effect of *Trachyspermum ammi* in DMBA induced Swiss albino mice skin carcinogenesis model at the initiation and promotion phases.

S.No.	Groups	Mode of treatment	Effective no. of animals	Number of mice with tumour (%)	No. of tumours/ mouse	% decrease in tumour multiplicity
1.	Control	-	10	0		
2.	DMBA (50 μg/50μl acetone)	Topical application	10	0		
3.	DMBA+2% croton oil	Topical application	14	12 (86)	5.083 ± 1.165	
4.	Modulator+DMBA +2% croton oil	Oral feeding of the modulator during the initiation phase	15	10 (66.66)	3.100±1.595 [a]	39%
5.	DMBA+2% croton oil+ modulator	Oral feeding of the modulator during the promotion phase	14	8 (57.1)	1.875 ± 0.835[b]	63.1%
6.	DMBA+2% croton oil+ modulator	Topical application of the modulator throughout the experiment	15	10 (66.66)	2.4 ± 1.075[b]	52.78%

Values are expressed as mean ± SD of 10-15 animals.

a(P<0.05), b (P<0.001) represent statistical significant difference between the experimental group and the control group.

Modulator used: 5% and 10% aqueous extract of *Trachyspermum ammi* for oral feeding and topical application respectively.

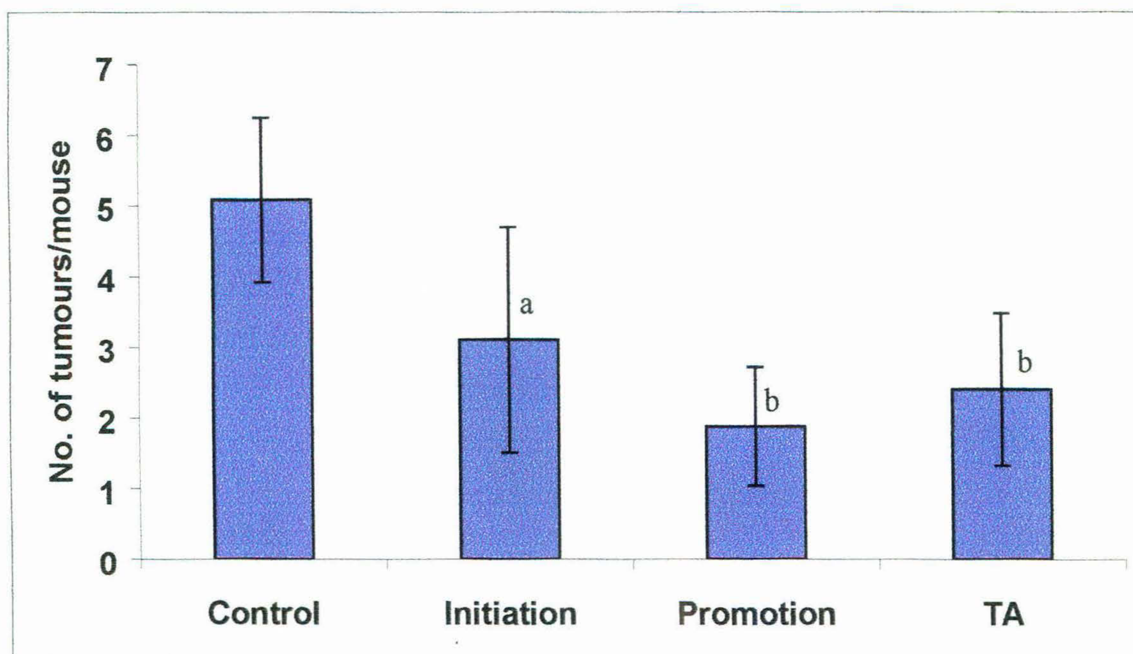


Figure 4: Effect of oral administration of 5% aqueous extract of *Trachyspermum ammi (*ajowan) on the initiation and promotion phase and the effect of topical application of 10% aqueous extract of ajowan on DMBA induced Swiss albino mice skin carcinogenesis model.

Error bars represent standard devitation.

Values are expressed as mean ± SD of 10-15 animals.

a(P<0.05), b (P<0.001) represent statistical significant difference between the experimental group and the control group.

TA= Topical application of 10% aqueous extract of ajowan.

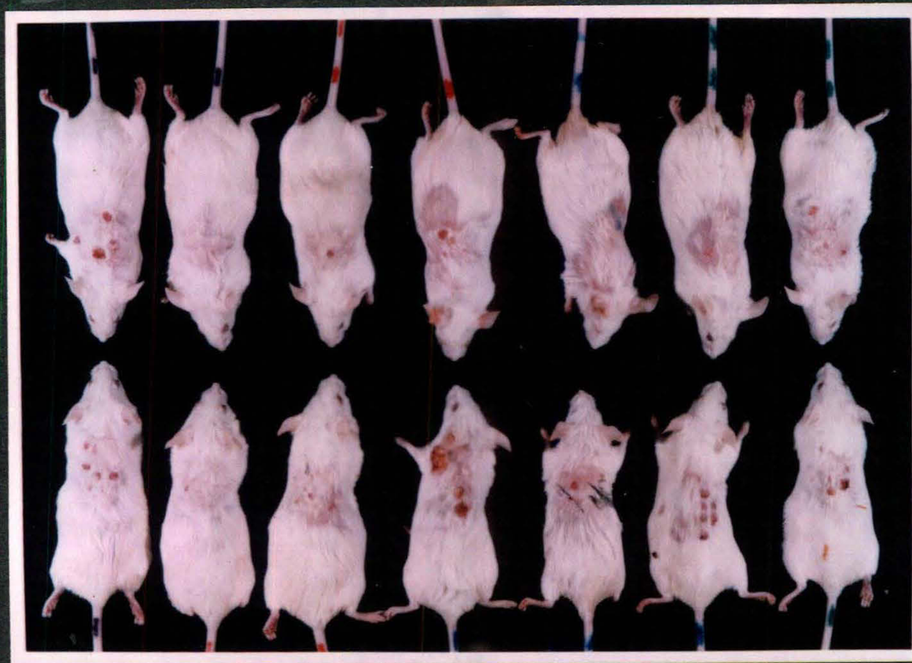


Plate 2. Induction of skin carcinogenesis in Swiss albino mice using DMBA as an initiator and 2% croton oil as a promoter. (Tumour incidence 86%)

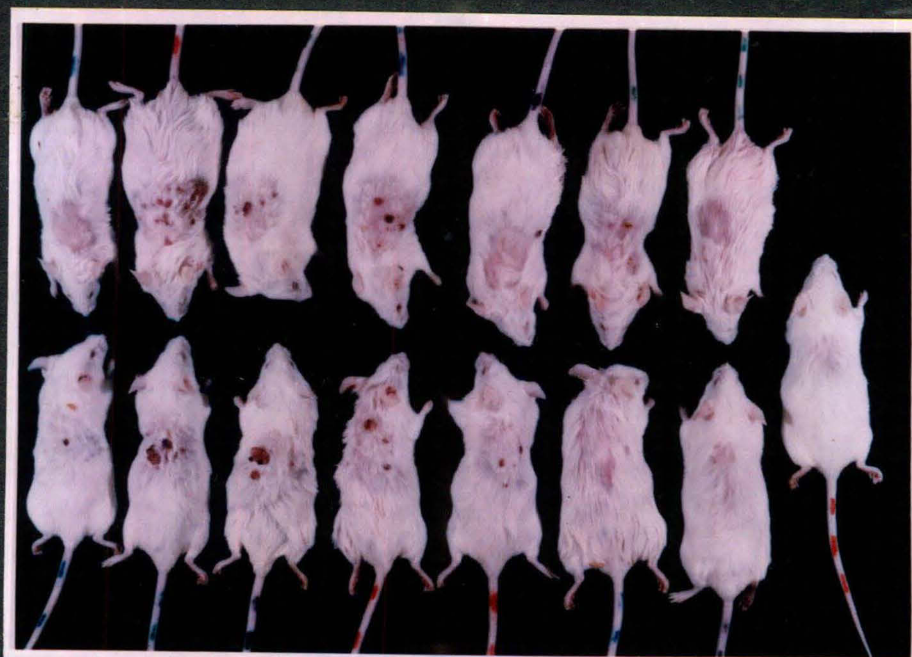


Plate 3. Effect of oral administration of 5% aqueous extract of *Trachyspermum ammi,* at the initiation phase of DMBA induced skin carcinogenesis in Swiss albino mice. (Tumour incidence = 66.66%)

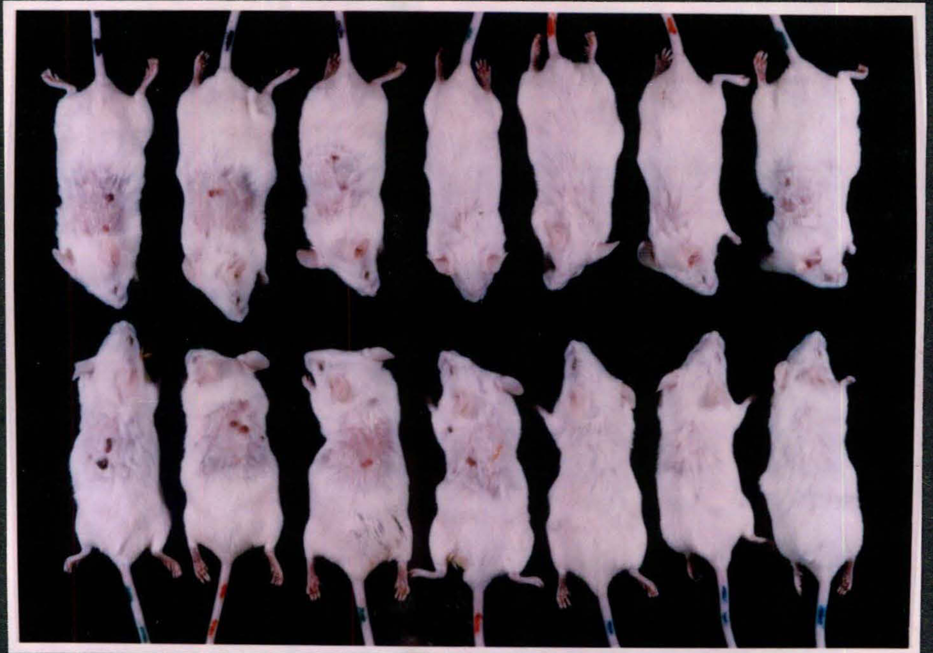


Plate 4. Effect of oral administration of 5% aqueous extract of *Trachyspermum ammi,* at the promotion phase of DMBA induced skin carcinogenesis in Swiss albino mice.

(Tumour incidence=57.1%)

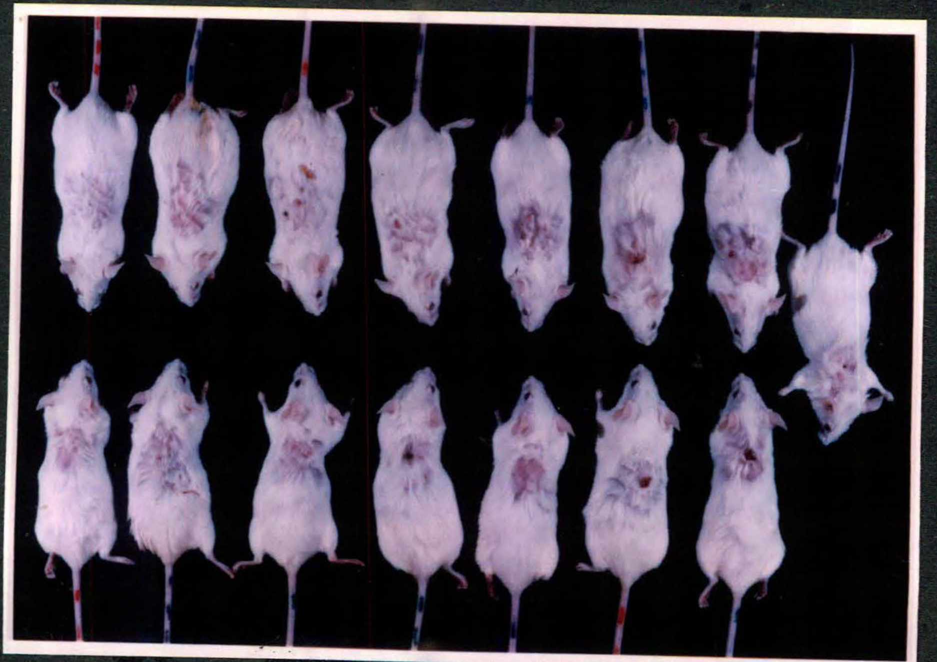


Plate 5. Effect of topical application of 10% aqueous extract of *Trachyspermum ammi,* in DMBA induced skin carcinogenesis in Swiss albino mice.

(Tumour incidence = 66.66%)

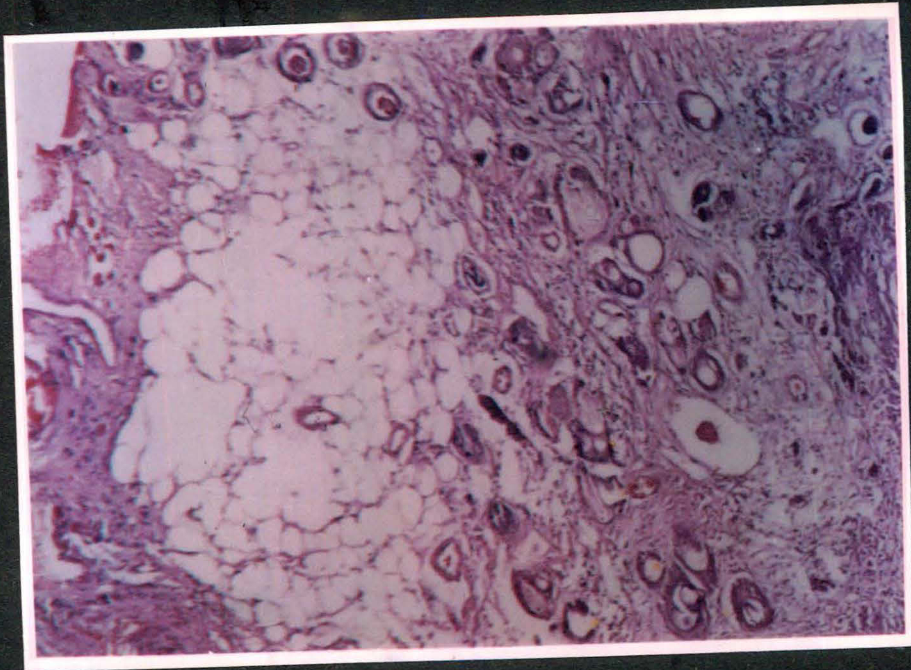


Plate 6. Histopathological section of normal skin with no pathological conditions (63X).

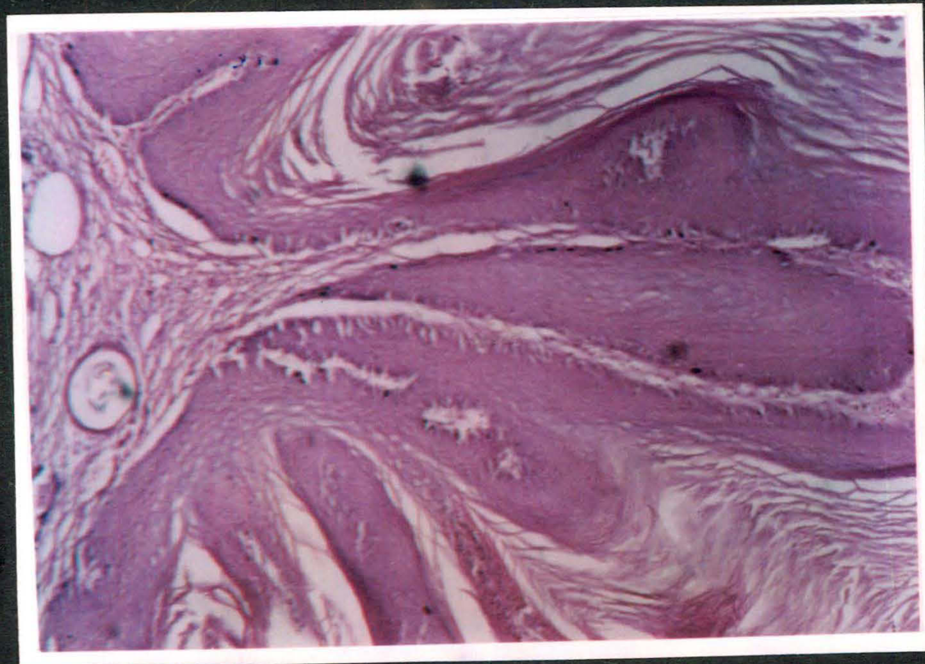


Plate 7. Histopathological section of skin with papilloma (63X).

Table 5: Effect of two different doses of *Trachyspermum ammi* on the specific activity of the mouse hepatic drug metabolizing enzymes (GST and DTD) and on the level of lipid peroxidation

Groups	GST ①	DTD ②	LP ③
Control (Normal Feed)	0.860 ± 0.106	0.0288 ± 0.0065	0.543 ± 0.0079
Low Dose (2.5%) Test diet	1.120 ± 0.138[a]	0.0301 ± 0.0033	0.464 ± 0.107
High Dose (5%) Test diet	1.003 ± 0.06183	0.0328 ± 0.0032	0.500 ± 0.0027[c]

Values are expressed as mean ± SD of 6-8 animals.

Values in parentheses represent relative change in parameters assessed (that is, levels of parameter assessed in livers of mice receiving test substance to that of control mice).

[a] ($p < 0.05$) and [c] ($p < 0.005$) represent statistical significant difference between the experimental group and the control group.

① μ mole of CDNB-GSH conjugate formed/min/mg protein.

② μ mole of DCPIP reduced/min/mg protein.

③ n mole of malondialdehyde formed/mg protein.

Abbreviations: GST: glutathione-S-transferase, DTD: DT diaphorase; LP: Lipid Peroxidation.

Treatment duration: 10 days

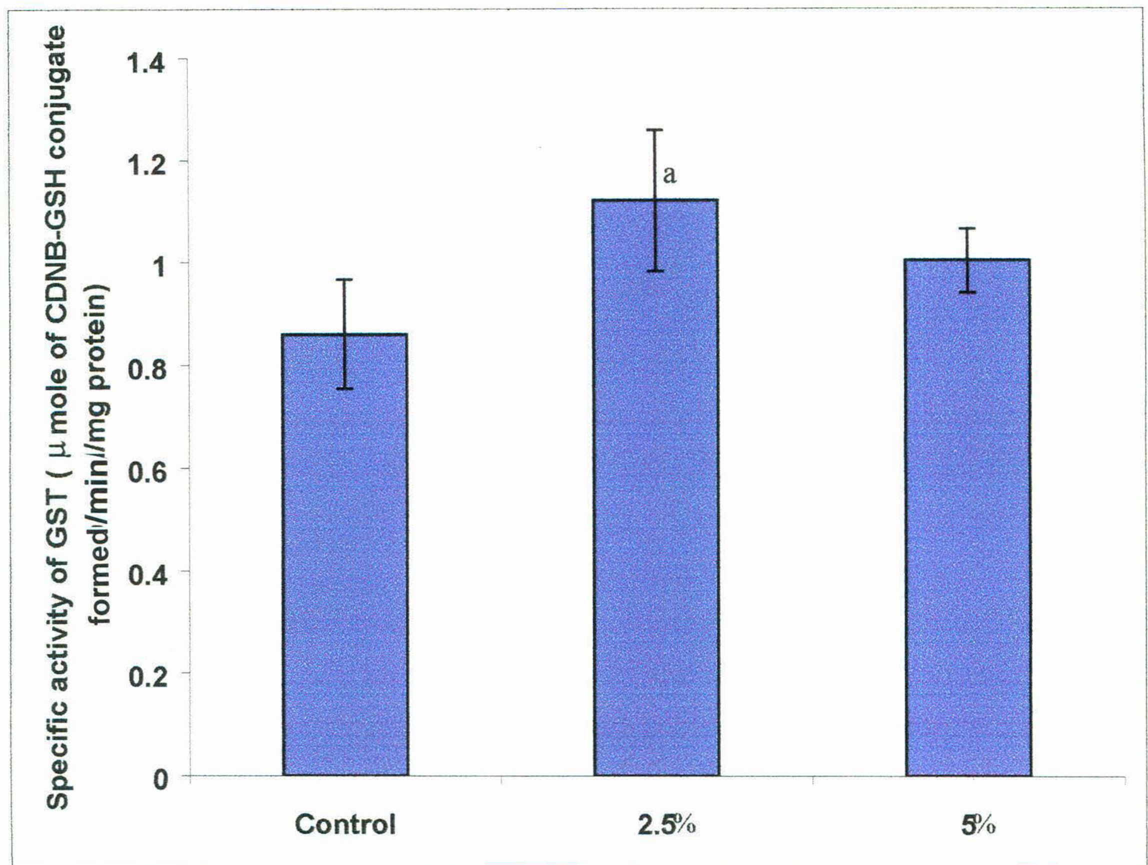


Figure 5: Effect of two different doses of *Trachyspermum ammi* (ajowan) in the test diet, on the specific activity of Glutathione-S-transferase in the liver of female Swiss albino mice.

Error bars represent standard deviation.

a ($P < 0.05$) represent statistical significant difference between the experimental group and the control group.

Treatment duration = 10 days.

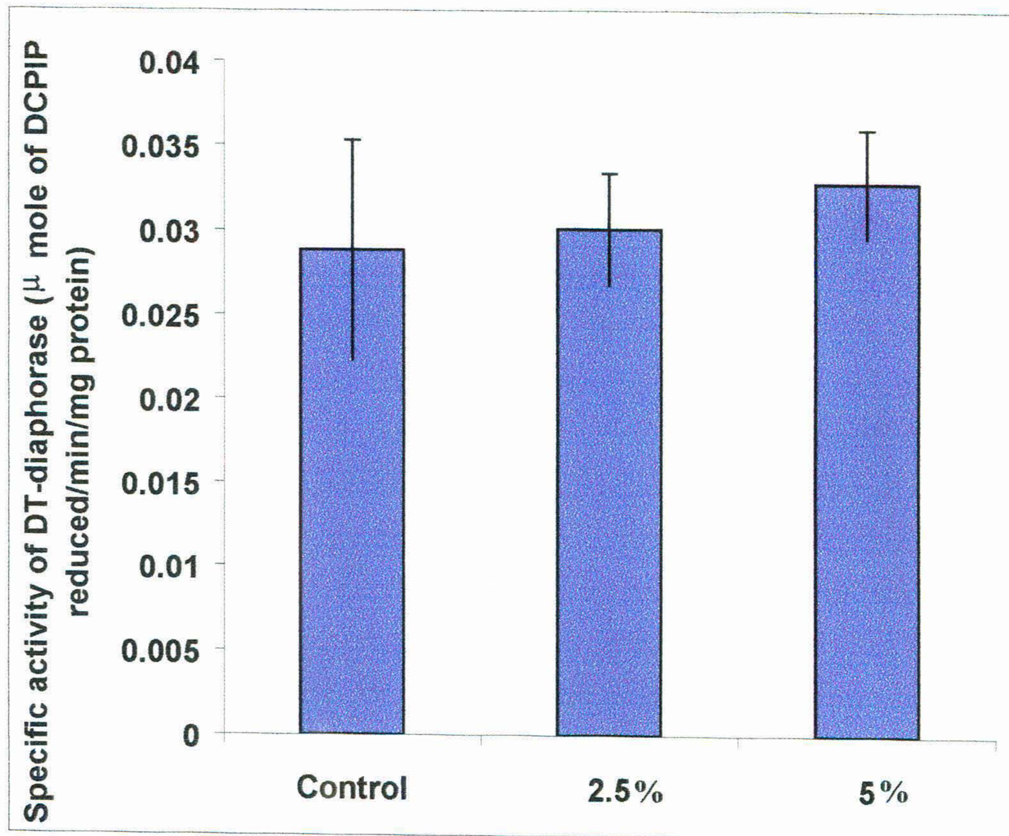


Figure 6: Effect of two different doses of *Trachyspermum ammi* (ajowan) in the test diet, on the specific activity of DT- diaphorase in the liver of female Swiss albino mice.

Error bars represent standard deviation.

Treatment duration = 10 days.

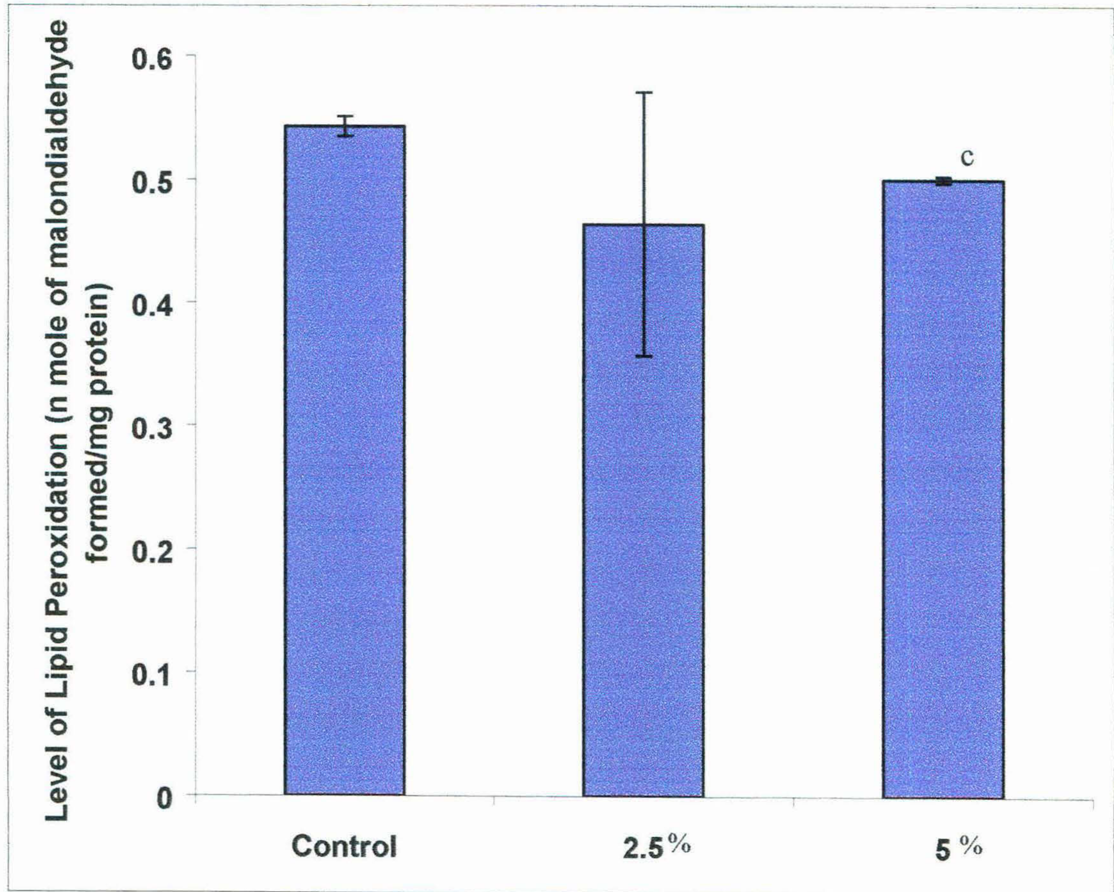


Figure 7: Effect of two different doses of *Trachyspermum ammi* (ajowan) in the test diet, on the level of lipid peroxidation in the liver of female Swiss albino mice.
Error bars represent standard deviation.
c (P< 0.005) represent statistical significant difference between the experimental group and the control group.
Treatment duration = 10 days.

5. DISCUSSION

Cancer chemoprevention is an exciting pharmaceutical cancer research involving the use of either naturally or synthetic agents to delay, inhibit or reverse the development of cancer in normal or preneoplastic conditions (Tanaka, 1994; Morse and Stoner, 1996; Pezzuto, 1997). Numerous experimental studies in rodents have identified certain food, food additives and plants with medicinal value as being effective in eliciting a chemomodulatory action on site specific tumour models (Rao *et al.*, 1980; Rao, 1984; Block *et al.*, 1992; Hayatsu *et al.*, 1993; Thorling, 1993; Rao and Hashim, 1995; Singh, 1999). A thorough understanding of the mechanisms through which the synthetic and naturally occurring compounds mediate protection from cancer is necessary, before the most effective dietary or pharmacologic strategies can be implemented to the general human population for prevention of cancer (Awasthi *et al.*, 1996).

The present study was an attempt to assess any possible cancer chemopreventive potential of *Anacardium occidentale* and *Trachyspermum ammi*. In case of *Anacardium occidentale* (cashew nut), it has been reported that the cashew nut shell oil exhibits a tumour promoting property in DMBA induced mice skin carcinogenesis model system (Banerjee and Rao, 1992). As compared to 1% croton oil which produced 94.7% tumours, the promoting efficacies of cashew nut shell oil at the dose levels of 1% and 2% were 45% and 55% respectively (Table 2). The cashew nut shell oil produced no tumours on its own indicating no solitary carcinogenic activity of the shell oil. (Table 2), (Banerjee and Rao, 1992).

Since the delicious edible cashew nut kernel are the processed products, possible contamination of the kernel with the shell oil cannot be overlooked. The present study was therefore undertaken to assess any possible tumour promoting property of the cashew nut kernel by employing a DMBA induced mouse skin carcinogenesis model system. Topical application of cashew nut kernel oil at both the dose levels (2.5% and 5%) did not produce any tumours, the tumour incidence being

nil as compared to the control group (86%) suggesting that the kernel oil does not have a promoting effect on DMBA induced mouse skin carcinogenesis model system. Moreover, the cashew nut kernel oil did not produce any tumours on its own during the study; indicating that the kernel oil does not exhibit any solitary carcinogenic activity. Further, the effect of oral administration of cashew nut kernel oil on the specific activity of some hepatic drug metabolising enzymes namely glutathione-S-transferase and DT-diaphorase and the level of lipid peroxidation were also assessed. Glutathione-S- transferases are multifunctional proteins and multiple forms of GST exist in most of the species. The main fucntion of GST is catalysis of the conjugation of electrophilic xenobiotics or carcinogens to endogenous GSH, for the protection of cellular components from these toxic compounds (Awasthi *et al.*, 1994). The enzyme is capable of intervening in lipid peroxidation at numerous points and are effective quenchers of oxidative stress.

As lipid peroxides generated from reactive oxygen species have been shown to be the substrates for α- GST isoenzymes, they are thus instrumental in the termination of the chain reaction of lipid peroxidation (Singhal *et al.*, 1992).

In the present study, oral administration of cashew nut kernel oil exhibited a significant ($P<0.05$),($P<0.005$) increase in the specific activity of hepatic Glutathione-S-transferase at both the low dose (50μl) and high dose (100 μl) levels respectively, when compared to the control group. GST belongs to the class of Phase II enzymes. It has been reported that many compounds which block the toxic mutagenic and neoplastic effects of carcinogens, share in common the ability to elevate the levels of Phase II detoxification enzymes (Wattenberg, L.W. 1983, 1985, 1992). The induction of Phase II enzymes is a causal mechanism for protection, since these enzymes divert ultimate carcinogens from reaction with critical cellular macromolecules (Talalay *et al.*, 1987). Hence cashew nut kernel oil may possibly be effective in reducing the electrophilic damage to the cell which might lead to initiation. Such modulators which

exhibit an induction in the level of Phase II enzymes like GST are classified as a type A inhibitor under blocking agents, according to the classification of chemopreventive agents by Wattenberg (Wattenberg, 1985).

DT diaphorase is another major Phase II enzyme. It is a flavoprotein which catalyses the two electron reduction of quinones, quinone imines, azodyes and other nitrogen oxides (Ernster, 1987). There was no significant change in the specific activity of DT diaphorase at both the dose levels of oral administration of cashew nut kernel oil.

Lipid peroxidation is a self propogating chain reaction, initiated in membrane lipids by hydroxyl radical and yields mutagenic reactive oxygen species, including singlet oxygen and is associated with the antioxidant depletion due to generation of reactive oxygen species (ROS). Oral administration of cashew nut kernel oil exhibited a significant decrease in the level of lipid peroxidation at the higher dose level (100 µl) thus suggesting that consumption of the same is not possibly creating any oxidative stress.

Among the Indian spices, ajowan *(Tachyspermum ammi)* finds its application in both culinary and medicinal purposes. Effect of oral administration of a 5% aqueous extract of ajowan at the initiation and promotion phases of DMBA induced skin carcinogenesis in mouse model system was studied. As compared to the percentage of tumour incidence of the control group (86%) the group of animals treated with the aqueous extract of ajowan at the initiation phase exhibited 66.6% tumour incidence and at the promotion phase, the percentage of tumour incidence was further reduced to 57.1%. Topical application of 10% aqueous extract of ajowan exhibited 66.66% tumour incidence. The mean number of tumours per tumour bearing mouse was the least (1.875 ± 0.835) in case of the group treated with oral administration of 5% aqueous extract of ajowan at the promotion phase and in the

control group it was (5.083 ± 1.165). In case of topical application, the mean number of tumours per tumour bearing animal was 2.4 ± 1.075. Oral feeding of 5% aqueous extract of ajowan during the promotion phase seems to be more effective in reducing the tumour burden of animals. The probable reason of the decrease in tumour burden may be due to the reversal of the promotion phase of carcinogenesis, in the DMBA induced skin papilloma of mice where 5% aqueous extract of ajowan seems to play a role as a blocking agent or an inhibitor preventing further progression of preneoplastic lesion to a neoplastic form.

Further, biochemcial assay was undertaken to study the effect of 2.5% and 5% test diet (ajowan powder) on the specific activity of hepatic drug metabolising enzymes (GST and DTD) and on the level of lipid peroxidation. There was a significant (P< 0.05) increase in the specific activity of hepatic Glutathione-S-transferase in case of the group treated with a lower dose (2.5%) of ajowan extract (1.120 ± 0.106). This suggests that *Trachyspermum ammi* exerts some protective effect by modulating the specific activity of the hepatic Glutathione-S-transferase.

There was no significant change in the specific activity of DT diaphorase.

The level of lipid peroxidation was significantly reduced to 0.500 ± 0.0027 (P<0.005) when compared to the level of lipid peroxidation in the control group (0.543 ± 0.0079). This suggests that oral administration of 5% aqueous extract of ajowan in mice did not create any oxidative stress.

In order to have a clear insight into the overall chemomodulatory effect of *Anacardium occidentale* and *Trachyspermum ammi,* the modulation in the specific activity of the Phase I enzymes and the antioxidant enzymes by these test substances have to be evaluated. The present study therefore provides a base for further detailed analysis on the mechanism of chemoprevention exerted by the above mentioned modulators which forms a part of regular diet.

6. SUMMARY AND CONCLUSION

Cancer is a devastating disease but is largely preventable. The goal of primary prevention is to avoid the development of cancer by reducing or eliminating exposure to cancer causing factors such as environmental carcinogens and lifestyle factors including nutrition and physical activity. Dietary habits appear to be the most promising area in this regard because the human diet comprises of not only a variety of carcinogens but also includes a plethora of natural and synthetic anti carcinogens and antimutagens.

In the present study, *Anacardium occidentale* (cashew nut) and *Trachyspermum ammi* (ajowan) were tested for their modulatory influence on the DMBA induced murine skin carcinogenesis model system. The effect of cashew nut kernel oil and aqueous ajowan extract on the specific activity of some of the drug metabolising enzymes namely glutathione-S- transferase and DT-diaphorase along with the effect on the level of lipid peroxidation was also evaluated.

Cashew nut *(Anacardium occidentale)* kernel is widely consumed with great relish. The cashew nut shell oil has been reported to exhibit a weak tumour promoting property (Banerjee and Rao, 1992). Since the edible kernel are the processed products, there is a possibility of contamination of the kernel by the shell oil, which might render it unfit for human consumption. Hence in the present study, by using a DMBA induced murine skin carcinogenesis model system, the possible promoting effect of cashew nut kernel oil was evaluated. Cashew nut kernel oil at a dilution of 2.5% and 5% with acetone was applied topically on the DMBA initiated, shaven dorsal scapular region of six weeks old female Swiss albino mice.

In the second set of experiments, cashew nut kernel oil was orally fed at two different dose levels (50 μl and 100 μl) to two different groups, and its effect on the specific activity of some of the drug metabolising enzymes like Glutathione-S-

transferase and DT diaphorase, alongwith the effect on the level of lipid peroxidation was evaluated.

Trachyspermum ammi (ajowan) forms an important spice ingredient for Indian culinary purposes. An attempt was therefore made to study the effect of oral administration of 5% aqueous extract of ajowan in DMBA induced murine skin carcinogenesis model system, at its initiation and promotion phases. Effect of topical application of a 10% aqueous extract of ajowan in DMBA induced mouse skin papillomagenesis was also analysed. Further the effect of a 2.5% and 5% test diet of ajowan powder on the specific activity of some of the drug metabolising enzymes namely Glutathione-S-transferase and DT-diaphorase alongwith their effect on the level of lipid peroxidation was studied.

Results obtained from the various experimental studies briefly described above can be summarised as below:

***Anacardium occidentale* (Cashew nut) kernel oil**

In the DMBA induced mouse skin papillomagenesis model system topical application of 2.5% and 5% cashew nut kernel oil in two separate experimental group of animals did not produce any tumours. Therefore, the tumour incidence was nil as compared to the positive control group of animals (DMBA + 2% croton oil topical application) which exhibited the tumour incidence as high as 86% with the mean number of tumours per tumour bearing animal as 4.917 ± 1.084.

There was a significant increase in the specific activity of Glutathione-S-transferase at low dose ($P< 0.05$) and high dose ($P< 0.005$) levels. There was no significant change in the specific activity of DTD at both the dose levels.

There was a significant ($P<0.05$) decrease in the level of lipid peroxidation at the higher dose.

***Trachyspermum ammi*(Ajowan)**

In the group treated with oral feeding of 5% aqueous extract of ajowan at the initiation and promotion phases, the tumour multiplicity was decreased by 39% and 63.11% respectively as compared to the control group. In case of topical application of 10% aqueous extract of ajowan, the tumour multiplicity was decreased by 52.78% as compared to the control group.

The specific activity of Glutathione-S- transferase was significantly increased ($P<0.05$) at high dose (5%)test diet only.

There was no significant change in the specific activity of DT diaphorase. The level of lipid peroxidation was significantly increased ($P<0.005$) in animals exposed to 5% test diet.

Hence, it can be concluded that the cashew nut kernel oil exhibits no tumour promoting property and is therefore safe for human consumption. Moreover, the kernel does not possess any solitary carcinogenic property. A significant increase in the specific activity of the Glutathione-S-transferase, suggests that the cashew nut kernel oil may have some chemopreventive properties through the modulation of the hepatic phase II enzymes. The oral administration of the kernel oil has significantly decreased the level of lipid peroxidation, therefore, the consumption of the same is not possibly creating any oxidative stress.

Further studies on the chemomodulatory action of *Anacardium occidentale* on the Phase I enzymes and the antioxidant enzymes are required to be done for a more detailed aspect of chemopreventive study of the same.

In case of *Trachyspermum ammi*, oral administration of 5% aqueous extract seemed to be more effective in reducing the tumour incidence and the tumour multiplicity at the promotion phase.

In this situation *Trachyspermum ammi* may possibly be playing a role of an inhibitor by reversing the effect of the promoter (2% croton oil) in the promotion phase of skin carcinogenesis.

Further, the significant increase in the specific activity of Glutathione –S-trnasferase at 5% test diet suggests that *Trachyspermum ammi* possibly exhibits some chemopreventive properties through modulation in the specific activity of hepatic GST. Further a significant decrease in the level of lipid peroxidation at the 5% test diet suggests that oral administration of the same at a dose of 5% is not creating any oxidative stress.

Similarly in this case, an insight into the activity of the Phase I enzymes alongwith the antioxidant enzymes is necessary to understand the mechanism of chemoprevention by the plant modulator.

Nevertheless, the plant modulators selected for this experimental study do not exhibit any effect which would encourage the process of carcinogenesis at the prescribed doses used in the experiment.

The present study forms a platform for further research into the Phase I metabolism and the antioxidant defense system, which can be induced or inhibited by the consumption of plant modulators employed in the present study. Consequently, a clear picture can be deciphered regarding their mechanism of action. The importance of the present study is the conclusive evidence for cashew nut kernel having no promoting action in papillomagenesis thus making it safe for human consumption. The oral consumption of cashew nut kernel oil is not producing any oxidative stress.

Regarding *Trachyspermum ammi,* a 5% aqueous extract possibly acts as an inhibitor of tumour promotion reducing the tumour incidence. It exerts some chemopreventive potential through modulation in the specific activity of hepatic GST. Consumption of a 5% test diet is not possibly creating any oxidative stress.

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