A STUDY OF THE ANTICANCER ACTIVITY OF SELECTED PLANTS IN APIACEAE FAMILY AND COMPARISON WITH THEIR CULTURED CALLUS

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CERTIFICATE

This is to certify that the thesis entitled "A study of the anticancer activity of selected plants in Apiaceae family and comparison with their cultured callus" submitted to the University of Calicut, is an authentic account of work carried out by Mr. Ajaikumar K.B. under my supervision and guidance for the award of "Doctor of Philosophy in Biochemistry" and no part of this thesis has been presented for any other degree, fellowship or any other titles, in any other university or society.

Dr. Jose Padikkala Ph.D
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Place : Amala Nagar
Date : 15.02.2006

DECLARATION

I hereby declare that the thesis entitled "A study of the anticancer activity of selected plants in Apiaceae family and comparison with their cultured callus" is a bonafied record of research work done by me during the course of Ph.D degree and that the thesis has not previously formed on the basis for the award of any other degree, diploma, associateship, fellowship or any other similar title, of any other university or society.

(Signature of the candidate)
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CONTENTS

Chapter 1

Introduction

Cancer is a disease which results from the abnormal growth and uncontrolled division of cells. Cancer is now considered as a genetic disease: that tumor cells originate from multiple genetic defects caused by exposure to environmental, dietary and infectious agents as well as other life style factors (Vay Liang et al, 2003). Among human tumors, most of the genetic alterations are acquired in the form of chromosomal translocations, deletion, inversion and amplifications and point mutations. Certain oncogenic viruses also play important roles in a few human tumors.

Molecular events controlling cell growth and differentiation are complex and involve an increasing array of intracellular pathways and molecules. Aberrations in such pathways and/or signal molecules may underlie the uncontrolled growth in cancer. There are two major classes of genes namely, proto-oncogenes and tumor suppressor genes which regulate the growth and differentiation of the normal cells (Morag and George, 1989). It is well established that mutation in these genes leads to uncontrolled cell growth characteristic of cancer. In non-circulatory tissues, such uncontrolled cell growths produce cell masses called tumors. Cancerous or malignant tumors are those, which detach from and migrate to other parts of the body giving rise to secondary tumors. The process is called metastasis (Gardner et al, 1991).

At the beginning of this new millennium, cancer remains the second leading cause of death in the World and the leading cause of death among the most productive age group, ie., those aged 45-64years (Vay Liang et al, 2001). According to a survey done by American Cancer Society, the newly diagnosed cases of cancer in America is about 1,368,030 in 2004 and the overall deaths is about 5,637,000 (Carmia Borek, 2004). In India, the cancer registry data estimated that half a million cancer cases are reported per year in the country. As the plateau of cancer death continues, the need for new approaches to prevent this hazardous disease became imperative.

Among the possible causes of cancer, damage to DNA and other cellular molecules, by reactive oxygen species ranks high as a major culprit in the onset and development of the disease. A current Scientific view indicates that damage to numerous regulatory genes ultimately results in the development of invasive and metastatic cancer, which is the culmination of the chronic disease process, carcinogenesis. Exposure of organisms to ionizing radiation and chemical carcinogens has long been known to favour development of cancer later in life which appears to involve the activation of oncogenes and inactivation of tumor suppressor genes. Some genetic damage by these carcinogens occurs by direct absorption of energy by DNA, but some is mediated by ionization of water and formation of highly reactive $OH^{\cdot}$. Hydroxyl radical attack upon DNA that generates a whole series of modified purine and pyrimidine bases, many of which are known to be mutagenic. Attack of $OH^{\cdot}$ upon deoxyribose also yields a multiplicity of products. The studies showed that ROS help in the formation of DNA adducts by carcinogens . RNS also attack DNA nitrating and deaminating bases to give mutagenic lesions. Oxidative damage to lipids and to proteins could also lead to mutagenic effects. In addition, low levels of ROS can stimulate cell proliferation. ROS can increase net protein phosphorylation and help to promote proliferation and the expression of immediate early genes such as *c-fos* and *c-myc*. In such cases the administration of antioxidants may have a protective role against these damages and carcinogenesis. Hence, current cancer research mainly focus on antioxidants for the prevention of this hazardous disease.

Primary prevention of cancer is one of the key approaches to the control of cancer. It includes i) avoiding exposure to known cancer causing agents, ii) enhancement of host defence mechanisms, iii) modifying lifestyle, and iv) chemoprevention.

Generally, cancer chemoprevention is recognized as the pharmacologic intervention with synthetic or naturally occurring chemicals to prevent, inhibit or reverse carcinogenesis or prevent the development of invasive cancer (Kelloff and Boone 1996; Kelloff et al, 1997; Mayne and Lippman 1997; Sporn 1991). Although still in its infancy, the new science of chemoprevention has been established as an

important approach to control malignancy. For the first time, it has been shown convincingly that the use of chemopreventive agents in men and women with premalignant lesions can substantially reduce the subsequent development of truly invasive cancer. Chemoprevention is now recognized as both a clinical and basic science.

Approximately five decades of systemic drug discovery and development have established a respectable armamentarium of useful chemotherapeutic agents (Yarbro, 1992; Chabner, 1991) as well as a number of important successes in the treatment and management of human cancer (Jessup et al, 1996). Although major advances have been made in the chemotherapeutic management of some patients, particularly haematologic malignancies, one half of all cancer patients, do not respond to therapy or relapse from the initial response and ultimately die from their metastatic disease. Thus, the continued commitment to the arduous task of discovering new antineoplastic therapeutic agents remains critically important (Greever *et al* 1992). Many of the existing antineoplastic agents share a common mechanism of action. Current research efforts are more diverse than ever, being driven by explosive discoveries in molecular biology and related areas to fully elucidate the development of the malignant process (eg:- factors controlling tumor angiogenesis and metastatic potential). The hope for improvement in treatment outcome for most patients with metastatic disease resides in continued research designed to discover novel therapeutic products that exploits differences in molecular targets between normal and tumor cells and to use them in combinations with biologic agents and immune therapies to eradicate systemic disease not curable by surgery or radiation.

Natural products are the most consistently successful source of drug leads. Approximately 60% of the world's population relies almost entirely on plants for medication (Fransworth, 1994) and natural products have long been recognized as an important source of therapeutically effective medicines. Newer developments based on natural products include the antimalarial drug artimisin and the anticancer agents taxol, docetaxel and camptothecin (Harvey, 1998, Verpoorte 1998 and Grabley 1999). Therefore, the use of natural products has been the single most successful strategy for the discovery of new medicines.

In addition to this historical success in drug discovery, natural products are likely to continue to be sources of new commercial viable drug leads. The chemical novelty associated with natural products is higher than that of any other sources. Despite the commonly held assumptions, natural products can be more economically source of chemical diversity than the synthesis of equivalent number of diverse chemicals. Additionally, natural products that are biologically active in assays are generally small molecules with drug- like properties. That is they are capable of being absorbed and metabolized by the body. Hence, the development cost of producing orally active medicines are likely to be much lower than with biotechnological products or with most compounds produced to date from combinatorial chemistry. Combinatorial chemistry might enable ligands for well established, tractable targets to be found more quickly, rather than being suited to the more signaling interactions that are associated with the majority or targets. For such targets, the chemical diversity of natural products is a major advantage.

Plant –derived substances have traditionally played important roles in the treatment of human diseases. Today, about 80% of the world population residing in third world countries still rely almost entirely on plant products for their primary healthcare. The remaining 20% of individuals living in the first world use, in more than 25% of cases, pharmaceuticals which have been directly derived from plant products (Fransworth, 1984; Cox, 1994). These range from common remedies such as aspirin (originally isolated from the Rosaceae, *Filipendula ulmaria*), to prescription drugs such as analgesic morphine and the cardiac glycoside digoxin (isolated from Papavaraceae, *Papaver somniferum*, and the Apocynaceae, *Digitalis purpurea*, respectively).

Plant-derived compounds were also of great significance in cancer therapy. It was for instance, only upon the addition of *Vinca* alkaloids vincristine or oncovin (isolated from *Catharanthus roseus*, Apocynaceae (Johnson et al, 1963)). To mechlorethamine, prednisone and procarbazine (the MOPP regimen) that the first curves in a human cancer (Hodgkin's disease) were achieved (Devita, 1970). The combination of the epipodophyllotoxin etoposide (derived from the mandrake plant *Podophyllum peltatum* and the wild chervil *P.emodi* (Berberidaceae (Stahelin, 1973), bleomycin, and cisplatin is currently a highly active and curative regimen in testicular

cancer (Williams et al, 1987). Etoposide is furthermore one of the most active agents against small cell lung carcinoma (Yasbro, 1992; chabner, 1991; Harvey, 1999; Williams et al, 1987).

The more recent development of the structurally and mechanistically novel taxanes (extracted from the bark of the Taxaceae; *Taxus brevifolia*, *T.canadensis* or *T.baccata* (Wani et al, 1971) and the camptothecins (derived from the bark and wood of the Nyssaceae, *Camptotheca accuminata* (wall et al, 1966) in the 1990s represented a landmark in Cancer research because of their significant anti-solid tumor efficacy. Paclitaxel is in many countries approved for the treatment of ovarian and breast carcinoma and also has important activity against non-small cell lung cancer (McGuire et al, 1996). Homoharringtonine, for instance is an alkaloid isolated from the Chinese tree *Cephalotaxus harringtonia* (Cephalotaxaceae) (Powell et al, 1970) and has shown efficacy against various leukemias (Kantajian et al, 1996). $-Ipomeanol is a pneumotoxic furan derivative isolated from the sweet potato, *Ipomea batatas* (Convolvulaceae) Rehm and devor, 1993) that has been under clinical evaluation as a lung-cancer-specific antineoplastic agent (Rowinsky et al, 1993). Elliptinium is a semi-synthetic derivative from ellipticine, which can be derived from Apocynaceae such as *Bleekeria vitensis* (Cragg, 1988) and which is presently used in Europe in the treatment of advanced cancer (Paoletti et al, 1980).

The family Umbelliferae (Apiaceae) contain around 200genera and 2,700 species. A number of plants in this family is used in traditional Indian practices for the treatment of various diseases such as leucoderma, urinary disorders, diseases of blood, bronchitis, inflammations, fevers, asthma, smallpox, ulcer, leprosy, dysentery etc. Some plants are used as immunostimulant. The important plants in this family include *Hydrocotyle asiatica*, *Eryngium coeruleum*, *Apium graveolens*, *Carum carvi*, *Carum copticum*, *Daucus carota*, *Ferula asafetida*, *Foeniculum vulgare*, *Coriandum sativum* etc. A number of chemicals have been isolated from these plants including cymene, dipentene, d-limone, phelandrene, pinene, terpinene. angelical, bornecol, geraniol, inositol, d-linalol, manitol, terpineol, anethol, apiol, methylchavicol, quinol, safrol, thymol, conhydrine, g- conhydrine, g-coniceine, coniine, daucine, N-methylconiine (Kirtikar and Basu, 2000).

Carum copticum, Pseucedanum vulgare, Corinadum sativum, and *Cuminum siminum* etc. is used in Traditional Indian medicine for the treatments of stomach, liver and kidney disorders. These are also used for the treatments of ascites, snake bite and scorpion sting. Due to these high medicinal properties we selected these plants for the present study.

Chapter 2

Review of Literature

Neoplasm literally means "new growth", and the new growth is a neoplasm. Willis (1967) defined "A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change". The term tumor was originally applied to the swelling caused by inflammation. Neoplasms also may induce swellings, but by long precedent, the non-neoplastic usage of tumor has passed into limbo; thus, the term is now equated with neoplasm. Tumors may be Benign or malignant

2.1.1 Benign tumors

Benign tumors ordinarily do not cause death, but there are rare exceptions to this rule. Death may result from synthesis of toxic levels of hormones by the benign tumor cells or by the position of the benign tumors in situations essential for life. Benign tumors are slow growing. The cells are well organized and well differentiated (the cells closely resemble those of the normal tissue). They may synthesize the gene products specific to the differentiated state. Normally, benign tumor cells synthesize fewer of these molecules per cell than those of the normal tissues, but when the tumor becomes large, toxic amounts of the molecules can be synthesized.

2.1.2 Malignant tumors

The cells of malignant tumors have the intrinsic ability to kill the host unless they are removed or killed. In contrast to benign tumors, numerous normal and abnormal mitotic figures are present in malignant tumors. These cells are programmed for proliferation and have large vesicular nuclei with large nucleoli. The cytoplasm contains many polysomes that are unattached to membranes and synthesize structural proteins required for cell division. The cells are pleiomorphic in size, shape, and staining reactions and have increased nucleocytoplasmic ratios. Malignant cells are less well differentiated than their benign counterparts, some so poorly that they defy histopathologic identification. Such tumors are said to be anaplastic. The anaplastic

cells invade and destroy the normal architecture of the organs, and replace it with masses of disorganized malignant cells.

The development of cancer is a multi step process. The stages of carcinogenesis, initiation, promotion and progression interact sequentially in the formation of malignancy.

2.2.1 Initiation

Initiation is the first step in which a carcinogen interacts with DNA (Freidwald and Rous, 1950). It is a rapid process that permanently alters cells. It alters the genetic material in an irreversible manner, leading the ability for transformation. Polycyclic aromatic hydrocarbons (PAHs) are major chemical class of carcinogens that has potent tumor initiating capacity. These agents are effective when administered topically, intragastrically, and intraperitoneally or transplacently. Tumor initiator bind covalently with DNA, RNA or proteins results in adduct formation (Cooper et al, 1995). DNA lesions are responsible for mutation and tumor induction. Studies on mouse skin epidermis have shown that stem cells are target for initiators.

Molecular analysis during initiation found that point mutations occur during initiation. Most of the genes in this category are ras family genes, comprising H-ras, K-ras and N-ras. These ras genes encode GTP binding proteins called p21, with intrinsic GTPase activity. The mutated gene product reduces GTPase activity, thereby dramatic changes made in the signal trnasduction pathways.

2.2.2 Promotion:

During promotion the expression of the mutated genes occur in the initiated cells, the mutated gene product enhances the proliferation of the initiated cells as foci. Promotion is a reversible process. Certain chemicals, physical trauma, UV-irradiation and silica fibres are some tumor promoting factors. 12-O-tetradeconyl phorbol 13-acetate (TPA) is a well known promoter. Application of TPA on mouse skin induces the infiltration of leukocytes (Scribner and Suss, 1978), hyperplasia of dark basal cells in the epidermis, keratinization and inflammatory changes. The biochemical alterations or increased rate of nucleic acid and protein synthesis (Balmin and Becker, 1974), increased phospholipid turnover and prostaglandin accumulation, increased the

synthesis and phosphorylation of histones and induction of ornithjine decarboxylase, an enzyme catalyses ornithin to putrescine.

Tumor promoter TPA also binds to protein kinase C (PKC) in competition with diacylglecerol, PKC stimulation results in phosphorylation of proteins that affect cell proliferation.

There are a number of reports suggesting the implication of free radicals during promotion. TPA promotes superoxide (O^{2-}) and other free radicals by polymorphonuclear leukocytes, by the activation of NADPH-oxidase system. There are cumulative evidences that TPA produces mutation through DNA strand break, chromosomal abnormalities and structural modifications of the bases in DNA. Tumor promotion study in mouse skin has shown that the selective clonal expansion of initiated cells into visible outgrowth or papillomas. This is due to the activation of H-ras and transforming growth factor (TGF) (Ciardiell et al, 1988).

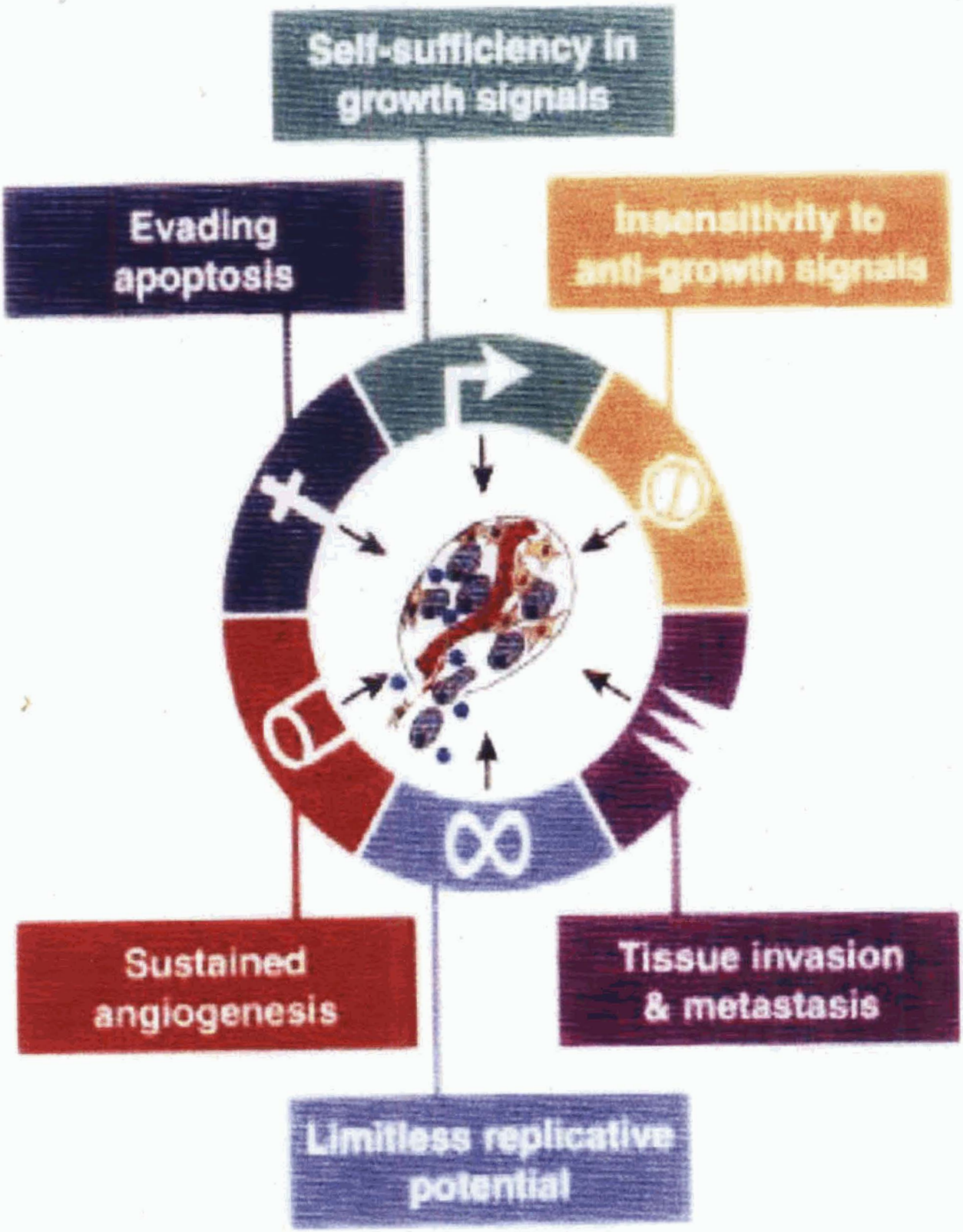
2.2.3 Progression:

Foulds (1969) defined progression as 'the gain or loss of unit characters leading to the autonomous state'. It is the final stage of carcinogenesis, which involves the accumulation of additional genetic changes in cells such as chromosomal changes leading to invasion, metastasis and increased rate of growth that progresses to the higher degrees of malignancy. The genetic changes include the activation of proto-oncogenes (H-ras and fos), aneuploid lesion, trisomia and chromosomal translocations. Mutation of tumor suppressor gene p53 is also noted (Callen and Ford, 1983).

2.2.4 Metastasis

Malignant cells have a capacity to grow along tissue spaces, nerves, and vessels, and finally penetrate lymphatics, venules and capillaries as well as the body cavities. Single or small clumps of tumor cells then break away from the original mass and are carried to distant organs, where they implant. This process of dissemination of malignant cells is called metastasis. The original tumor is called primary tumor, and the process of metastasis establishes secondary tumors in distant organs where they incite the development of a stroma and develop into new tumors,

Figure 2.1



Different aquired capabilities of cancer

which in turn invade and metastasize. Metastasis has been defined by Willis (1967) as the discontinuous growth of tumor cells.

2.3 Angiogenesis

Neovascularization is a prerequisite for the local expansion of tumor colonies beyond the size restricted by oxygen and nutrient diffusion. New capillaries also provide cancer cells with conduits for entry into the circulation. Extravasated cancer cells will later require neovascularization in order to grow and form new metastatic foci. Therefore angiogenesis is necessary at the beginning and end of the metstatic cascade. The process of blood vessel formation is functionally similar to tumor cell invasion and can be considered as a form of regulated invasion, with the independent events of adhesion, proteolysis and migration that characterize the spreading of cancer cells also displayed by endothelial cells (Kohn and Liotta, 1990; Liotta et al, 1991). Interstitial collagenase (MMP-1), MMP-2 and urokinase have been shown to be secreted by endothelial cells in vitro and are necessary for vessel formation in vivo; inhibition of collagenolytic activity inhibits angiogenic activity (Herron et al, 1986; Kohn et al, 1995; Moscatelli, 1986). Several of these angiogenic factors may cause a pleiotropic response of enzyme production, endothelial cell migration, and proliferation. Basic fibroblast growth factor is the most potent angiogenic factor described and is involved in many aspects of neovascularization. FGF-2 stimulates endothelial cell growth, protease secretion and motility of endothelial cells in vitro and may be involved in the pathogenesis of a variety of human cancers (Galves 1983; Sato and Rifkin, 1988; taylor et al, 1993).

Histologic and ultrastructural analysis of tumor vessels have revealed pronounced differences in tumor vessels as compared with normal vessels found in mature tissues. The distinction includes differences in cellular composition of tumor vessels, the basement membrane composition and integrity and differences in permeability (Furcht, 1986). Due to a discontinuous basement membrane, tumor vessels are leaky and easily penetrated by cancer cells entering the circulation at high rate (millions of cells a day) (Gajdusek, 1993). Recently, a positive correlation between the number of tumor microvessels in cancer specimens and patient outcome was demonstrated for a number of cancer cell types, including ovarian cancer, breast

cancer, melanoma and prostate cancers (Brawer, 1994; Hollingsworth et al, 1995; Srivastava et al, 1988; Weidner et al, 1992; Weidner, 1991).

2.4 Oncogenes

. Oncogenes are pathologically altered versions of normal cellular genes called proto-oncogenes, that regulate cellular growth processes such as proliferation, differentiation, and programmed cell death (Cooper, 1990). Oncogenes exert their malignant action in an autosomal dominant fashion through a gain in function mechanism. More than 100different oncogenes have been discovered thus by far various methods (Levine, 1995). Most of these oncogenes were identified in an experimental animal model of cancer (Varmus, 1989). Only a small subset of these genes, however, seem to play a role in human cancer. A few proto-oncogenes, notably those of the ras and myc families, have been implicated repeatedly in the initiation and progression of various human tumors. Proto-oncogenes are converted into oncogenes by the genetic mechanism of mutation, gene amplification and chromosomal rearrangement (Bishop, 1989). Proto-oncogenes encode proteins that are involved in the control of cell growth. Alteration of the structure and/or expression of the proto-oncogenes can activate them to become oncogenes capable of transforming susceptible cells into the neoplastic phenotype. Proto-oncogenes are classified into five groups based on the functional and biochemical properties of their protein products. These groups are as follows

2.4.1 Growth factors

Growth factors are secreted polypeptides that function as extracellular signals to stimulate the proliferation of target cells (Cross and Dexter, 1991; Weinberg, 1989). The growth factors include Platelet derived growth factor (PDGF), Nerve growth factor, Epidermal growth factor and fibroblast growth factor. The link between growth factors and retroviral oncogenes was revealed by study of the *sis* oncogene of simian sarcoma virus, a retrovirus first isolated from a monkey fibrosarcoma. Sequence analysis showed that *sis* encodes the beta chain of PDGF (Doolittle et al, 1983). This discovery established the principle that inappropriately expressed growth factors could function as oncogenes. The constitutive expression of the sis gene product appears to cause neoplastic transformation by the mechanism of autocrine stimulation, resulting in self-sustained aberrant cell proliferation. Another

example of a growth factor that can function as an oncogene is *Int-2*, a member of the fibroblast growth factor family. *Int-2* is sometimes activated in mouse mammary carcinomas by MMTV insertional mutagenesis (Peters et al, 1986).

2.4.2 Growth factor receptors

Some viral oncogenes are altered versions of normal growth factor receptors that possess intrinsic tyrosine kinase activity (Yarden and Ullrich, 1988; Gill, 1989). Examples include *erb* B, *erb* B-2, *fms*, *kit*, *met*, *ret*, *ros* and *trk*. Mutation or abnormal expression of growth factor receptors can convert them into oncogenes (Segatto et al, 1988). For example, deletion of the ligand-binding domain of *erb* B is thought to result in constitutive activation of the receptor in the absence of ligand binding (Wells and Bishop, 1988). Point mutation in the tyrosine kinase domain and deletion of intracellular regulatory domains can also result in the constitutive activation of receptor tyrosine kinases. Increased expression through gene amplification and abnormal expression in the wrong cell type are additional mechanisms through which growth factor receptors may be involved in neoplasia. The identification and study of altered growth factor receptors in experimental models of neoplasia have contributed much to our understanding of the normal regulation of cell proliferation.

2.4.3 Signal transducers

Mitogenic signals are transmitted from growth factor receptors on the cell surface to the cell nucleus through a series of complex interlocking pathways collectively referred to as the signal transduction cascade (Ullrich and Schlessinger, 1990). This relay of information is accomplished in part by the stepwise phosphorylation of interacting proteins in the cytosol. Signal transduction also involves guanine nucleotide-binding proteins and second messengers such as the adenylate cyclase system (Casey and Gilman, 1988; Gilmon, 1987). The first retroviral oncogene discovered, *src*, was subsequently shown to be involved in signal transduction. Many proto-oncogenes are members of signal transduction pathways (Cantley et al, 1991; Boevne and DeFranco, 1989). These consist of two main groups: nonprotein receptor kinases and GTP-binding proteins. The nonreceptor protein kinases are subclassified into tyrosine kinases (eg: abl, lck and src). GTP-binding proteins with intrinsic GTP-ase activity are subdivided into monomeric and heterotrimeric groups (Kaziro et al, 1991). Monomeric GTP-binding proteins are

members of the important ras family of proto-oncogenes that includes H-ras, K-ras and N-ras (Lowy and Willumsen, 1993). Heterotrimeric GTP-binding proteins implicated as proto-oncogenes currently include gsp and gip. Signal transducers are often converted to oncogenes by mutations that lead to their unregulated activity, which in turn leads to uncontrolled cellular proliferation (Rodrigues and Park, 1994).

2.4.4 Transcription factors

Transcription factors are nuclear proteins that regulate the expression of target genes or gene families (Mitchell and Tjian, 1989). Many proto-oncogenes are transcription factors that were discovered through their retroviral homologues (Lewin, 1991). Examples include erb A, ets, fos, jun, myb, and c-myc. Together, fos and jun from the AP-1 transcription factor, which positively regulates a number of target genes whose expression leads to cell division (Chiu, 1988; Angel and Karin, 1991). Erb A is the receptor for the T3 thyroid hormone, triiodothyronine (Damm et al, 1989; Privalsky, 1992). Proto-oncogenes that function as transcription factors are often activated by chromosomal translocations in haematologic and solid neoplasms (Cleary, 1991). An important example in human cancer is the c-myc gene, which helps to control the expression of genes leading to cell proliferation (Marcu et al, 1992).

2.4.5 Programmed cell death regulators

The only proto-oncogene thus far shown to regulate programmed cell death is bcl-2. Bcl-2 was discovered by the study of chromosomal trnslocations in human lymphoma (Tsujimoto et al, 1984; Cleary et al, 1986). Experimental studies show that bcl-2 activation inhibits programmed cell death in lymphoid cell populations (Koremeyer, 1992). The dominant mode of action of activated bcl-2 classifies it as an oncogene. The bcl-2 gene encodes a protein localized to the inner mitochondrial membrane, endoplasmic reticulum, and nuclear membrane. The normal function of bcl-2 requires interaction with other proteins, such as bax, also thought to be involved in the regulation of programmed cell death. It is unlikely that bcl-2 is the only apoptosis gene involved in neoplasia, although additional proto-oncogenes await identification.

Table No.2.1 Lists examples of proto-oncogenes according to their functional categories.

2.4.7 Mechanism of Oncogene Activation

Three genetic mechanisms activate oncogenes in human neoplasms; mutations, gene amplification and chromosomal rearrangements

2.4.7.1 Mutation

Mutations activate proto-oncogenes through structural alterations in their encoded proteins. These alterations, which usually involve critical protein regulatory regions, often lead to the uncontrolled continuous activity of the mutated protein. Various types of mutations, such as base substituitions, deletions and insertions, are capable of activating proto-oncogenes (Bishop, 1991). Retroviral oncogenes, for example, often have deletions that contribute to their activation. Examples include deletions in the amino terminal ligand – binding domains of the erbB, kit, ras, met, and ret oncogenes (Cooper, 1990). Point muatations are frequently detected in the ras family of proto-oncogenes (K-ras, H-ras and N-ras) (Rodenhuis, 1992). It has been estimated that as many as 15 to 20% of unselected human tumors may contain a ras mutation (Table..). Mutations in the K-ras predominate in carcinomas (Stebos et al, 1990; Forrester et al, 1987; Almonguera et al, 1988). N-ras mutations are predominantly found in haematological malignancies, with upto a 25% incidence in acute myeloid leukemias and myeloid dysplastic syndromes (liu et al, 1987; Lyans et al, 1988).

2.4.7.2 Gene amplification

Gene amplification refers to the expansion in copy number of a gene within the genome of a cell. Gene amplification was first discovered as a mechanism by which some tumor cell lines can acquire resistance to growth-inhibiting drugs (Alt et al, 1978). The process of gene amplification occurs through redundant replication of genomic DNA, often giving rise to karyotypic abnormalities called double-minute chromosomes (DMs) and homogenous staining regions (HSRs) (Cowell, 1982). DMS are characteristic 'minichromosome" structures without centromeres. HSRs are segments of chromosomes that lack the normal alternating pattern of light and dark

Table No. 2.1 Examples of proto-oncogenes and their function

Proto-oncogene		Function of protein product	Method of identification
Growth factors			
	sis	Platelet derived growth factor	Retroviral homologue
	int-2	Growth factor	Insertional Mutagenesis
Growth factor receptors			
	erb B	Tyrosine kinase/EGF receptor	Retroviral homologue
	erb B-2 (neu/HER-2)	Tyrosine kinase	Transfection
	fms	Tyrosine kinase/CSF-1 receptor	Retroviral homologue
	kit	Tyrosine kinase/steel receptor	Retroviral homologue
	trk	Tyrosine kinase/NGF receptor	Transfection
	ret	Tyrosine kinase	Transfection
	met	Tyrosine kinase/hepatocytes	Transfection
Growth factor receptor			
	sea	Tyrosine kinase	Retroviral homologue
	ros	Tyrosine kinase/CSF-1 receptor	Retroviral homologue
	mas	Angiotensin receptor	
Signal transduction			
	abl	Tyrosine kinase	Retroviral homologue
	fes	Tyrosine kinase	Retroviral homologue
	fgr	Tyrosine kinase	Retroviral homologue
	lck	Tyrosine kinase	Insertional mutagenesis

src	Tyrosine kinase	Retroviral homologue
yes	Tyrosine kinase	Retroviral homologue
raf	Serine/threonine kinase	Retroviral homologue
mos	Serine/threonine kinase	Retroviral homologue
pim	Serine/threonine kinase	Insertional mutagenesis
H- ras	Binds GDP/GTP	Retroviral homologue
K- ras	Binds GDP/GTP	Retroviral homologue
N- ras	Binds GDP/GTP	Transfection
gsp	G protein	Mutation
gip	G protein	Mutation

Transcription factors

erb A	T_3 receptor/DNA binding	Retroviral homologue
ets	DNA binding	Retroviral homologue
fos	DNA binding/AP-1 complex with jun	Retroviral homologue
jun	DNA binding/AP-1 complex with fos	Retroviral homologue
myb	T_3 receptor/DNA binding	Retroviral homologue
c-myc	DNA binding	Retroviral homologue
L-myc	DNA binding	Amplification
N-myc	DNA binding	Amplification
rel	DNA binding	Retroviral homologue
ski	DNA binding	Retroviral homologue

Programmed cell death regulation

bcl-2	Membrane protein/apoptosis	Chromosomal translocation

staining bands. Both DMs and HSRs represent large regions of amplified genomic DNA containing up to several hundred copies of a gene. Amplification leads to the increased expression of genes, which in turn can confer a selective advantage for cell growth.

The frequent observation of DMs and HSRs in human tumors suggested that the amplification of specific proto-oncogenes may be a common occurrence in neoplasia (Alitale and Schwab, 1986). Studies then demonstrated that three proto-oncogene families –myc, erbB, and ras – are amplified in a significant number of human tumors. About 10 – 20% of breast and ovarian cancers show c-myc amplification s found in some types of squamous cell carcinomas (Brison, 1993). N-myc was discovered as a new member of the myc-proto-oncogene family through its amplification in neuroblastomas (Schwah and Alitalo, 1983). Amplification of N-myc coreelates strongly with advanced tumor stage in neuroblastoma, suggesting a role for this gene in tumor progression (Seeger et al, 1985). L-myc was discovered through its amplification in small cell carcinomas of the lung, a neuroendocrine –derived tumor (Nau et al, 1985). Amplification of erbB, the epidermal growth factor receptor, is found in up to 50% of glioblastomas and in 10- 20% of squmaous carcinomas of the head and neck (Brison, 1993). Approximately 15 to 30% of breast and ovarian cancers have amplification of the erbB-2 (HER-2neu) gene. In breast cancer, erbB-2 amplification correlates with advanced stage and poor prognosis (Slamon et al, 1989). Members of the ras gene family, including K-ras and n-ras, are sporadically amplified in various carcinomas.

2.4.7.3 Chromosomal rearrangements

Recurring chromosomal rearrangements are often detected in haematologic malignancies as well as in some solid tumors (Croce, 1987; Solomon et al, 1991; Rabits, 1994). These rearrangements consists mainly of chromosomal translocations and less frequently, chromosomal inversions. Chromosomal rearrangements can lead to haematologic malignancy by two different mechanisms (a) the transcriptional activation of proto-oncogenes, or (b) the creation of fusion genes. Transcriptional activation, sometimes referred to as gene activation, results from chromosomal rearrangements that move a proto-oncogene close to an immunoglobulin or T-cell receptor gene. Transcription of the proto-oncogene then falls under control of

regulatory elements from the immunoglobulin or T-cell receptor gene locus. This circumstance causes deregulation of proto-oncogene expression, which can then lead to neoplastic transformation of the cell.

Fusion genes can be created by chromosomal rearrangements when the chromosomal break points fall within the loci of two different genes. The resultant juxtaposition of segments from two different genes gives rise to a composite structure consisting of the head of one gene and the tail of another gene. Fusion genes encode chimeric proteins with transforming activity. In general, both genes involved in the fusion contribute to the transforming potential of the chimeric oncoprotein. Mistakes in the physiologic rearrangement of immunoglobulin or T-cell receptor genes are thought to give rise to many of the recurring chromosomal rearrangements found in haematologic malignancy (Haluska et al, 1986). In some cases, the same proto-oncogene is involved in several different translocations.

2.5 Tumor suppressor genes

2.5.1 Rb gene

This is the first tumor suppressor gene discovered (Kaelin, 1997; Lin, 1996). PRb, the product of the Rb gene, is a nuclear phosphoprotein that plays a key role in regulating the cell cycle. It is expressed in every cell type examined and an inactive hyperphosphorylated state. In its active state, pRb serves as a brake on the advancement of cells from the G1 to S phase of the cell cycle. When the cells are stimulated by growth factors, the Rb protein is inactivated by phosphorylation (pRb-p), the brake is released, and the cells transverse the G1 to S check point. Once the cells enter S phase, they are committed to divide without additional growth factor stimulation. During the ensuing 'M' phase, the phosphate groups are removed from pRb by cellular phosphatases, thus generating the dephosphorylated form of pRb. Rb germ-line mutations fail to predispose to more common cancers, despite the fact that somatic Rb mutations have been observed in breast, small cell lung, bladder, and prostate cancers (Bookstein et al, 1990; Harbaur et al, 1988; Horowitz et al, 1989; Lee, 1988).

The Rb gene is often inactivated in tumors other than retinoblastoma, such as breast cancer, lung cancer and genitourinary cancer, but it may not play an essential role in regulation of cell growth in many tissue types. Patients with germ line mutations of RB1 are prone to osteosarcoma, soft tissue sarcoma and perhaps melanoma and brain tumors, but not breast, lung and genitourinary cancers (Bouguski and McCormick, 1993). In the latter group of neoplasms, loss of RB1 may contribute to malignant characteristics, but apparently is not essential to growth control in tissues.

2.5.2 p53

Studies in the late 1970s revealed that a cellular phosphoprotein with a relative molecular mass of about 53,000 formed a tight complex with SV40 T antigen, and hence the p53 protein was so named (Lane and Crawford, 1979; Linzer and Levine, 1979). Additional studies established that p53 also complexed with other viral oncogene products, including adenovirus EIB, and that p53 was present at low levels in normal and tumor cell lines (Crawford, 1984; Lane and Benchimol, 1990; Sarnow, 1982).

The p53 gene was inactivated by DNA rearrangements in some viral-induced murine erythroleukemias, the HL-60 promyelocytic leukemia cell line, and some osteosarcomas (Ahua et al, 1989; Lane and Benchimol, 1992; Masuda et al, 1987; Mowat et al, 1985; Wolf et al, 1984). Based on the types of tumors in which p53 mutations have been found and the prevalence of p53 mutations in those tumor types, p53 is believed to be among the most frequently mutated genes in human cancer (Vogelstein and Kinzler, 1992). Although gross rearrangements of the p53 gene are seen in some pediatric tumors such as osteosarcoma and rhabdomyosarcoma, and splicing mutations are seen in some SCCLs, the vast majority of the somatic mutations in p53 are missense mutations (Greenblatt et al, 1994; Mulligan, 1990).

2.5.3 *WT1* gene

The WT1 gene was identified in 1990 by virtue of mutations inactivating the gene in patients with the WAGR syndrome, as well as of somatic mutations in the gene in tumors from a minority of patients with unilateral Wilm's tumor and no

associated cogenital malformations (Bonetta et al, 1990; Call et al, 1990; Gessler et al, 1990). WT1 is encoded by 10exons and its transcripts are subject to alternative splicing (Haber, 1991). Its mRNAs encode proteins with molecular masses of 45,000 to 49,000 and zinc finger motifs. Based on the predicted aminoacid sequence, WT1 proteins were suspected to function in transcriptional regulation (Haber, 1992; Rauscher, 1993). In particular, the WT1 proteins have been found to suppress the expression of a number of growth – inducing genes; including the early growth response (EGR1), insulin like growth factor 2 (IGF-2), and platelet derived growth factor A chain (PDGFA) genes. The ability of WT1 to regulate the expression of growth inducing genes may account, at least in part, for the function of WT1 gene as a tumor suppressor genes (Haber, 1993). In contrast to the rather ubiquitous expression of the RB1 and p53 genes, high levels expression of the WT1 gene appears to be restricted to embryonic kidney and a small subset of other tissues (Haber and Housman, 1992; Pritchard –Jones, 1990; Rauscher, 1993).

2.5.4 The *APC* gene

APC is very large, encoding a 2843 aminoacid protein that is expressed in all tissues studied (Groden etal, 1991; Kinzler et al, 1991; Polakis, 1995). The central region of the APC protein binds to b-catenin, a cytoplasmic protein that is presumed to link the cytoskelton to E-cadherin, an adhesion molecule on the cell surface (Polakis, 1995; Rubeinfeld et al, 1993; Su, 1993). Hence, the APC protein may function in relaying signals generated by cell-cell and/or cell extracellular matrix interactions to the growth-controlling pathways within the cell.

Germ line mutations of APC have been identified in more than two-thirds of the polyposis kindreds studied (Miyoshi et al, 1992; Nagase et al, 1992; Nagase and Nakamura, 1993; Polakis, 1995; Powell et al, 1993). Almost all of the germline mutations identified in familial adenomatous polyposis (FAP) patients cause premature truncation of the APC protein product, as a result of either nonsense mutations or of small deletions or insertions.

FAP is a relatively common genetic disease with a prevalence of about 1 in 7,000 individuals, and it accounts for about 0.5% to 1% of the annual colorectal cancer (CRC) causes in the United States. The importance of APC gene, however, is

not limited to FAP. Present findings suggest that virtually all colorectal cancers, whether they occur in patients with inherited predisposition to polyposis or in "sporadic" cases, harbor mutant APC alleles (Miyoshi et al, 1992; Nagase and Nakamura, 1993; Polakis, 1995; Powell, 1992). In FAP, one mutant APC allele is inherited in every colonic epithelial cell, and the first stage of colonic neoplasia is initiated when the APC allele from the unaffected parent is inactivated. IN sporadic CRC cases, somatic mutations in both APC alleles arise in rare single cells within the colon. As nearly half of the adult population of the Western world will develop an adenoma, and as each of these lesions is likely to harbor mutations in one or both APC alleles. APC mutations may be the most common functional genetic alterations arising in humans.

2.5.5 The p16 gene

The p16 was originally discovered during investigations of the cell cycle and was found to encode a protein that inhibited the cyclin-dependent kinases CDK-4 and CDK-6. Subsequent studies revealed that subtle mutations in one allele of the p16 gene were present in some patients with familial melanoma (hussussian et al, 1994; Kamb et al, 1994; Wainwright, 1994) and that somatic mutations of both p16 alleles were present in a significant fraction of many different cancer types, including but not limited to, melanomas, gliomas, bladder cancers, and leukemias (Bonnetta, 1994; Cairns et al, 1994; caldas et al, 1994; He, 1994; Spruck et al, 1994). In some of these tumors, deletions of the p16 gene were also found to involve a nearby gene termed p15 or MTS2.

2.5.6 The *NF1* and *NF2* gene

Neurofibromatosis types 1 and 2 are diseases with autosomal dominant inheritance in which neurofibromas of the peripheral or central nervous system, respectively, are the major neoplastic features. In addition to peripheral neurofibromas, NF1 patients also manifest café-an-lant spots on the skin and are at risk for several cancers; including pheochromocytomas, neurofibrosarcomas, gliomas and leukemia. The NF1 gene on chromosome 17q was identified through a positional cloning approach that was guided by linkage analysis and detailed characterization of

the chromosome 17q sequences affected by chromosomal rearrangements in two unusual patients with typel neurofibromatosis (Barker et al, 1987; Cawthon et al, 1990; Fountain et al, 1989; Li et al, 1992; O'Connell et al, 1989, Ponder, 1990; Seizinger et al, 1987; Viskochil et al, 1990; Wallace et al, 1990). NF1 is a large gene with over 50 exons, encoding a protein with a mass greater than 3,00,000 (Viskochil et al, 1993). Perhaps in part because of the large size and complexity of the NF1 gene, germ-line mutations in those with type 1 neurofibromatosis have been identified in only about a third of affected families (Upadhyaya et al, 1992; Viskochil et al, 1993). Inactivation of both NF1 alleles has been demonstrated in leukemias arising in children with type 1 neurofibromatosis (Shannon et al, 1994) establishing that NF1 behaves like other tumor suppressor genes. Somatic mutations in the NF1 gene have been seen in a few cancer types arising sporadically, including colorectal cancer, neuroblastoma, melanoma and leukemia (Johnson et al, 1993; Seizinger, 1993; The et al, 1993; Viskochil et al, 1993). The protein encoded by the NF1 gene termed neurofibromin – appears to function as a GTPase activating protein (GAP) for the RAS-protooncogene family (Ballester et al, 1990; Gangfeng et al, 1990; Martin et al, 1990; McCormick, 1995; Xu et al, 1990).

NF2 patients develop Schwannomas of the eighth cranial nerve, and occasionally other brain tumors, such as meningiomas and ependymomas. The gene responsible for NF2 was localized to chromosome 22q by linkage analysis and LOH studies in meningiomas (Rouleau et al, 1987; Seizinger et al, 1986; Seizinger et al, 1987a; Seizinger et al, 1987b). The NF2 gene was ultimately identified by a positional cloning approach (Rouleau et al, 1993). In addition to germ-line mutations in the NF2 gene in those with central neurofibromatosis, somatic mutations of both NF2 alleles have been identified in meningiomas and ependymomas arising sporadically (144, 201, 238). The NF2 gene encodes a protein termed merlin (formoesin-, ezrin-, radixin-like protein) or Schwannomin (Rouleau et al, 1993; Trofatter et al, 1993). Based on the similarity of its sequence to that of other proteins involved in the organization of the cells cortical actin cytoskelton, merlin has been hypothesized to function in transducing growth regulatory signals generated by cell-cell and cell-extracellular matrix interactions.

2.5.7 The *VHL* gene

Linkage studies assigned the VHL gene to chromosome 3p and the gene was subsequently identified by positional cloning (Barker et al, 1987; Latif et al, 1993; Seizinger et al, 1988). Germ line mutations in the gene have been found in the majority of those with the VHL syndrome. In addition, somatic mutations in both alleles of the VHL gene have been identified in the great majority of sporadic renal cell cancers of the clear cell type, but have not been seen in those of the papillary type (Gnarra et al, 1994; Shuin et al, 1994; Whaley et al, 1994).

Although the function of the VHL gene is not well known, recent studies suggest that it encodes a protein with a molecular weight of about 20,000. The VHL protein has been implicated in regulating the efficiency and processivity of the cell's transcriptional machinery.

2.5.8 The *BRCA1* and *BRCA2* genes

The BRCA1 gene recently was isolated (Miki et al, 1994). It has at least 24 exons and encodes a zinc-finger protein that has been suggested to function in transcriptional regulation. Germ-line mutations in BRCA1 have been identified in a number of patients with premenopausal breast cancer and/or ovarian cancer, and the gene is presumed to function as a tumor suppressor gene (Futreal, et al, 1994; Miki, 1994; Smith, 1991). At present, it is estimated that about one-half of the families with apparent autosomal dominant transmission of breast and ovarian cancer susceptibility and average age of onset of less than 45 years may harbor germ-line mutations in BRCA1 (Easton et al, 1993). Unexpectedly, the studies carried out thus far suggest that somatic mutations in the coding regions of BRCA1 – may be infrequent in non-familial breast cancers and ovarian cancers (Futreal et al, 1994). Another gene, termed BRCA2, that predisposes predominantly to premenopausal breast cancer was recently localized to chromosome 13q, but has not yet been identified (Wooster et al, 1994). Together, germ-line mutations in BRCA1 and BRCA2 may account for the majority of cases of inherited pre-menopausal breast cancer. In addition those carrying germ-line mutations of the BRCA1 and BRCA2 genes may be at increased risk for the development of prostate and colorectal cancer.

2.6 DNA Repair genes and Cancer

The inactivating mutations in the DNA repair genes presumably have only indirect effects on cell growth. Specifically, defects in DNA repair lead to an accelerated rate of mutations for all genes, including oncogenes and tumor suppressor genes. Thus, defective DNA repair accelerates the tumorigenic process, leading to a more rapid acquisition of the multiple mutations necessary to convert a normal cell into a malignant one.

DNA repair defects have been implicated more recently in cancer predisposition syndromes with autosomal dominant modes of inheritance. Hereditary nonpolyposis colorectal cancer (HNPCC) was one of the first cancer predisposition syndromes to be described.

2.7 DNA methylation and Cancer

Several investigators have examined the role of DNA methylation (eg: at CpG islands) in the production of cancer and in the maintenance of the cancerous state. This question has generally been addressed through the use of two techniques (a) the sensitivity of certain restriction endonucleases to a methyl group (eg: Msp1 will cut CCGG independent of whether the middle C is methylated, while HpaII will digest the DNA at this tetramer only when the middle CpG is unmethylated), and (b) the utilization of 5-azacystidine, an inhibitor of DNA methyl trnsferase, the enzyme that catalyses the methylation of the CpG islands.

Kuo and colleagues have reported less methylation of the CpG sequences in the α-fetoprotein gene in hepatoma DNA when compared to normal liver DNA (Lonov et al, 1993). On the other hand, Baylin and colleagues have noted hypermethylation of specific regions of human chromosomes in tumor cells, particularly through the use of chromosome 11probes (Baylin et al, 1986; deBustros et al, 1985). The latter studies raise the interesting possibility of increased methylation associated with the silencing of tumor suppressor genes. Frost and colleagues were first to report an alteration in tumorigenesity by changing the immunogenecity of tumor after exposure to 5-azacytidine (Frost et al, 1983; Frost et al, 1984). In conclusion the methylation of DNA, principally at the CpG islands plays a role in the

tumorigenesis process and in tumor progression. However, that role is not a simple one and may be unique for each individual tumor.

2.8 Telomerase and Cancer

Telomeres, the terminal capping DNA sequences of chromosomes, play an important role in maintaining chromosomal stability (Blackburn, 1991; Greider, 1991). A shortening of the telomeric ends has been observed with each round of DNA replication within normal cells. Consequently, increasing age, such as in bone marrow and epithelial cells results in a reduction in the telomere restriction fragment length (Harley et al, 1990; Hastie et al, 1990). Sequence analysis of telomeric DNA in a number of eukaryotic systems has demonstrated the presence of hundreds to thousands of tandemly repeated bases, TTAGGG (Blackburn, 1992). The number of copies of these sequence determines the dividing capacity of a cell. Unlike normal cells, in which a continuing loss of telomeric TTAGGG repeats occurs, many neoplastic cells maintain their tandem sequences; examples include human malignant hematopoietic cells (Nilsson et al, 1994), ovarian carcinoma cells (Cleaver, 1968), and many others (Kim et al, 1994). The maintenance of the telomeric terminal repeats is the responsibility of a ribonucleoprotein enzyme, telomerase. This remarkable enzyme contains a RNA with a complementary sequence to the terminal repeat, thus allowing hybridization to the telemetric DNA and subsequent polymerization of deoxyribonucleotides of the TTAGGG units. In normal cells, telomerase activity appears highest in the early stages of development and is, therefore, responsible for the apparent immortalization of reproductive cells; telomerase activity diminishes with age. Cancer cell, on the other hand, maintain their levels of telomerase, which leads to continuous replenishment of the telomeric terminal repeats. The result is the immortalization of the cancer cell. The existence of the telomerase in cancer cells provides an avenue of opportunity for the development of more specific chemotherapeutic agents.

2.9 CHEMICAL CARCINOGENESIS

The chemical origin of human malignancies was recognized by observations of unusual cancer incidences in persons in certain occupational groups. The capacity for chemicals to cause cancer was subsequently confirmed in numerous experimental animal studies. The extent to which chemical exposures contribute to cancer incidence

was not appreciated fully until population – based studies documented differing organ-specific cancer rates among geographically distinct populations. Changes in cancer frequency among migrating ethnic groups, high cancer rates associated with specific occupations, and the high risk of smoking – associated cancers confirmed that environmental and lifestyle exposures were major determinants of human cancer risk. Current data indicate that changing lifestyles and exposures can modify cancer risk (Wingo et al, 1999). Individual genetic factors also can influence cancer risk in several ways. In hereditary cancer syndromes, genetic factors dictate a very high cancer risk for a small group of individuals. However, the general population carriers hereditary susceptibility genes that increase cancer risk for particular exposures. Thus, most human cancer is not simply a genetically determined sequela of aging but rather the manifestation of personal and cultural behavior superimposed on individually determined hereditary susceptibility. The experimental induction of tumors in animals, neoplastic transformation of cultured cells by chemicals and analysis of environmentally induced human tumors have revealed important concepts regarding the pathogenesis of cancer (Yuspa and Poirier, 1988; Harris, 1991; Stanley, 1995; Lawley, 1994). Often, chemical carcinogens are organ-specific, target epithelial cells, and cause genetic damage (ie; are genotoxic). Chemically related DNA damage can occur either directly from environmental exposures or indirectly by activation of endogenous mutagenic pathways (eg:- nitric oxide and oxyradicals) (Wink, 1998).

Analysis of the chemical induction of cancer in animal models and human populations has had a major impact on human health. Experimental studies have been instrumental in validating hypothesis generated from human studies. Animal experiments confirmed the carcinogenic and tumor-promoting properties of cigarette smoke and identified the active chemical and gaseous components (Hecht, 1999). The transplacental carcinogenesity of diethylstilbestrol and the hazards of specific occupational carcinogens (eg:- vinyl chloride, benzene, aromatic amines, and bis(chloromethyl)ether) led to the removal of the suspected human carcinogens from the environment and reduction of the cancer rate.

A wide variety of chemicals and chemical classes can cause cancer in animals and human (Report on Carcinogenesis, 1998), yet the process is very specific. Most chemicals are not known to be carcinogenic. Within chemical classes, sterioisomeres

may vary widely in carcinogenesity. Carcinogens can be genotoxic, nongenotoxic or both. Genotoxic carcinogens have high chemical reactivity (such as alkylating agents) or can be metabolized to reactive intermediates by the host. They form covalent adducts with macromolecules and target DNA in the nucleus and mitochondria (Lawley, 1994; Dipple, 1995).

A number of chemicals that cause cancers in the laboratory rodents are not demonstrably genotoxic (Jackson et al, 1993; Ashby and Paton, 1993; Tennant, 1993). Synthetic pesticides and herbicides fall within this group, as do a number of natural products that are ingested (Ames and Gold, 1998). In general, these agents are carcinogenic at high doses in laboratory animals and require prolonged exposure. The mechanism of action is controversial and may be related in some cases to toxic cell death and regenerative hyperplasia (Tennant, 1993; Tennant, 1991). Induction of endogenous mutagenic mechanisms, such as DNA oxy radical damage (Guyton and Kensler, 1993), depurination, and deamination of 5-methylcytosine by exposure to non-genotoxic carcinogens, may contribute to carcinogenesity of these agents. In other cases, nongenotoxic carcinogens may have hormonal effects, influencing hormone-dependent tissues directly (Davis et al, 1993; Dunnick et al, 1995). Though the contribution of nongenotoxic carcinogens to human cancer causation is not certain, they may serve also as modifiers in concert with genotoxic agents.

2.9.1 ORGANIC COMPOUNDS

2.9.1.1 Alkylating agents:

Alkylating agents are chemicals that transfer alkyl groups, often methyl or ethyl groups, to nucleotides to form DNA adducts. The N-nitroso compounds, especially the nitrosamines, are perhaps the most insidious and therefore potentially most hazardous of the various carcinogens. Activation of these compounds often requires biotransformation either enzymatically by oxidation (as for dimethylnitrosamine) or directly by alkali-mediated hydrolysis (as for the direct-acting carcinogen methylnitrosourea). In either case, a methyl group (CH_3-) or an ethyl group (CH_3CH_2-), depending on the chemical, is available for the modification of a DNA base. Methylation occurs predominantly at exocytic oxygen moieties or ring nitrogens.

Numerous naturally occurring toxins have also been characterized, many of which may function as natural pesticides or antibiotics for plants and fungi and also may exhibit carcinogenic activity (Ames, Magaw and Gold, 1987). Over three hundred mycotoxins have been identified, some of which are strong mutagens and carcinogens. The potent liver carcinogen aflatoxin B_1, produced by the common mold *Aspergillus flavus*, can heavily contaminate grains, vegetables, and nuts of which the mold thrives. Metabolic activation of aflatoxin B_1 involves oxidation of the aflatoxin molecule by the P-450 mixed-function oxidases to generate an intermediate which reacts with a cyclic nitrogen in guanine, forming the adduct 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B_1 (Essigmann et al, 1982).

2.9.1.2 Arylkylating agents:

These are chemicals that transfer aromatic or multiringed compounds to a nucleotide to form an adduct. Polycyclic aromatic hydrocarbons (PAH), the principal group of aralkylating agents, remain an occupational problem in several industries. More recently, the crude oils used in cotton spinning (Heller, 1930) or tool setting (Waldron, Waterhouse and Tessema, 1984) were reported to cause scrotal cancer in workers whose pants became saturated with the oils. Structurally similar compounds have been found in combustion products (Grimmer and Misfeld, 1983) and as such are added continuously to the environment. This includes products such as benzo[a]pyrene and the potent 7, 12-dimethylbenz[a]anthracene, which are generated in cigarette smoke and on charcoal-grilled meats (Lijinsky and Shubik, 1964). These compounds readily induce tumors in laboratory animals, causing rapid tumorigenesis in rat mammary tissue following ingestion (Huggins, Grand and Brillantes, 1961), the major route of exposure for humans.

2.9.1.3 Arylhydroxylamines:

These are chemicals that transfer aromatic amines to nucleotides to form adducts. The carcinogenic activity of the aromatic amines, a major group of arylhydroxylamines, was established from epidemiologic studies of workers in the dyestuff industry. Occupational exposures to aniline dyes caused a high incidence of baldder cancer (Rehn, 1895), and the etiologic agents most responsible were eventually identified as 2-naphthylamine and benzidine (Case et al, 1954). In a study of workers involved in the distillation of 2-naphthylamine, nearly all heavily exposed

individuals subsequently developed bladder cancer (Case, 1969). Similarly. 2-naphthylamine was implicated in the high incidence of bladder tumors in the manufacturing of rubber (Case and Hosker, 1954). Bioassays for carcinogenic activity in rats and dogs showed these compounds to be active primarily in the bladder following dietary exposures (Radonski, 1979).

The arylhydroxylamines, like PAH, require metabolism by the P-450 mixed function oxidase enzymes, and commonly undergo N-oxidation to generate reactive intermediates. In the case of 2-naphthylamine, the chemical is first enzymatically oxidized to generate an N-hydroxyl intermediate, which may be further metabolized in liver or kidney to form a stable glucuronide conjugate that is passed to the bladder (Kadlubar, Miller and Miller, 1977). In the bladder, the final activated electrophile, presumed to be a nitrenium cation, is formed, which can react with DNA in bladder epithelia (Orzechowski, Schrenk and Bock, 1992).

2.10.2 INORGANIC COMPOUNDS:

Certain inorganic metals and minerals exhibit carcinogenic activities or are associated with elevated risks for cancer in humans. These include arsenic, nickel, chromium, and asbestos. The unequivocal effect of asbestos on tumorigenesis and its extensive presence in the environment such as in cement construction materials, insulation, and fireproofing, have established it as a significant health hazard. The term "asbestos" encompasses a variety of silica fibre types. Although one form of asbestos, the serpentine magnesium-containing chrysotile ($Mg_6Si_4O_{10}$ $(OH)_8$), represents more than 90% of the mined asbestos in the United states, carcinogenic acivity is generally associated with the amphibolic iron-bound crocidolite ($Na_2(Fe^{3+})_2(Fe^{2+})_3$ $Si_8O_{22}(OH)_2$) or calcium-containing tremolite ($Ca_2Mg_5Si_8O_{22}$ $(OH)_2$). Asbestos workers present with either lung carcinomas or mesotheliomas of the pleura or peritoneum. As many as 20-25% of heavily exposed workers develop lung cancer (Lemen, Dement and Wagoner, 1980). Although all types of asbestos fibers can cause chromosomal aberrations (Sincock, Delhanty and Casey, 1982; Hei et al, 1992) as well as lung tumors in rats (Davis et al, 1978), studies of fiber biodistribution in asbestos workers with mesothelioma suggest that the lung burden of amphiboles is significantly greater than chrysotile fibres (MacDonald and MacDonald, 1987; Wagner et al, 1988).

2.11. IONIZING RADIATION:

The hazards of exposure to ionizing radiation were recognized shortly after Roentgen's discovery of X-rays in 1895. Acute skin reactions were observed in many individuals working early X-ray generations, and by 1902 the first radiation-induced cancer was reported arising in an ulcerated area of the skin. Within a few years, a large number of such skin cancers had been observed, and the first report of leukemia in five radiation workers appeared in 1911 (Upton, 1986).

The ionizing event involves the ejection of an orbital electron from a molecule, producing a positively charged or ionized molecule. These molecules are highly unstable and rapidly undergo chemical change. This change results in the production of free radicals, atoms or molecules containing unpaired electrons. These free radicals are extremely reactive and may lead to permanent damage of the affected molecule, or the energy may be transferred to another molecule. Most of the energy deposited within a cell results in the production of aqueous free radicals, since approximately 80% of the cell is water.

Studies of transformation induced by radiations indicate that it is a progressive, stimuli-step process by which normal cells acquire the various phenotypic characteristics of cancer cells. There appears to be three major independent stages in the malignant transformation of cells in vitro: the development of morphologic changes, cellular immortality.

2.11.1 Molecular mechanisms:

a) DNA damage

It is well recognized that DNA damage is central to the initiation phase of carcinogenesis induced by ionizing or ultraviolet radiation (Weinstein, 1988). The cellular enzyme protein kinase 'C' (PKC) plays a critical role in growth control and appears to be involved in the promotional phase of radiation carcinogenesis (Hei et al, 1994). PKC, activated by phorbol ester tumor promoters such as TPA, can produce a cascade of events resulting in alterations in gene expression, membrane function and ultimately cellular differentiation and proliferation (Weinstein, 1988). In addition, radiation can directly induce changes in gene expression via transcriptional or post translational mechanisms (Little, 1986). From studies of radiation induced

carcinogenesis in human populations and experimental systems, it appears that radiation acts primarily as an initiating agent by its ability to damage DNA. Radiation can induce both specific base damage and DNA strand breaks, and mammalian cells possess efficient enzymatic mechanisms for repairing these types of damage. Although it has long been assumed that unrejoined double strand breaks are the critical DNA lesions responsible for cell killing by radiation, it now appears that incorrectly rejoined DNA double strand breaks are important mutagenic and carcinogenic lesions. This DNA mispair appears to lead to chromosomal deletions and rearrangements. DNA structural analysis of radiation induced mutants at specific gene loci in human cells indicate that most mutations arise as a result of such large-scale genetic and chromosomal changes (Li et al, 1992).

b) Oncogenes and radiations:

The role of specific oncogenes activation in radiation-induced cancer is less clear (Bowden et al, 1990; Cox and Little, 1992). Activation of ras oncogene occurs, though in relatively low frequencies, in mouse lymphomas induced by radiation (65) and a specific codon 146 ras mutation has been described in a small fraction of neutron-induced thymic lymphomas (Sloan et al, 1990). Activation of C-Ki-ras as well as amplification of c-myc has been reported in some radiation-induced rat skin tumors (Sawey et al, 1987), but not in mouse skin tumors (Bowden et al, 1990). Amplification and rearrangement of c-myc has been reported in a small fraction (6-30%) of radiation-induced murine osteosarcomas (Sturm et al, 1990).

c) Radiation and tumor suppressor genes

In terms of radiation carcinogenesis, much recent interest has centered on the p53 gene, as it appears to play an important role in cell cycle control, radiosensitivity, the development of genetic instability leading to cell trasformation, and perhaps in the response of human tumors to radiation. P53 mutations have been found in a wide spectrum of human cancers (Greenblatt, 1994) and in mouse skin tumors induced by ionizing radiation (Dostuyama et al, 1994).

RB is a tumor suppressor gene that is associated with retinoblastoma, a malignant eye tumor of children. Interestingly, patients with the hereditary type of retinoblastoma appears to be at an unusually high risk for the development of

radiation-induced secondary tumors, primarily osteogenic sarcomas occurring in the treatment field (Eng et al, 1993). The fact that activation of a tumor suppressor gene may result from large-scale genetic changes such as deletions, genomic rearrangements and recombinational events suggest that tumors that arise as a result of the loss of suppressor gene activity may be particularly susceptible to induction by irradiation.

2.12 VIRAL CARCINOGENESIS

The belief that viruses participate in carcinogenesis has now gone full circle from the days in the early 1970s, when the NCI director declared that cancer would be cured in five years, presumably by a vaccine to tumor viruses, to the mid-1970s when retrovirologist and Nobel laureate Howard Temin proposed that the only tumor induced by a vieus was the nonmalignant wart, to the discovery of cellular oncogenes incorporated into the genomes of retroviruses. The literature is replete with associations of viral sequences in certain tumors (eg; Epstein-Barr virus and Burkitt's lymphoma or hepaptitis B virus and liver cancer), but direct proof of their involvement in carcinogenesis has remained somewhat elusive. Perhaps the best case has been demonstrated for the human papilloma viruses (HPV) and anogenital cancers, especially cervical cancer. Epidemiological studies have long implicated a venereal component in cervical carcinoma (Kessler, 1976), but initial attempts to correlate the now reputed etiologic agent HPV viral types. Some 65 different HPVs have been reported (DeVilliers, 1989), of which 10 are categorized as "high risk" types due to their involvement in cases of severe dysplasia and neoplasia (Werness, Munger and howley, 1991). Recent examination of cervical carcinomas now has shown that nearly 85% of these cancers contain "high risk" HPV sequences (Riou et al, 1990). Molecular fragmentation of the "high risk" forms has revealed two viral oncoproteins that interact with one of the cellular growth suppressor gene proteins pRb or p53 (Munger et al, 1989; Scheffner et al, 1990), important regulatory factors for cell cycle progression. The HPV oncoproteins are capable, either independently or in cooperation with other transforming genes, of immortalizing (Hawley –Nelson et al, 1989) or transforming (Phelps et al, 1988) a variety of cell types in vitro. Although these observations in no way prove that HPVs play a role in human cervical cancers, the epidemiologic reports combined with the molecular findings provide compelling

evidence that they function in the neoplastic process and, on the basis of their late detectability, probably do so during the tumor progression stage.

Clearly, viral participation in carcinogenesis is a rare occurrence and therefore probably impacts few individuals; however, viral studies have provided among the most important observations in carcinogenesis of the 1980s and 2000s through the discovery of oncogene sequences in tumor retroviruses. These are regulatory genes that normally function within vertebrate cells but have been modified and incorporated into retroviral genomes, converting the viruses to oncogenic forms.

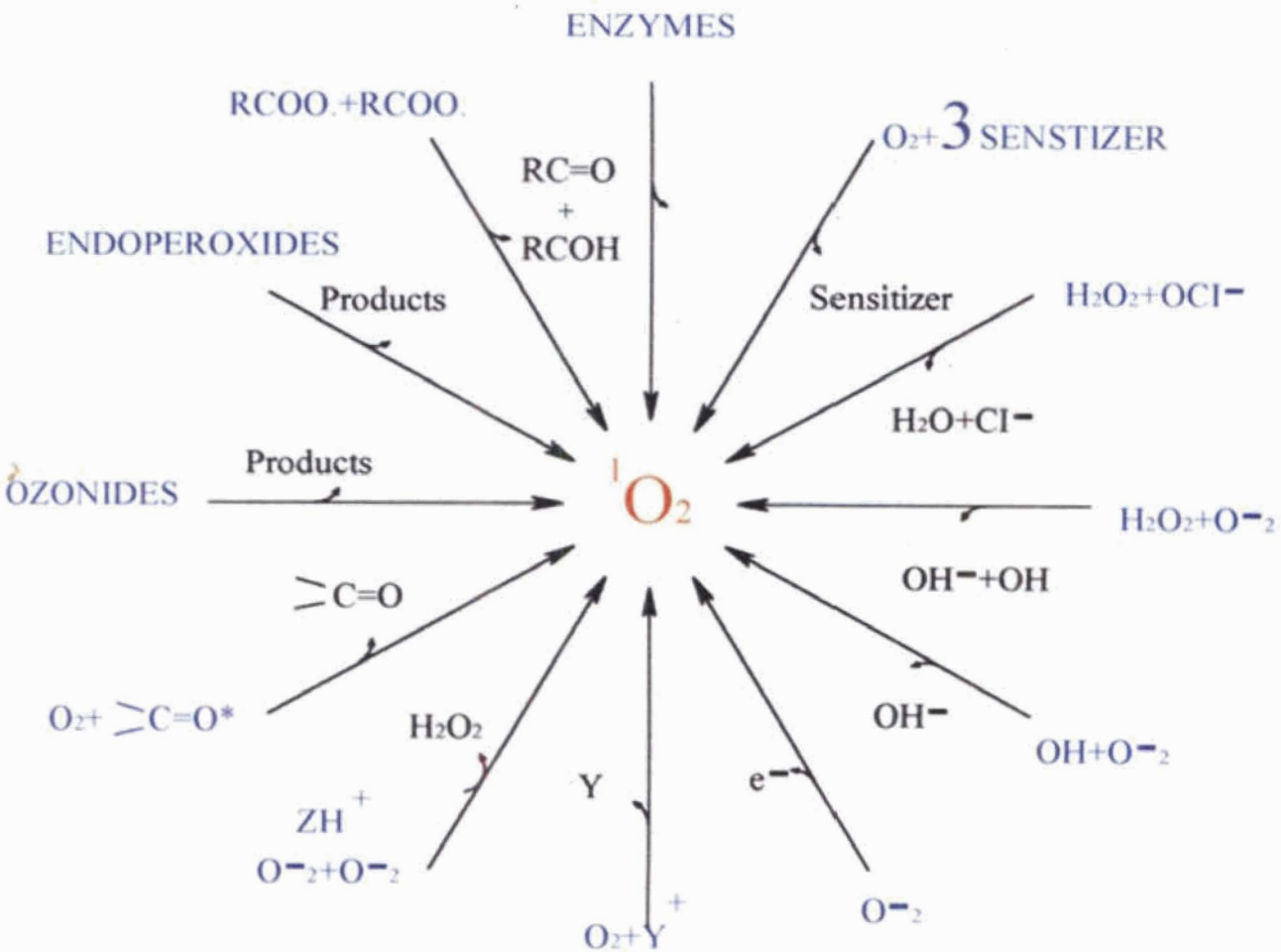
2.13 REACTIVE OXYGEN SPECIES AND CANCER

During the last decade, considerable attention has been focused on the involvement of oxygen free radicals (OFR) in various diseases (Halliwell et al., 1992; Aemes et al., 1993; Cerutti and Trump, 1991; Guyton and Kensler, 1993). OFR are continuously generated in cells exposed to an aerobic environment during the course of normal metabolism. Despite the presence of strong antioxidant defence mechanism to counteract the OFR and minimize the plausible oxidative damage (Jansen et al., 1993; Yu, 1994), OFR and minimise the plasible oxidative damage (Davies, 1993). OFR dependent damage of proteins (Hussain et al., 1994), DNA another biomolecules accumulate during the lifetime of organisms. It has been postulated that age-dependent diseases as atheroscelorosis, arthritis, neuro-degenerative disorders and cancer involve OFR at least at some stage of their development.

Cancer development is now commonly recognized as a micro-evolutionary process that requires the cumulative action of multiple events. These events may occur in a single cell clone and can be explained by a simplified three-stage model. These stages include (a) the induction of DNA mutation in a somatic cell known as initiation, (b) stimulation of the initiated cell and its clonal expansion referred as promotion, and (c) malignant conversion of the benign tumour into cancer termed as progression. OFR have been shown to stimulate cancer development by playing a role at all The three stages namely, initiation, promotion and progression (Cerutti, 1994; Pryor, 1987).

Figure 2.2

Different ways in which singlet oxygen can be formed



2.13.1 Aetiology of oxygen-free radicals

The term OFR refers to forms of oxygen exhibiting high reactivity and having at least one unpaired election. However, other reactive forms of oxygen are also known which non-free radical. Both of these forms are collectivity referred as reactive oxygen species (ROS) and include singlet oxygen, superoxide anion, hydrogen peroxides, hydroxyl radical, etc. singlet oxygen is formed by the transfer of radiant energy to the oxygen molecule which is triplet and paramagnetic at ambient temperatures. The stepwise univalent reduction of oxygen leads to the formation of superoxide anion, hydrogen peroxide and hydroxyl radical (Fig.1.2). The oxidation potential and reactivity of various ROS may be given in the following order (Fridovich, 1978):

$$\cdot O_2 < H_2O_2 < {}^1O_2 < \cdot OH$$

OFR are short-lived species that are generated in situ in normal cells under pathological conditions. In addition, the metabolism of xenobiotics and/ or exposure to ionizing radiation also generate these species. An important feature of free radical reactions with non-radical species is the formation of new radical species. The free driven reactions are usually chain reactions (Halliwell and Gutteridge, 1984).

2.13.2 Oxidative stress in carcinogenesis

In aerobic biota, the OFR are formed in normal cell metabolism from molecular oxygen. Despite tight antioxidant defences, these OFR cause constant damage to oxidizable molecules, which are repaired or replaced in a dynamic equilibrium. The condition of cellular oxidative stress arises either from the overproduction of OFR or from the deficiency of antioxidant defences or repair mechanism(s) and results in reversible tissue injury. Examples of short-term oxidative stress are the ischemia reperfusion injury syndrome, acute inflammation and hyperoxia (due to the exposure to hyperbaric oxygen). I addition, important endogenous manifestation of chronic oxidative stress is inflammatory disorders (Cerutti and Trump, 1991).

The role of OFR in different stages of carcinogenesis is described as follows:

2.13.3 Role of ODFR in initiation

Evidences have accumulated to suggest that ROS play an important role in tumour initiation by enhancing or facilitating the metabolic activation and/or initiating

effects of carcinogens. Out of many mechanisms described for the chemical initiation of tumourigenesis, a number of them may involve free radicals in the cascade of reactions (Pryor, 1987). Initiators are in general metabolized to an ultimate carcinogen, which usually forms adduct(s) with DNA. A number of initiators have been shown to produce free radicals by themselves (Floyd et al., 1978; Ames, 1983; Nakayama et al., 1983). DNA is the potential target for initiators of carcinogenesis. Free radicals cause DNA base damage, DNA single strand breaks, cross-linking between DNA and proteins or DNA and chromosomal aberration. One of the major products of base damage in DNA is thymine glycol (Kaneko and Leadon, 1987). For example, N-hydroxy-2-napthylamine and benzo(a) pyrene have been shown to produce thymine glycol in cultured fibroblasts or DNA in solution (Leadon, 1987). Superoxide anion generated from hypoxanthine-xanthine oxidase system induces chromosomal aberration in cultured Chinese hamster cells (Sofuni and Ishidate, 1984) and V79cells (Iwata et al., 1984). In addition, it has also been shown to act as a weak complete carcinogen in other model systems (Zimmerman and Cerutti, 1984). Hydrazine and its derivatives dimethyl hydrazine and isoniazid, which produce ROS have been shown to induce sister chromatid exchange in CHO cells (Macrae and Stich, 1979). Chromosomal breakage, rearrangement and sister chromatid exchange are also formed as result of photochemical or enzymatic generation of superoxide radicals. It has been implicated that H_2O_2 and hydroxyl radical, which are formed within the cells of cultured human IMR-90 fibroblasts by the exposure to fluorescent light were responsible for the induction of DNA damage (Parshad et al, 1980). H_2O_2 has also been implicated as a causative agent in the induction of chromosomal damage (Bradely and Erickson, 1981) and found associated with the induction of Cancer in animal model system (Shamberger, 1972; Ito et al., 1981). H_2O_2 induces molecular damage leading to induction of transformation in the normal cells in vitro (Kennedy et al., 1984).

Strong evidence for the involvement of free radicals in free radicals-induced DNA damage and consequent carcinogenesis comes from the fact that these processes can be abolished by free radicals scavengers/ antioxidants (Yi Sun, 1990; Machlin and Bendich, 1987). Potent inhibitors of skin tumour initiation in mice include antioxidants (butylated hydroxytoluene, butylated hydroxyanisole and selenium), flavones (7, 8-benzoflavone and quercetin) and vitamins (A, C and E) etc. some of the flavones and antioxidants appear to inhibit skin carcinogenesis by inhibiting the metabolism of a

procarcinogen to its ultimate carcinogenic from (Hocman, 1988). The antioxidant, butylated hydranisol (BHA) is widely used as food preservative and has been shown to inhibit chemically induced lung, liver, mammary, fore stomach, colon and liver cancer in experimental animals. Similarly, inhibitory effects of selenium and vitamins E and C have been demonstrated (Ito and Hirose, 1987; Katiyar *et al.*, 1993) have shown that the possible inhibition of N-nitrosodiethylamine and benzo(a)pyrene induced forestomach and lung tumourigenesis by some polyphenolic fraction isolated from green tea, is due the induction of antioxidant enzymes by these fractions.

2.13.4 Free radical – generating compounds as tumour promoters

There is a long list of oxidants with tumour promoting properties. H_2O_2, peroxyacetic acid, chlorobenzoic acid, benzoyl peroxide, decanoyl peroxide, cumene hydroperoxide, lauryl peroxide, dicumyl peroxide, *p*-nitroperbenzoic acid and periodic acid are demonstrated to be tumour promoters in cultured in mouse epidermal cell and in mouse skin models (Gimenez- Conti et al., 1991). H_2O_2 has also been reported to be a complete promoter in a rat duodenum (Hirota and Yokoyama, 1981). Promoting potentials of these peroxides is attributed to their ability to generate ROS within the biological system. Keratinocytes and epidermal cells exposed to hydroperoxide tumour promoters have been shown to generate free radicals (Timmins and Davies, 1993; Taffe et al., 1987; Taffe and Kensler, 1986). Taffe et al. have demonstrated the generation of alkyl radical and alkoxyl radical by incubating different organic hydroperoxide with isolated mouse keratinocytes (Taffe et al.,1987).

The generation of free radicals from a variety of oxidant tumour promoters has also been studied in other biological systems such as liver microsomes and cytosolic fraction (Davies, 1989; Greenley and Davies, 1990; Davies, 1993). Antipsoriatic agent, anthrone derivatives such as anthralin and chrysarobin that can generate directly free radical by the simple oxidation in presence of air and light, have been shown to be mouse skin tumour promoters. The ultimate promotional ability of these compounds has been suggested to be due to the production of super oxide radical (Block and Burns, 1963; Ashton et al., 1983; Kruszewski et al., 1985). The metabolism of benzo(a)pyrene has been shown to be accompanied initially by the generation O_2 and subsequently by

H_2O_2 and OH, and the involvement of these radicals in tumour promotion has also been suggested (Lesko et al., 1975).

2.13.5 Activation of reactive oxygen by tumour promoters

It is now known that tumour promoters act at least in part by inducing a cellular prooxidant state. Many tumour promoters accentuate the elaboration of ROS by the endogenous sources to create a cellular prooxidant state. TPA stimulates leukocytes and macrophages for the increased consumption of oxygen and in turn generates ROS. It has been suggested that ROS derived from these pro-inflammatory cells are critical component of the tumour promotion processes (Keisari et al., 1984). Another potential source ROS in some target tissues is xanthine oxidase. Reiners et al.(1987) have observed a 2-3 fold increase in xanthine oxidase activity in kerationocytes following TPA treatment (Reiners *et* al.,1987). Keratinocytes exposed to hydroperoxide tumour promoters generate free radicals (Perchellet et al., 1988; Perchelet and Perchellet, 1989; Gali et al., 1992). The levels of hydroperoxides are increased gradually by successive applications of TPA until a plateau is reached (Perchelet and Perchellet, 1989). In vivo, TPA stimulates the infiltrations of neutrophils and as a result myeloperoxidase activity in dermis is enhanced. It is known that it also enhances the formation of H_2O_2 and oxidized DNA bases in epidermis (Wei and Frenkel, 1991; Wei et al., 1993). Tumour promotion process is mediated through the interaction of TPA with the PKC receptor. The tumour promoting activity of different analogs of TPA was found to be in concordance with their ability to act as stimulators of PKC (O, Brian et al., 1988; Gopalakrishna and Anderson, 1989; Larson and Cerutti, 1989). The other classes of epidermal tumour promoters such as mezerein and indole alkaloids, which are chemically different from TPA, also activate PKC and stimulate O_2 production. The weak endogenous skin tumour promoter, diacylglycerol also stimulates superoxide generation from neutrophils (Fujita et al., 1984). BPO does not bind to PKC, but activates it indirectly (Donnelly et al., 1987). Some tumour promoters such as anthralin, indolacetic acid and tween 60, which do not activate PKC, do not evoke this response. Further, other tumour promoters such as polytoxin and thapsigargain also evoke oxidative burst in neutrophils but apparently not through the PKC, other protein kinases have been shown to get activated under the predisposed conditions of oxidative stress. For example, oxidants have been found to activate the rat insulin receptor kinase (Chan

et al., 1986; Hayes et al., 1987) and stimulate phosphorylation of the ribosomal protein through Ca^{2+}-dependent event (Larson and Cerutti, 1988).

2.13.6 Modulation of cellular antioxidant defense systems by tumour promoters

In addition to the production of free radicals, many tumour promoters have been shown to modulate the cellular antioxidant mechanism. Solanki et al (1981). Have shown that in mouse epidermis TPA, anthralin, non-phorbol-ester tumour promoter, etc cause a rapid and sustained decrease in the activities of superoxide dismutase and catalase. Similarly, the TPA-mediated changes in glutathione peroxidase activity were found to be time-dependent and are transiently increased within 30 min of TPA treatment which is followed by a depression between 1 to 12 hr. glutathione reductase activity was also depressed throughout the multiple exposure of TPA (Taffe and Kensler, 1986). Consistent with these enzymatic changes, a four-fold increase in the levels of oxidized glutathione was observed (Perchellet and Perchellet, 1989) In mouse epidermal JB6 cells also showed that TPA treatment reduces SOD, CAT and GPX activities (Kolde et al, 1976). A known liver tumour promoter, clofibrate modulates antioxidant enzyme activities. Catalase activity per unit volume of peroxisome has been shown to decrease continuously during the treatment of clofibrate in the male rat liver. Ciriolo et al. (1982) have reported that clofibrate and other peroxisome proliferator tumour promoter, fenofibrate lowered the activities of GPX, and SOD.

2.13.7 Antioxidant as antipromoters

Kensler et al. (Nakamura et al., 1985) have postulated that if tumour promotion is related to the increase in the intracellular free radical level, the application of free radical scavenger or antioxidant could conceivable modulate tumour growth. Indeed, exogenous addition to mouse skin of a lipophilic biomimetic SOD, Cu (II) (3, 5-dispporpil salicylic acid), Cu DIPS, inhibits TPA-induced ODC activity and tumour formation (Enger and Kensler, 1985; Nakamura et al., 1985). In experiments by Friedmen and Cerutti, 1983, scavengers of ROS (SOD, CAT and mannitol) displayed similar effects by antagonizing PMA-induced ODC activity in mouse mammary tumour cells.

Antioxidants such as GSH, cysteine, and α-tocopherol were shown to prevent the TPA-mediated decrease in the ratios of reduced to oxidized glutathione in mouse epidermal cells. In the same system, these antioxidants inhibit TPA-induced ODC activity as well as tumour growth (Perchellet and Perchellet, 1989). Oberley and Oberley (1988) have demonstrated the role of antioxidant enzymes in cell immortalization, transformation and carcinogenesis. Furthermore, the free radical scavengers such as BHA and BHT, which are known inhibitors of lipid peroxidation, were shown to inhibit TPA- and BPO-induced ODC activity and tumour promotion in mouse skin (Kozumbo et al., 1983). Additionally, these compounds and their analogs were found positive for their inhibitory effects of TPA-stimulated chemiluminescence in PMNs. Various antioxidants were found to prevent Fe-NTA-mediated induction of ODC and [^{3}H] thymidine incorporation both in liver and kidney cells (Athar and Iqbal, 1998; Iqbal et al., 1995). There is also evidence that contrary to their antioxidant properties, these antioxidants under certain circumstances may act tumour promoter. For example, BHA has been shown to promote forestomach and urinary bladder tumourigenesis, while it was found to inhibit lever and mammary gland carcinogenesis. BHT on the other hand, promoted carcinogenesis of urinary bladder, thyroid and esophagus, while it inhibited ear duct and mammary gland carcinogenesis. It has been reported that BHT and vitamin E inhibited the promoting action of polyunsaturated fatty acids in mammary gland carcinogenesis in rats initiated with DMBA the effect has been proposed to be caused by their ability to block prostaglandin synthesis or by inhibition generation of oxidative products of fatty acids, which may play a role in tumour promotion by blocking oxidative metabolism of polyunsaturated fatty acids. The naturally occurring plant products such as nordihydroguaiaretic acid and diallyal sulfide having anti oxidant properties abrogate the tumour promoting effects of BPO in murine skin (Athar et al, 1990). Ascorbic acid (Smart et al., 1987) and α-tocopherol have been shown to have inhibitory effects on the mouse skin tumour promotion by TPA. Sarcophytol, a marine product has been reported to inhibit the two-stage carcinogenesis in DMBA initiated telocidile/TPA promoted mouse skin (Fujiki et al., 1989). The anti-tumour promotional activity is proposed to be due to their ability to suppress the PMN infiltration, ROS generation and oxidative DNA damage. Several free radical scavengers have been shown to inhibit chyrosarobin-and anthralin-induced tumour promotion. The known renal tumour promoter, potassium bromate-mediated DNA damage is inhibited by glutathione, cysteine and vitamin C (Sai et al., 1992). The dietary antioxidant, tannic acid

suppresses DMBA-induced skin and benzo(a)pyrene-induced lung and forestomach tumourigenesis (Athar et al.,1989).

2.13.8 Role of ROS in tumour progression

Rotestein et al. (1987) have shown that low frequency of conversion of papillomas to carcinomas can be increased by generating compound, benzoyl peroxide. Theses results promoted many other researchers to further investigate the role of ROS in the progression stage of carcinogenesis. It has been shown that BPO enhances the invasiveness of mouse epidermal carcinoma cell lines Athar et al., 1989; Warren et al., 1993). Many initiators also have potential to enhance the conversion of papilloma to carcinoma (Hennings et al., 1983). In a two-stage carcinogenesis model, exposure to ionizing radiation as source of free radicals augmented the malignant conversion although, it did not alter the incidence of papillomas. Athar et al. (1989) have demonstrated the efficacy of number of different free radical generating compounds to enhance the malignant conversion of benign papillomas into caricinoma. They have also suggested that the effectiveness of these compounds may be related to the type radicals produced in to the biological system. Further, it has been shown that organic hydroperoxides are metabolized into free radicals by normal mouse skin keratinocytes and by the human carcinoma keratinocytes. These observations suggest that prooxidant compounds having ability to be metabolized in to free radicals may enhance the rate of progression of benign tumours to malignant neoplasms involving free radicals. These findings suggest that tumour progression rate may be sensitive to the ROS-mediated genetic alterations. It was also postulated that progression of cancer might involve free radical-induced clastogenic changes leading to the activation and /or inactivation of various cellular genes in the experimental carcinogenesis. Ruggeri et al. (1991) showed inactivation of p53 tumour suppressor gene during the progression stage. This inactivation may lead to onset genes coding metalloproteases, which are involved in the inviasion and / or metastasis (Matrisian et al., 1986; Ostrowski et al., 1988). Athar et al. (1991) have reported the protective effect of all *trans*-retinoic acid (RA) in the free radicals-mediated conversion of chemically-induced and UV-B radiation-induced skin papillomas to carcinomas (Ostrowski et al., 1988).

2.14 CANCER THERAPY

The different strategies used in cancer treatment are surgery, radiation and chemotherapy. The physical removal of tumour mass is the foundation of surgery. Radiotherapy and chemotherapy are exposure to toxic ionizing radiation or cytotoxic chemicals respectively to destroy cancer cells without having to find and remove them.

2.14.1 Surgery

Surgery is the oldest treatment for cancer and until recently, was the only treatment that could cure patients with cancer. Surgical procedures are used to physically remove malignant tissues, and it remains one of the important modality of treatment for malignant tumours

2.14.2 Radiation therapy

Ionizing radiation continues to be a curative option for many patients alone or in combination with other modalities. Radiation therapy for cancer originated in the finding that X-rays sterilize rams by killing the proliferating germ cells in the tests that maintain spermatic genesis. Radiation is most toxic to proliferating cells, higher doses are required to kill cells that are capable of proliferating, but are not actively dividing (quiescent cells) at the time of exposure. Mammalian cells are most sensitive to radiation induced damage in the late G_2 and M-phase of the cell cycle, cellular damage produced by radiation therapy is an indirect results of ionizing chemicals in the cell to very reactive compounds. Cytotoxicity is primarily caused by Oxygen derived free radicals such as H_2O_2, superoxide and hydroxyl radicals. Synthesis of radio sensitizors, which augment the amount of injury and induced by radiation to hypoxic cells that are relatively resistant, has increased the effectiveness of radiation therapy.

2.14.3 Radioprotectors

Radiotherapy is the most common modality for treating human cancers. Eighty percent of cancer patients need radiotherapy at some time or other, either creative or palliative purpose. To obtain optimum results, a judicious balance between the total dose of radiotherapy delivered and the threshold limit of the surrounding normal critical tissues is required. In order to obtain better tumour control with a higher dose, the normal tissues should be protected against radiation injury. Thus the

role of radio protective compounds is very important in clinical radiotherapy (Nair et.al, 2001).

Ionizing radiation causes damage to living tissues through a series of molecular events, such as photoelectric, compton and Auger effects, depending on the radiation energy. Because human tissues containing 80% water, the major radiation damage is due to the aqueous free radicals, generated by the action of radiation on water. The major free radicals resulting from aqueous radiolysis are OH, H, Caq, HO_2, H_3O^+, etc.

These free radicals react with cellular macro molecules, such as DNA, RNA, proteins, membrane etc., and cause cell dysfunction and mortality. These reactions take place in tumour as well as normal cells when exposed to radiation.

Radio protecting agents can be classified into three groups. (a) Radio protectors (b) adaptogens and (c) absorbents. These include several myelo, entero and cerebro protectors. Adaptogens act as stimulators of radio resistance. These are natural protectors, which offer chemical protection under low levels of ionizing radiation. These are generally entracted from cells of plants and animals and have least toxicity. Absorbents protect the organisms from internal radiation and chemicals. These include drugs which prevent the incorporation of radioiodine by thyroid gland and absorption of radionucleides ^{137}Cs, ^{90}Sr, ^{239}Pu etc. Different Radioprotectors and their mechanism of action were shown in table 1.1.

2.14.4 Chemotherapy

Cancer chemotherapy had its roots in the work of Paul Ehrlich, who coined the word Chemotherapy. The era of modern chemotherapy may be started in 1948 with the introduction of nitrogen mustard. Most agents currently in use appear to exert their effects primarily on the cell multiplication and tumour growth. Because cell multiplication is characteristic of normal cells as well as cancer cells, most of the chemotherapeutic agents also have toxic effects on the normal cells. Inhibition of cell multiplication and tumour growth can take at several levels within the cells are: (1) macromolecular synthesis and function (2) cytoplasmic organization and (3) cell membrane synthesis and function. Most chemotherapeutic agents particularly those that

Table 2.2 Different Radioprotectors and their mechanism of action

	Radioprotectors	Mechanism of Action
A.	*Sulfhydryl compounds* Cysteine, Cysteamine, Glutathione, AET. WR 2127 and other WR compounds.	Free radical scavenging, donation of H atom.
B.	*Antioxidants* Tempace, Hoechst 33342, Vitamin A, E & C, TMG, Melatonin, etc.	Free radical scavenging
C.	*ACE inhibitors* Captopril, Elanopril, Pencilamine, Pentoxyfylline, L-158, 809, etc.	Protease inhibition through rennin angiotensin system, anti-oxidation collagen synthesis inhibition.
D.	*Cytoprotective agents* Mesna, Dexrazoxane, Amifostin (WR2127).	Reduced toxicity of chemotherapeutic drugs, decrease of urothelial toxicity and nephrotoxicity
E.	*Metalloelements* Manganese chloride, Cadmium salts, Bismuth Subnitrate, etc.	Metallothionine induction
F.	*Immunomodulators* Gamma-interferon, Polysaccharides AM5, AM218, Heat killed Lactobacillus cells, Broncho-Vaxom, Trehalose dicorynomycolate, AS101.	Immune stimulation, increased production of cytokines.
G.	*Lipopolysaccharides and Prostaglandins*	Prostaglandin synthesis, elevated levels of cyclic AMP, DNA repair.
H.	Plant extracts and isolated compounds Curcmin, Orientin, Vicinin	Free-radical scavenging, anti-oxidation.

affect macromolecular synthesis can be grouped according to whether they depend on the cell being in the cycle. (Wilson, 1975; Teng, 1977).

2.14.5 Chemotherapeutic agents

Chemotherapeutic agents can be classified into two groups based on their source/ origin:

1. Non-plant derived anticancer agents
2. Plant derived anticancer agents.

2.15.6 Non- Plant derived Antineoplastic therapeutic agents

a) Antifolates:

The folate-dependent enzymes represent attractive targets for antitumor chemotherapy because of their critical role in the synthesis of the nucleotide precursors of DNA. In 1948, Farber et al, were the first to show that aminopterin, a 4-aminoacid anlogue of folic acid, could inhibit the proliferation of leukemic cells and produce remissions in acute leukemia. Their findings ushered in the era of antimetabolite chemotherapy and generated great interest in the antifolate class of agents. Since then, the clinical value of antifolate compounds has been proven in the treatment of the leukemias, breast cancer, head and neck cancer, lymphomas and choriocarcinoma (De Vita et al, 1993). Various heterocyclic compounds with the 2,4-diamino structural configuration have antifolate activity and include pyrimidine analogues such as pyrimethamine and trimethoprim (Baggot et al, 1986; Morrison and Allegra, 1989; Chu et al, 1990; Lyons et al, 1990; Bertino et al, 1979; O'Dwyer et al, 1985; Sigel et al, 1987; Sirotnak et al, 1984; Kamen et al, 1984; Jones et al, 1981), classic pteridines such as aminopterin and methotrexate (De Vita et al, 1993), and compounds with replacement of the nitrogen at either the 5, 8, or 10 position with a carbon atom, such as the quinazolines (Bertino et al, 1979; O'Dwyer et al, 1985; Sigel et al, 1987) and 10-ethyl-10-deazaaminopterin (10-EDAM, Edatrexate) (Sirotnak et al, 1984).

b) 5-Fluropyrimidines:

The 5-fluorinated pyrimidines were first of all synthesized by Heidelberger et al,1957. The impetus for synthesis of fluorinated pyrimidines came from the

observation that rat hepatomas more avidly utilize radiolabelled uracil than do nonmalignant tissues (Rutman et al, 1954). This finding implied that the enzymatic pathways for utilization of uracil, and possibly analogues of uracil, differ between malignant and normal cells and represent a possible target for antimetabolite chemotherapy. These drugs have shown the predicted biochemical action and have become useful in the treatment of human solid tumors, including breast cancer, gastrointestinal adenocarcinomas, and squamous cell carcinomas arising in the head and neck. They have invoked interest not only because of their inherent antitumor activity but also because of their synergistic interaction with other antitumor agents, irradiation, physiologic nucleosides, and leucovorin (LCV).

c) Cytidine Analogues:

Nucleoside analogues compete with their physiologic counterparts for incorporation into nucleic acids and have earned an important place in the treatment of acute leukemia. The most important of these are the arabinose nucleosides, a unique calss of animetabolites originally isolated from the sponge *Cryptothethya crypta* (Bergmann and Feeney, 1951) but now produced synthetically (Walwick et al, 1959). They differ from the physiologic deoxyribonucleosides by the presence of a β-OH group in the 2^1 posiotion of the sugar. Several arabinose nucleosides have useful antitumor and antiviral effects, the most active cytotoxic agent of this class being arabinosyl – cytosine (araC). A related nucleoside, arabinosyladenine, has antitumor and antiviral action (Lee et al, 1960), and its analogue, 2-fluoro-araAMP, has strong activity in lymphomas and in chronic lymphocytic leukemia (Warrell and Berman, 1986).

d) Purine Antimetabolites

6-Mercaptopurine was introduced as an antineoplastic agent 50years ago (Burchenal). It remains an important drug in maintenance therapy for children with acute lymphoblastic leukemia (ALL). 6-Thioguanine (6-TG) is a second – line agent for remission induction and maintenance therapy of acute myelogenous leukemia (AML). Azathiprine, a prodrug of 6-MP with better immunosuppressive activity than 6-MP (Murray et al, 1963), is used widely as an immunosuppressant in clinical transplantation. 6-MP is converted to 6-MP ribose triphosphate, which is incorporated

into DNA and RNA. 6-MP kills cells primarily by incorporation into DNA in the form of thiodeoxyguanosine. Cells incorporating 6-MP survive, but their abnormal DNA template compromises subsequent replication (Ling et al, 1992). Although both inhibition of purine interconversion and the effects of metabolite incorporation into DNA contribute to the antitumor activity of 6-MP metabolites present in DNA best correlates with cytotoxicity (Tidd and Patterson, 1974).

e) Hydroxyurea

Hydroxyurea was originally synthesized in Germany in 1869 (Dresler and Stein, 1869), but its potential biologic significance was not recognized until 1928 when leukopenia and megaloblastic anemia were observed in experimental animals treated with this compound (Rosenthal et al, 1928). In the 1950s the drug was evaluated in a large number of experimental murine tumor tumor models and was found to have broad antitumor activity against both leukemia and solid tumor models (Tarnowski and Stock, 1958; Stearns et al, 1963). It has also been evaluated in a number of advanced solid tumors, and although responses were seen in early studies of diseases including malignant melanoma, squamous cell cancer of the head and neck, renal cell carcinoma, transitional cell carcinoma of the urothelium, and prostate cancer, the level of activity was modest, and hydroxyurea cannot be considered to be standard therapy either as a single agent or as part of the standard chemotherapy regimen for any solid tumor (Donehower, 1992).

f) Alkylating agents

The alkylating agents are antitumor drugs that act through the covalent bonding of alkyl groups to cellular molecules. Historically, the alkylating agents have played an important role in the development of cancer chemotherapy. The nitrogen mustards, mechlorethamine (HN_2, "nitrogen mustard") and tris(β-chloroethyl)amine (HN_3), were the first nonhormonal agents to show significant antitumor activity in humans (Rhoads, 1946; Jacobson et al, 1946; Goodman et al, 1946). This compound was found to produce lymphoid aplasia in addition to the expected irritation of the lungs and mucous membranes and was evaluated as an antitumor agent (Adair and Bagg, 1931). The related but less reactive, bischloroethylamines were found to be less toxic and to cause regressions of lymphoid tumors in mice. At present, alkylating

agents occupy a central position in cancer chemotherapy, both in conventional combination regimens and in high-dose protocols with bone marrow transplantation. Because of their linear dose-response curve in cell culture experiments, these drugs have become the primary tools used in allogenic transplantation protocols for acute leukemia and in autologous transplantation for lymphomas and breast cancer.

g) Nonclassic alkylating agents

Compounds with diverse chemical structures are also capable of covalent binding to biologic macromolecules, and they too have important clinical antitumor activity. These compounds, referred to as the nonclassic alkylating agents, include procarbazine, dacarbazine, hexamethylmelamine, pentamethylmelamine, and temozolomide. Although these agents lack bifunctionality, they share a common structural feature, an N-methyl group, which is important for activity (Newell et al, 1987). These agents are essentially prodrugs and must undergo complex metabolic transformation to active intermediates; their precise cellular mechanisms of action and clinical pharmacology are not completely understood, but they are clinically useful, and indeed, procarbazine and dacarbazine are part of curative regimens for lymphomas.

h) Bleomycin

In a search for new antimicrobial and antineoplastic agents, Umezawa and collegues (Umezawa et al, 1966) isolated a number of small glycopeptides from culture broths of the fungus *Streptomyces verticillus*. The most active antitumor agent found in these broths was, infact, a mixture of peptides now known in clinical usage as bleomycin, a drug that has important activity against Hodgkin's disease, non-Hodgkin's lymphoma, testicular cancer, malignant pleural effusions, cancers of the cervix and penis, and head and neck cancer. Bleomycin used in combination with vinblastine and cis-dichlorodiammineplatinum has produced a high rate of cure in patients with germinal neoplasms of the testis, and in fact, deletion of bleomycin from this regimen compromises therapeutic efficacy (Levi et al, 1993).

i) Anthracyclines and Anthracenediones

The anthracycline antibiotics doxorubicin and daunorubicin, initially discovered over 30 years ago (DiMarco et al, 1964; Arcamone et al, 1969) are among

the most widely used antineoplastic agents in current clinical practices; their antineoplastic spectrum of action compares favorably with those of the alkylating agents and the taxanes. Doxorubicin and daunorubicin are especially active against the hematopoetic malignancies such as acute lymphocytic and acute myelogenous leukemia, Hodgkin's and non-Hodgkin's lymphoma and multiple myeloma, as well as carcinomas of the breast, lung, ovary, stomach and thyroid, sarcomas of bone soft-tissue origin, and various childhood malignancies. In the clinic, the anthracyclines doxorubicin and daunorubicin have no known antagonistic interactions with any of the other commonly used anticancer agents (Legha et al, 1982a; Legha et al, 1982b; Gianni et al, 1983; Johnson et al, 1986).

2.15. 8 Plant- derived antineoplastic therapeutic agents

2.15.8.1 Antimicrotubule agents

a) Vinca alkaloids

The Vinca alkaloids are naturally occurring or semisynthetic nitrogenous bases that are present in minute quantities in the pink periwinkle plant *Catharanthus roseus* G. Don. The Vinca alkaloids were demonstrated to induce cytotoxicity by disrupting microtubules. Until 1994, only two Vinca alkaloids, vincristine (VCR) and vinblastine (VBL), were approved for treatment of malignant diseases in the United States. A third Vinca lakaloid, vindesine (VDS, deasacetyl VBL, carboxymide), a semisynthetic derivative and human metabolite of VBL, was introduced in the 1970s. Other *Vinca* alkaloids with antitumor activity include vinleurosine and vinrosidine, however, further clinical development of these compounds has been abandoned due to their unpredictable toxicities. Recently, two semisynthetic derivatives of VBL, vinorelbine and vinzolidine have undergone clinical evaluation (Budman, 1993).

b) Taxanes:

The taxanes are an important new class of anticancer agents that exert their cytotoxic effects on microtubules by a unique mechanism of action. Both paclitaxel, the prototypical taxane, which has been approved worldwide for the treatment of several malignancies, and docetaxel, a potent semisynthetic analogue, have significant activity in a broad range of tumor types that are generally refractory to conventional therapies, including chemotherapy resistant epithelial ovarian cancer, advanced breast

cancer, small and non-small cell lung cancer, bladder cancer, germ cell malignancies, and head and neck cancer (Rowinsky et al, 1992).

2.15.8.2. Homoharringtonin (BHT):

BHT is a class of cephalotoxin esters, was first isolated by from the bark of the species of cephalotaxus. Cephalotoxins include harringtonine, isoharringtonine and deoxyharringtonine. It acts on the ribosome to inhibit the protein synthesis.

2.15.8.3 Camptothecin

Camptothein analogues are a promising family of anticancer agents with a unique mechanism of action, the inhibition of DNA unwinding enzyme topoisomerase I. The parent compound camptothecin, is a naturally occuring alkaloid formed in the bark and wood of the Chinese tree, *Camptotheca accuminata.* In 1966 Wall et al identified camptothecin as the active agent. Because of the promising preclinical activity, the drug entered clinical trials in the early 1970s under National Cancer Institute sponsership. Because of its insolubility in aqueous solutions, camptothenic was formulated as its sodium salt (NSC-100880). Camptothecins are the only well characterized inhibitors of topoisomerase I. The elucidatioin of this novel mechanism of action led to successful attempts to develop more soluble, less toxic topoisomerase I inhibitors with even greater preclinical anti cancer activity. The other camptothein derivatives are irinotecan (CPT II) and topotecan have clinical anticancer activity (Slichenmyer et al., 1993, Potmesil, 1994, Burris and Fields, 1994). Four camptothecin analogues are currently undergoing clinical evaluation, including irinotecan, topotecan, 9-aminocamptothecin and GG 211.

In the presence of camptothecins, the topoisomerase reaction is altered, resulting in a drug induced stabilization of the cleavable complex (Hsiang et al., 1985). Camptothecins interact non-covalently with the DNA bound topoisomerase I and inhibit the realization step of the reaction. So there is an accumulation of stabilized cleavable complexes and persistence of single stranded DNA breaks. This DNA damage alone is not toxic to cell because the lesions are highly reversible and can be repaired rapidly once the drug is removed (Covey et al., 1989).

2.15.8.4 Topoisomerase II inhibitors (Epipodophyllotoxins and Ellipticines):

Podophyllotoxins originally isolated from mayapple plant (Cragg and Suffness, 1988). A number of semisynthetic derivatives of podopyllotoxin have been made. Two glycosidic derivatives, VM-26 or teniposide and VP-16 or etoposide are active against a number of human malignancies. Zetoposide (Vp-16) was introduced in clinical trials in 1971 and was approved by the Food and Drug Administration (FDA). Teniposide (VM-26) has been used in Europe for several years was approved by FDA in 1992 for refractory childhood leukemia (Senter et al, 1988; Haisma et al, 1992).

Etoposide is extremely active in non-Hodgkin's lymphoma and has been incorporated into several aggressive regimens for diffuse non-Hodgkin's lymphoma. The combination of etoposide and ifosamide has striking activity against pediatric sarcomas. The drug also has been used in the treatment of refractory acute myelogenous leukemia, non-small cell lung carcinoma and Kaposi's sarcoma.

Teniposide (VM-26) is highly active in combination in pediatric hematologic malignancies including both acute myelocytic leukemias (AML) and acute lymphocytic leukemias (ALL). Teniposide is highly effective salvage therapy for initial induction failures in childhood ALL and also has been incorporated in salvage therapy for both Hodgkin's and non-Hodgkin's lymphoma. Teniposide has shown activity against bladder cancer, neuroblastoma, and small cell lung cancer and tumors of the central nervous system.

Ellipticine is an alkaloid derived from the Apocynaceae family, including *Ochrosia*, *Bleekeria vitensis* and *Aspidosperma subincanum* (Cragg and Suffness, 1988). Despite its promising preclinical activity, severe toxic effects observed in animal studies hampered the progress of allipticine toward clinical trials. The semisynthetic derivative 2-N-methyl–9- hydroxyellipticine acetate, however, exhibited good activity in vivo in various mouse and rat systems while also lacking hematopoietic toxicity (Paoletti et al, 1980). Clinical trials of ellipticine were initiated in 1977. Presently, ellipticine is used in the treatment of advanced breast cancer in Europe.

2.16 CANCER CHEMOPREVENTION

Primary prevention of cancer is one of the key approaches to the control of cancer. It includes i) avoiding exposure to known cancer causing agents, ii) enhancement of host defence mechanisms, iii) modifying lifestyle, and iv) chemoprevention.

Generally, cancer chemoprevention is recognized as the pharmacologic intervention with synthetic or naturally occurring chemicals to prevent, inhibit or reverse carcinogenesis or prevent the development of invasive cancer (Kelloff and Boone 1996; Kelloff et al, 1997; Mayne and Lippman 1997; Sporn 1991). Although still in its infancy, the new science of chemoprevention has been established as an important approach to control malignancy. For the first time, it has been shown convincingly that the use of chemopreventive agents in men and women with premalignant lesions can substantially reduce the subsequent development of truly invasive cancer. Chemoprevention is now recognized as both a clinical and basic science.

2.17 MECHANISMS OF ACTION OF CHEMOPREVENTIVE AGENTS

Findings obtained so far from carcinogenesis experiments, chemical/biological sciences, pathology and epidemiology have promoted our understanding of the basic mechanisms of chemoprevention. Cancer chemoprevention may target various processes such as those listed in Table 2.3, originally proposed by Kelloff et al. (Kelloff et al, 1996).

2.17.1 Carcinogen-blocking activities *inhibition of carcinogen uptake*

As an example of this mechanism, calcium is listed. It inhibits colon tumor formation and colonic mucosal hyperproliferation in rats. The effect was attributed to a combination of calcium ions and excess bile and free fatty acids in the colonic lumen (Pence, 1993). This experimental observation also suggests that other chelating agents may have chemopreventive potential by inhibiting carcinogen uptake to the body.

a) Inhibition of carcinogen formation/activation

A very good example of this mechanism is inhibition of the formation of carcinogenic nitrosamines by water-soluble vitamin C or oil-soluble vitamin E, which

Table 2.3 Possible mechanisms of action of chemopreventive agents

Action	Candidate agent
Carcinogen-blocking activities	
Inhibit carcinogen uptake	Calcium
Inhibit formation or activation of carcinogen	Arylalkyl isothiocyanates, DHEA, NSAIDs, polyphenols
Deactivate/detoxify carcinogen	Oltipraz, other GSH-enhancing agents
Prevent carcinogen binding to DNA	Oltipraz, polyphenols
Increase level or fidelity of DNA repair	NAC, protease inhibitors
Antioxidant activities	
Scavenge reactive electrophiles	GSH-enhancing agents
Scavenge oxygen radicals	Polyphenols, vitamin E
Inhibit arachidonic acid metabolism	Glycyrrhetinic acid, NAC, NSAIDs, polyphenols, tamoxifen
Antiproliferation/antiprogression activities	
Modulate signal transduction	Glycyrrhetinic acid, NSAIDs, polyphenols, retinoids, tamoxifen
Modulate hormonal/growth factor activity	NSAIDs, retinoids, tamoxifen
Inhibit oncogene activity	Genistein, NSAIDs, monoterpenes
Inhibit polyamine metabolism	DFMO, retinoids, tamoxifen
Induce terminal differentiation	Calcium, retinoids, vitamin D
Restore immune response	NSAIDs, selenium, vitamin E
Induce programmed cell death (apoptosis)	Butyric acid, genistein, retinoids, tamoxifen
Correct DNA methylation imbalances	Folic acid
Inhibit angiogenesis	Genistein, retinoids, tamoxifen
Inhibit basement membrane degradation	Protease inhibitors
Activate antimetastasis genes	

block the reaction between secondary amines and nitrite under very acidic conditions in the stomach (Mirvish, 1986). Carcinogen formation can also be inhibited by preventing metabolic activation of a procarcinogen. Many chemopreventive agents (e.g. allylic sulfides, arylalkyl isothiocyanates, carbamates and flavonoids) exhibit this type of activity. Several compounds that inhibit aromatase also inhibit chemical carcinogenesis in estrogen-sensitive tissues (Lubert et al, 1994).

b) Deactivation/detoxication of carcinogens

Carcinogen deactivation and detoxication constitute a very important mechanism of chemoprevention. Two metabolic pathways are related. One is the phase I metabolic enzymes, which are primarily the microsomal mixed-function oxidases. The polar groups of chemicals (e.g. hydroxyl group) become substrates for this conjugation. The other pathway is by the phase II metabolic enzymes related to conjugation and formation of glucuronides, glutathione (GSH) conjugates and sulfates. GSH is a prototype carcinogen scavenger. It reacts spontaneously or via catalysis by GSH S-transferases (GSTs) with numerous activated carcinogens such as MNNG, AFB1 and B[a]P diol epoxide and other activated polycyclic aromatic hydrocarbons (Mandel et al, 1987). A number of promising chemopreventive agents belong to this category. Prominent compounds among them are the allylic sulfides, which are natural products found in onion, garlic and other *Allium* genus vegetables. Oltipraz [5-(2-pyrazinyl)- 4-methyl-1,2-dithiol-3-thione] is a potent GST inducer with a wide spectrum (Poebuck et al, 1991). *N*-Acetyl-L-cysteine (NAC) is essentially a precursor of GSH (Kelloff et al, 1996).

c) Prevention of carcinogen binding to DNA

After activation and formation of a carcinogen in the body, a DNA–carcinogen adduct is formed, which is another important target process for chemoprevention. Oltipraz prevents the formation of AFB1–DNA adducts. This reaction is attributed to increased detoxication of AFB1 by GST and, as a consequence, formation of AFB1–DNA adducts is inhibited (Kelloff et al, 1996).

d) Enhancement of DNA repair

The DNA-damage modulating enzyme poly(ADP-ribosyl) transferase (ADPRT) is decreased by carcinogens; for example, NAC prevents the decrease in ADPRT caused by the carcinogen 2-acetylaminofluorence (Kelloff et al, 1996).

2.17.2. Antioxidant/anti-inflammatory activities

a) Scavenging of oxygen radicals

Oxygen radicals such as singlet oxygen, peroxy radicals, superoxide anion and hydroxyl radicals are closely associated with carcinogenesis acting in any phase, namely initiation, promotion and progression (Roebuck et al, 1991). Oxygen radicals exert a mutagenic effect by oxidizing DNA bases, and radicals also cause DNA strand breaks and chromosome deletions/ rearrangements. Many compounds scavenging activated oxygen species exhibit chemopreventive effects. As an example, NAC is known to react with hydroxyl radicals (Kensler et al, 1992). The reaction of β-carotene with singlet oxygen is well known. Phenolic antioxidants are known to scavenge peroxy radicals. Particularly, vitamin E (α-tocopherol) is known to scavenge peroxy radicals, singlet oxygen and superoxide radicals.

b) Inhibition of arachidonic acid (AA) metabolism

AA is metabolized by oxidative enzymes to prostaglandins (PGs), thromboxanes, leukotrienes and so forth. Aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) are useful chemopreventive agents for colon cancer. Those drugs are potent inhibitors of cyclooxygeneses, enzymes that catalyze the synthesis of prostaglandins from AA. These drugs have attracted much attention from the viewpoint of the relationship between cancer and inflammation (Zenser et al, 1992).

2.17.3 Antiproliferation/antiprogression activities *modulation of signal transduction*

It is of interest to regard carcinogenesis as a disorder of signal transduction. Hormones and growth factors that regulate cell growth, proliferation and differentiation communicate across cell membranes, via receptors and receptor-associated enzymes. Second messengers which convey information between the cell membrane and the nucleus through cytoplasm include cyclic adenosine

monophosphate, inositol (1,4,5)-trisphosphate, diacylglycerol, prostaglandins and regulatory proteins such as mitogen-activated protein (MAP) kinases. The various steps of signal transduction are potential target sites for chemoprevention in restoring normal cellular controls (Powis et al, 1994).

a) Modulation of hormonal/growth factor activity

The regulation of cell growth and proliferation may occur at multiple levels: hormones and growth factors, membrane/cytoplastic/ nuclear receptors (e.g. estrogen, progesterone, retinoid, glucocorticoid, vitamin D and thyroid receptors). Transforming growth factor (TGF-α) has antiproliferative activity in both normal and neoplastic cells (Glick et al, 1991). Insulin-like growth factor 1 (IGF-1) stimulates cell replication in various tumors. These steps are all targets of chemoprevention.

b) Inhibition of polyamine metabolism

Polyamines are one of the essential factors in cell proliferation, differentiation and malignant transformation (Pegg, 1988). A critical step in polyamine biosynthesis is the ornithine decarboxylase (ODC)-catalyzed formation of putrescine from ornithine. Chemical agents that inhibit induction of ODC thus possess chemopreventive potential. Difluoromethylornithine (DFMO) is a specific and irreversible inhibitor of ODC.

c) Restoration of immune response

There are many reports regarding the importance and usefulness of antibodies to oncogene products or oncoproteins (De Flora and Ramel, 1988). Prostaglandin E2 is known to suppress the immune response in certain tumor cells and inhibitors of cyclooxygenase diminish this immune suppression. Chemopreventive retinoids work as immuno-stimulants. In fact, retinoic acid enhances cell-mediated and natural killer cell cytotoxicity. Immunostimulative effects of selenium and α-tocopherol have been reported, which may explain part of their action as chemopreventive agents.

2.18 *Carum copticum* Linn.

Carum copticum Linn. is a herb belonging to the family Apiaceae is cultivated throughout India, Baluchistan etc. It is commonly called as Bishop's weed.

Figure 1.1

***Carum copticum* Habit**

***Carum copticum* seeds**

Ajwain promotes digestion alleviates gas, promotes kidney function and generally benefits the nerves. It is considered to benefits the respiratory system and promotes kidney function. The seeds are considered to be powerful detoxifying agents. The seeds are bitter and hot, carminative, diuretic, galactogogue, tonic, expectorant, cure weakness of limbs, and paralysis, chest pains, improves speech and eyesight, stimulate the intestine, good for ear boils, liver, spleen, vomiting, dyspepsia, kidney troubles, inflammations (Unani) (Mahaskar, 1935). The fruits are much valued for their antispasmodic, stimulant, toxic, carminative properties. The roots are diuretic and carminative. It is used in febrile affections and in stomach disorders. The seeds are described for snake bite and scorpion sting (Sushrutha, Harithasamhita). The essential oil of *Carum copticum* Linn. contains not less than 40- 50% of Thymol brown as Ajwain-Ka-phol (Crude thymol) which is antihelmintic. Ajwain oil is shown to be toxic at different dilutions to pathogenic bacteria and is shown inhibitory to various microorganisms. It is also applied to retrieve rheumatoid and neuralgic pain. The seeds are also immune enhancing.

Chapter 3

3.1.1 Plant material

Authenticated seeds of *Carum copticum*, *Psecedanum vulgare*, *Coriandum sativum* and *Cuminum ciminum* were obtained from Amala Ayurveda Hospital and Research Centre, Thrissur, Kerala, India.

3.1.2 Chemicals

Minimal Essential Medium (MEM)	Himedia Laboratories Pvt Ltd. India
Rosewell Park Memorial Institute medium (RPMI-1640)	Himedia Laboratories Pvt Ltd. India
Trypsin	E.Merck India Ltd
Steptomycin	E.Merck India Ltd
Agar agar	Qualigens India
Nitro blue tetrazolium(NBT)	Sigma Chemical Co. St Luis, USA
MTT	Sigma Chemical Co. St Luis, USA
Deoxyribose	E.Merck India Ltd
Cyclophosphamide (CTX)	Dabur India Ltd, India
Dimethyl suphoxide (DMSO)	E. Merck India Ltd
5,5'-dithio-bis 2-nitrobenzoic acid (DTNB)	Sisco Research Laboratories, India
Thiobarbituric acid (TBA)	Sisco Research Laboratories, India
Aspirin	German remedies Ltd.
Ranitidin	Torrent pharmaceuticals, India
Tamoxifen	Cadila Pharmaceuticals, India
Azoxymethane	Sigma Chemical Co. St Luis,USA
2,4-Dichloro phenoxy acetic acid (2,4-D)	Sigma Chemical Co. St Luis, USA
Naphthalene acetic acid (NAA)	Sigma Chemical Co. St Luis, USA
Kinetin (KN)	Sigma Chemical Co. St Luis, USA
Silica gel G	Qualigens India
Silica gel for column chromatography	Qualigens India (60-120 mesh)
Murashige and Skoog (MS) medium	Himedia Laboratories Pvt Ltd. India

3.1.3 Instruments

Laminar Flow chamber	Kemi Pvt Ltd, India
Refrigerated centrifuge	Remi Pvt ltd, India
Freeze drier	Labconco, USA
Microscope	Meiji, Japan
Inverted microscope	Willvert Will
UV chamber	Commag
UV spectrophotometer	Elico India Ltd.
Elisa Plate reader	Awareness Technology Inc. USA

3.1.4 Cell lines

Dalton's Lymphoma Ascites (DLA) cells	Adayar Cancer Institute
Ehrlich ascites tumour (EAC) cells	Adayar Cancer Institute
Mouse lung fibroblast (L929) cells	National facility for animal cell and tissue culture, Pune, India.

3.1.5 Maintenance of Cell lines

The cells (DLA and EAC) were maintained in the intraperitoneal cavity of mice. At first $1X10^6$ (100µl) cells were injected into the intraperitoneal cavity of mice. After 15 days the cells were aspirated using a 1ml syringe and Phosphate Buffered Saline (PBS). The cells were washed three times in PBS to remove the impurities. The number of cells were counted using a haemocytometer and make up as 100µl PBS containing $1X10^6$ cells. Then the cells were injected into the intraperitoneal cavity of another mice and it continued in every 15days intervals. The L929 cells were maintained in in vitro culture using animal cell culture medium and maintained in 3% CO_2 atmosphere.

3.1.6 Animals

Swiss albino mice	College of Veterinary and Animal Sciences, Thrissur, India
Balb/c mice	College of Veterinary and Animal Sciences, Thrissur, India

Wistar Rats College of Veterinary and Animal Sciences, Thrissur, India

Sprague dawley rat College of Veterinary and Animal Sciences, Thrissur, India

3.2.1 Preparation of 70% methanolic extract of seeds

The seeds were air dried, powdered and extracted with 70% methanol. The extract was evaporated to dryness. The yield of extract was noted. The residue suspended in distilled water and used for further experiments.

3.2.2 Cytotoxicity assays

3.2.2.1 Determination of 3 h- time cytotoxicity of the extract

Cytotoxicity studies were carried out using DLA and EAC tumour cells. Different concentrations of the extract (10- 1000 μg/ml) were incubated with tumour cells (10^6) suspended in phosphate- buffered saline (PBS, pH 7.2) and cytotoxicity was determined after 3 hours using the trypan blue exclusion method (Babu et al., 2002)

3.2.2.2 Determination of 72 h-time cytotoxicity of the extract

Cytotoxicity of the extract in culture was determined using L-929 cells (Anis and Kuttan.,1999). Cells (10^4) were plated in a 96 well flat-bottomed titre plates and maintained with MEM (Minimum Essential Medium) containing 10% goat serum. Various concentrations of the extract (10-100 μg/ml) were added into the wells and incubated for 72 h. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5%, 20 μl) was added to the wells 4 h before the end of incubation. Medium and reagents were aspirated, 98% dimethylsulphoxide (DMSO) was added and after shaking for 15 min, the absorbance was measured at 545 nm with a reference wavelength of 630 nm using an Elisa plate reader(Awarness Technology Inc.). The percentage cytotoxicity was calculated and compared with untreated controls.

3.2.3 Superoxide scavenging activity

It was determined by the light induced superoxide generation by riboflavin and subsequent reduction of nitroblue tetrazolium as described by McCord and

Fridovich (1969). The reaction mixture contained EDTA (6μM) containing 3 μg NaCN, riboflavin (2μM), NBT (50μM), various concentrations of the test material and phosphate buffer in a final volume of 3 ml. The tubes containing the reaction mixture were uniformly illuminated with an incandescent lamp for 15 min and the optical density was measured at 530 nm before after illumination. The percentage inhibition was evaluated by comparing the absorbency value of the control tubes and experimental tubes.

3.2.4 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the test material was measured by studying the competition between deoxyribose and test compounds for hydroxyl radicals generated from the Fe^{3+}/ ascorbate/EDTA/H_2O_2 system (Fenton reaction). The hydroxyl radicals attack deoxyribose which eventually results in the formation of thiobarbituric acid reacting substances (Elizabeth and Rao, 1990). The reaction mixture containing deoxyribose (2.8 mM), ferric chloride (0.1μM), EDTA (0.1 mM), H_2O_2 (1mM), ascorbate (0.1 mM) KH_2PO_4 -KOH (20 mM, pH 7.4) and various concentrations of the sample in a volume of 1 ml was incubated for 1 h at 37^0C. Deoxyribose degradation was measured as thiobarbituric acid reactive substances by the method of Ohkawa et al.,(1979). The inhibition produced by different concentrations of the sample as well as the concentration required for 50% inhibition was calculated.

3.2.5 Lipid peroxidation assay

Lipid peroxidation was induced in rat liver homogenate by the method described Bishayee and Balasubramonian (1971) in the presence of different concentrations of the test material and estimated by thiobarbituric acid reactive substances by the method Ohkawa et al, (1979). Different concentrations of the test material was incubated with 0.1 ml of rat liver homogenate (25%) containing 30 mM KCl, Tris- HCl buffer (0.04 M, pH 7.0), ascorbic acid (0.06mM) and ferrous ion (0.16 mM) in a total volume 0.5 ml for 1h. At the end of the incubation period, 0.4 ml of the reaction mixture was treated with 0.2 ml SDS (8.1%), 1.5 ml thiobarbituric acid (0.8%) and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml by adding distilled water and kept in water bath at 95 ^{0}C for 1h. After cooling, 1ml

distilled water and 5 ml butanol-pyridine mixture (15:1 v/v) was added. After vigorous shaking, the tubes were centrifuged and the upper layer containing the chromophore was read at 532 nm. The percentage inhibition was calculated and the concentration required for 50 % inhibition was calculated.

3.2.6 Nitric oxide radical scavenging activity

Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction (Green et al., 1982; Marcocci et al., 1994a). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the extract (from 1 to 5000 µg/ml) was incubated at 25° C for 150 min. After incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbance of the chromophore formed was reviewuated at 546 nm.

3.2.7 Preparation of tissue homogenates (liver, kidney and mucosa)

Animals were sacrificed. Liver, kidney and stomach were excised and rinsed thoroughly in ice-cold saline to remove the blood. They were then gently blotted between the folds of a filter paper and weighed in an analytical balance. 25 % of homogenate was prepared in 0.05 M phosphate buffer (pH 7) using a polytron homogeniser at 4°C. A part of this homogenate was used for the determination of reduced glutathione, glutathione peroxidase, malondialdehyde. Rest of the homogenate was centrifuged at 10, 000 rpm for 20 min for removing the cell debris, unbroken cells, nuclei, erythrocytes and mitochondria. The supernatant was used for the estimation of superoxide dismutase, and catalase.

3.2.8 Determination of tissue reduced glutathione (GSH)

Reduced glutathione in the tissue was determined according to the method of Moron et al., (1979).

Principle

The acid soluble sulfhydryl groups (non-protein thiols of which more than 93% is reduced glutathione) forms a yellow colored complex with dithionitrobenzene (DTNB). The absorbance of the colored complex was measured at 412 nm.

Procedure

0.5 ml of the tissue homogenate was mixed with 0.1 ml of 25 % TCA and kept on ice for few minutes. These were then subjected to centrifugation at 3000 g for few minutes to settle the precipitate. 0.3 ml of the supernatant was mixed with 0.7 ml of 0.2 M sodium phosphate buffer (pH 8) and 2 ml of 0.6 mM DTNB (prepared in 0.2 M buffer, pH 8). The yellow color obtained was measured after 10 min at 412 nm against a blank which contained 0.1 ml of 5% TCA in place of the supernatant. A standard graph was prepared using different concentrations (10-50 nmoles) of GSH in 0.3 ml of 5 % TCA. The GSH content was calculated with the help of this standard graph and expressed as nmol/mg protein.

3.2.9 Determination of tissue superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined according to the method of Mc Cord and Fridovich (1969).

Principle

Illumination of riboflavin solution in the presence of EDTA causes a reduction of the flavin. It then re-oxidizes and simultaneously reduces oxygen to O_2^-, which is allowed to react with a detector molecule NBT, reduced the NBT to a formazan blue. The SOD in the sample will inhibit the formazan production.

Procedure

0.01 ml of the homogenate was mixed with 0.2 ml of 0.1 M EDTA (containing 0.0015% NaCN), 0.1 ml of 1.5 mM NBT and phosphate buffer (67 mM, pH 7.8) in a total volume of 2.6 ml. After adding 0.05 ml of riboflavin, the absorbance of the solution was measured against distilled water at 560 nm. Illuminated all the tubes uniformly for 15 min and absorbance of the blue color formed were measured again. Percent of inhibition was calculated after comparing absorbance of sample with the absorbance of control (the tube containing no enzyme activity). The volume of the sample required to scavenge 50 % of the generated superoxide anion was considered as 1 unit of enzyme activity and expressed in U/ mg protein.

3.2.10 Determination of tissue catalase (CAT) activity

Tissue Catalase activity was determined according to the method of Beers and Sizer (1952).

Principle

Catalase catalyses the decomposition of H_2O_2. In the ultraviolet range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in extinction at 240 nm.

$$2H_2O_2 \rightarrow 2\ H_2O + O_2$$

Procedure

0.1 ml of the tissue homogenate (approximately 0.1 mg protein) was mixed with 1.9 ml of the phosphate buffer (0.5 M, pH 7). The decrease in extinction was measured at 240 nm, 1 min interval for 3 min immediately after adding 1 ml of 11 mM H_2O_2 solution in buffer. A sample control was placed in the reference cuvette containing 0.1 ml of tissue homogenate and 2.9 ml of the buffer. Activity of catalase was calculated using the molar extinction coefficient of 43.6 cm^{-1}.

mmoles of H_2O_2 decomposed/min/mg protein

$$\text{or (U/mg protein)} = \frac{\Delta A/\text{min} \times 1000 \times 3}{43.6 \times \text{mg protein in sample}}$$

3.2.11 Determination of tissue glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was determined according to the method of Hafemann et al., (1974).

Principle

The activity of GPx was determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and NaN_3.

$$H_2O_2 + 2\ GSH \rightarrow 2H_2O + 2\ GSSG$$

Procedure

Tissue homogenate (approximately 0.5 mg protein) was incubated with 0.1 ml of 5mM GSH, 0.1 ml of 1. 25 mM H_2O_2, 0.1ml of 25 mM NaN_3 and phosphate buffer (0.05 mM, pH 7) in a total volume of 2.5 ml at 37°C for 10 min. The reaction was stopped by adding 2 ml of 1.65 % HPO_3^{2-} and the reaction mixture was centrifuged at 1500 rpm for 10 min. 2 ml of the supernatant was mixed with 2 ml 0.4 M Na_2HPO_4 and 1ml of 1mM DTNB. The absorbance of the yellow colored complex was measured at 412 nm after incubation for 10 min at 37°C against distilled water. A

sample without the tissue homogenate processed in the same way was kept as the non-enzymatic reaction.

One unit of enzyme activity was defined as decrease in log GSH by 0.001/min after subtraction of the decrease in log GSH per minute for the non-enzymatic reaction and is expressed as units/mg protein.

3.2.12 Determination of tissue lipid peroxidation

The level of lipid peroxidation was measured as malondialdehyde (MDA) according to the method of Ohkawa et al (1979).

Principle

The tissue malondialdehyde was allowed to react with TBA. The MDA-TBA adduct formed during the reaction in acidic medium was extracted to the organic layer and the absorbance was measured at 532 nm.

Procedure

4 ml of reaction mixture containing 0.4 ml of the tissue homogenate, 1.5 ml of 0.8 % TBA, 1.5 ml of acetic acid (20 %, pH 3.5) and distilled water was kept for 1 h in a boiling water bath at 95°C. After 1 h, the reaction mixture was removed from the water bath, cooled and added 1 ml of distilled water. 5 ml of butanol: pyridine mixture (15:1) was added to the reaction tube, mix thoroughly and centrifuged at 3000 rpm for 10 min. Absorbance of the clear supernatant was measured at 532 nm against butanol:pyridine mixture. The MDA was calculated with the help of a standard graph made by using different concentrations (1-10 nmol) of 1'1'3'3'-tetramethoxypropane in 1 ml distilled water and is expressed as nmol of MDA/mg protein.

3.2.13 Determination of tissue protein

Protein content in the tissue was determined according to the method of Lowry *et al.* (1951)

Principle

The blue color developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan in the protein plus the color developed by the biuret reaction of the protein with the alkaline cupric tartrate were measured at 660 nm.

Procedure

0.01 ml of the homogenate was mixed with 0.990 ml of distilled water, 5 ml of alkaline $CuSO_4$ (0.5 % $CuSO_4$ in 1 % sodiumpotassium tartrate and 2% Na_2CO_3 in 0.1 N NaOH mixed in the ratio 1:50) was kept for 10 min at room temperature. 0.5 ml of 1 N Folin phenol reagent was added and absorbance was measured after 20 min at 660 nm against the reagent blank. Protein content was calculated from the standard graph prepared using different concentrations (0.1-0.5 mg/ ml) of bovine serum albumin (BSA).

3.2.14 Determination of serum glutamate oxaloacetate transaminase (SGOT) activity

SGOT activity was determined according to the method of Reitman and Frankle (1957).

Principle

Serum containing glutamate oxaloacetate transaminase catalyses the reaction between L-aspartate and α-ketoglutarate, to form oxaloacetate and glutamate. The unstable oxaloacetate is converted to pyruvate and reacts with 2,4,-dinitrophenylhydrazine. The absorbance of the resultant brown colored phenylhydrazone is measured at 505 nm under alkaline conditions.

Procedure

Reagents used were from Span diagnostic kit. 0.1 ml of serum was added to 0.5 ml of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-aspartate in 100 ml phosphate buffer 0.1M, pH 7.4) at 37°C and incubated for 60 min. After the incubation, 0.5 ml of dinitrophenylhydrazine (19.8 mg/dl 1 N HCl) was added, mix well and kept at room temperature for 20 min. 0.4 ml of NaOH was added and read the absorbance after 10 min at 505 nm using the reagent blank. A control tube containing buffered substrate was treated with serum after the incubation at 37° C was also followed in the same manner. The enzyme activity was calculated from the standard (sodium pyruvate, 2 mM) calibration curve. The enzyme activity (U/ml) is converted to IU/l by multiplying with 0.483.

3.2.15 Determination of serum glutamate pyruvate transaminase (SGPT) activity

SGPT activity was determined according to the method of Reitman and Frankle (1957).

Principle

Serum containing glutamate pyruvate transaminase catalyses the reaction between L-alanine and α-ketoglutarate, to form pyruvate and glutamate. The pyruvate thus formed was treated with 2,4,-dinitrophenylhydrazine. The absorbance of the resultant brown colored phenylhydrazone is measured at 505nm under alkaline condition.

Procedure

Reagents used were from Span diagnostic kit. 0.1 ml of serum was added to 0.5 ml of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-alanine in 100 ml phosphate buffer 0.1M, pH 7.4) at 37°C and incubated for 30 min. After the incubation, 0.5 ml of dinitrophenylhydrazine (19.8 mg/dl 1 N HCl) was added, mixed well and kept at room temperature for 20 min. 0.4 ml of NaOH was added and read the absorbance after 10 min at 505 nm using the reagent blank. A control tube containing buffered substrate was treated with serum after the incubation at 37° C was also followed in the same manner. The enzyme activity was calculated from the standard (sodium pyruvate, 2 mM) calibration curve. The enzyme activity (U/ml) is converted to IU/l by multiplying with 0.483.

3.2.16 Determination of serum alkaline phosphatase (ALP) activity

Serum ALP activity was determined according to the method of Kind and King (1954).

Principle

ALP in the serum reacts with disodium phenyl phosphate under alkaline pH 10 release phenol. Phenol reacts with 4-aminoantipyrene in the presence of alkaline oxidizing agent to give a red colored complex, which is measured at 510 nm against reagent blank.

Procedure

Reagents used were from Span diagnostic kit. 0.05 ml of serum was incubated with 0.5 ml of the buffered substrate (1ml of 0.254 g of disodium phenyl phosphate

dihydrate/dl water mixed with 1ml of the carbonate buffer pH 10) and 1.54 ml of distilled water at 37°C for 15 min. After the incubation, 2 ml chromogen (1ml of 0.6 g 4-aminoantipyrene/dl water and 1ml of potassium ferricyanide 2.4 g/dl water) reagent was added and measured at 510 nm. Phenol (10 mg %) was used as the standard for the calibration curve. The activity (KA/dl) is converted to IU/l by multiplying with 7.1.

$$\text{Serum ALP (IU/l)} = \frac{O.D_T - O.D_C \times 10 \times 7.1}{O.DS}$$

3.2.17 Determination of serum γ-glutamyl transpeptidase (GGT) activity

Serum GGT activity was determined according to the method of Szas (1976).

Principle

GGT in the serum reacts with L-gamma-glutamyl-3-carboxy-4-nitroanilide and glycylglycine to form L-gamma-glutamyl-glycylglycine and 5-amino-2-nitrobenzenoate. The rate of the reaction is measured per minute for 3 min at 405 nm against distilled water.

Procedure

Reagents used were from Agappe diagnostic kit. 1 ml of the working reagent (reconstituted reagents tris buffer 182 mM, pH 8.25 and L-gamma-glutamyl-3-carboxy-4-nitroanilide 2.97 mM containing 85 mM glycylglycine) was mixed with 0.1 ml serum. After 1 min, changes in absorbance were measured per minutes for 3 min at 405 nm using distilled water as blank.

$$\text{Serum GGT (U/l)} = (\Delta A/\text{min}) \times 1158$$

3.2.18 Determination of serum creatinine

Serum creatinine was determined according to method of Brod and Serota as described in Text book of Clinical Biochemistry, Varley (1980).

Principle

Creatinine forms a yellow-orange compound in alkaline medium with picric acid. The intensity of the color is measured at 500 nm. The concentration of the dyestuff formed over a certain reaction time is a measure of the creatinine concentration.

Procedure

Reagents used were from Merk diagnostic kit. 0.2 ml of serum was mixed with 0.5 ml of buffer (313 mM NaOH and 12.5 mM phosphate, pH 8) and 0.5 ml of 8.73 mM picric acid. The absorbance is measured immediately after 1 min ($O.Dt_1$) and exactly after 5 min ($O.Dt_2$) at 500 nm. A standard creatinine solution (1 mg/dl) was treated in the same way.

$$\text{Creatinine concentration (mg/dl)} = \frac{O.Dt_2 - O.Dt_1}{O.Ds_2 - O.Ds_1}$$

3.2.19 Determination of serum total protein

Serum protein was determined by the method of Reinhold, as described in Text book of Clinical Biochemistry, Varley (1980).

Principle

Protein reacted with cupric ions in alkaline medium to form a violet colored complex. The intensity of the complex was measured at 530 nm.

Procedure

The reagents used were from Span Diagnostic kit. 1 ml of the working Biuret reagent was mixed with 0.01 ml of serum and absorbance was measured at 530 nm against reagent blank. 0.01 ml of the standard solution was treated in the same way.

$$\text{Serum total protein (g/dl)} = \frac{O.D_T \times 6}{O.Ds}$$

3.2. 20 Determination of serum albumin

Serum protein was determined using bromocresol green (Dumas and Peters, 1979).

Principle

Albumin in serum bound with bromocresol green at pH 4.2 to form green colored complex. The intensity of the color was measured at 640 nm.

Procedure

Reagents used were from Ranbaxy diagnostic kit. 0.01 ml of serum was mixed with 1 ml of BCG reagent (Succinate buffer 75 mM pH 4.2 and Bromocresol green 0.14 g/l). The absorbance was measured at 628 nm against reagent blank. Human albumin (3.8 mg/dl) was used as the standard.

$$\text{Albumin (g/dl)} = \frac{O.D_T \times 3.8}{O.D_S}$$

3.2.21 Determination of Superoxide dismutase (SOD) activity in blood

Blood SOD activity was determined according to the method of Mc Cord and Fridovich (1969) after removing the haemoglobin by the method of Minami and Yoshikawa (1979).

Principle

Blood was haemolysed by cold water at 4°C and the haemoglobin was removed by chloroform-ethanol mixture. The supernatant containing superoxide dismutase was determined.

Procedure

0.1 ml of the heparinised blood was haemolysed by 0.9 ml of cold water (4°C). The haemolysate was treated with 0.25 ml of $CHCl_3$ and 0.5 ml of ethanol with vigorous mixing to remove the haemoglobin. The mixture was centrifuged at 15000 rpm for 60 min. The 0.025 ml of the clear supernatant was used for the SOD assay as described in the section 3.2.4. The volume of the sample required to scavenge 50 % of the generated superoxide anion was considered as 1 unit of enzyme activity and was expressed as U/g Hb.

3.2.22 Determination of catalase (CAT) activity in blood

Catalase activity in the blood was determined according to the method of Aebi (1974).

Principle

The catalase activity was measured from the decomposition of H_2O_2.The decomposition of H_2O_2 was measured by the decrease in extinction at 240 nm. The difference in extinction per unit time is a measure of the catalase activity.

Procedure

Erythrocyte sediment was prepared from the heparinised blood and washed 3 times with isotonic saline. A stock haemolysate containing approximately 5 g. Hb/dl was prepared by the addition of 4 parts by volume of distilled water. A 1:500 dilution of this concentrated haemolysate with sodium-potassium phosphate buffer (0.05 M,

pH 7) was prepared immediately before the assay. Reference cuvette contained 1 ml of buffer and 2 ml of haemolysate and test cuvette contained 2 ml diluted haemolysate. The reaction was started by addition of 1 ml of H_2O_2 (30 mM in the buffer) to the test cuvette, mixed well and the decrease in extinction was measured at 240 nm for 30 sec. by 15 sec. interval. Catalase activity was calculated using the formula and expressed as k/g Hb, where k is a rate constant of 1st order reaction.

$$\text{Catalase (k/g Hb)} = \frac{2.3 \times (\log E_1 - \log E_2) \times \text{dil. factor}}{15 \times \text{g Hb/ml of blood}}$$

$$= \frac{0.153 \times 1000 \times (\log E_1 - \log E_2)}{\text{g Hb/ml of blood}}$$

E_1 is E_{240} at t=0 and E_2 is E_{240} at t=15 sec.

3.2.23 Determination of glutathione (GSH) content in blood

Reduced glutathione in blood was determined according to the method of Moron (1979) after preparing a haemolysate in water.

Principle

Reduced glutathione forms a yellow colored complex with dithionitrobenzene (DTNB). The absorbance of the colored complex was measured at 412 nm.

Procedure

A 20 % haemolysate of heparinised blood was prepared in distilled water and proceeded for the glutathione determination as described in the section 3.2.3. The GSH level was expressed as micromoles/ml of blood.

3.2.24 Determination of glutathione peroxidase (GPx) activity in blood

Glutathione peroxidase activity was determined according to the method of Hafemann et al., (1974).

Principle

The activity of GPx was determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and NaN_3.

Procedure

0.02 ml of heparinised blood was treated with 0.1 ml of 5mM GSH, 0.1 ml of 1. 25 mM H_2O_2, 0.1ml of 25 mM NaN_3 and phosphate buffer (0.05 mM, pH 7) in a total volume of 2.5 ml at 37°C for 10 min. The reaction was stopped by adding 2 ml

of 1.65 % HPO_3^{2-} and the reaction mixture was centrifuged at 1500 rpm for 10 min. 2 ml of supernatant was used for the estimation according to the procedure given under tissue GPx determination (section 3.2.6). The result was expressed as U/g Hb.

3.2. 25 Determination of haemoglobin (Hb) in blood

Haemoglobin was determined according to the method of Drabkin and Austin (1932).

Principle

Haemoglobin was treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogenphosphate. The ferricyanide forms methaemoglobin, which is converted to cyanmethaemoglobin by the cyanide. The intensity of the color formed is measured at 546 nm against reagent blank. The optical density is directly proportional to the amount of haemoglobin present in the blood.

Procedure

The reagents used were from Agappe diagnostic kit. 0.02 ml of fresh whole blood was mixed with 5 ml of the cyanmeth reagent. The optical density was measured at 546 nm against reagent blank after 5 min incubation at room temperature. The O.D of standard solution corresponding to 60 mg/dl haemoglobin at 546 nm was read against reagent blank used for calculating the concentration of haemoglobin in the blood.

$$\text{Haemoglobin (g/dl)} = \frac{O.D_T \times 60 \times 0.251}{O.D_S}$$

3.2.26 Determination of total white blood cell (WBC) count

Total WBC count was determined using haemocytometer as described in the textbook of practical Physiology, Chaudhari (2000 b).

Principle

The whole blood was diluted using a diluent which haemolyses red cells. Leaving all the nucleated cells intact. The number of white cells in a known volume and known dilution were counted using a counting chamber.

Procedure

Added 0.02 ml blood to 0.38 ml of diluting fluid charged the neubauer counting chamber with the well-mixed diluted blood. Counted the total number of white blood cells in the four large corner squares of chamber after 3-4 min.

Total number of WBC = Number of cells counted x 50 count/mm^3

3.2. 27 Determination of Bone marrow cellularity

Bone marrow cellularity was determined by the method of Sreedni et al (1992). The animals were sacrificed, bone marrow was collected from the femur using a jet of PBS containing serum using a syringe. Bone marrow was made into single cell suspension and the cell number was determined using hamocytometer and expressed as total number of live cells/femur.

3.2.28 Statistical analysis

Student's '*t*' test was used for the statistical evaluation of the data. To determine the statistical significance between two valued in the control (X) and treated (Y)group, '*t*' value was calculated using the equation.

$$T = \frac{\bar{X} - \bar{Y}}{S\sqrt{[(1/nx)+(1/ny)]}}$$

Where X and Y are the means of the two samples X and Y; nx and ny are the sample S was found out using the equation,

$$S = \sqrt{\frac{(nx-1)\,Sx^2 + (ny-1)\,Sy^2}{nx+ny-2}}$$

Where 'Sx' and 'Sy' are the standard deviation of the two samples. Statistical significance was deduced from '*t*' distribution table.

Chapter 4

Cytotoxic and antitumor properties of selected medicinal plants from Apiaceae family

4.1 INTRODUCTION:

Cancer is the second largest single cause of death in children and adults, claiming more than 6 million live each year worldwide. Among the methods that exist for the treatment of cancer in modern medicine-chemotherapy, radiotherapy and surgery-chemotherapy is considered as the most effective method of cancer treatment. Intervention with chemopreventive agents at the early stage in cancer is theoretically more rational than attempting to eradicate fully developed tumors. The plants belonging to the family Apiaceae possess high medicinal properties. Some plants like *Centella asiatica*, *Daucus carota*, *Ferula asafoetida* L., *Hydrocotyl sibthopioides* Lam and *Hydrocotyl maritima* Honda etc are known to have anticancer activities. Other plants of this family are used in Ayurvedic and Homeopathic system of medicine as well as spices. The present study is a detailed investigation of the 4 Apiaceae Taxa's (*Carum copticum*, *Coriandum sativum*, *Cuminum siminum* and *Pseucedanum graveolens*) cytotoxic potential with in vitro chemosensitivity assays and in vivo tumor models.

4.2 MATERIALS AND METHODS

4.2.1 PREPARATION OF CRUDE EXTRACTS

The seeds of the plants (*Carum copticum*, *Coriandum sativum*, *Cuminum siminum* and *Pseucedanum graveolens*) were obtained from Amala Ayurveda Hospital, Thrissur, Kerala, India. The seeds were dried, powered, and extracted twice with 70% methanol. The filtrate was evaporated, dried using a vaccum evaporator. The extracts were dissolved in water and subjected to in vitro cytotoxicity assay.

4.2.2 Short term in vitro cytotoxicity assay (Trypan Blue Exclusion method)

Cytotoxicity studies were carried out using DLA and EAC tumour cells. Different concentrations of the extract (10- 1000 µg/ml) were incubated with tumour cells ($1X10^6$) suspended in phosphate- buffered saline (PBS, pH 7.2) and cytotoxicity was determined after 3 hours using the trypan blue exclusion method (Babu et al., 2002)

4.2.3 Brine shrimp cytotoxicity (BST) assay

4.2.3.1 Hatching the Shrimp

Brine shrimp eggs were hatched in a shallow rectangular dish (22×32 cm) filled with artificial sea water prepared with a commercial salt mixture and double distilled water. A steel divider with several 2 mm holes was clamped in the dish to make two unequal compartments.The eggs (20 mg) were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. After 48 h phototropic nauplii were collected by pipette from the lighted side, having been seperated by the divider from their shells.

4.2.3.2 Sample preparation

Samples were prepared by dissolving the compound in strile double distilled water and different concentrations were transferred to 1.25 cm discs of filter paper (Whatman No.1) . The discs were dried in air for 1 hour, placed in dram vials. Control discs were prepared using only sterile double distilled water. Five replicates were prepared for each dose level.

4.2.3.3 Bioassay

Ten shrimps were transferred to each sample vial using pipette, and artificial sea water was added to make 5 ml. The nauplii can be counted macroscopically in the stem of the pipette against the light background. A drop of yeast suspension (3 mg/ 5 ml) was added as food to each vial. The vials were maintained under illumination , after 6 and 24 hours the percent death at each dose and the control were determined, using the formula

[(Test - Control) / Control]×100 (Mayer *et al.*, 1982).

4.2.4 Toxicity Studies of *Carum copticum.*

The animals (female Swiss albino mice) were divided into 3 groups of 8 animals in each group. The treatment schedule is as follows

Group I - Normal without any treatment

Group II - *C. copticum* 1gm/kg (Oral administration)

Group III - *C. copticum* 2gm/kg (Oral administration)

The animals were sacrificed after 48hours of *C.copticum* extract administration and the GOT (3.2.14), GPT (3.2.15), ALP (3.2.16) and creatinine (3.2.18) levels were estimated in serum. The liver and kidney tissues were subjected to histopathological analysis.

4.2. 5 Solid tumor studies of *Carum copticum* 70% methanolic extract

The animals (female Swiss albino mice) were divided into 4 groups of 10 animals in each group. The solid tumor was induced in all groups of animals by intramuscular injection of $3X10^6$ Dalton's Lymphoma Ascites Cells (DLA) in the hind limb of mice. The treatment schedule is as follows

Group I –Vehicle – (0.1ml normal saline)

Group II - Cyclophosphamide (CTX) 4mg/kg (i.p, for 14 consecutive days)

Group III - *Carum copticum* 50mg/kg (for 14 consecutive days)

Group IV - *Carum copticum* 100mg/kg (for 14 consecutive days)

4.2.6 Synergistic action of *C. copticum* 70% methanolic extract along with radiation:

The animals (female Swiss albino mice) were divided into 4 groups of 10 animals in each group. The solid tumor was induced in all groups of animals by intramuscular injection of $3X10^6$ Dalton's Lymphoma Ascites Cells (DLA) in the hind limb of mice. The treatment schedule is as follows

Group I -Vehicle – (0.1ml normal saline)

Group II - Radiation (100rad/animal) (single dose, 7^{th} day after tumor induction)

Group III - *Carum copticum* 50mg/kg

Group IV - Radiation (100rad/animal) + *Carum copticum* 50mg/kg

4.2.7 Synergistic action of *C.copticum* 70% methanolic extract along with cyclophosphamide:

The animals (female Swiss albino mice) were divided into 4 groups of 10 animals in each group. The solid tumor was induced in all groups of animals by submuscular injection of $3X10^6$ Dalton's Lymphoma Ascites Cells (DLA) in the hind limb of mice. The treatment schedule is as follows

Group I -Vehicle – (0.1ml normal saline)
Group II - Cyclophosphamide (CTX) 4mg/kg
Group III - *Carum copticum* 50mg/kg
Group IV - CTX 4mg/kg + *Carum copticum* 50mg/kg

4.2.8 Synergistic action of *C.copticum* 70% methanolic extract along with radiation and cyclophosphamide:

The animals (female Swiss albino mice) were divided into 5 groups of 10 animals in each group. The solid tumor was induced in all groups of animals by intramuscular injection of $3X10^6$ Dalton's Lymphoma Ascites Cells (DLA) in the hind limb of mice. The treatment schedule is as follows

Group I -Vehicle – (0.1ml normal saline)
Group II - Cyclophosphamide (CTX) 4mg/kg
Group III - Radiation 100rad/animal
Group IV - *Carum copticum* 50mg/kg
Group V - CTX 4mg/kg + Radiation (100rad/animal) + *Carum copticum* 50mg/kg

The tumor diameter and weight of animals were measured at 5 days intervals and the tumor volume was calculated by using the formula $\mathbf{4/3\ \pi\ r^2_1.r^2}$

4.2.9 Ascites tumor studies of *Carum copticum* 70% methanolic extract

The animals (Female Swiss albino mice) were divided into 3 groups of 10 animals in each group. The ascites tumor was induced in all groups of animals by

intraperitoneal injection of $1X10^6$ Dalton's Lymphoma Ascites Cells (DLA) in the hind limb of mice. The treatment schedule is as follows

Group I – Vehicle (0.1ml Normal saline)
Group II- *Carum cotpicum* 50mg/kg
Group –III- *Carum copticum* 100mg/kg

The % of increase in life span was calculated using the formula

$$\frac{\text{Control- Treated}}{\text{Control}} \times 100$$

4.3 RESULTS AND DISCUSSION:

The results of the cytotoxic studies using DLA and EAC cell lines showed that the *Carum copticum* methanolic extract is more cytotoxic than other three plants (Table No 4.1). Hence we eliminated the other three plants and continued our work on *Carum copticum.*

The 70% methanloic extract of *C. copticum* showed brime shrimp toxicity in a dose dependent manner (Table No. 4.2). The extract showed toxicity in L929 cell lines at a concentration of 20µg/ml. The toxicity studies of the 70% methanolic extract showed no considerable change in the level of GOT, GPT, ALP and creatinine compared to the normal group (Table No. 4.3). The histo pathological studies of the liver and kidney of the animals showed that the extract is non toxic to the animals (Figure No. 4.1, 4.2; $p<0.01$) The administration of the methanolic extract of *Carum copticum* significantly inhibited the formation of solid tumor in a dose dependent manner (Table No. 4.4, Figure No. 4.3, 4.4; $p<0.01$). The extract also showed synergistic activity when administered along with radiation and cyclophosphamide (Table No.4.5, 4.6, 4.7, Figure No. 4.5, 4.6, 4.7). In certain cancers single drug treatment is not effective. In this case the combination therapy using two or more drugs is a better alternative. In the present study the *Carum copticum* methanolic extract showed more activity when administered along with cyclophosphamide. It is widely accepted that radiation may interact with a single drug in several ways, resulting in a sub additive, additive or supra additive response. Radiotherapy is an

Table No. 1 Cytotoxic activity of selected medicinal plants from Apiaceae family

Plants	IC_{50} µg/ml	
	DLA	EAC
Carum Copticum	52±2.1	84±4.6
Coriandum sativum	-----	------
Pseucedanum graveolens	-----	------
Cuminum siminum	-----	------

Table No. 4.2 Brime Shrimp Toxicity of *C. copticum* (After 48hour)

	Concentration µg/ml
50%	0.07 µg/ml
100%	0.11µg/ml

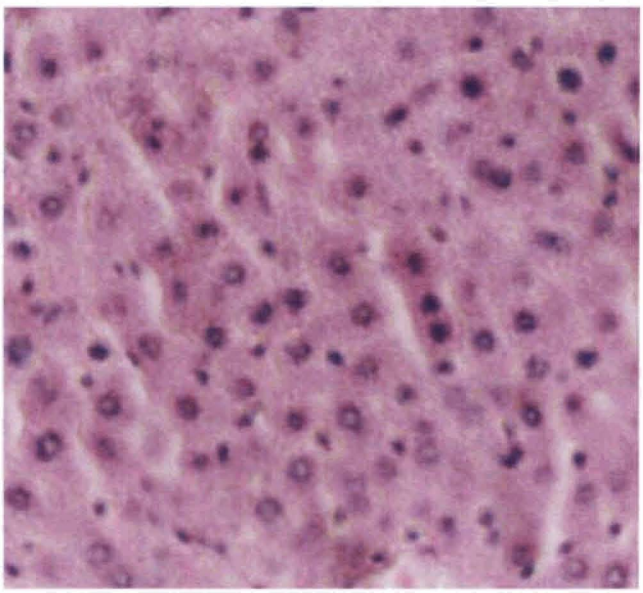
Table No. 4.3 Toxicity studies of *C. copticum* (GOT, GPT, ALP and Creatinine levels)

	SGOT IU/l	SGPT IU/l	ALP IU/l	Creatinine IU/l
Normal	44.68±2.09	52.74±3.87	37.39±2.79	0.91±0.07
***C. copticum* 1g/kg**	46.21±3.58	51.60±4.28	35.96±3.01	0.95±0.05*
***C.copticum* 2g/kg**	43.89±3.76	57.60± 7.18	34.68±2.36*	0.93±0.09*

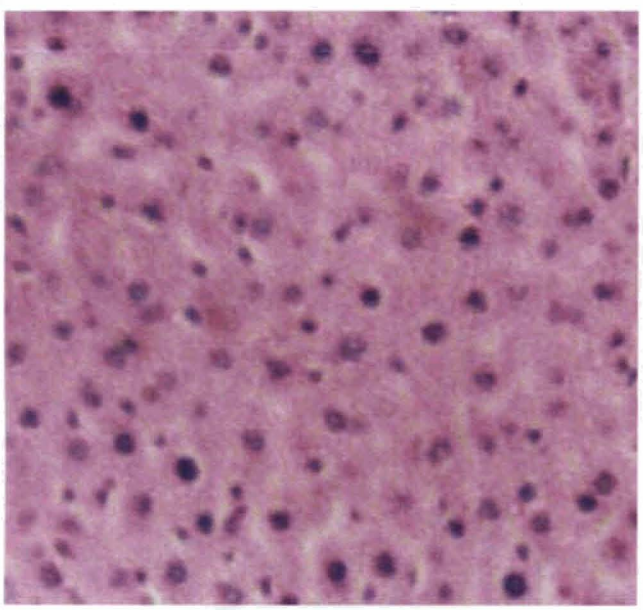
P<*0.01. Values are Mean ± SD of 8 animals in each group

Figure 4.1

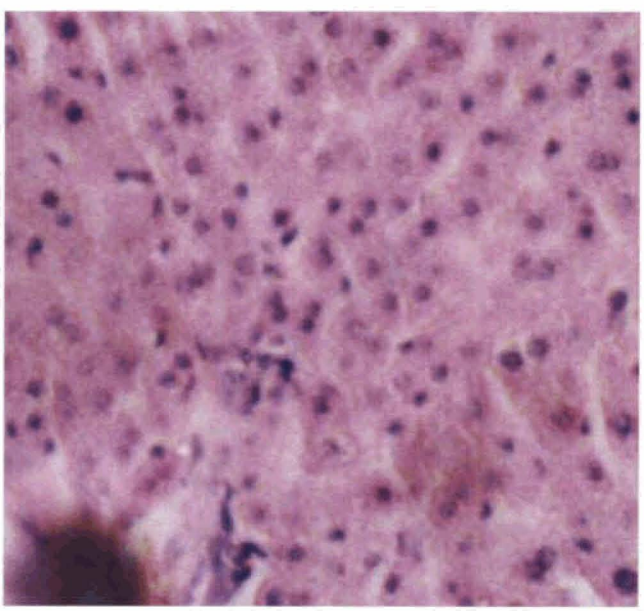
Toxicity studies of 70% methanolic extract of *C.copticum* (Histopathological analysis of the liver)



Normal



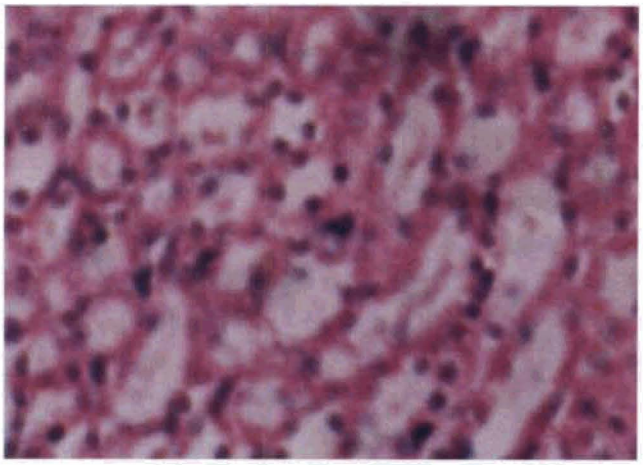
***C.copticum* 1g/Kg**



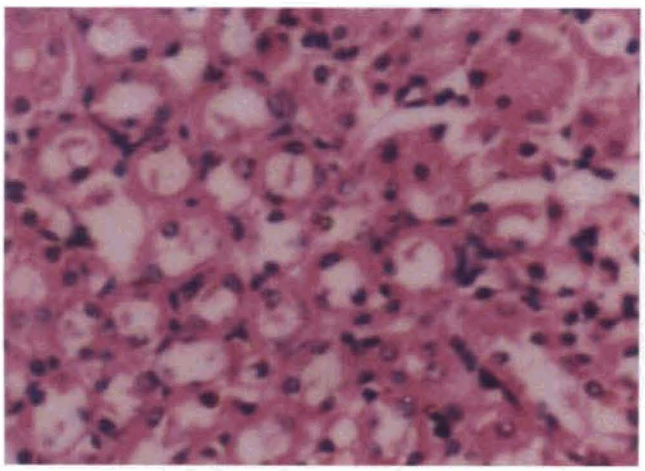
***C.copticum* 2g/Kg**

Figure 4.2

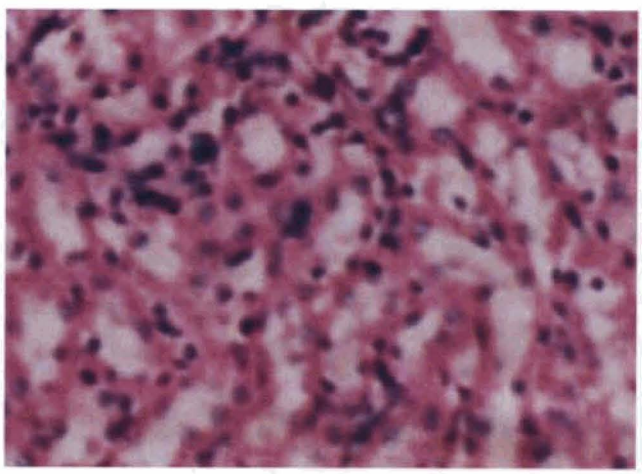
Toxicity studies of *C.copticum* 70% methanolic extract (Histopathological analysis of kidney)



Normal



***C.copticum* 1g/Kg**



***C.copticum* 2g/Kg**

Fig No. 4.3 Effect of *C. copticum* on DLA induced solid tumor model (Tumor volume)

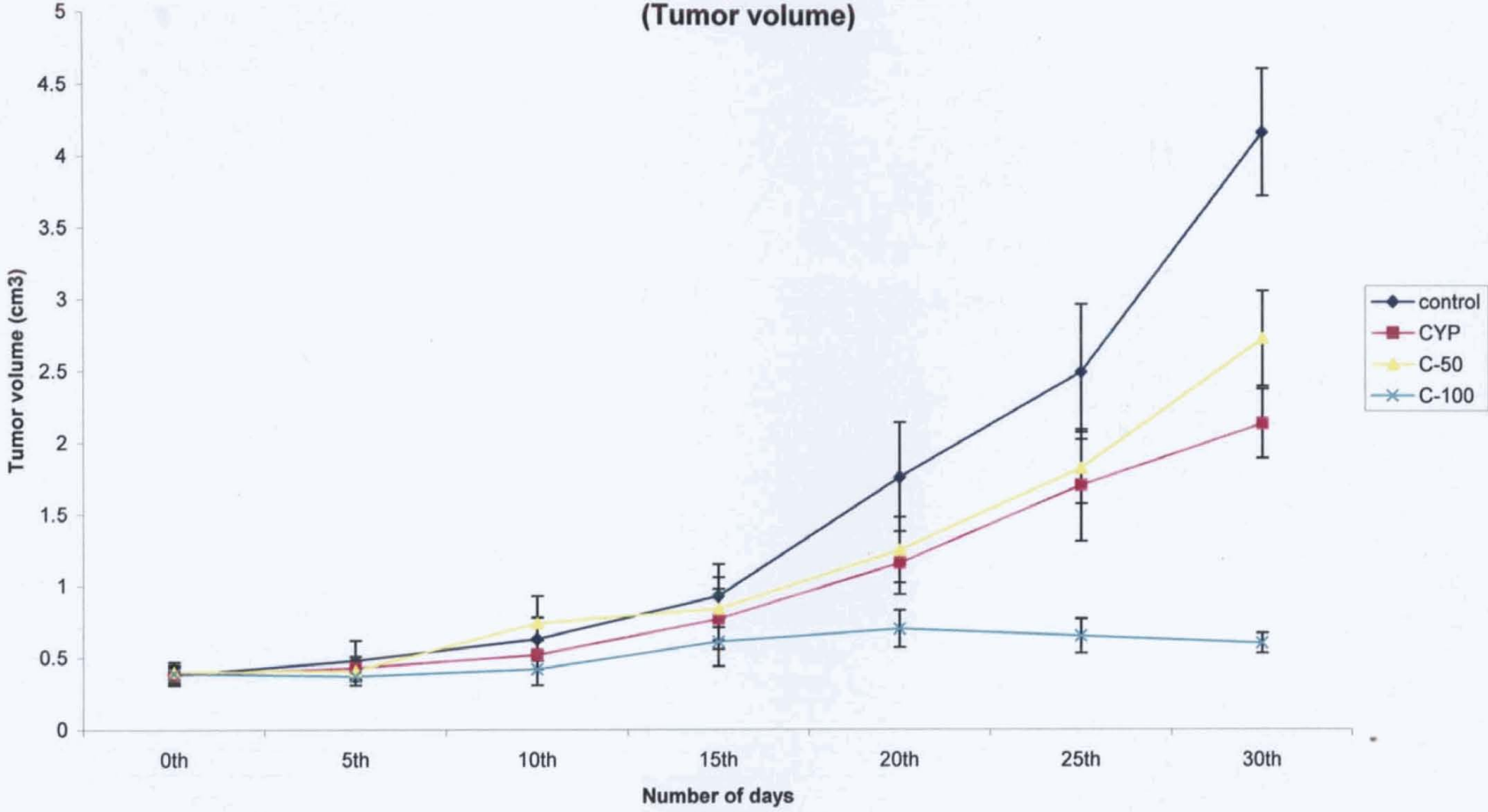
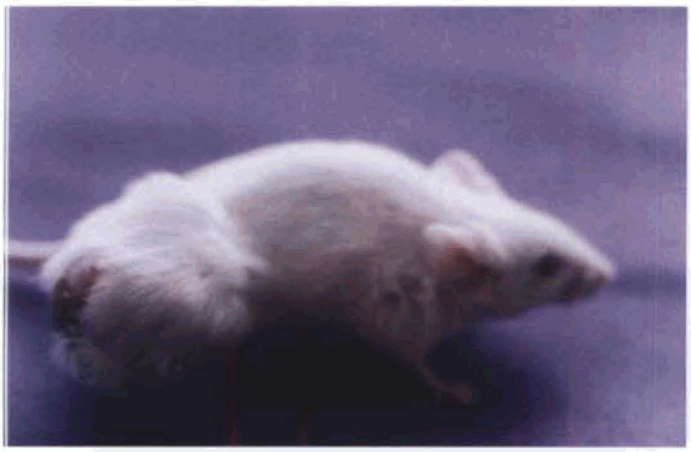
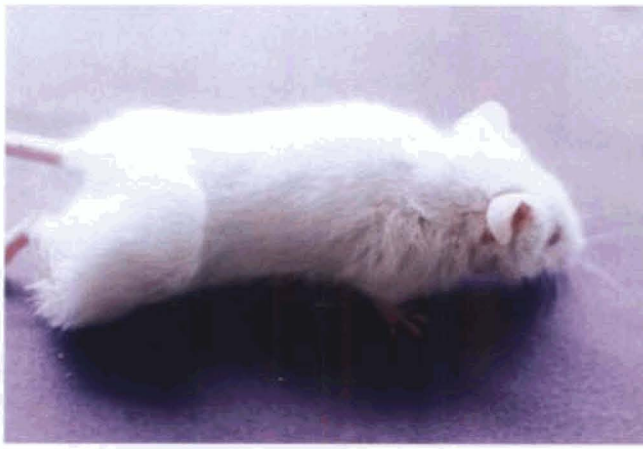


Figure 4.4

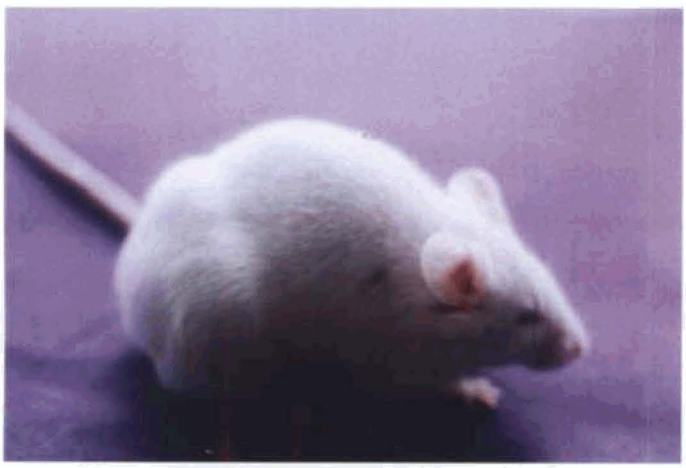
Effect of *C.copticum* on DLA induced solid tumor



Control



CTX 4mg/Kg



***C.copticum* 50mg/Kg**

***C.copticum* 100mg/Kg**

Table No. 4.4 Effect of *C. copticum* on DLA induced solid tumor model (Body weight)

	0th day	5th day	10th day	15th day	20th day	25th day	30th day
Control	21.34±0.76	21.94±0.74	22.48±0.79	23.62±0.84	24.96±0.83	26.04±0.88	27.96±0.93
CTX 4mg/kg	22.09±0.72*	23.39±0.70*	23.81±0.73*	24.42±0.72*	24.86±0.68*	25.13±0.71*	25.81±0.73*
***C.copticum* 50mg/kg**	21.76±0.61*	22.19±0.65*	22.88±0.66*	23.57±0.69*	24.64±0.68*	25.03±0.68*	26.17±0.71*
***C. copticum* 100mg/kg**	22.68±0.82	23.02±0.93	23.46±0.84*	23.92±0.99	24.56±0.82*	25.01±0.86*	25.09±0.84*

P<*0.01. Values are Mean ± SD of 10animals in each group

Fig No. 4.5 Synergistic action of *C. copticum* along with CTX (Tumor volume)

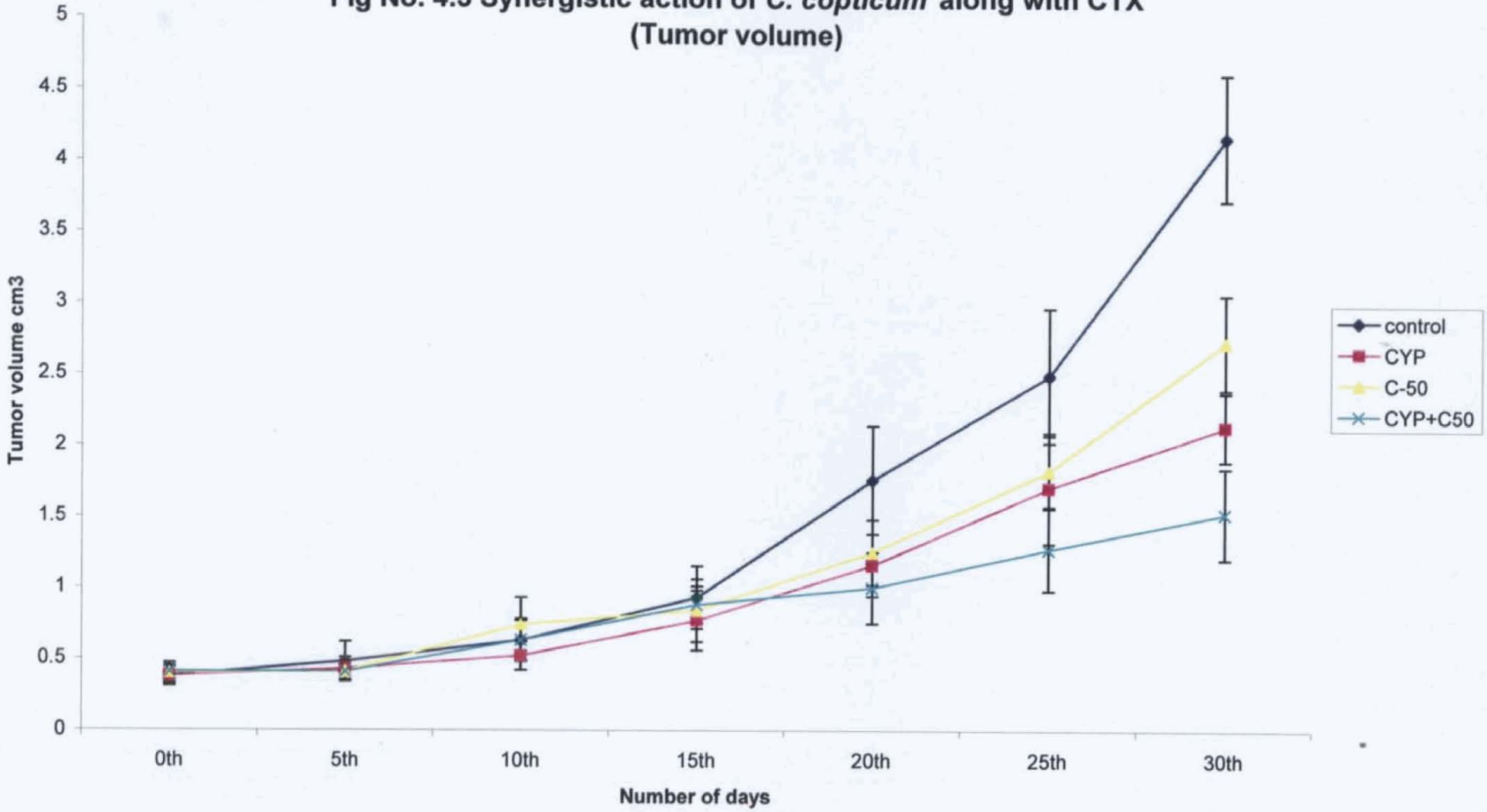


Table No. 4.5 Synergistic action of *C. copticum* along with cyclophosphamide on DLA induced solid tumor model (Body weight)

	0th day	5th day	10th day	15th day	20th day	25th day	30th day
Control	21.34±0.76	21.94±0.74	22.48±0.79	23.62±0.84	24.96±0.83	26.04±0.88	27.96±0.93
CTX 4mg/kg	22.09±0.72*	23.39±0.70*	23.81±0.73*	24.42±0.72*	24.86±0.68*	25.13±0.71*	25.81±0.73*
C.copticum 50mg/kg	21.76±0.61*	22.19±0.65*	22.88±0.66*	23.57±0.69*	24.64±0.68*	25.03±0.68*	26.17±0.71*
CTX + *C. copticum* 50mg/kg	22.98±0.64*	23.27±0.65*	23.73±0.61*	24.09±0.64*	24.38±0.68*	24.66±0.67*	25.13±0.69*

P<*0.01. Values are Mean ± SD of 10animals in each group

Fig No.4.6 Synergistic action of *C. copticum* along with radiation (Tumor volume)

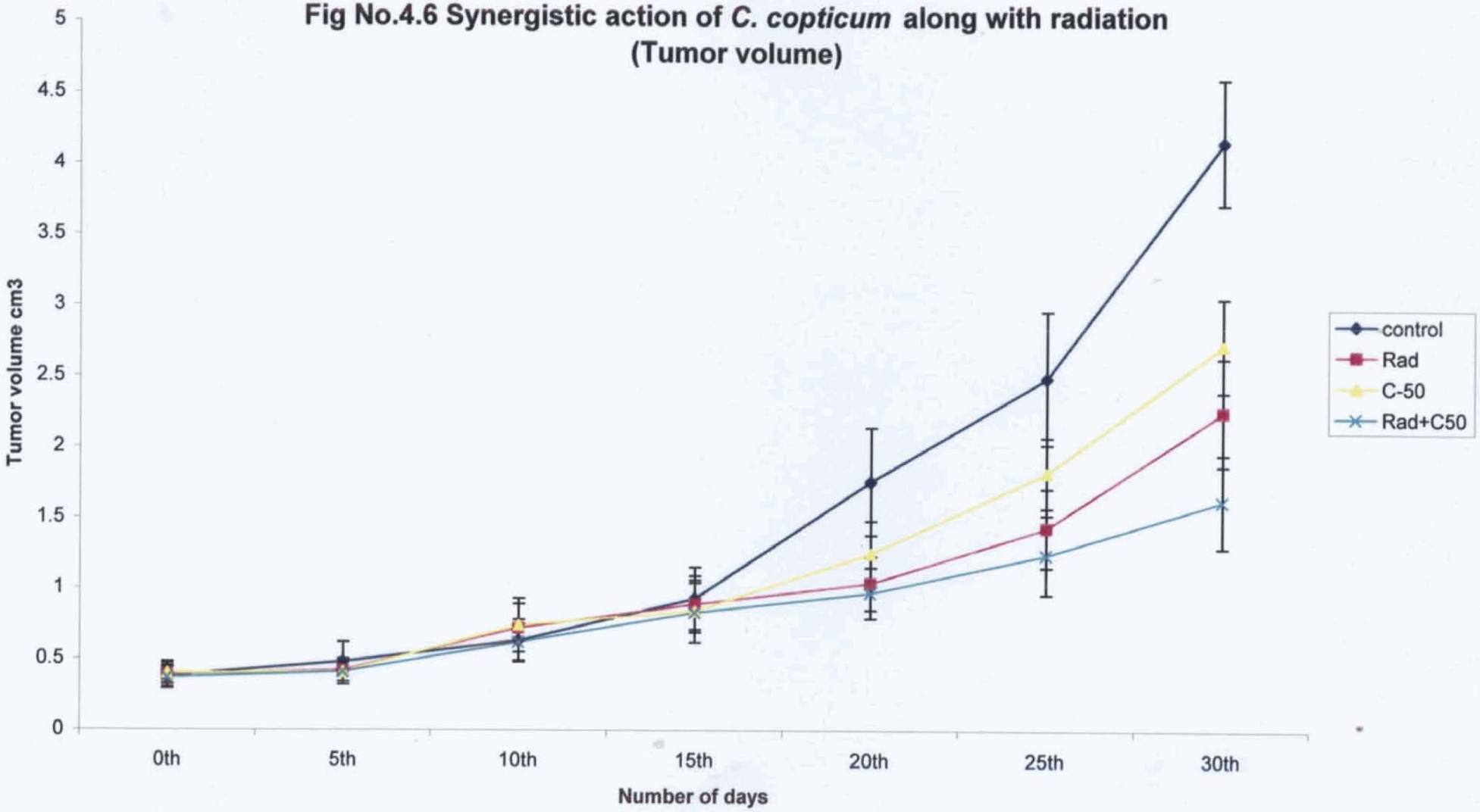


Table No. 4.6 Synergistic action of *C. copticum* along with radiation on DLA induced solid tumor model (Body weight)

	0th day	5th day	10th day	15th day	20th day	25th day	30th day
Control	21.34±0.76	21.94±0.74	22.48±0.79	23.62±0.84	24.96±0.83	26.04±0.88	27.96±0.93
Radiation alone	23.24±0.67*	23.65±0.68*	24.03±0.66*	24.64±0.71*	25.18±0.73*	25.92±0.76*	26.53±0.72*
***C.copticum* 50mg/kg**	21.76±0.61*	22.19±0.65*	22.88±0.66*	23.57±0.69*	24.64±0.68*	25.03±0.68*	26.17±0.71*
Rad+ *C.copticum* 50mg/kg	23.18±0.71*	23.42±0.73*	23.83±0.74*	24.27±0.75*	24.61±0.71*	25.06±0.72*	25.43±0.74*

P< *0.01. Values are Mean ± SD of 10animals in each group

Fig No.4.6 Synergistic action of *C. copticum* along with radiation (Tumor volume)

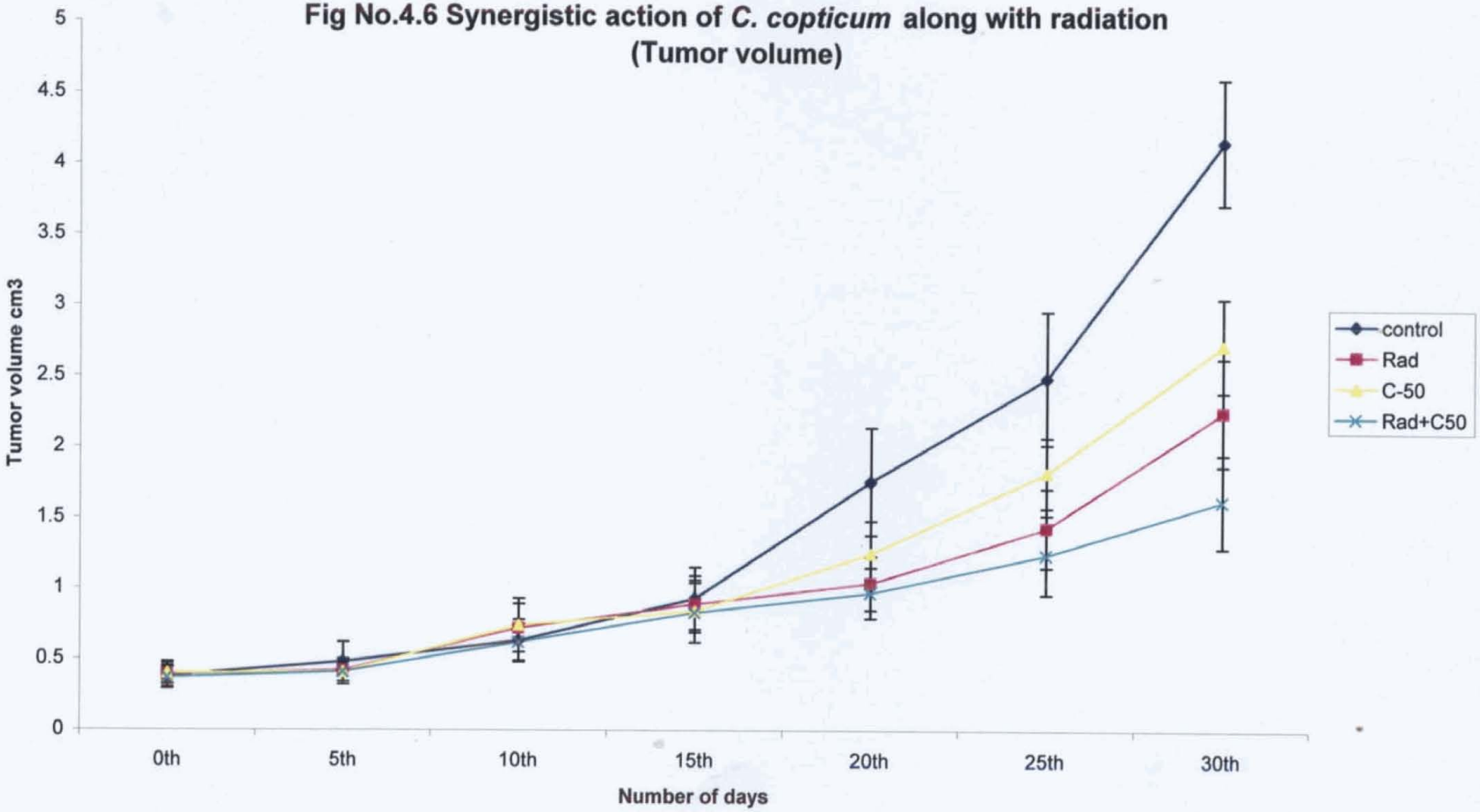


Table No. 4.7 Synergistic action of *C. copticum* along with cyclophosphamide and radiation on DLA induced solid tumor model (Body weight)

	0th day	5th day	10th day	15th day	20th day	25th day	30th day
Control	21.34±0.76	21.94±0.74	22.48±0.79	23.62±0.84	24.96±0.83	26.04±0.88	27.96±0.93
Radiation alone	23.24±0.67*	23.65±0.68*	24.03±0.66*	24.64±0.71*	25.18±0.73*	25.92±0.76*	26.53±0.72*
CTX 4mg/kg	22.09±0.72*	23.39±0.70*	23.81±0.73*	24.42±0.72*	24.86±0.68*	25.13±0.71*	25.81±0.73*
***C.copticum* 50mg/kg**	21.76±0.61*	22.19±0.65*	22.88±0.66*	23.57±0.69*	24.64±0.68*	25.03±0.68*	26.17±0.71*
CTX+Rad+ *C.copticum* 50mg/kg	21.53±0.85	21.82±0.82	22.14±0.81	22.47±0.86	22.91±0.86	23.43±0.89	23.85±0.90

P<*0.01. Values are Mean ± SD of 10animals in each group

Fig No 4.7 Synergistic action of *C. copticum* along with radiation and cyclophosphamide (Tumor volume)

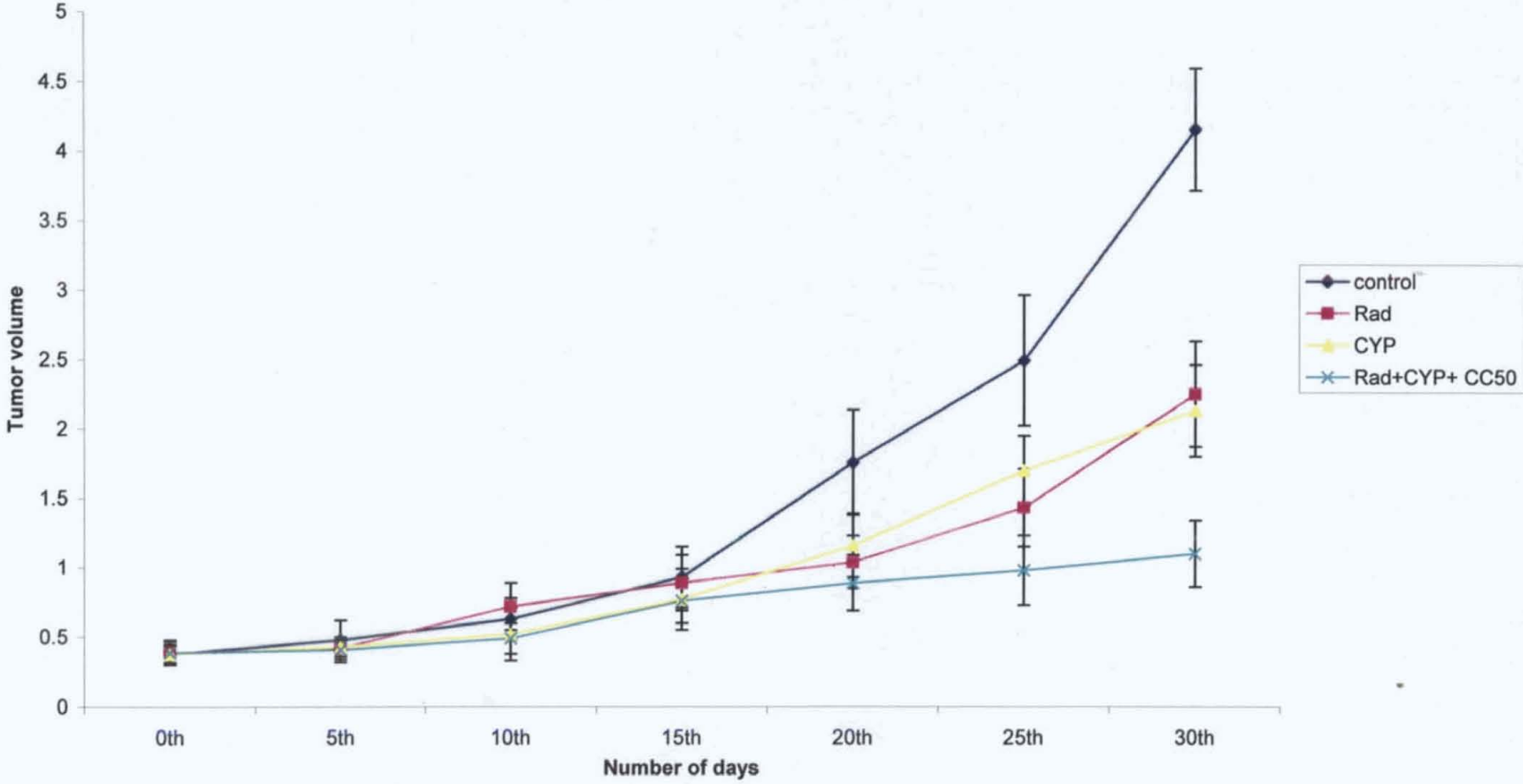


Table No. 4.8 Effect of *C. copticum* on EAC induced ascites tumor

Treatment	No. of days survived	% increase in life span
Control	19.25±2.05	----
50mg/kg b.wt	38.67± 3.37*	100.89
100mg/kg. b.wt	57.00± 6.11*	196.10

P<*0.01. Values are Mean ± SD of 10animals in each group

important modality in the treatment of maliganat diseases as a curative and palliative procedure as well as an adjuvant to other forms of treatment. Radiotherapy selectively destroys or alters the growth potential of the neoplastic cells in situ without significantly compromisiong the normal tissue within the tumor. *C.copticum* methanolic extract may affect as adjuvant response modifier with radiation and cyclophosphamide. The administration of the extract (50mg/kg and 100mg/kg) showed a significant increase in the number of survival days of EAC induced ascites tumor animals in a dose dependent manner (Table No. 4.8).

Nowadays several plant extracts are reported to have antitumor activity. A number of antineoplastic phytochemicals are reported from these plants. The preliminary phytochemical analysis of *Carum copticum* methanolic extract showed the presence of flavinoids. These classes of compounds show high biological activities. Hence, in the present study it can be concluded that the antitumor activity of *C. copticum* may be due to the presence of these compounds.

Chapter 5

Antioxidant and Hepatoprotective activity of Carum copticum

5.1.1 INTRODUCTION:

Free radicals are chemical species that possess an unpaired electron in the outer shell of the molecule. This is the key factor in the structure of this species (Greenwald, 1991; Halliwell, 1995) and is the reason why they are highly reactive in nature. They are highly reactive means that they have low chemical specificity, ie; they can react with most molecules in its vicinity which consists of lipids, proteins, carbohydrates and DNA that can initiate a chain of events which results in the onset of several diseases like cancer, liver cirrhosis, ageing, ischemia, reperfusion, acute hypertension etc. (Chessman and Slater, 1993). It also means that in trying to gain stability by capturing the needed electron they don't survive in their original state for very long and quickly react with their surroundings. Hence free radical attack the nearest stable molecule, "stealing" in it's electron. When the "attacked" molecule loses it's electron, it becomes a free radical itself beginning a chain of reaction. Once the process is started it can cascade, finally resulting in the disruption of a living cell. Free radicals are produced continuously in cells either as by-products of metabolism or deliberately as in phagocytosis (Chessman and Slater, 1993). The most important free radicals in the body are the radical derivatives of oxygen better known as reactive oxygen species. These molecules in it's triplet state or singlet state, superoxide anion, hydroxyl radical, nitric oxide, peroxynitrite, hypochlorous acid, hydrogen peroxide, alkoxy radical and the peroxyl radicals. Others are carbon-centered free radical (CCl3) that arises from the attack of an oxidizing radical on an organic molecule.

The compounds that can scavenge free radicals are collectively called as antioxidants and have great potential in ameliorating several diseases. Human body has inherent mechanisms to reduce free radicals induced injury by the action of enzymes such as superoxide dismutase, glutathione peroxidase, catalase etc. by non-enzymatic means involving ascorbic acid, Vit. E etc. Interestingly several medicinal

plants have been reported to have antioxidant activity due to the presence of Flavonoids, terpenes and polyphenols (Irshad and Chaudari, 2002).

The liver protects the body from potentially injurious substances (endotoxins) absorbed from the intestinal tract, as well as the toxic byproducts of metabolism. The most important in the detoxification process is that of the microsomal drug metabolizing system of the liver. A large number of xenobiotics are reported to be potentially hepatotoxic due to the generation of free radicals, which can undergo a variety of secondary reactions. Some examples are acetaminophen, tetracycline, antineoplastic agents, ethanol and carbon tetrachloride. Hepatotoxins may react with the basic cellular constituents-proteins, lipids, RNA and DNA and induce almost all types of lesions of the liver (Guillouzo, 1998). Most of the chemicals must undergo metabolic activation by phase I enzyme to form electrophilic reactants, which can interact with nucleophilic group in the macromolecules including DNA. The incidence of hepatocellular carcinomas correlated with the incidence of hepatic disease in high-risk areas of China, Southeast Asia and Africa (Benson et al., 1979 and Zhu et al., 1988). Since the damaging effect of this chemical is thought to be mainly due to the free radical interaction, it could be worth giving attention to some antioxidants capable of scavenging these reactive species.

Several herbal drugs have been prescribed as 'liver tonics' for the reduction of toxicity due to ingested xenobiotics (Groombridge, 1992). Numerous medicinal plants and their active components such as phenolic antioxidants, indoles, isothiocynates, coumarins, flavonones, allylsulfides etc. are used for liver diseases in ethnomedical practices as well as traditional systems of medicine. (Kensler, 1997). Administration of antioxidants such as vitamin E, promethazine, propyl gallate and reduced glutathione (GSH) or of the compound SKS-525A, which inhibits microsomal drug metabolism, has been shown to decrease CCl_4 toxicity in animals (Halliwell and Gutteridge, 1985a). The present study evaluated the in vitro antioxidant and hepatoprotective activity of *C. copticum* against CCl_4 and Paracetamol induced hepatic damage.

5.1.2 MATERIALS AND METHODS:

5.1.2.1 Superoxide radical scavenging activity (3.2.3).

5.1.2.2 Hydroxyl radical scavenging activity (3.2.4)

5.1.2.3 Nitric oxide radical scavenging activity (3.2.6)

5.1.2.4 Inhibition of in vitro tissue lipid peroxidation (3.2.7)

5.1.2.5 Effect of *C. copticum* on CCl_4 induced hepatic damage:

Female Wistar rats (180-200gm) were used for the present study. The animals were randomized into 4 groups of 10animals in each group. The treatment schedule was as follows

Group I - Normal without any treatment
Group II -CCl_4 alone
Group III -CCl_4 + *C.copticum* 250mg/kg
Group IV -CCl_4 + *C.copticum* 500mg/kg

Group III and IV were received *C. copticum* 70% methanolic extract (250mg/kg and 500mg/kg) respectively for 6 consecutive days. On 6^{th} day the group II, III and IV animals were received CCl_4 at a dose of 1.5ml/kg in liquid paraffin (1:1). After 48hours of CCl_4 administration the animals were sacrificed blood and liver was collected and subjected to biochemical and histopathological analysis.

5.1.2.6 Effect of *C.copticum* on Paracetamol induced hepatic damage:

Female Wistar rats (180-200gm) were used for the present study. The animals were randomized into 4 groups of 10animals in each group.

Group I - Normal without any treatment
Group II -Paracetamol alone
Group III - Paracetamol + *C.copticum* 250mg/kg
Group IV - Paracetamol + *C.copticum* 500mg/kg

Group III and IV were received *C. copticum* 70% methanolic extract (250mg/kg and 500mg/kg) respectively for 4 consecutive days. On 4^{th} day the group

II, III and IV animals were received paracetamol at a dose of 2gm/kg After 48hours of paracetamol administration the animals were sacrificed blood and liver was collected and subjected to biochemical and histopathological analysis.

5.1.2.7 BIOCHEMICAL ANALYSIS:

Liver was removed and washed thoroughly in ice-cold saline and homogenate (10 %) was prepared in PBS (50 mM, pH 7.0) (section 3.2.7). A part of the homogenate was used for the estimation of reduced glutathione (GSH) (section 3.2.8). The remaining homogenate was centrifuged at 10,000 rpm for 10 min in a cooling centrifuge at 4°C, after removal of the cell debris; supernatant was used for the assay of superoxide dismutase (SOD) (section 2.2.9), catalase (CAT) (section 3.2.10) and glutathione peroxidase (GPx) (section 3.2.11). In serum GOT (section 3.2.14), GPT (section 3.2.15) and ALP (section 3.2.16) was estimated. Protein was determined by the method of Lowry *et al.* (section 3.2.13)

Non-coagulated (heparin) blood was used for the determination of antioxidant status. SOD (section 3.2.22), CAT (section 3.2.23), GSH (section 3.2.24), and GPx (section 3.2.26) were estimated in erythrocyte lysate. Haemoglobin was determined using Drabkin's reagent (section 3.2.27).

5.1.3 RESULTS AND DISCUSSION:

The methanolic extract of *Carum copticum* showed in vitro Superoxide, Hydroxyl and nitric oxide radicals scavenging activity. The extract also inhibited the lipid peroxidation in vitro (Table No. 5.1.1).

The CCl_4 treated as well as paracetamol treated groups of animals showed an increase in liver marker enzyme levels such as SGOT, SGPT and ALP which is an indicative of hepatic damage. While the administration of the methanolic extract of *C. copticum* significantly reduced the levels of these enzymes in a dose dependent manner (Table No. 5.1.2, 5.1.5). The in vivo antioxidant status of the extract treated groups of animals showed an increase in SOD, Catalase, GSH and GPx (Both in liver and blood) levels which is an indicative of the free radical scavenging activity of *C. copticum* generated during CCl_4 and paracetamol administration (Table No. 5.1.3, 5.1.4, 5.1.6, 5.1.7)

Table No. 5.1.1 In vitro scavenging activity of *C. copticum*

Assays	IC_{50}µg/ml
Superoxide radical	67.13±3.4
Hydroxyl radical	132.40±6.98
Nitric oxide radical	45.97±2.86
In vitro lipid peroxidation	148.0±8.9

Table No. 5.1.2 Effect of *C.copticum* on CCl_4 Induced Hepatic damage

	SGOT (IU/L)	SGPT (IU/L)	Serum ALP (IU/L)
Normal	65.33±9.04	159.21±19.61	42.79±5.67
CCl_4 alone (1.5ml/kg)	254.37±22.08*	689.45±85.89*	189.54±12.39*
CCl_4 + *C.copticum* 250mg/kg	206.19±11.28*	479.49±18.04*	143.28±10.42*
CCl_4 + *C.copticum* 500mg/kg	164.05±14.53*	245.71±12.54*	98.86±10.31*

P< *0.01. Values are Mean ± SD of 10 animals in each group

Table No. 5.1.3 Effect of *C.copticum* on CCl_4 Induced Hepatic damage (Blood antioxidant levels)

	SOD U/g Hb	CAT k/g Hb	GSH umol/ml	GPX U/g Hb
Normal	1546.0 ± 80.60	95.18±6.61	3.72±0.18	5094.0±505.74
CCl_4 alone (1.5ml/kg)	908.0± 54.89*	66.28±2.93*	3.26±0.11*	2581.5±236.0*
CCl_4 + *C.copticum* 250mg/kg	1227.0±61.60*	74.13±2.74*	3.56±0.06*	3529±205.50*
CCl_4 + *C.copticum* 500mg/kg	1457.5±70.10*	92.75±4.15*	3.73±0.12*	4962.0±513.75

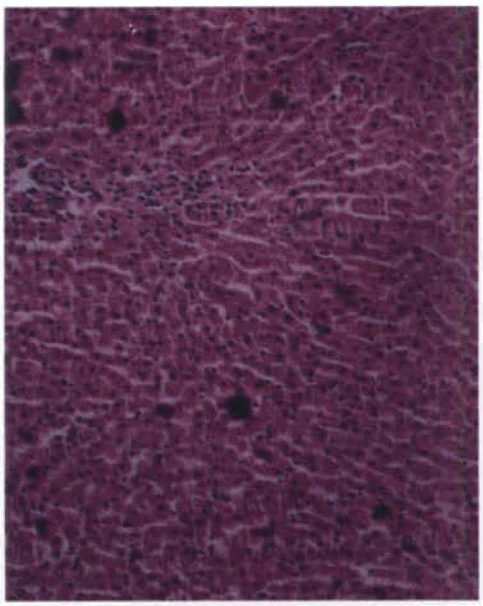
P<*0.01. Values are Mean ± SD of 10 animals in each group

Table No. 5.1.4 Effect of *C.copticum* on CCl_4 Induced Hepatic damage (Tissue parameters)

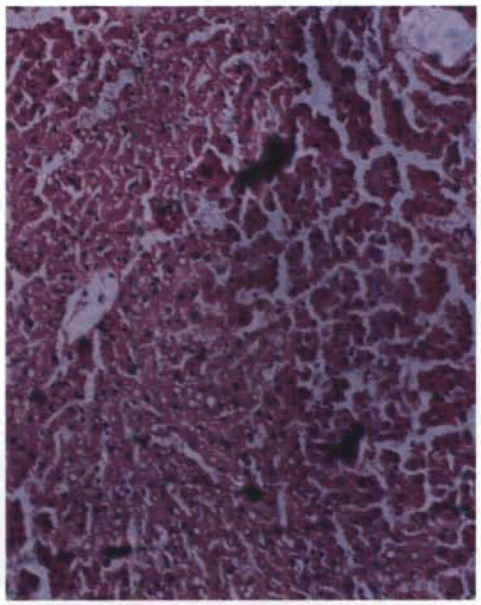
	SOD U/mg Protein	**CAT U/mg Protein**	**GSH nmol/mg**	**GPx U/mg protein**	**Tissue Lipid Peroxidation nmol/mg**
Normal	21.20±2.33	65.52±3.22	13.85±1.00	26.98±1.60	1.72±0.26
CCl_4 alone (1.5ml/kg)	15.87±1.34*	43.78±2.86*	9.43±0.87*	16.55±1.55*	3.46±0.39*
CCl_4 + *C.copticum* 250mg/kg	20.95±1.94*	56.52±3.24	11.70±0.54*	23.02±1.80	2.81± 0.27*
CCl_4 + *C.copticum* 500mg/kg	23.15±1.83*	64.37±4.01	12.68±0.87*	27.28±1.48*	2.14±0.25*

P< *0.01. Values are Mean ± SD of 10 animals in each group

Effect of *C.copticum* on CCl_4 induced hepatotoxicity (Histopathology of the liver)

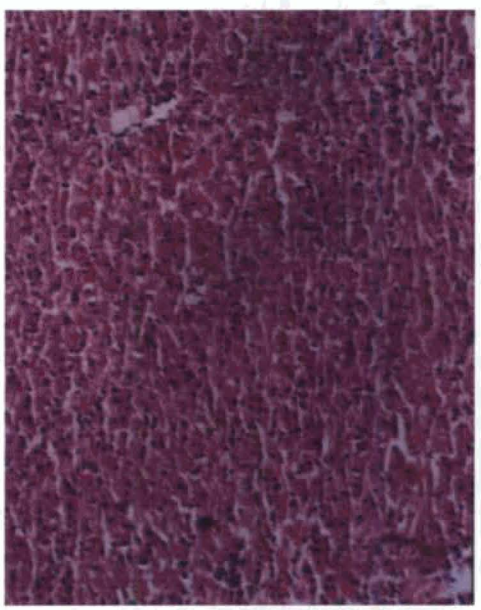


Normal



CCl_4 alone

CCl_4+ *C.copticum* 250mg/Kg



CCl_4+ *C.copticum* 500mg/Kg

Table No. 5.1.5 Effect of *C.copticum* on Paracetamol Induced Hepatic damage

	SGOT (IU/L)	SGPT (IU/L)	Serum ALP (IU/L)
Normal	66.37±6.06	165.49±21.22	45.72±7.51
Paracetamol (2.0gm/kg)	262.89±18.38*	714.77±60.5*	167.49±13.28*
Paracetamol + *C.copticum* 250mg/kg	173.78±9.04*	491.89±36.63*	132.54±12.63*
Paracetamol + *C.copticum* 500mg/kg	156.05±5.27*	260.79±29.50*	96.83±5.89*

P< *0.01. Values are Mean ± SD of 10 animals in each group

Table No. 5.1.6 Effect of *C.copticum* on Paracetamol Induced Hepatic damage (Blood antioxidant levels)

	SOD U/g Hb	CAT K/g Hb	GSH umol/ml	GPX U/mg Hb
Normal	1381.67±103.20	98.77±4.93	4.16±0.36	4886.5±517.50
Paracetamol (2.0gm/kg)	914.60±39.05*	65.15±4.29	3.56±0.13*	2521.25±209.00*
Paracetamol + *C.copticum* 250mg/kg	980.0±45.50*	80.97±4.32*	3.71±0.06*	3217.0±262.50*
Paracetamol + *C.copticum* 500mg/kg	1307.5±69.75*	94.63±5.46	3.88±0.07*	5189.5± 386.50*

P< *0.01. Values are Mean ± SD of 10 animals in each group

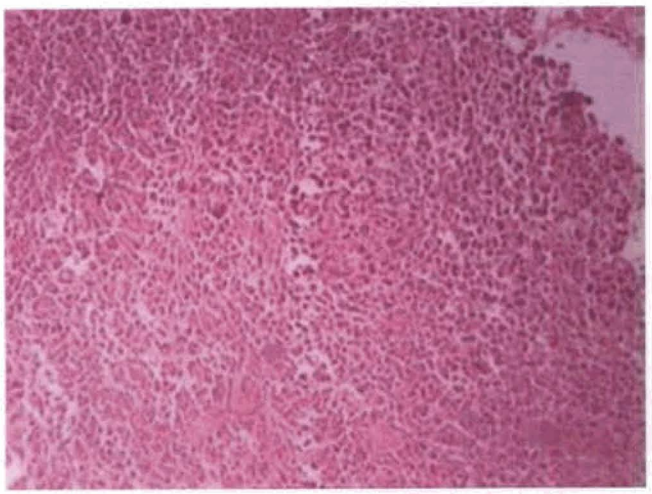
Table No. 5.1.7 Effect of *C.copticum* on Paracetamol Induced Hepatic damage (Tissue parameters)

	SOD U/mg Protein	**CAT U/mg Protein**	**GSH Nmol/mg**	**GPx U/mg protein**	**Tissue Lipid Peroxidation nmol/mg protein**
Normal	23.27±2.04	72.87±3.57	14.55±1.52	24.68±2.04	1.65±0.31
Paracetamol (2.0gm/kg)	15.68±1.41*	45.85±2.12*	7.43±0.71*	16.40±0.70*	3.21±0.47*
Paracetamol + *C.copticum* 250mg/kg	19.02±1.10*	63.50±4.39	10.19±0.91*	17.95±0.90*	2.81±0.34*
Paracetamol + *C.copticum* 500mg/kg	25.67±1.43*	71.23±3.41*	13.03±0.87*	24.02±2.02*	2.14±0.18*

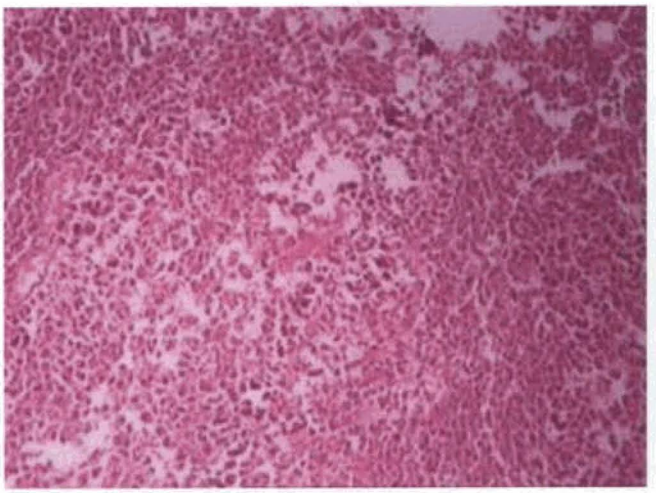
P< 0.01. Values are Mean ± SD of 10 animals in each group

Figure 5.1.2

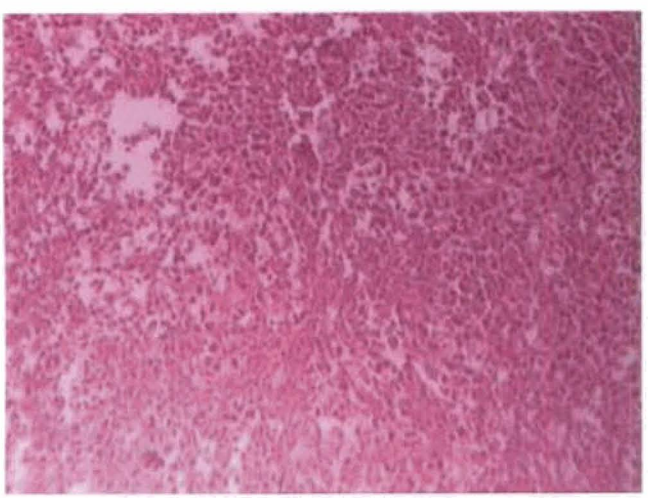
Effect of *C.copticum* on paracetamol induced hepatotoxicity (Histopathological analysis of the liver)



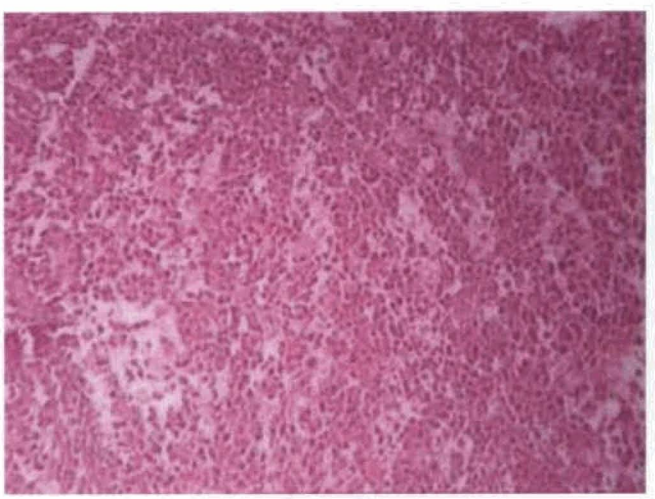
Normal



Paracetamol 2g/kg



Paracetamol+ *C.coptcum* 250mg/Kg



Paracetamol+ *C.coptcum* 500 mg/Kg

Histopathological examination of the liver challenged with acute doses of CCl_4 and paracetamol showed centrilobular necrosis, inflammatory infiltration of lymphocytes and fatty changes. The liver sections of rats treated with the extract (250 and 500 mg/kg) plus CCl_4 or paracetamol showed well-preserved architecture (Fig. 5.1.1, 5.1.2), where as liver challenged with doses of CCl_4 or paracetamol showed severe necrosis, fatty infiltration, fibrosis, ballooning degeneration and lymphocytes infiltration. The effects were moderate to low in the liver of extract plus CCl_4 or paracetamol (250 and 500 mg/kg) treated animals.

Results of the present study reveal the significant hepatoprotective activity of the methanolic extract of *C. copticum*, against CCl_4 and paracetamol hepatotoxicity. The activities of transaminases (SGPT and SGOT) were elevated significantly in the serum of CCl_4 or paracetamol alone treated animals. The elevation of SGPT in the serum indicates the hepatocyte necrosis. The altered ratio of SGPT to SGOT in animals administered with CCl_4 or paracetamol alone clearly confirms the hepatotoxic status of the animals. The elevated serum ALP activity was due to biliary system abnormalities, which was reduced significantly in the extract treated animals. The pretreatment of the extract prevented the elevation of SGPT, SGOT and serum ALP consequent to CCl_4 or paracetamol treatment, which indicates the hepatoprotective activity of the extract.

The antioxidant status of the hepatocytes was altered in CCl_4 as well as paracetamol treated animals. The treatment of *C. copticum* extract prior to the CCl_4 injection or paracetamol admistration effectively protected the decline of hepatic antioxidant status. CCl_4 is metabolized in Cyt P-450 system to give the trichloromethyl radical ($CCl_3^{\cdot}$). Trichloromethyl radical reacts with oxygen to form trichloroperoxy radical (CCl_3O_2), both these products induce the peroxidation of lipids (Ahr et al., 1982). The products of peroxidation are known to inhibit protein synthesis and activity of certain enzymes. Liver contains high concentrations of both CAT and GPx. The chronic treatment of the CCl_4 decreased the activity of CAT and GPx. Further the activity of SOD and the level of GSH were also declined in the liver. The declined antioxidant status is responsible for the increased lipid peroxidation, which leads to loss of membrane fluidity, integrity and finally cell functions of liver (Halliwell and Gutteridge, 1989 and Smith et al., 1987). This may results in the

leakage of enzymes and toxic metabolites to circulation. The treatment of the extract prior to the CCl_4 injection increased the hepatocyte SOD, CAT and GPx activity and effectively prevented the radical mediated loss of membrane integrity. Hence the extract treated animals showed reduced transaminase activity and MDA level in serum.

The role of GSH in the formation of conjugates with electophilic drug metabolites most often formed by cytochrome P-450 linked monooxygenase is now well established (Rana et al., 2002). Studies with a number of model show that the hepatotoxicity of xenobiotics is often produced by GSH depletion (Mitchell et al., 1973 and Jollow et al., 1974). The decreased concentration of GSH increases the sensitivity to oxidative and chemical injury. Exogenous GSH could offer protection against CCl_4 induced injury in rats (Rana and Tayal, 1981). CCl_4 induced cirrhosis produced a decrease in the components of the hepatic glutathione antioxidant system (Altomare et al., 1998 and Cabre et al., 2000). Results of the present study also support these findings. The treatment of extract before the CCl_4 injection prevented the decline of hepatic GSH level. More over, GSH protect hepatocyte by forming the substrate of the GPx and react directly with various aldehyde produced during peroxidation of membrane lipid. Treatment of rat with extract plus CCl_4 enhances the activity of Se-GPx (selenium dependent GPx) compared to the CCl_4 alone treated animals. The enhanced GPx activity could partially explains the protection of bio-membrane from oxidative attack. The protective role of Se-GPx against CCl_4 hepatotoxicity has been reported in rat (Rana and Rastogi, 1993). The fat accumulation in the liver of CCl_4 treated animals is due to blockage in the synthesis of lipoproteins that carry triglyceride away from liver. Histopathological observation of the CCl_4 alone treated liver clearly shows the level of fatty infiltration and necrosis due to radical mediated cell cytotoxicity. The CCl_4 plus extract treated liver showed lesser degree of fatty changes and necrosis.

Since liver is very active in GSH biosynthesis and its translocation to blood, significant reduction of extrahepatic tissue and blood GSH level indicate the hepatic damage (Rana and Kumar, 2002). This is evident from the decreased level of GSH in the blood of animals treated with CCl_4 alone. The level was enhanced in the extract (50 mg/kg) plus CCl_4 treated animals. The declined activity of the SOD, CAT and Se-GPx in the erythrocytes of CCl_4 treated animals indicated the fragility of

erythrocyte to the toxic metabolites and radicals released from liver. Erythrocytes are regularly subjected to high oxygen tension as they are among the first cells exposed to exogenous oxidative substance that are ingested, injected or inhaled (Halliwell and Gutaridge, 1985a). The pretreatment of extract prior to CCl_4 challenge protects the liver; decline the release of toxic metabolites and radicals that may normalize the antioxidant enzyme activity in the erythrocytes. The declined antioxidant status in erythrocytes partially explains the enhanced lipid peroxidation level (MDA) in the serum of CCl_4 treated animals. The level of serum MDA was lowered in the extract plus CCl_4 treated animals in a dose dependent manner due to the enhanced antioxidant status in the liver as well as in the blood. Erythrocytes contain high concentrations of polyunsaturated fatty acids (PUFA), ferrous ions and molecular oxygen, which makes them highly vulnerable to oxidative stress (Yadav et al., 1997).

The damage due to paracetamol administration is also due to the generation of free radicals. The liver as well as blood SOD, Catalase, GSH, GPx as well as Tissue lipid peroxidation levels in the control as well as treated animals give a strong evidence for this. Moreover, the results of the present study revealed the in vivo antioxidant activity of *C. copticum*.

Antiinflammatory and Gastroprotective activity of Carum copticum

5.2.1 INTRODUCTION:

Gastric ulceration in the stomach due to various factors is considered as one of the major human sufferings today affecting nearly 5% of the global population (Debashis et al 2002). Ulcer develops when a balance between some defensive and offensive factors is lost. The defensive factors are either endogenous or exogenous in origin. The endogenous damaging factors are hydrochloric acid, reflexed bile, pepsin, leukotriens and reactive oxygen species (ROS) such as O^{2-}, H_2O_2 and $OH^{\cdot}$ The exogenous damaging factors mainly include alcohol, steroidal and non-steroidal antiinflammmatory drugs and drugs which stimulate gastric acid and pepsin secretion, stress and tension and *Helicobactor pylori.* The mucosal defence against these defensive factors is contributed by mucus-bicarbonate barrier, surface active phospholipid, prostaglandin, mucosal blood flow, cell renewal and migration, antioxidants and antioxidant enzymes and some growth factors.

Inflammation, a fundamental protective response; may be harmful in conditions such as life threating hypersensitive reactions to insect bites, drugs, toxins and in chronic diseases such as rheumatic arthritis, atherosclerosis, lung fibrosis and cancer (Collins, 1999). Inflammation can accelerate the development of cancer (Wiseman and Halliwell, 1996). Chronic inflammation is a risk factor for epithelial carcinogenesis (Weitzman and Gordan, 1990). Prostaglandins (PGs) generated during the inflammation appear to be important in the pathogenesis of cancer due to their effect on mitogenesis, cellular adhesion, immune surveillance and apoptosis. Increased production of PGs from arachidonic acid in transformed cells is associated with up regulation of COX-2 (Subbaramaiah et al., 1996). Many sources of inflammation are effective, including that caused by viral, bacterial and parasitic infections. In colon cancer, predisposing sources of chronic inflammation include

ulcerative colitis and infection with the parasite *Scistosoma japonicum* (Wiseman and Halliwell, 1996). Infection with *Schistosoma haematobium* produces chronic bladder inflammation and is associated with increased cancer at this site (Kawai, 1994). Tumor-promoting phorbol esters induce COX-2 gene expression (Mestre, 1997). A notable activity of tumor promoters is their ability to recruit inflammatory cells and to a strong relationship between the effect of tumor promoters to stimulate inflammatory cells to release ROS/RNS and their capacity to promote tumors.

Management of these painful diseases, their prevention or cure is one the challenging problem today. A number of drugs have been proved useful in controlling inflammation and ulceration such as aspirin, indomethacin, ranitidine etc, but their long term uses are not devoid of disturbing side effects. Hence, the search is still on to find a drug possessing antioxidant, antiinflammatory and antiulcer properties which will serve as a powerful therapeutic agent to cure gastric ulceration. The search has also been extended to herbal drugs for new and novel molecules, which afford better protection and decrease the incidence of relapse.

In traditional Indian medicine several plants and herbs have been used to treat gastro-intestinal disorders, including gastric ulcer and inflammation. The present study investigated the anti-inflammatory and antiulcer activity of *C. copticum.*

5.2.2 MATERIALS AND METHODS

5.2.2.1 Antiinflammtory activity of *C. copticum* methanolic extract against carrageenan induced paw odema

The animals were divided into 4 groups of 6 animals in each group. The inflammation was induced by injecting 0.1% of carrageenan In the right hind paw of mice and the paw thickness was calculated In every 1hr intervels. The % of inhibition was calculated.

Group I – Control
Group II- Diclofenac 4mg/kg (i.p)
Group III- *C. copticum* 250mg/kg
Group IV- *C. copticum* 500mg/kg

5.2.2.2 Antiinflammtory activity of *C. copticum* methanolic extract against dextran induced paw odema

The animals were divided into 4 groups of 6 animals in each group. The inflammation was induced by injecting 0.1% of Dextran In the right hind paw of mice and the paw thickness was calculated In every 1hr intervels. The % of inhibition was calculated.

Group I – Control
Group II- Diclofenac 4mg/kg (i.p)
Group III- *C. copticum* 250mg/kg
Group IV- *C. copticum* 500mg/kg

5.2.2.3 Antiinflammtory activity of *C. copticum* methanolic extract against formalin induced paw odema

The animals were divided into 4 groups of 6 animals in each group. The inflammation was induced by injecting 1% of formalin In the right hind paw of mice and the paw thickness was calculated In every day upto 7 days. The % of inhibition was alculated.

Group I – Control
Group II- Diclofenac 4mg/kg (i.p)
Group III- *C. copticum* 250mg/kg
Group IV- *C. copticum* 500mg/kg

5.2.2.4 Antiulcer activity against EtOH induced gastric ulcer

Female Wistar rats were used for the present study. The animals were divided into 5 groups of 6 animals in each group. The treatment schedule was as follows

Group I- Normal
Group II – EtOH (5ml/kg)
Group III- EtOH + Ranitidin 50mg/kg

Group IV – EtOH + *C. copticum* 250mg/kg

Group V- EtOH + *C. Copticum* 500mg/kg

To 36 hr fasted animals EtOH was given orally. The drug was administered 1hr prior to the induction of ulcer. The animals were sacrificed after 4hr of EtOH administration. Ulcer index was calculated. The stomach was subjected to biochemical and histopathological analysis.

5.2.2.5 Antiulcer activity against Aspirin induced gastric ulcer

Female Wistar rats were used for the present study. The animals were divided into 5 groups of 6 animals in each group.

Group I- Normal

Group II – Aspirin (400mg/kg) alone

Group III- Aspirin + Ranitidin 50mg/kg

Group IV – Aspirin + *C. copticum* 250mg/kg

Group V- Aspirin + *C. Copticum* 500mg/kg

To 36 hr fasted animals aspirin was given orally. The drug was administered 1hr prior to the induction of ulcer. The animals were sacrificed after 4hr of aspirin administration. Ulcer index was calculated. The stomach was subjected to biochemical and histopathological analysis.

5.2.2.6 Determination of Ulcer Index (U.I):

The ulcerative index was calculated by severity of gastric mucosal lesions graded as follows.

Erosions	**Score**
1mm or less	1
1-2mm	2
More than 2mm	3

The U.I. was calculated by using the formula

U.I = 1X (number of lesions of grade 1) + 2X (number of lesions in grade 2) + 3X (number of lesions in grade 3)

Then the overall score was divided by a factor 10, which was designated as ulcer index (Main and Whittle, 1975)

5.2.2.7 BIOCHEMICAL ANAYISIS

Stoamch was removed and washed thoroughly in ice-cold saline, the mucosd was taken using a blunt knife and homogenate (10 %) was prepared in PBS (50 mM, pH 7.0) (section 3.2.2). A part of the homogenate was used for the estimation of reduced glutathione (GSH) (section 3.2.8). The remaining homogenate was centrifuged at 10,000 rpm for 10 min in a cooling centrifuge at 4°C, after removal of the cell debris; supernatant was used for the assay of superoxide dismutase (SOD) (section 3.2.9), catalase (CAT) (section 3.2.10) and glutathione peroxidase (GPx) (section 3.2.11). Protein was determined by the method of Lowry *et al.* (section 3.2.13)

5.2.3 RESULTS AND DISCUSSION :

Results of the study reveal that all the extract of *C. copticum* inhibited the inflammation induced by carrageenan, dextran and formalin (Table No. 5.2.1, 5.2.2, 5.2.3) and ulceration induced by aspirin and ethanol in a dose dependent manner (Table No. 5.2.4, 5.2.5, 5.2.6, 5.2.7)

The extract shows significant decrease in rat mucosal injury induced by aspirin and ethanol in a dose dependent manner. Administration of 80% of ethanol to 36hr fasted animals resulted in severe gastric damage visible from the outside of the stomach as thick reddish- black lines. After opening, the stomach lesions were found in the mucosa and consisted of elongated bands, 1-10mm long, usually parallel to the long axis of the stomach. They were located mostly in the corpus (the portion of the stomach secreting acid and pepsin) whereas no gross lesions were developed in the fore stomach (the non-secretary part of the stomach) (Figure No. 5.2.1, 5.2.2).

Ethanol administration to rats produced gastric damage with an ulcer index of 6.78±0.725, while the treated animals showed significant decrease in ulcer index with a percentage of inhibition of 39.97, 73.89, and 95.32 in the ranitidine, 250mg/kg, 500mg/kg groups of animals respectively. All animals treated with ethanol alone and 4 animals in ranitidine treated groups shows intraluminal bleeding in the glandular

Table No. 5.2.1 Effect of *C. copticum* on Carrageenan induced inflammation

	Initial paw thickness (mm)	Paw thickness on 3rd hour (mm)	Increase in paw thickness (mm)	% of inhibition
Control	19.33±1.63	36.83±1.47	17.5	----
Diclofenac 4mg/kg	20.0±1.41	25.83±1.04*	5.83	66.69
Diclofenac 4mg/kg + *C. copticum* 250mg/kg	19.17±1.47*	30.33±1.03*	11.16	36.23
Diclofenac 4mg/kg + *C. copticum* 500mg/kg	19.37±1.21*	26.50±1.05*	7.17	59.03

P<*0.01. Values are Mean ± SD of 6animals in each group

Table No. 5.2.2 Effect of *C. copticum* on Dextran induced inflammation

	Initial paw thickness (mm)	Paw thickness on 3rd hour (mm)	Increase in paw thickness (mm)	% of inhibition
Control	19.50±1.38	38.33±1.03	18.33	-----
Diclofenac 4mg/kg	19.17±1.47	30.16±0.75*	10.99	40.04
Diclofenac 4mg/kg + *C. copticum* 250mg/kg	19.50±1.05*	33.16±1.69	13.66	25.48
Diclofenac 4mg/kg + *C. copticum* 500mg/kg	19.17±1.07*	26.83±1.17	7.66	58.21

P< *0.01. Values are Mean ± SD of 6animals in each group

Table No. 5.2.3 Effect of *C. copticum* on Formalin induced inflammation

	Initial paw thickness (mm)	Paw thickness on 6th day (mm)	Increase in paw thickness (mm)	% of inhibition
Control	19.33±1.21	44.83±1.94	25.5	----
Diclofenac 4mg/kg	19.50±1.38	32.67±1.51*	13.17	48.35
Diclofenac 4mg/kg + *C. copticum* 250mg/kg	19.66±1.03*	34.33±2.42	14.67	42.47
Diclofenac 4mg/kg + *C. copticum* 500mg/kg	19.34±1.63	30.83±1.72	11.50	54.90

P< *0.01. Values are Mean ± SD of 6animals in each group

Table No.5.2.4 Stabilization of gastric mucosa by *C.copticum* in Ethanol induced gastric ulcer

	Normal	EtOH alone (80%)	EtOH & Ranitidine (50mg/kg b.wt)	EtOH & *C.copticum* (250mg/kg b.wt)	EtOH & *C.copticum* (500mg/kg b.wt)
Ulcer index	0	6.78± 0.72	3.27±0.50	1.77±0.37	0.37±0.41
Inhibition% of Ulcer index	100	0	51.57	73.89	95.32
Intraluminal bleeding	0	6	4	0	0

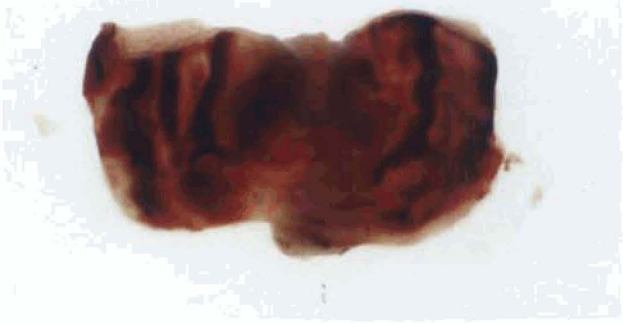
P< *0.01. Values are Mean ± SD of 6animals in each group

Table No. 5.2.5 *C.copticum* mediated cytoprotection in Ethanol induced gastric ulcer

	Normal	EtOH alone (80%)	EtOH & Ranitidine (50mg/kg b.wt)	EtOH & *C.copticum* (250mg/kg b.wt)	EtOH & *C.copticum* (500mg/kg b.wt)
SOD U/mg protein	7.22±0.54	5.0±0.49*	8.25±0.74	9.43±0.60*	10.18±0.94*
Catalase U/mg protein	28.51±1.94	18.17±1.35*	24.32±1.62*	27.47±1.57 *	27.25±1.98
Tissue Lipid Peroxidation nmol/mg	2.88±0.44	5.97±0.85*	4.13±0.54*	3.16±0.62	2.50±0.51
GSH nmol/mg	31.80±2.45	17.48±1.87	20.60±2.04	29.3±1.65*	35.07±2.16*
GPx nmol/mg	36.02±1.98	14.13±1.42	19.50±2.24	29.47±2.04	36.77±2.39
Total Protein nmol/mg	1.70±0.17	2.40±0.22*	2.37±0.24	2.42±0.14*	2.57±0.20**

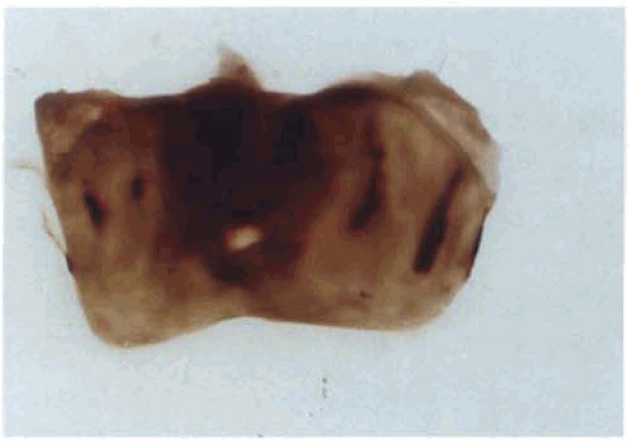
P< *0.01. Values are Mean ± SD of 6animals in each group

Effect of *C.copticum* on ethanol induced gastric ulceration (Morphology of the stomach)



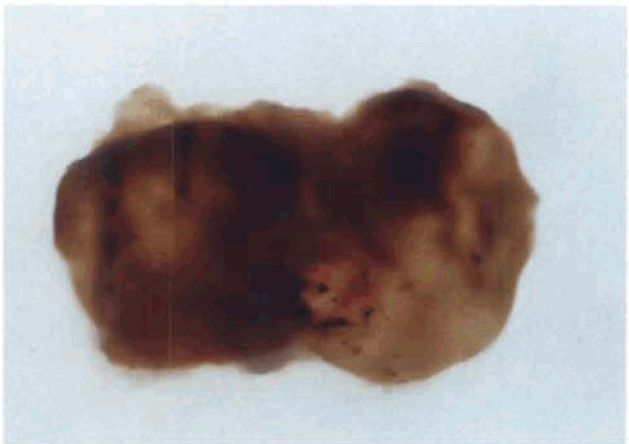
Control

Normal



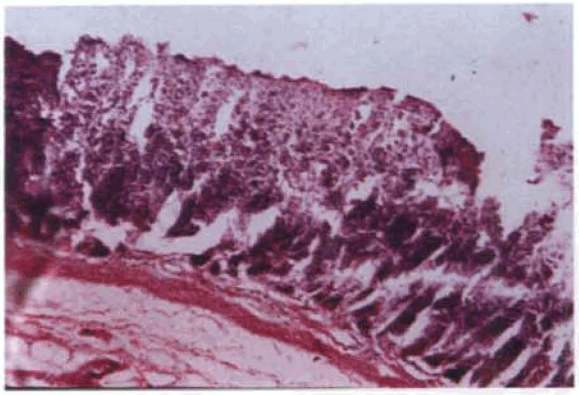
Ranitidine

***C.copticum* 250mg/Kg**



***C.copticum* 500mg/Kg**

Effect of *C.copticum* on ethanol induced gastric ulceration (Histopathological analysis of the stomach)



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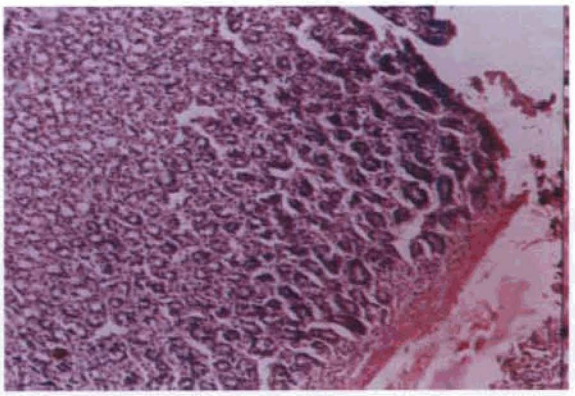
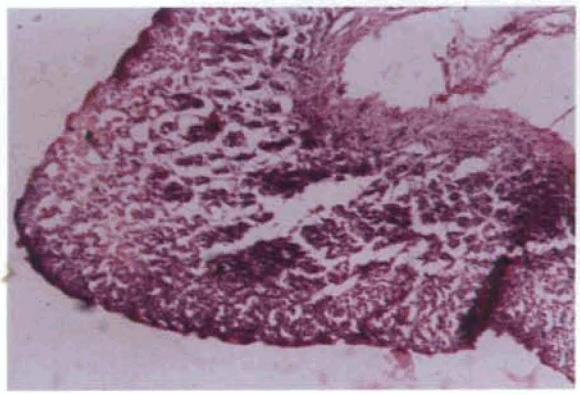
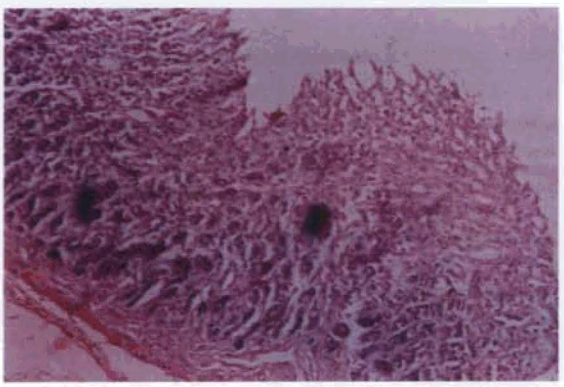


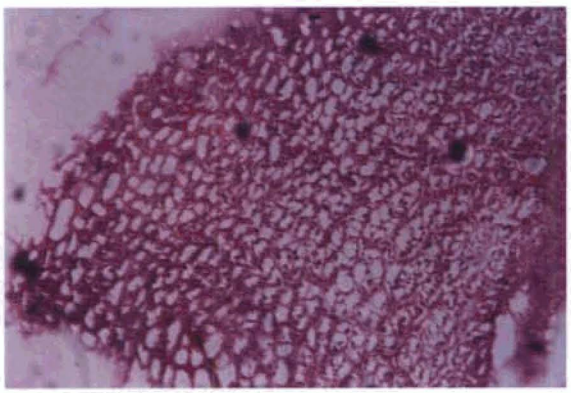
Normal



Ranitidine



***C.copticum* 250mg/Kg**



***C.copticum* 500mg/Kg**

Table No.5.2.6 Stabilization of gastric mucosa by *C.copticum* in Aspirin induced gastric ulcer

	Normal	ASP alone (400mg/kg b. wt)	ASP & Ranitidine (50mg/kg b.wt)	ASP & *C.copticum* (250mg/kg b.wt)	ASP & *C.copticum* (500mg/kg b.wt)
Ulcer index	0	4.68± 0.67	2.35±0.53	1.78±0.33	0.1±0.2
Inhibition% of Ulcer index	100	0	49.79	61.97	97.86
Intraluminal bleeding	0	6	3	0	0

P< *0.01. Values are Mean ± SD of 6animals in each group

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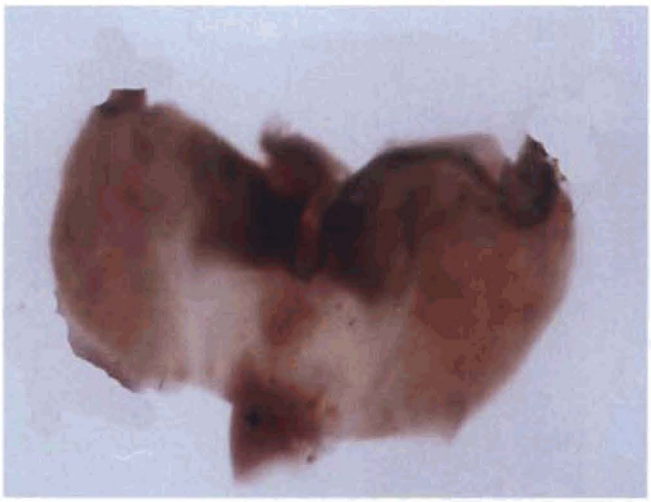
Table No. 5.2.7 *C.copticum* mediated cytoprotection in Aspirin induced gastric ulcer

	Normal	ASP alone 400mg/kg b. wt)	ASP & Ranitidine (50mg/kg b.wt)	ASP & *C.copticum* (250mg/kg b.wt)	ASP & *C.copticum* (500mg/kg b.wt)
SOD U/mg protein	7.22±0.54	5.37±0.81	6.01±0.48*	7.0±0.49*	7.90±0.43*
Tissue Lipid Peroxidation nmol/mg	2.88±0.44	4.92±0.49*	3.83±0.48*	3.25±0.48*	2.50±0.51
GSH nmol/mg	31.80±2.45	19.78±1.93	27.27±1.72*	28.37±2.01*	30.91±1.66*
GPx nmol/mg	36.02±1.98	18.55±2.08	24.20±2.09	27.78±1.96	34.78±2.41
Total Protein nmol/mg	1.70±0.17	2.32±0.14*	2.45±0.10*	2.52±0.16*	2.67±0.17*

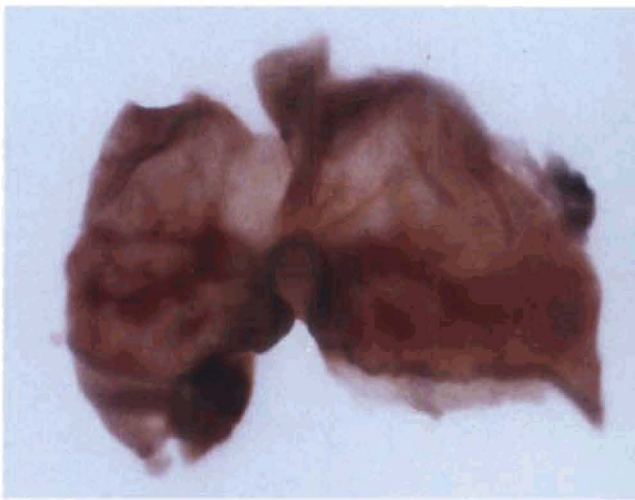
P< *0.01. Values are Mean ± SD of 6animals in each group

Figure 5.2.3

Effect of *C.copticum* on aspirin induced gastric ulcer (Morphology of the stomach)



Normal



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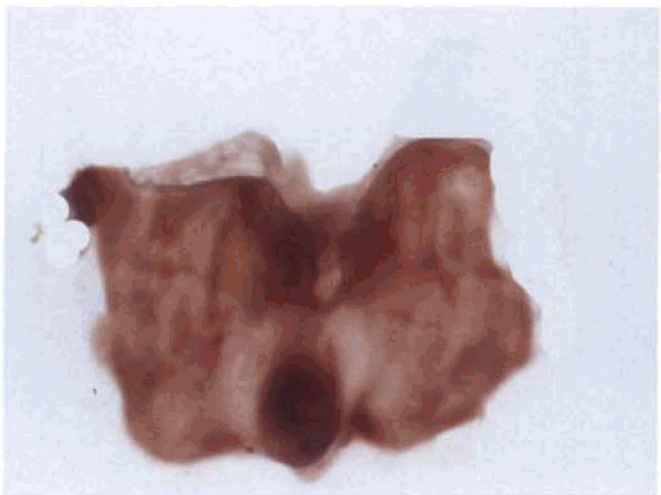
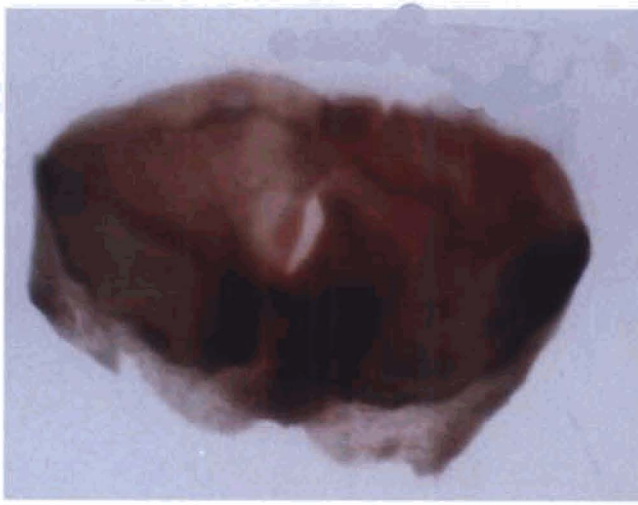
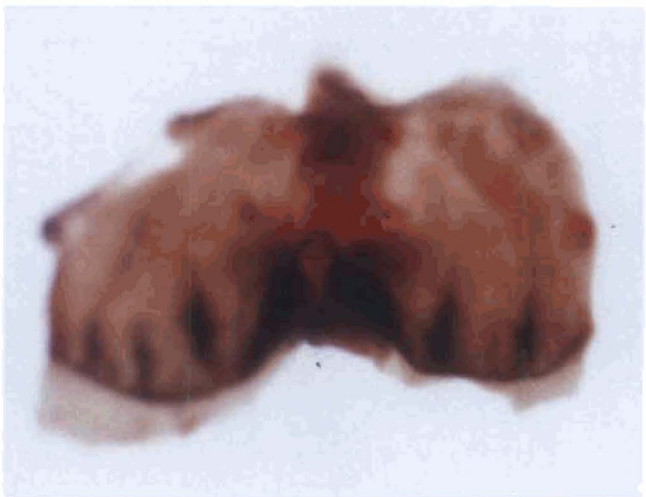


Ranitidine



***C.copticum* 250mg/Kg**

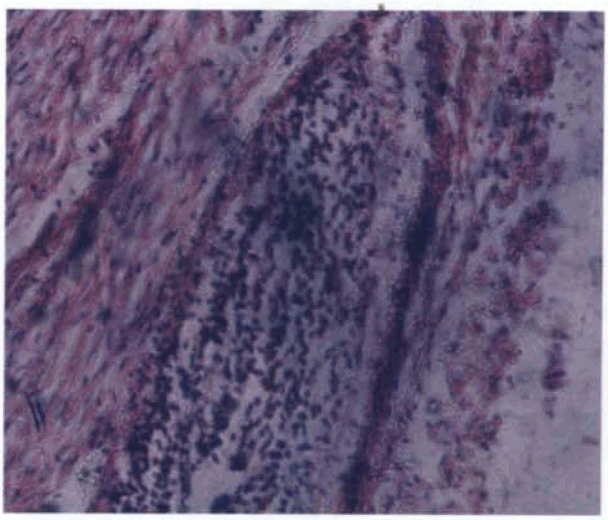


***C.copticum* 500mg/Kg**

Figure 5.2.4

Effect of *C.copticum* on aspirin induced gastric ulcer (Histopathology of the stomach)

Normal



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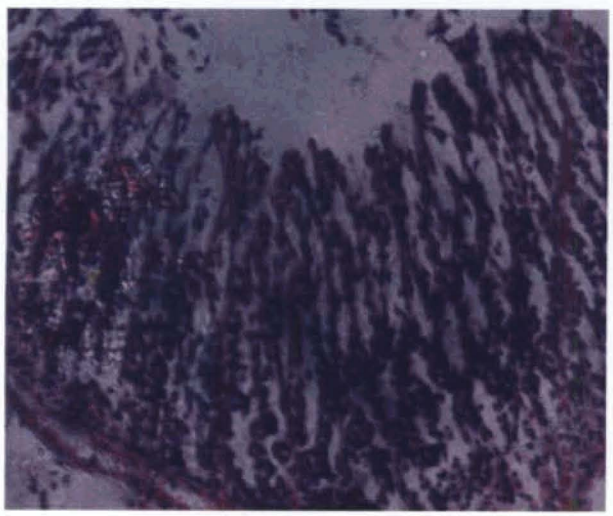
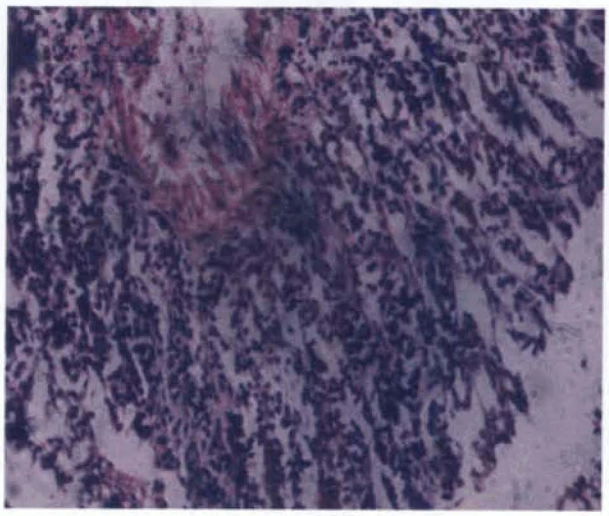
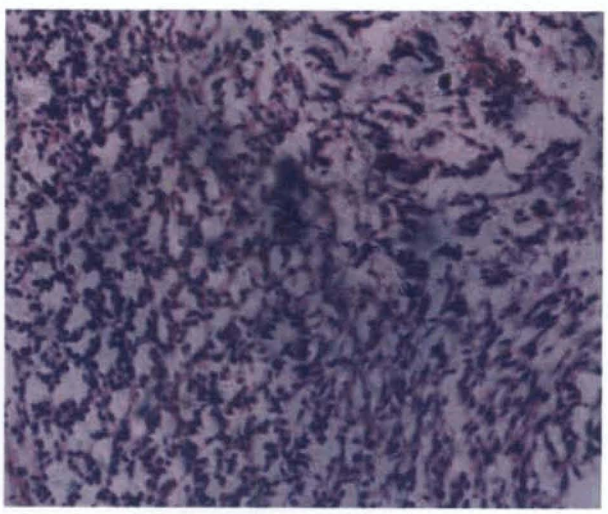


Ranitidine



***C.copticum* 250mg/Kg**



***C.copticum* 500mg/Kg**

portion of the stomach, while all animals in the *C.copticum* (250mg/kg, 500mg/kg) pretreated group were protected from intraluminal bleeding. In 500 mg/kg treated group out of six animals, the ulcer index is found in only two animals, the rest is devoid of any gastric lesions. The biochemical analysis shows that the pretreatment of the plant extract produced an increase in SOD, Catalase, GSH, GPX levels, whereas it produced a decrease in tissue lipid peroxidation.

Administration of aspirin produced severe gastric lesions in control animals whereas a significant decrease in ulcer index values were found in treated groups of animals. All the animals in the controlled group and 3 animals in ranitidine treated group showed intraluminal bleeding whereas no intraluminal bleeding is found in other animals. The *in vivo* antioxidant status of the animals also shows the cytoprotective activity of the extract. In treated groups the SOD, Catalase, Tissue lipid peroxidation, GSH, GPX levels are coming closer to the normal levels as shown in table No. 4. In 500 mg/kg treated group out of 6 animals only 2 animals shows ulcer index, the rest is devoid of any gastric lesions.

Ulceration of the stomach mainly develops in the antral region due to lesions in the gastric mucosa, when a balance between offensive and defensive factors is lost. Among various causes of gastric ulceration, lesions caused by stress (Phull et al, 1995, Yoshikawa et al, 1987), alcohol consumption (Szelenyl and Bruno, 1988, Pihan et al, 1987), *Helicobactor pylori* infection (Davies et al, 1994) and use of non-steroidal anti-inflammatory drugs (Vaananann et al, 1991, Yoshikawa et al, 1993) have been shown to be mediated largely through the generation of Reactive oxygen species (ROS). ROS decreases the levels of endogenous antioxidants such as GSH, α-tocopherol and ascorbate, and make the mucosa more prone to oxidative damage (Phull et al, 1995). Yoshikawa (Yoshikawa et al, 1989) reported suppression of gastric mucosal injury induced by ischemia-reperfusion, after administration of SOD, Catalase, indicating the role of ROS in the damage.

Ethanol consumption lowers the concentration of non-protein sulphydryls especially by glutathione (Szabo et al,1985) thereby exerting ulcerogenic effect by increasing ROS formation (Szelenyl et al 1988, Pihan et al, 1987) especially $^{\cdot}OH$

radicals. The generation of hydroxyl radicals causes lipid peroxidation which causes loss of membrane fluidity, impaired ion transport and membrane integrity and finally loss of cellular functions (Halliwell and Gutteridge, 1989, Smith et al, 1987) and results in increased gastric lesions induced by ethanol. $^{\cdot}OH$ generated in vivo not only peroxidizes membrane lipids but also oxidatively damages the critical cellular proteins, thus impairing the vital cellular functions. It is reported that, GSH with its sulphydryl groups functions in the maintenance of sulphydryls groups of other molecules (especially proteins), as a catalyst for detoxification of foreign compounds, hydrogen peroxide and free radicals (Meister et al, 1976). The present investigation shows increased levels of SOD, catalse and GSH whereas a significant decrease in lipid peroxidation levels in treated animals i.e.; the administration of the extract results in the increased levels of antioxidant enzymes to detoxify the free radicals generated during ethanol administration. The decreasing values of lipid peroxidation in treated animals show the ability of the extract to scavenge $^{\cdot}OH$ radicals and protect the mucosa against ethanol induced gastric ulceration. Studies focusing on the pathogenesis of ethanol induced gastric mucosal injury suggest that an initial event is disruption of the vascular endothelium resulting in increased vascular permeability oedema formation and epithelial lifting. In the treated group of animals a significant decrease in the ulcer index values are obtained as compared to control group and the protection of ethanol induced gastric ulceration in the present investigation suggest the involvement of cytoprotection by the *Carum copticum* methanolic extract.

Non- steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, indomethacin, ibuprofen etc, which are commonly used as pain killers in the treatment of rheumatoid arthritis and many other acute and chronic inflammatory conditions cause gastric mucosal damage (Ivey, 1988). The best studied drug, aspirin, by inhibiting prostaglandin synthesis, interferes with protective mechanism such as mucus and bicarbonate secretion, surface epithelial hydrophobicity and mucosal blood flow (Langman et al, 1991). These changes permit back diffusion of acid through the breached surfaces to destroy cells, capillaries and vein causing hemorrhagic ulcer. Enhancement of leukotriene synthesis by NSAIDs exhibits damaging effect. The changes brought about by NSAIDs, as described above, in totality can induce gastric damage through the generation of ROS (Vaananann et al, 1991) and inhibiting cell

proliferation (Vaananann et al, 1991, Yoshikawa et al, 1993). NSAIDs also inhibit gastric peroxidase and increase mucosal H_2O_2 and $^{\cdot}OH$ level to cause oxidative mucosal damage (Banerjee, 1990). This $^{\cdot}OH$ causes lipid peroxidation and increases gastric lesions induced by aspirin (Pihan et al, 1987). These lipid peroxidation causes decrease in levels of GSH in the gastric mucosa. The present study shows increasing levels of antioxidant enzymes like SOD, Catalase, GSH, GPx and decreasing levels of lipid peroxidation. These results revealed the ability of the extract to scavenge the ROS generated by aspirin administration and make the mucosa more resistant to oxidative damage. The significant inhibition in ulcer index values in the present investigation also shows the potent activity of the extract against the inhibition of prostaglandin synthesis, enhancement of leukotriene synthesis, decrease in the mucosal ATP synthesis and cell turnover process and inhibition of GPx by NSAIDs.

The histopathological studies of ethanol and aspirin induced ulceration models shows severe erosion of gastric mucosa, with necrotic patches, sub-mucosal edema and neutrophils infiltration in control animals (Figure No. 5.2.3, 5.2.4) . The control group also shows the presence of necrotic debri in the laminar proper of the mucosa infiltered with polymorpho nuclear leukocytes. The depth of the injury extends up to the muscularis with RBC extravasations. All of these symptoms were found to be normal in treated groups of animals. The protection against ulcerogenesis as manifested in significant reduction in ulcer index as well as protection to the gastric mucosal GSH, GPx and antioxidant status of ulcerated animals was clearly confirmed the cytoprotective effect of *C.copticum* 70% methanolic extract against aspirin and ethanol induced gastric ulceration.

Carrageenan induced acute inflammation in animals is one of the most suitable model to screen anti-inflammatory agents. The development of carrageenan-induced edema is biphasic; the first phase is attributed to the release of histamine, 5-hydroxytryptamin and kinins, while the second phase is related to the release of prostaglandins (Larsen and Henson, 1992; Brooks et al., 1991 and Vane and Booting, 1987). Extracts of *C.copticum* significantly inhibit the inflammation resulting in the decrease of paw edema. How ever, the anti-inflammatory activity of ethyl acetate extract was higher than the methanol and aqueous extracts. The dextran induced paw edema is known to mediate both by histamine and serotonin (Ghosh, 1963). Dextran

induces fluid accumulation because of mast cell degranulation with little protein and few neutrophills while, carrageenan induces a protein rich exudates containing large number of neutrophils (Lo et al., 1982). The maximum increase in paw thickness in the control group of animals was at 3rd hour after the carrageenan or dextran injection, hence this time period was selected for calculating the increase in paw thickness. The paw thickness is decreasing gradually in all the group of animals after 3rd hour but more effective decrease was found in the extract or diclofenac treated group of animals.

Formalin induced paw edema is one of the most suitable test procedure to screen chronic anti-inflammatory agents as it closely resembled human arthritis (Greenwald, 1991). The effect of formalin is also biphasic, an early neurogenic component followed by a later tissue mediated response (Wheeler-Aceto et al., 1991). The extracts of *C.copticum* effectively inhibited the inflammation induced by formalin. The maximum edema formation is on 6th day after the formalin injection in all the groups and decreased there after with more effectively in the extract or diclofenac treated group of animals. Treatment of extracts at 100 mg/kg body wt more effectively decreased the formalin induced edema. The result suggests the usefulness of *C.copticum* extracts in the treatment of inflammation associated with arthritis.

Oxygen free radicals and nonradical reactive oxygen intermediates released by neutrophils and other phagocytes have been increasingly implicated in inflammation/immune disorders (Ward et al., 1991). Superoxide is known to participate in the formation of chemotactic factors and recruitment of PMNs (Murakami et al., 2000). *In vitro* free radical scavenging activity of extract of *C.copticum* showed the significant superoxide radical scavenging activity. Hence, the extract can directly scavenge the superoxide anion that might inhibit the recruitment of PMNs and inflammation. Interaction between superoxide and nitric oxide radical regulates the vascular tone or inflammation. More over the coupling product of superoxide and nitric oxide enhances COX-2 activity (Landino et al., 1976), involving inflammatory process. Extracts of *C.copticum,* is a dual inhibitor of both O_2^- and $NO^\bullet$ radical, reduce the inflammation.

Antimutagenic activity of Carum copticum

5.3.1 INTRODUCTION:

The contemporary view of cancer is that a tumor arises and progress through the accumulation of serial genetic changes, including successive mutations, which involve activation of protooncogenes and inactivation of tumor suppressor genes, leading to the uncontrolled proliferation of progeny cells. Molecular genetic analysis of tumor samples suggests that the accumulation of multiple genetic changes is essential for a normal cell to progressively acquire malignant phenotypes. Somatic mutations have been detected in genes related to several forms of human diseases. Mutations in various oncogenes and tumor supperssor genes have been identified in various types of cancers (Fearon and Vogelstein, 1990). Mouse skin is among the first models in which carcinogen-specific oncogene mutation patterns were demonstrated. Point mutations of K-*ras*, *p53* and *APC* were most commonly found in human tumors (Wright and Williams, 1993; Tahara, 1990 and Bos, 1989). Mutations of K-*ras* and *APC* can occur in early lesions, while alterations of *p53* and *DCC* often occur in advanced tumors (Mao and Sidransky, 1994). Average spontaeneous mutation frequencies per base pair in human cells are estimated to be in the range of 10^{-8} to 10^{-10} and increase 10–1000 fold upon exposure to a mutagen. Chemicals and radiation are known to induce mutations. Damage to DNA by oxygen free radicals is frequently postulated to cause mutations that are associated with the initiation and progression of human cancers. As mutations are one of the important factors contributing to oncogenesis, discovery of natural antimutagenic substances is a promising step to select the cancer chemopreventive agents.

5.3.2 MATERIALS AND METHODS

5.3.2.1 Determination of antimutagenic activity

Antimutagenic activity was determined by the method of Ames (1983) using *Salmonella typhimurium* strains.

5.3.2.1.1 Principle

The test measures the reverse mutation from histidine auxotrophy to prototrophy in several specially constructed mutants of *S. typhymurium*. The compounds to be tested is mixed with mutagen and bacterial strain and incubated in histidine deficient medium for 48 h at 37°C. All bacteria those who have reverted back to wild type will grow as colonies. The antimutagenic activity is determined as the decrease in number of colonies after comparing the reversion in the plate of mutagen alone.

5.3.2.1.2 Reagents

1. 0.5 mM histidine/ biotin solution- dissolved 12.36 mg of biotin in 100 ml of hot distilled water. 9.6 mg of histidine is added to the solution after cooling. The solution autoclaved at 121°C for 20 min.
2. Spizizen's salt solution (10x) – 0.2 g of Mg Cl_2 7 H_2O, 1 g trisodium citrate, 14 g of anhydrous K_2HPO_4, 6 g KH_2PO_4 and 2 g of $(NH_4)_2$ SO_4 were dissolved in 70 ml of distilled water and made up to 100 ml. Autoclaved the solution at 121°C for 20 min.
3. 40 % glucose- 40 g of glucose was dissolved in 100 ml of distilled water and autoclaved at 121°C for 20 min.
4. Top agar- 600 mg of agar and 500 mg of NaCl were dissolved in 100 ml of distilled water and 2 ml of this was poured into test tubes and autoclaved at 121°C for 20 min. Before pouring the top agar onto minimal agar 0.2 ml of the sterilized histidine/biotin solution was added to each tube.
5. Minimal agar plates-1.5 g of agar was dissolved in 85 ml of distilled water. The solution was autoclaved at 121°C for 20 min. After the sterilization, 10 ml of sterilized Spizizen's salt (10x) and 5 ml of 40 % glucose were added. 20 ml of this solution was poured into sterile petri plates under sterile condition.

Confirming genotype of Salmonella typhimurium strains

Genotype of the *Salmonella strains* was evaluated by the method of Maron and Ames (1983).

5.3.2.1.3 Histidine requirement

Histidine requirement of the tester strains TA 98 and TA 100 were confirmed by streaking across the histidine/biotin plate and across the biotin control plate. The

plates were incubated overnight at 37°C and examined for growth on the histidine/biotin plates.

All the tester strains showed growth on the histidine/biotin plate and no growth on the control (biotin alone) plate indicate the histidine requirement of the strains.

5.3.2.1.4 rfa mutation

Strains having *rfa* mutation was tested using crystal violet sensitivity test. 0.1 ml of each tester strains TA 98 and TA 100 was mixed with 2 ml of molten agar at 45°C and poured on nutrient agar plate. The plates were tilted and rotated to distribute the top agar evenly. Sterile filter paper disc, (8 mm diameter) containing 10 µl of a 1mg/ml crystal violet, transferred to the strain seeded plates. After 12 h incubation measured the zone of inhibition.

The zone of inhibition was approximately 13 mm appears around the crystal violet disc indicating the presence of rfa mutation in all the tester strains.

5.3.2.1.5 uvrB mutation

The uvrB mutation was tested by UV sensitivity test. With a sterile cotton swabs, streak the tester strains TA 98, and TA 100 were standard across a nutrient plate, in parallel strips.. A piece of cardboard was placed over the uncovered the plate so that half of each bacterial streak was covered. Irradiate the plate with a 15 W germicidal lamp at a distance of 33 cm. TA 98 and TA 100 were irradiated for 8 sec. The irradiated plates incubated at 37°C for 12-24 h. Strains with uvrB deletion will grow only on the un-irradiated side of the plate.

TA 98 and TA 100 showed growth only on the un-irradiated side of the plate which indicated the absence of excision repair enzymes.

5.3.2.1.6 R-factor

R-factor was tested by ampicillin resistance on ampicillin plate. R-factor of the tester strains TA 98 and TA 100was confirmed by streaking across the surface of ampicillin plate. The plates were incubated overnight at 37°C and examined for growth.

TA 98 and TA 100 strains showed growth in the ampicillin plate indicated the ampicillin resistance.

5.3.2.2 Antimutagenic assay *C. copticum*

Antimutagenic activity was determined by the method of Maron and Ames (1983). NaN_3 (2 μg/plate), MNNG (1 μg/plate), doxorubicin (10 μg/plate), and NPDA (20 μg/plate) were used as mutagens.

Freshly grown overnight cultures of *Salmonella* tester strains (TA 98 and TA 100) in nutrient broth (0.1 ml, approximately 10^9 bacterial cells/ml) were mixed with 0.1 ml of various concentrations of extract of *C.copticum* (1, 2 and 4mg/plate), 0.2 ml of 0.5 mM histidine/biotin solution and 0.01 ml of mutagen in 2 ml of molten top agar at 45°C. The mixture was poured onto minimal glucose agar plate (section 3.2.30) and incubated for 48 h at 37°C. After the incubation period, number of revertants per plate were counted using a colony counter.

All the experiments were repeated twice in triplicate. The percent inhibition was calculated using the formula $\{(1-(R_2-SR)/ (R_1-SR)\}$ X100, where R_1 is the average number of revertants in the presence of mutagen alone, R_2 the average number of revertants in the presence of mutagen plus extract and SR is average number of revertants in the plate without extract or mutagen (spontaneous revertants). To evaluate the toxicity of the extract plates without mutagens but with extract, (2 5 mg/plate *C.copticum* extract) treated in the similar manner was employed.

5.3.3 RESULTS AND DISCUSSION

70% methanolic extract of *C. copticum* showed significant inhibition of mutagenicity induced mutagens (NaN_3, MNNG, doxorubicin, and NPDA) (Table No.5.3.1, 5.3.2, 5.3.3) The antimutagenic activity of the extract against mutagens probably may be due to the direct inactivation of the mutagens. MNNG induces a wide spectrum of mutations by alkylating purines and pyrimidines. The major adduct reported was O^6-methylguanine. Among the antimutagenic activity tested extract showed maximum activity against the doxorubicin-induced mutation of TA 98 and TA 100. Doxorubicin and related quinoines are capable of undergoing an electron

Table No. 5.3.1 Antimutagenic activity of *C. copticum* against NaN_3 and MNNG

Groups	NaN_3		MNNG	
	No. of colonies/plate	% of inhibition	No. of colonies/plate	% of inhibition
Control	795.0±60.5	----	652.0±32.5	----
***C.copticum* 1mg/plate**	693.0±37.5*	12.45	527.0±11.0*	19.17
***C.copticum* 2mg/plate**	522.0±26.0*	34.33	421.0±15.5*	35.42
***C.copticum* 4mg/plate**	309.0±15.3*	61.13	265.0±19.6*	59.35

P< *0.01. Values are Mean ± SD of 4 plates in each group

Table No. 5.3.2 Antimutagenic activity of *C. copticum* against NPDA

Groups	NPDA			
	TA 98 No. of colonies/plate	TA 98 % of inhibition	TA100 No. of colonies/plate	TA 100 % of inhibition
Control	2054±87.0	----	137.0±5.0	----
C.copticum 1mg/plate	1799±46.6*	12.41	120±6.5	12.41
C.copticum 2mg/plate	1285±48.5*	37.43	92±6.0	32.85
C.copticum 4mg/plate	935±43.2*	54.48	56±5.6	59.12

P< *0.01. Values are Mean ± SD of 4 plates in each group

Table No. 5.3.3 Antimutagenic activity of *C. copticum* against Doxorubicin

Groups	Doxorubicin			
	TA 98 No. of colonies/plate	**TA 98 % of inhibition**	**TA100 No. of colonies/plate**	**TA 100 % of inhibition**
Control	214.0±17.5	----	263.0±6.5	----
***C.copticum* 1mg/plate**	168.0±15.5	21.49	231.0±7.6	12.17
***C.copticum* 2mg/plate**	134.0±8.0*	37.38	167.0±4.5*	36.50
***C.copticum* 4mg/plate**	82.0±5.6*	61.68	118.0±7.0	55.13

P< *0.01. Values are Mean ± SD of 4 plates in each group

reduction and generation of free radical species including activated oxygen species. These free radical species may be involved in the reverse mutation of the *Salmonella* tester strains. *C. copticum* extract may possibly also interfere in the intercalation of doxorubicin to DNA or scavenge the generated free radical.

Chemoprotective activity of Carum copticum against Cyclophosphamide induced toxicity

5.4.1 INTRODUCTION:

The era of modern chemotherapy can be considered to have began in 1948 with the introduction of nitrogen mustard (Chabner et al, 1996). In the past three decades, chemotherapy has evolved as one of the major therapeutic disciplines of clinical homology. (Benham et al, 1983). Anti neoplastic agents used in chemotherapy act by interfering with essential molecular process in the cancer cell. Most of the drugs exert their effect by modifying or inhibiting various cellular biochemical processes, thereby inhibiting metabolic or replicative mechanisms (Heskel, 1990). Eventhough it is the most commonly used method, which often produces numerous toxic side effects. The chemotherapeutic drugs currently available are highly toxic and hence the drug dose which can be used for cancer therapy is limited by normal tissue tolerance. Tissue injuries in target and non-target cells, especially cells of immune system, represent one of the factors limiting the administration of chemotherapeutic drugs in cancer treatment (Tannock et al, 1989).

Minimizing the damage to normal tissues caused by chemotherapy has been instigated developments of methods to improve the therapeutic index (Uma devi, 1998). Several agents like WR-2721 (Kemp et al, 1996), N-acetyl cysteine, mercaptoethane sulfonate (MESNA) (Khojasteh et al, 2000) and mercaptoproponyl glycine have been tried as chemoprotecting agents but toxicity produced after repeated administration limited their clinical significance.

The present study investigated the chemoprotective activity of C. copticum methanolic extract against Cyclophosphamide (CTX) induced toxicities.

5.4.2 MATERIALS AND METHODS

Female BALB/c mice were used for the present study. The animals were divided into 4groups of 16 animals in each group. The treatment schedule was as follows

Group I - Normal
Gropup II- CTX 25mg/kg (ip upto 10 days)
Group III- CTX 25mg/kg +*C. copticum* 250mg/kg (oral, daily)
Group IV- CTX 25mg/kg+ *C. copticum* 500mg/kg (oral, daily)

The TC (section 3.2.26) and bone marrow cellularity (section 3.2.27) were calculated at 5, 10, 15 and 30 days. The animals were sacrificed at 5, 10, 15 and 30 days and biochemical parameters such as SOD, CAT, GSH, GPx and tissue lipid peroxidation were estimated.

5.4.2.1 BIOCHEMICAL ANALYSIS

Evaluation of antioxidant enzymes and GSH in Tissue

Liver and Kidney was removed and washed thoroughly in ice-cold saline and homogenate (10 %) was prepared in PBS (50 mM, pH 7.0) (section 3.2.2). A part of the homogenate was used for the estimation of reduced glutathione (GSH) (section 3.2.8) and tissue lipid peroxidation (3.2.12). The remaining homogenate was centrifuged at 10,000 rpm for 10 min in a cooling centrifuge at 4°C, after removal of the cell debris; supernatant was used for the assay of superoxide dismutase (SOD) (section 3.2.9), catalase (CAT) (section 3.2.10) and glutathione peroxidase (GPx) (section 3.2.11).

5.4.2.3 Evaluation of antioxidants enzymes and GSH in blood

Non-coagulated (heparin) blood was used for the determination of antioxidant status. SOD (section 3.2.21), CAT (section 3.2.22), GSH (section 3.2.23), and GPx (section 3.2.24) were estimated in erythrocyte lysate. Haemoglobin was determined using Drabkin's reagent (section 3.2.25).

5.4.3 RESULTS AND DISCUSSION

The present investigation showed the chemoprotective activity of the 70% methanolic extract of *C. copticum* against CTX induced toxicity. The main side effects of CTX include myelosuppression, alopecia etc. The control groups of animals (CTX alone) showed a decrease in TC and Bone marrow cellularity (Figure No. 5.4.14, 5.4.15; $p<0.01$) levels whereas the *C. copticum* extract treated groups of animals showed an increase in TC and Bone marrow cellularity levels. The in vivo antioxidant status of the animals were carried out in blood, liver and kidney. The antioxidant status of the control animals showed a decrease in the levels of SOD, CAT, GSH and GPx whereas an increase in *C. copticum* treated groups (Figure No. 5.4.3-5.4.14; $p<0.01$). The tissue lipid peroxidation in control animals is very high compared to the *C. copticum* extract treated groups of animals.

Alkylating agents were among the first compounds identified to be useful in cancer chemotherapy. All the alkylating agents have a common property of dissociating a positive charged, electrophilic alkyl group capable of attacking negatively charged electron rich, nucleophilic sites on most of the biological molecules. The chemotherapeutic usefulness derives from their ability to form a variety of DNA adducts that sufficiently alter DNA structure or function or both so as to have a cytotoxic effect. Many of them undergo a very complex activation process before it can generate reactive intermediates. Initial activation reaction of CTX carried out by microsomal oxidation system in liver produces 4-hydroxy CTX, a cytotoxic metabolite, which diffuses from hepatocytes into plasma and distributed throughout the body. 4-hydroxy CTX is then further converted to some other cytotoxic metabolites and acrolein and phosphoramide mustard are among them (Berger, 1993; Grochow, 1996). Phosphoramide mustard is known to cause myelosuppression. In the present study the myelosuppression caused by CTX is effectively prevented by *C. copticum* seed extract. The Extracts treated groups also showed enhanced levels of bone marrow cellularity, which indicate that *C. copticum* stimulate the haemaotopoitic system.

The metabolism of CTX in the body produces highly reactive electrophiles and the decreased value of GSH in CTX treated group is probably due to electrophilic burden on the cells and also due to and also due to the formation of acrolein, which is known to deplete GSH content and DNA alkylation (McDiarmed et al, 1991).

Fig No. 5.4.1 Effect of *C.copticum* on CTX induced toxicity (Blood SOD level)

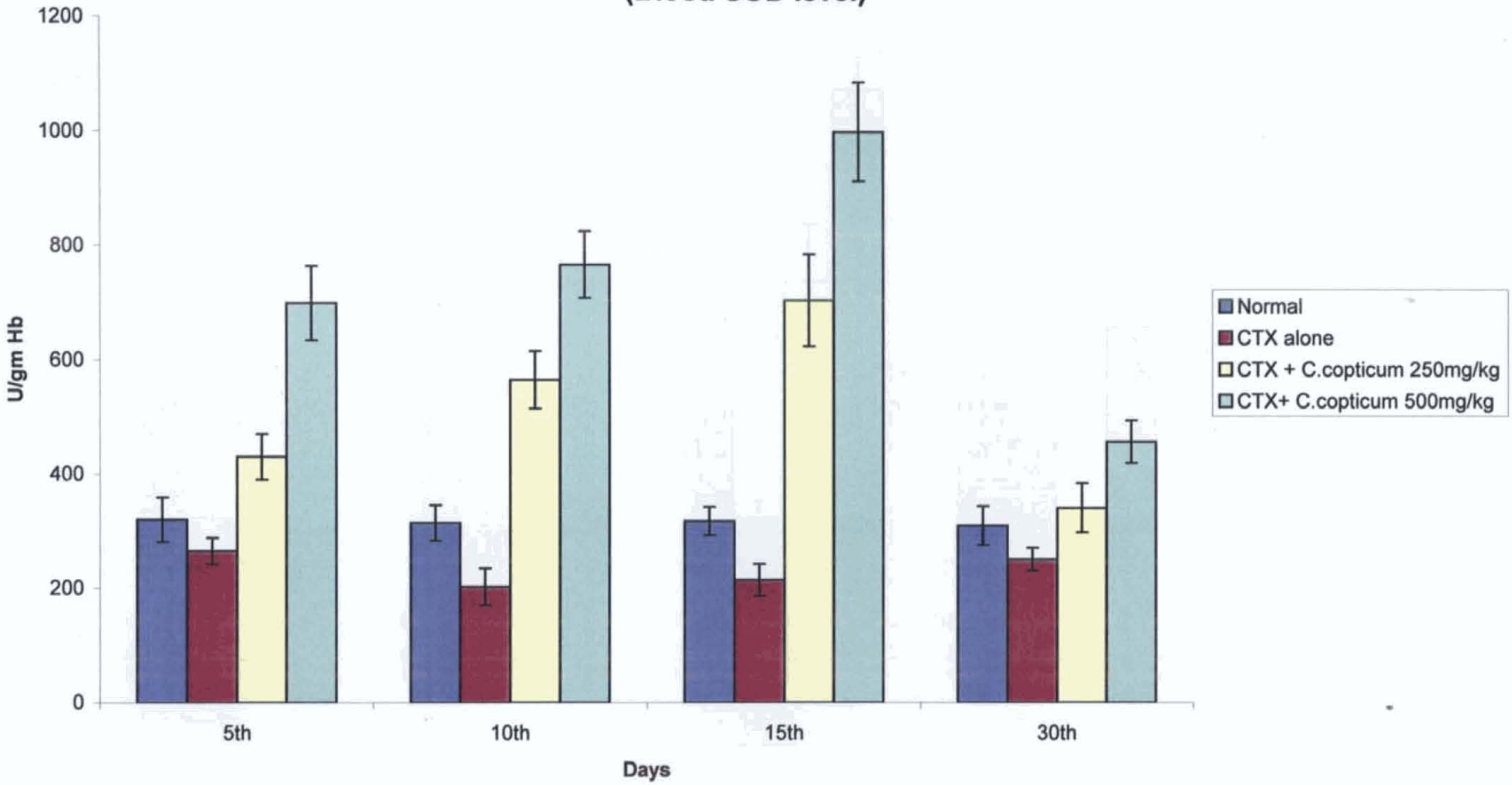


Fig No. 5.4.2 Effect of *C.copticum* on CTX induced toxicity (Liver SOD level)

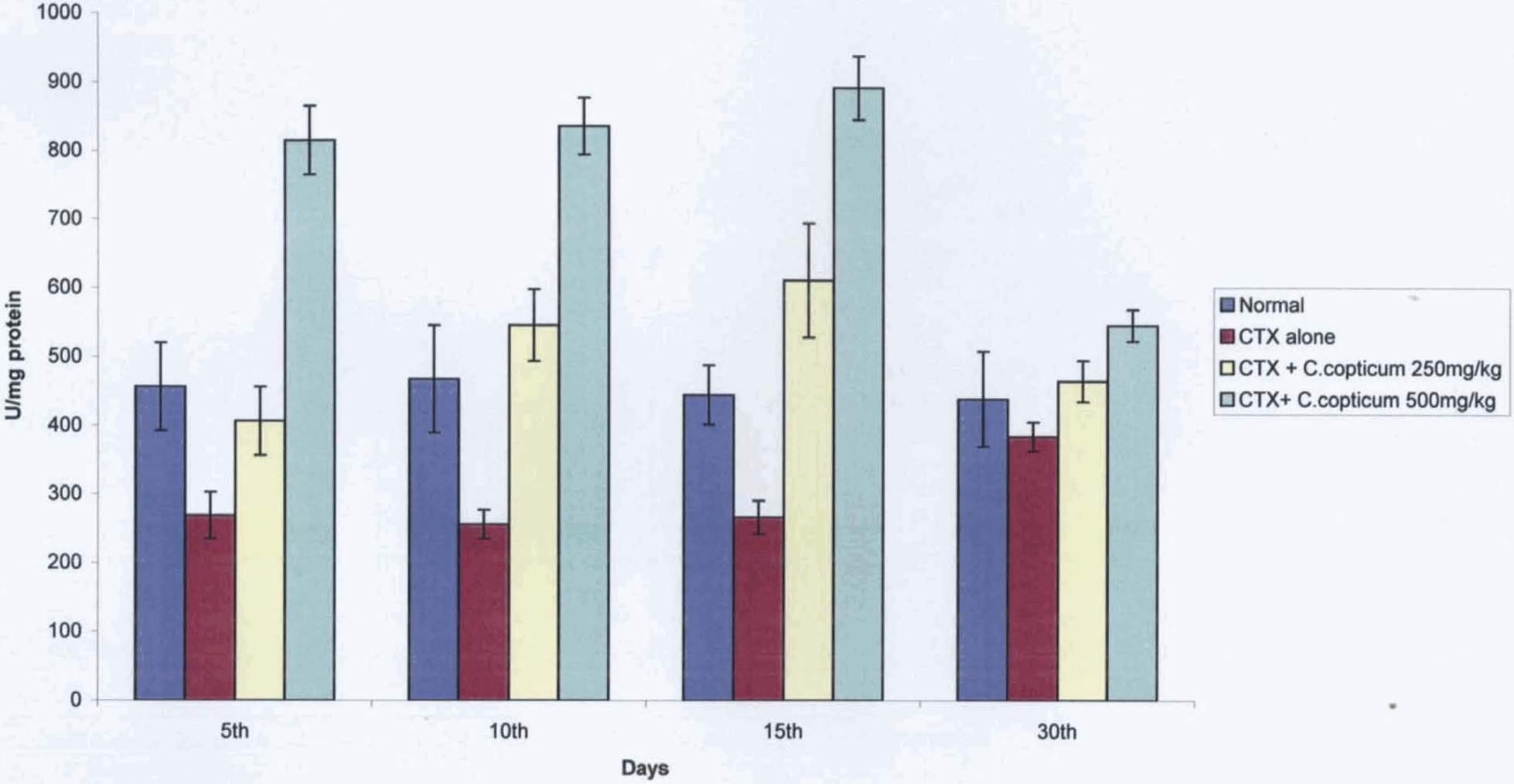


Fig No. 5.4.3 Effect of *C.copticum* on CTX induced toxicity (Kidney SOD level)

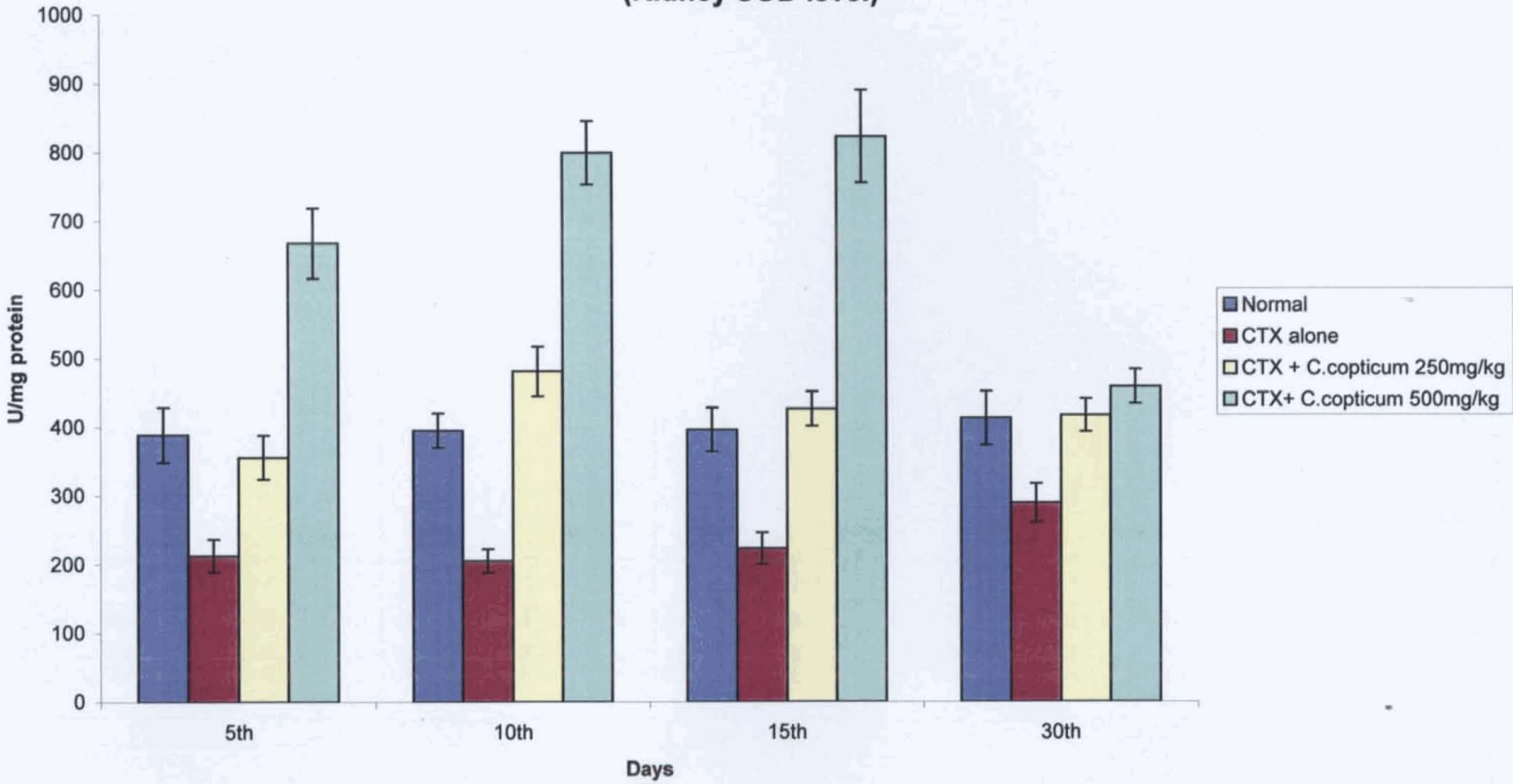


Fig No. 5.4.4 Effect of *C.copticum* on CTX induced toxicity (Blood CAT level)

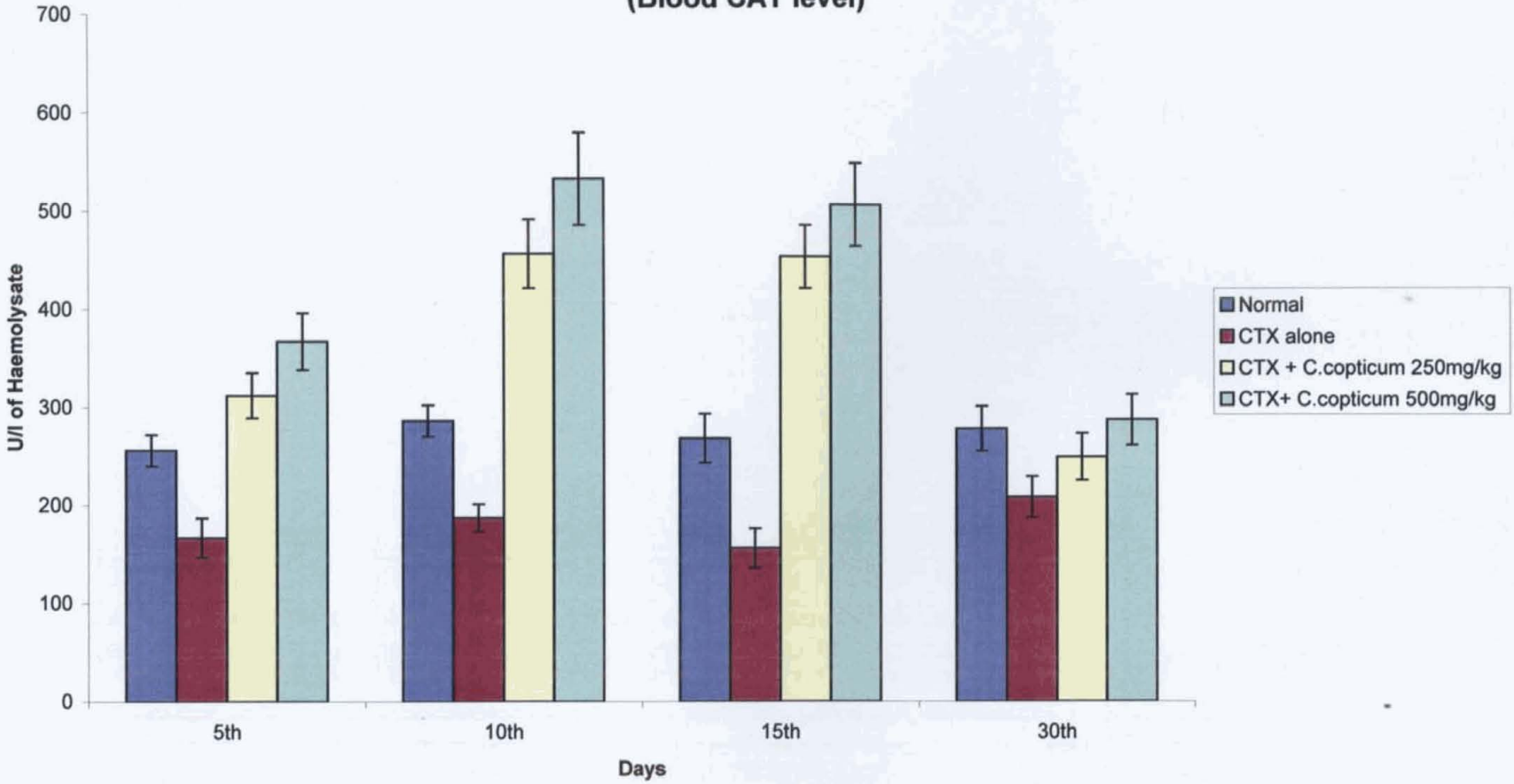


Fig No. 5.4.5 Effect of *C.copticum* on CTX induced toxicity (Liver CAT level)

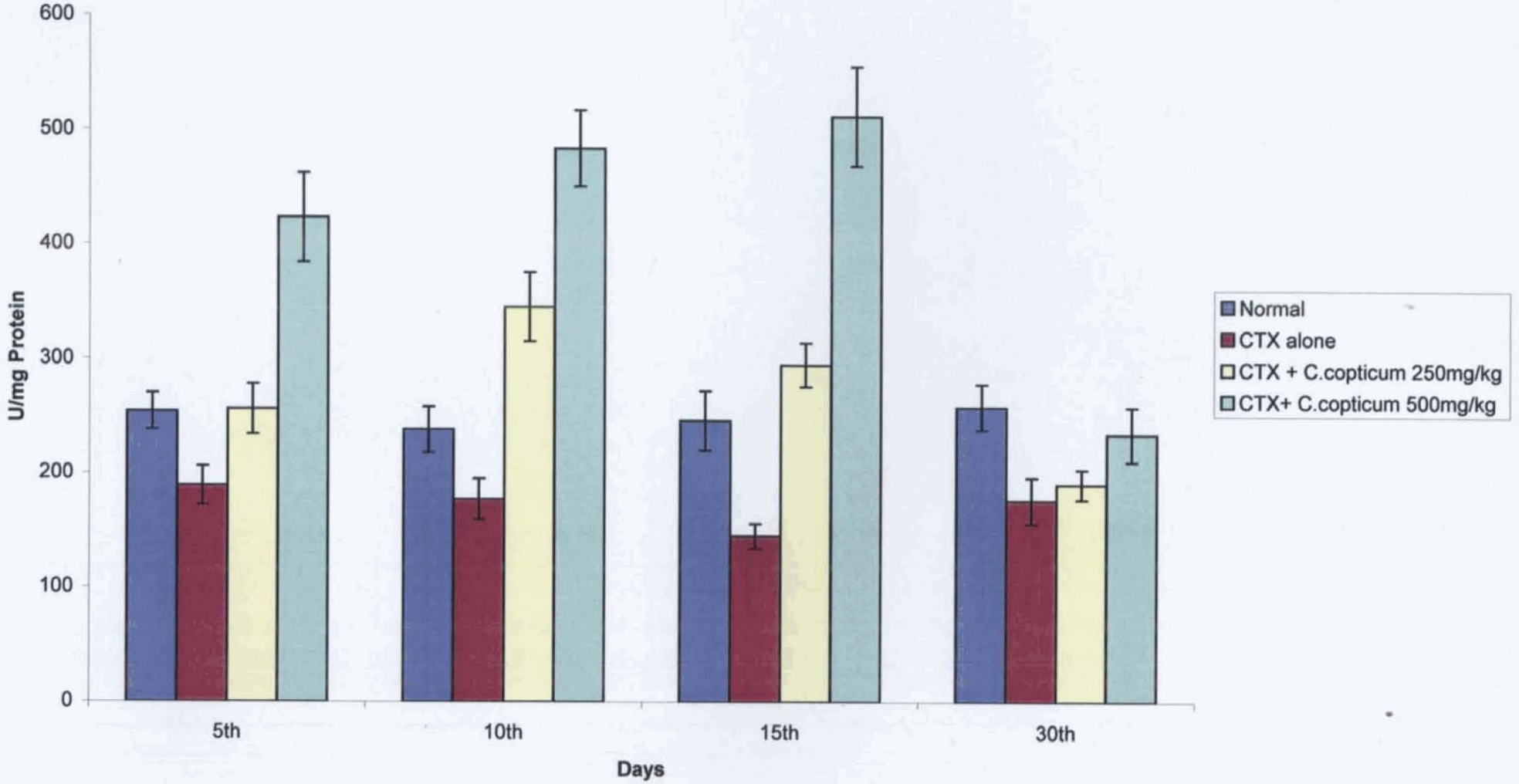


Fig No. 5.4.6 Effect of *C.copticum* on CTX induced toxicity (Kidney CAT level)

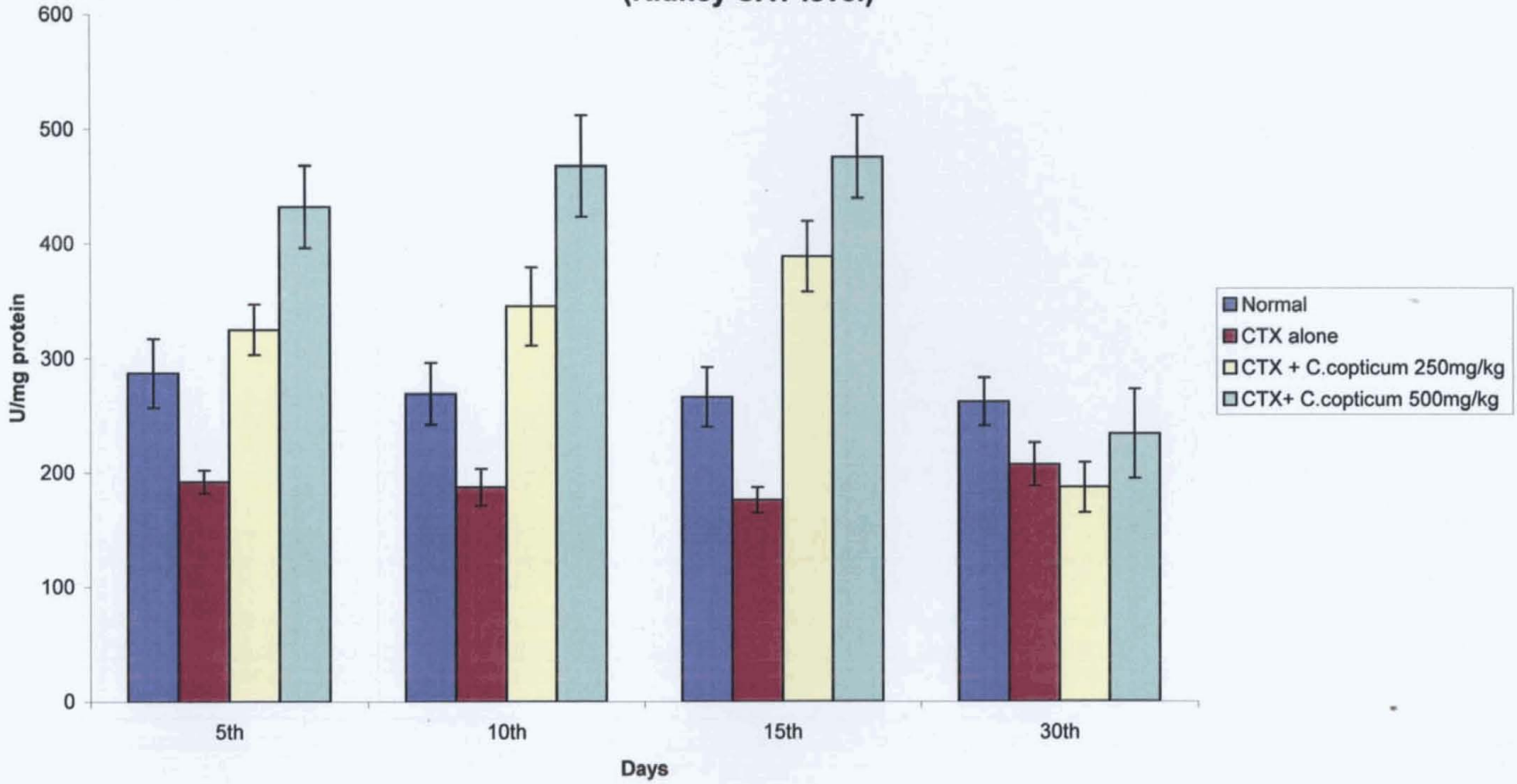


Fig No. 5.4.7 Effect of *C.copticum* on CTX induced toxiocity (Blood GSH level)

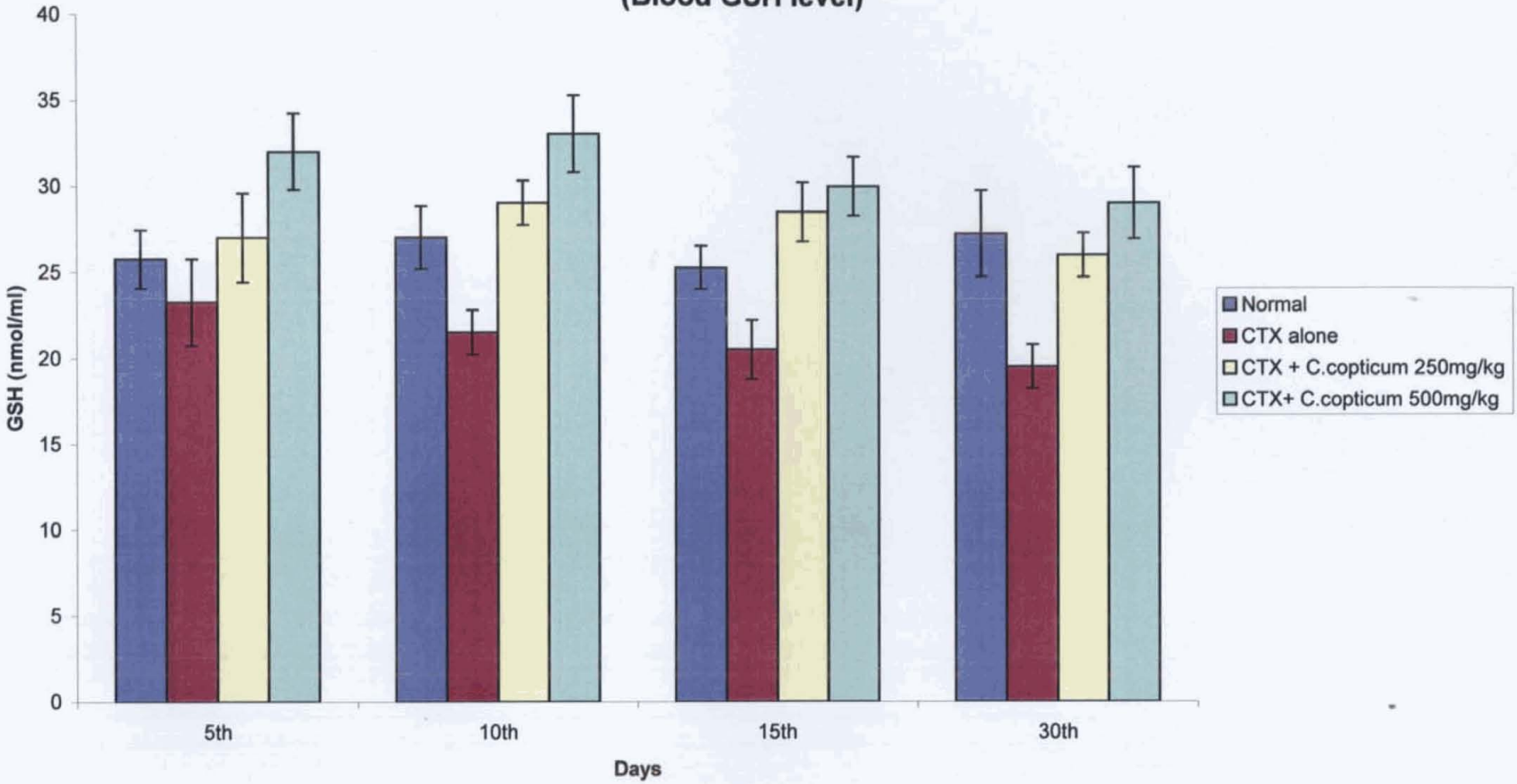


Fig No. 5.4.8 Effect of *C.copticum* on CTX induced Toxicity (Liver GSH level)

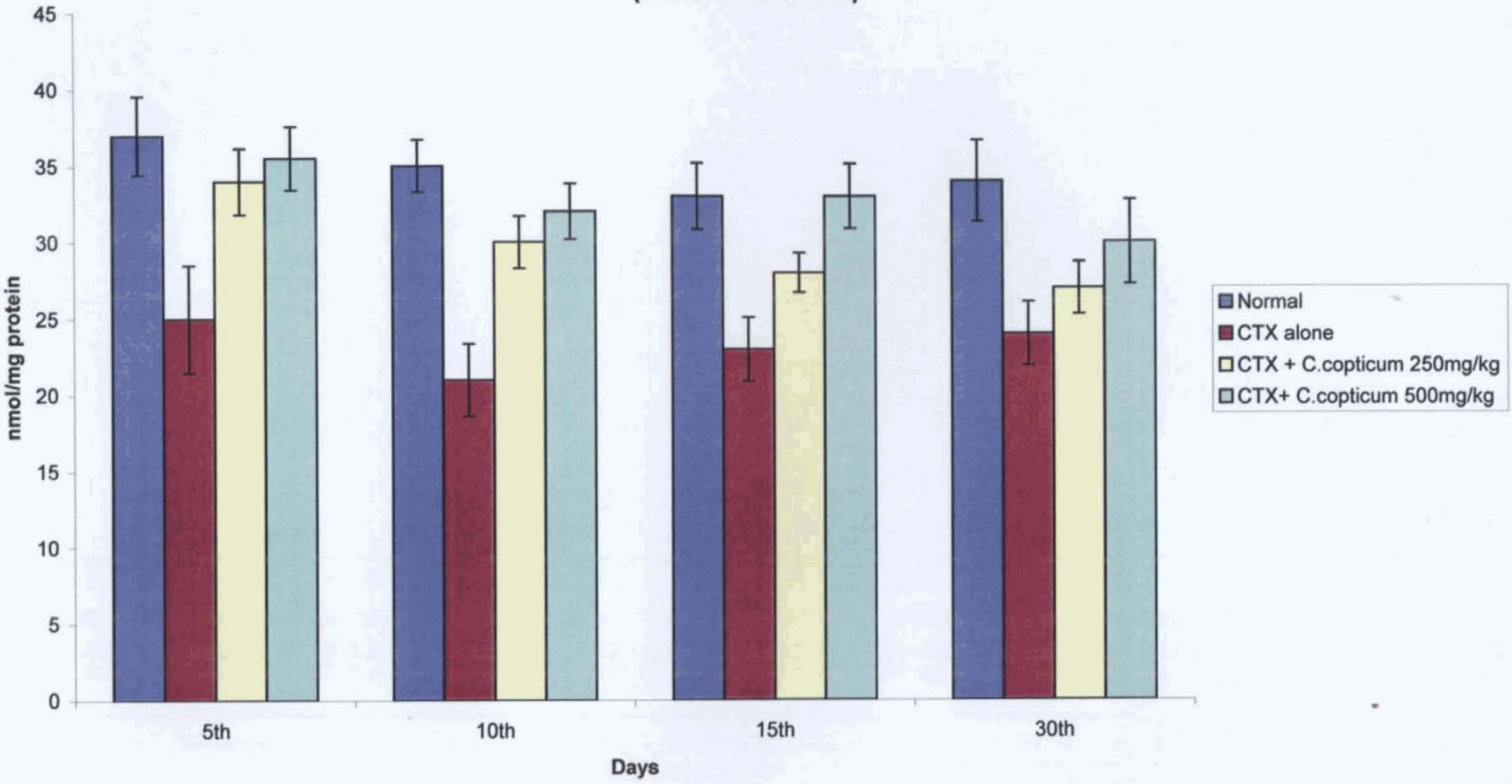


Fig No. 5.4.9 Effect of *C.copticum* on CTX induced toxicity (Kidney GSH level)

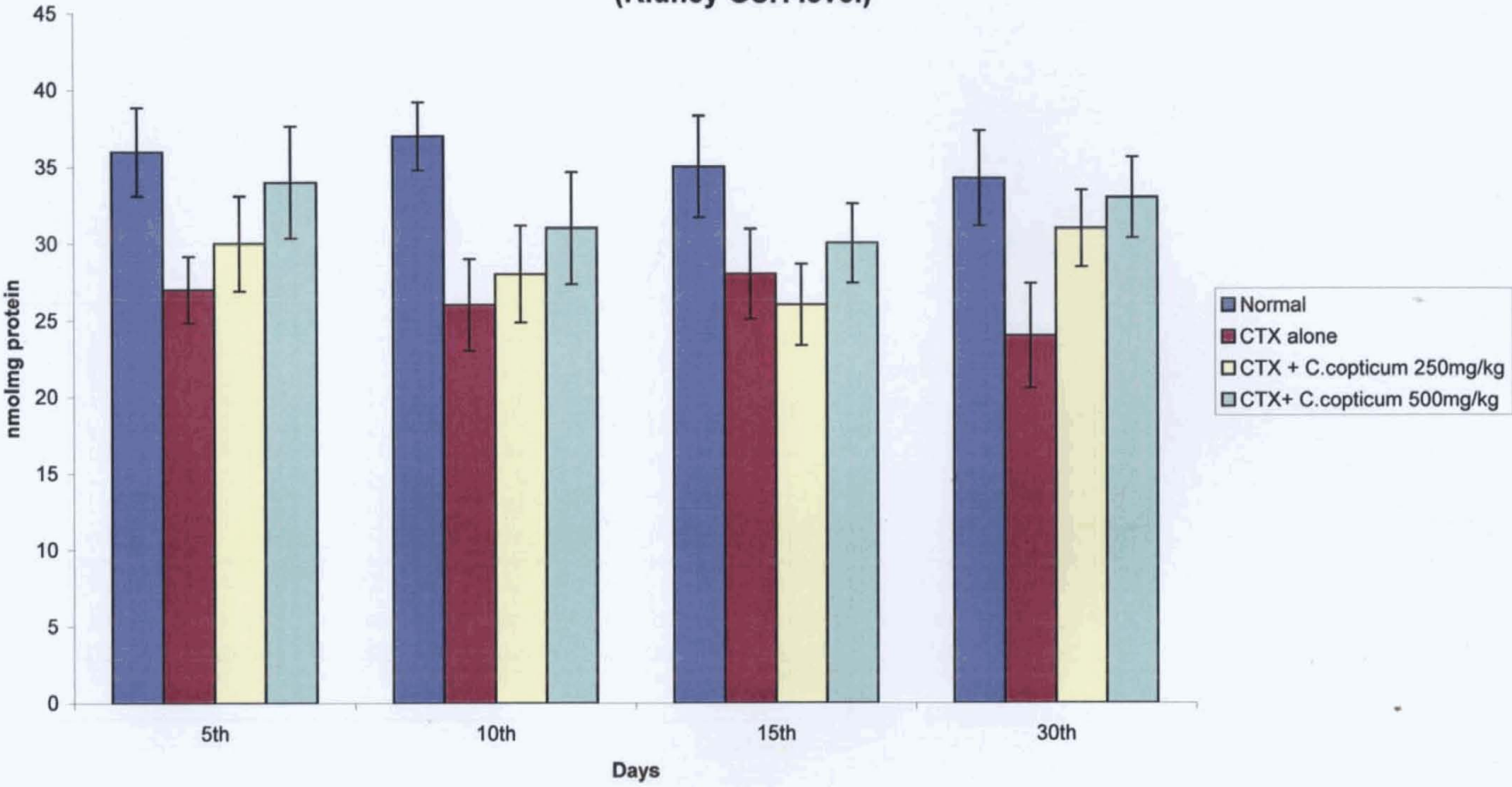


Fig No. 5.4.10 Effect of *C.copticum* on CTX induced damage (Blood GPX level)

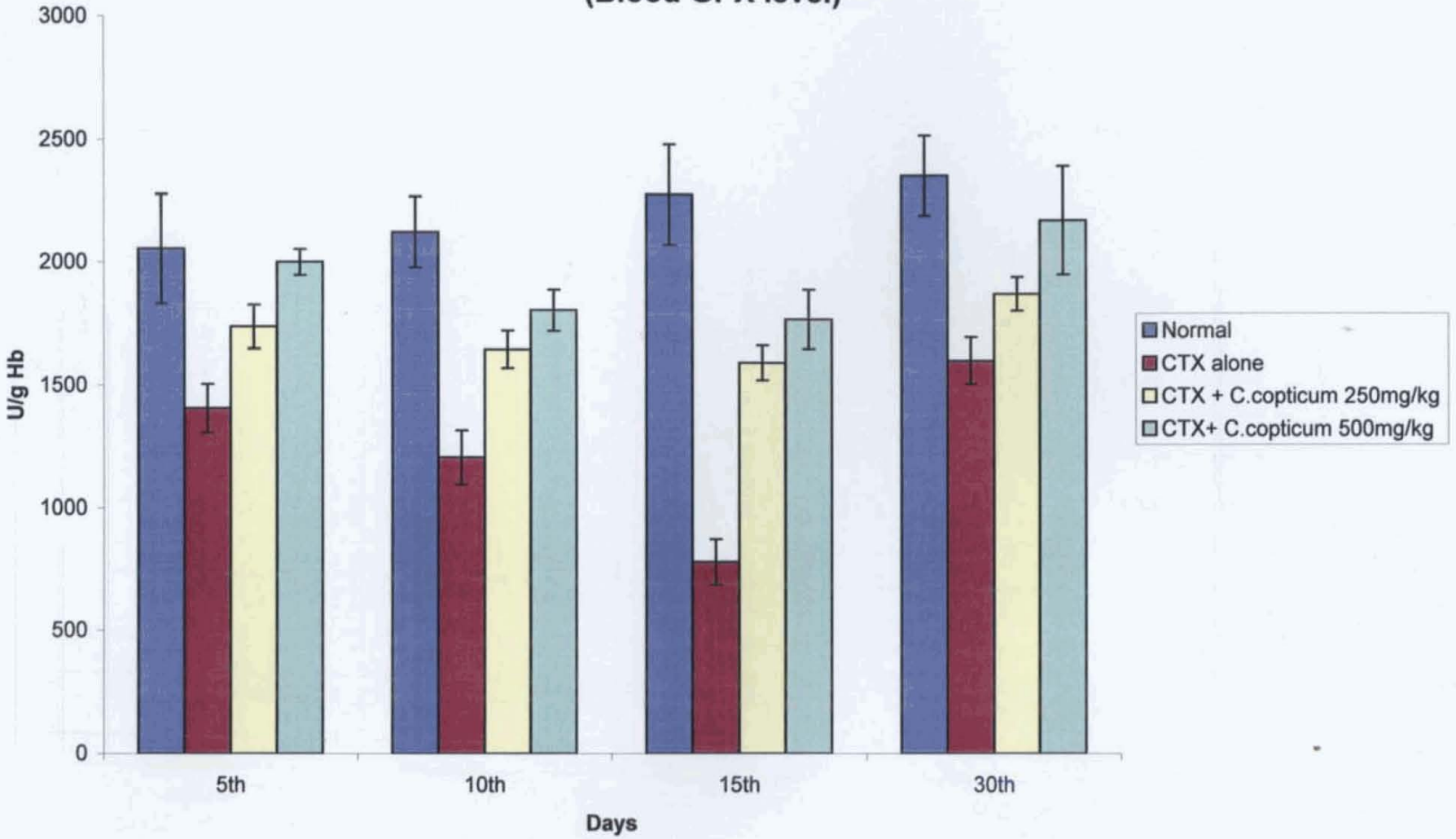


Fig No. 5.4.11 Effect of *C. copticum* on CTX induced toxicity (Liver GPx level)

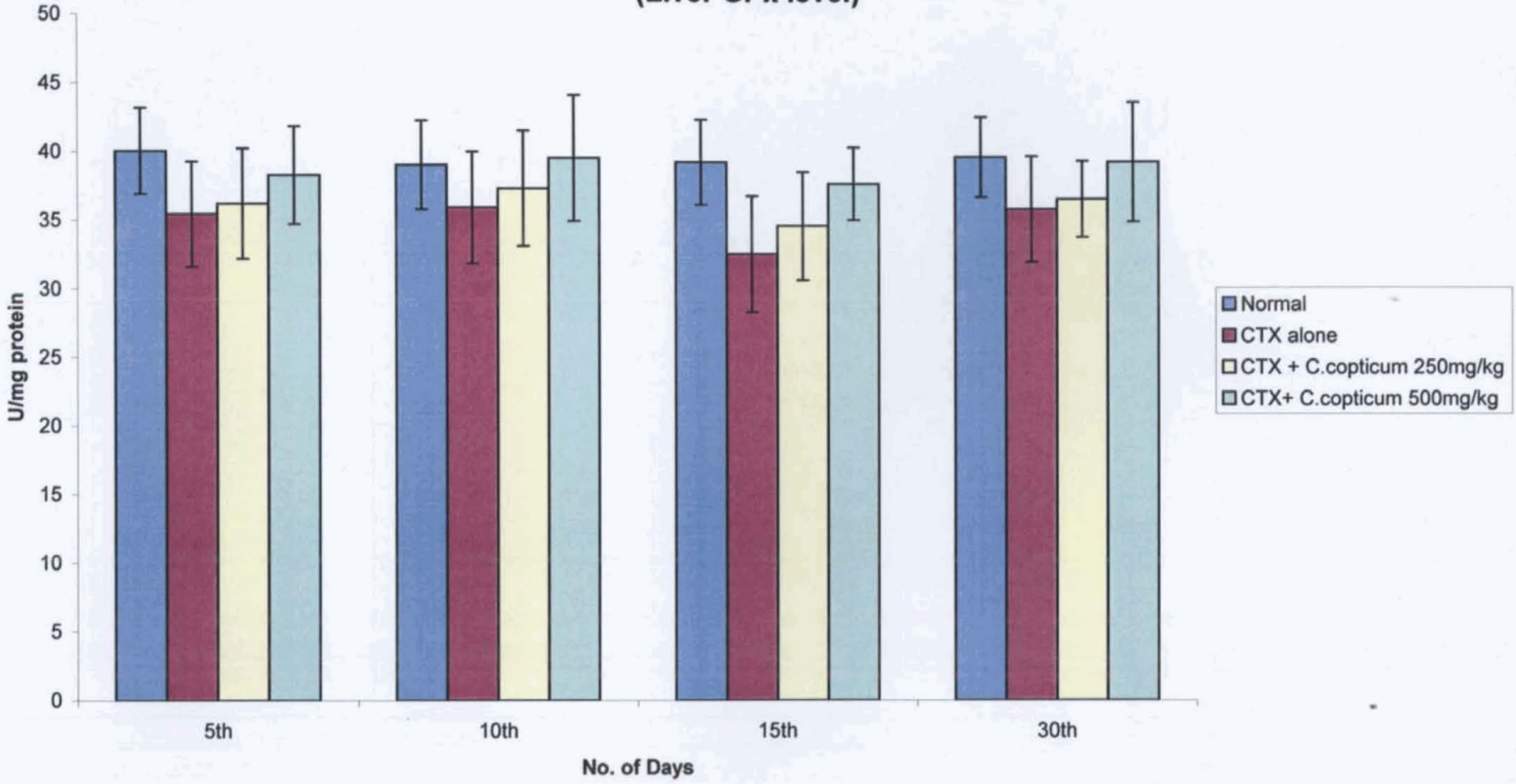
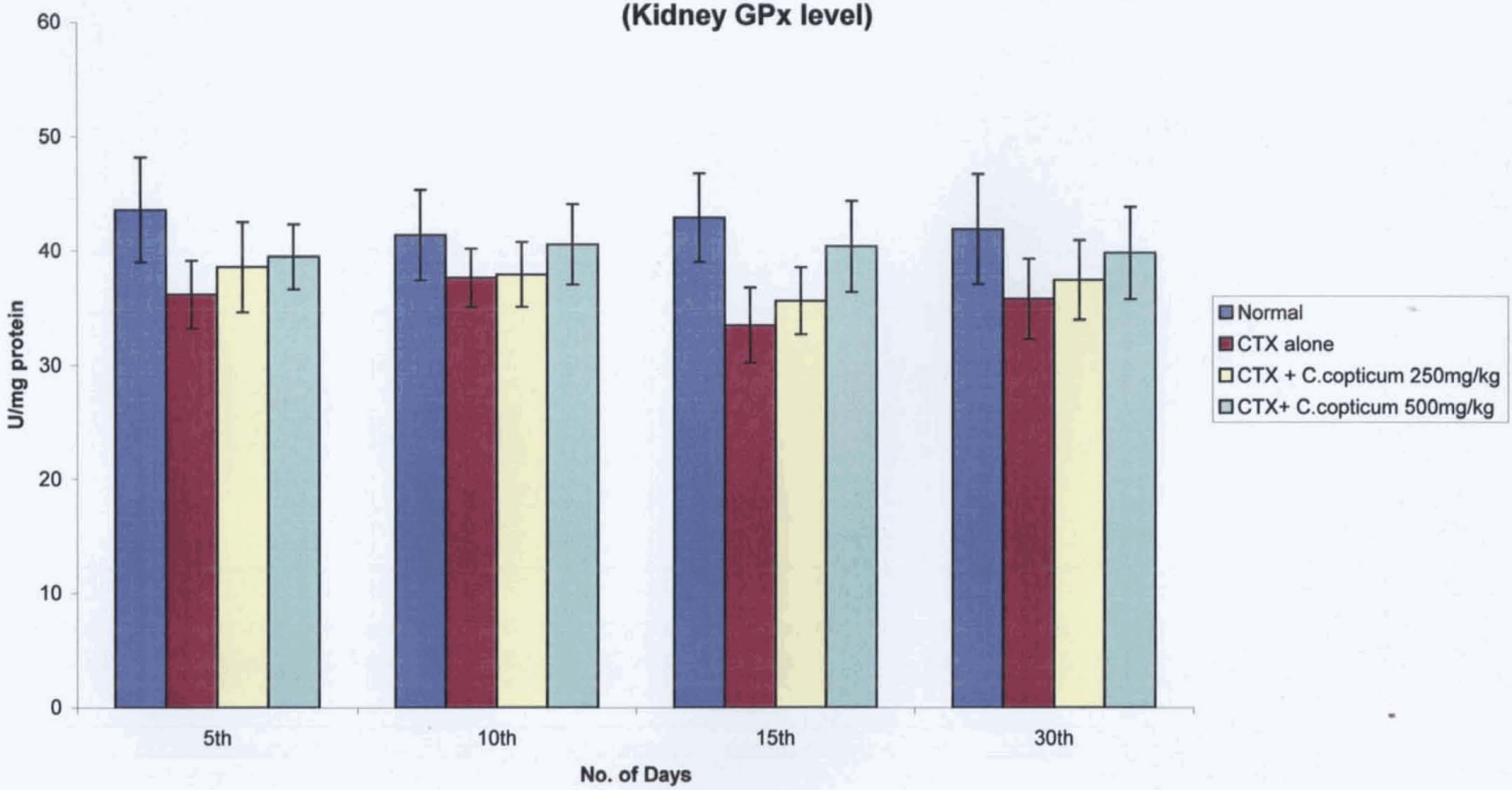


Fig No.5.4.12 Effect of *C. copticum* on CTX induced damage (Kidney GPx level)



5.4.13 Effect of *C. copticum* on CTX induced toxicity (Liver MDA level)

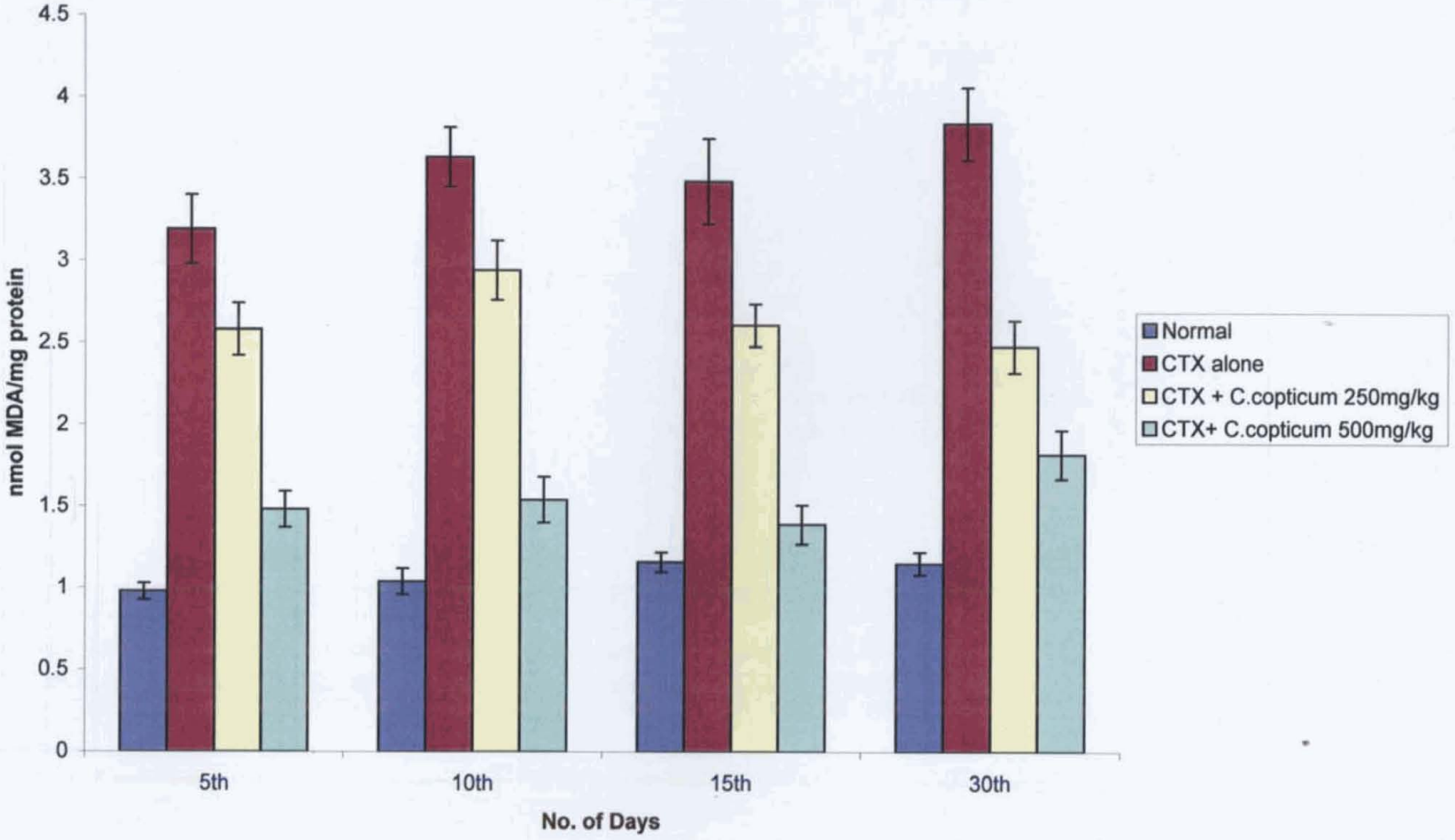


Fig No. 5.4.14 Effect of *C. copticum* on CTX treated animals (Bone marrow cellularity)

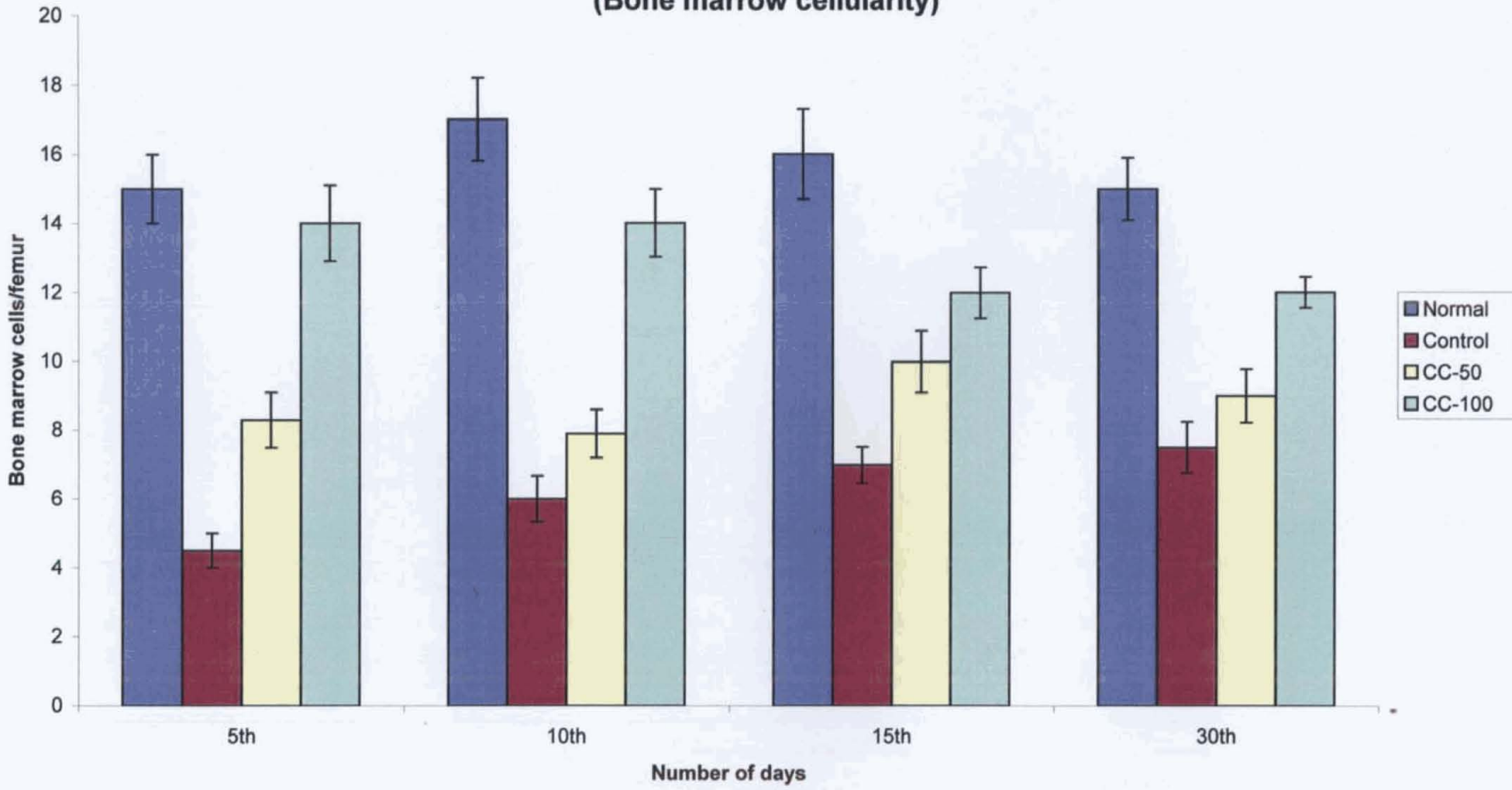
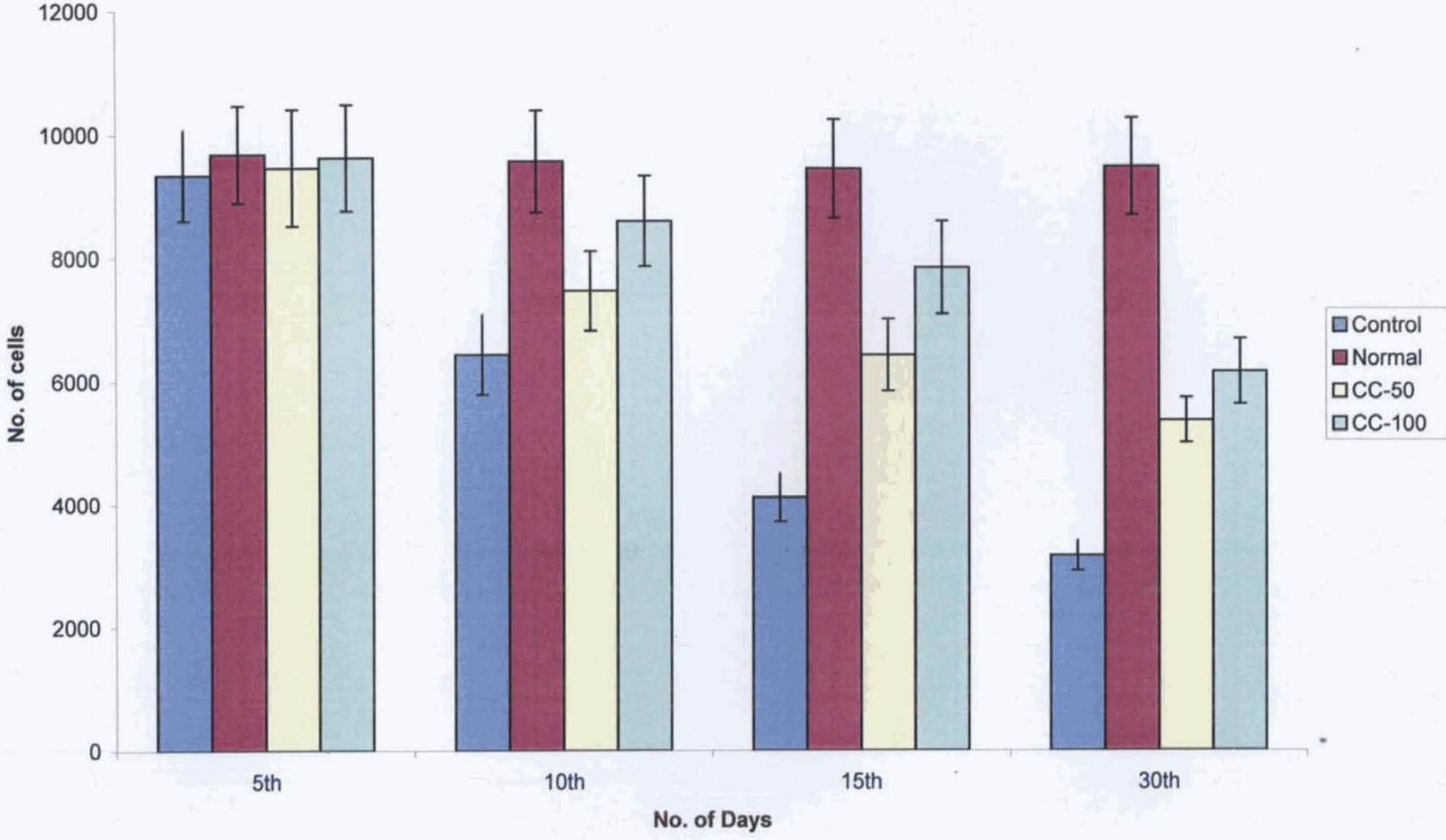


Figure No. 5.4.15 Effect of *C. copticum* in CTX induced Toxicity (WBC count per ml)



Treatment with *C. copticum* reduces the electrophilic burden and thereby increases the GSH levels. The increased levels of in vivo antioxidant systems revealed that extract is also act as an antioxidant in vivo to reduce the toxicity induced by CTX.

Radioprotective activity of Carum copticum against γ- radiation

5.5.1 INTRODUCTION

The modern era of radiation therapy is considered to have begun with the development of the cobalt machine, a simpler approach to the products of high-energy radiation (Benham et al, 1983). Radiotherapy is the most common modality for treating human cancers. The therapeutic doses of radiation are used to kill cancerous cells. Radiations used for the treatment of cancer are mainly the X-rays and gamma rays. The ability of ionizing radiations to kill cancerous cells through the induction of cell damage makes this an important modality in the therapeutic approach against cancer in humans.

Radiation therapy has numerous side effects. Normal human tissues are not immune to the damaging effects of ionizing radiations. Their interaction with biological systems results in the generation of many highly reactive short lived reactive oxygen species mainly due to the hydrolysis of water. The major free radicals generated are OH^-, H, H_3O^+, H_2O_2. These free radicals react with cellular macromolecules such as DNA, RNA, proteins, membrane etc and cause cell disfunction and mortality. These reactions take place in tumor as well as normal cells when exposed to radiation. Other major side effects of this therapy are depression of peripheral blood count, nausea and vomiting.

The present study investigated the radioprotective activity of *C. copticum* against γ-radiation induced damage.

5.5.2 MATERIALS AND METHODS

Female BALB/c mice were used for the present study. The animals were divided into 4groups of 16 animals in each group.

Group I - Normal

Gropup II- Radiation 600rad/animal (single dose)

Group III- Radiation 600rad/animal + *C. copticum* 250mg/kg

Group IV- Radiation 600rad/animal + *C. copticum* 500mg/kg

The administration of the extract were started 5 days before irradiation. The TC and bone marrow cellularity were calculated at 5, 10, 15 and 30 days. The animals were sacrificed at 5, 10, 15 and 30 days and biochemical parameters such as SOD, CAT, GSH, GPx and tissue lipid peroxidation were estimated.

5.5.2.1 BIOCHEMICAL ANALYSIS

5.5.2.1.1 Evaluation of antioxidant enzymes and GSH in Tissue

Liver and Kidney was removed and washed thoroughly in ice-cold saline and homogenate (10 %) was prepared in PBS (50 mM, pH 7.0) (section 3.2.2). A part of the homogenate was used for the estimation of reduced glutathione (GSH) (section 3.2.8) and tissue lipid peroxidation (3.2.12). The remaining homogenate was centrifuged at 10,000 rpm for 10 min in a cooling centrifuge at 4°C, after removal of the cell debris; supernatant was used for the assay of superoxide dismutase (SOD) (section 3.2.9), catalase (CAT) (section 3.2.10) and glutathione peroxidase (GPx) (section 3.2.11).

5.5.2.1.2 Evaluation of antioxidants enzymes and GSH in blood

Non-coagulated (heparin) blood was used for the determination of antioxidant status. SOD (section 3.2.21), CAT (section 3.2.22), GSH (section 3.2.23), and GPx (section 3.2.24) were estimated in erythrocyte lysate. Haemoglobin was determined using Drabkin's reagent (section 3.2.25).

5.5.3 RESULTS AND DISCUSSION

The ability of ionizing radiations to kill cancer cells through the induction of cell damage makes this an important modality in the therapeutic approach against cancer in humans. But normal human tissues are not immune to the damaging effects of ionizing radiations. The degree of cell damage induced by radiations depends on numerous factors, including the radiation dose, its scheduled administration, the stage

Fig No. 5.5.1 Effect of *C. copticum* on irrdiated animals (Blood SOD level)

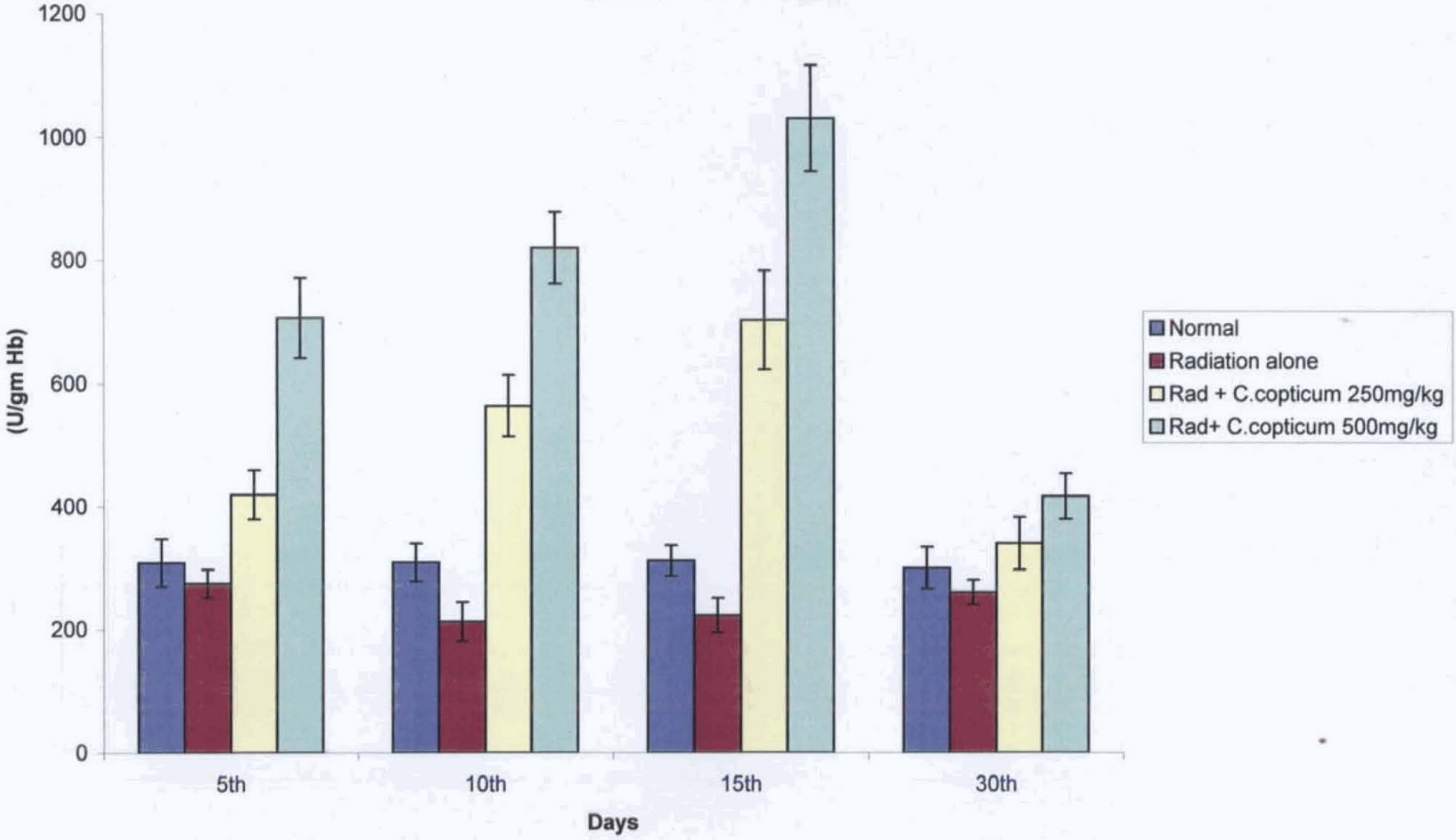


Fig No. 5.5.2 Effect of *C.copticum* On irrdiated animals (Liver SOD level)

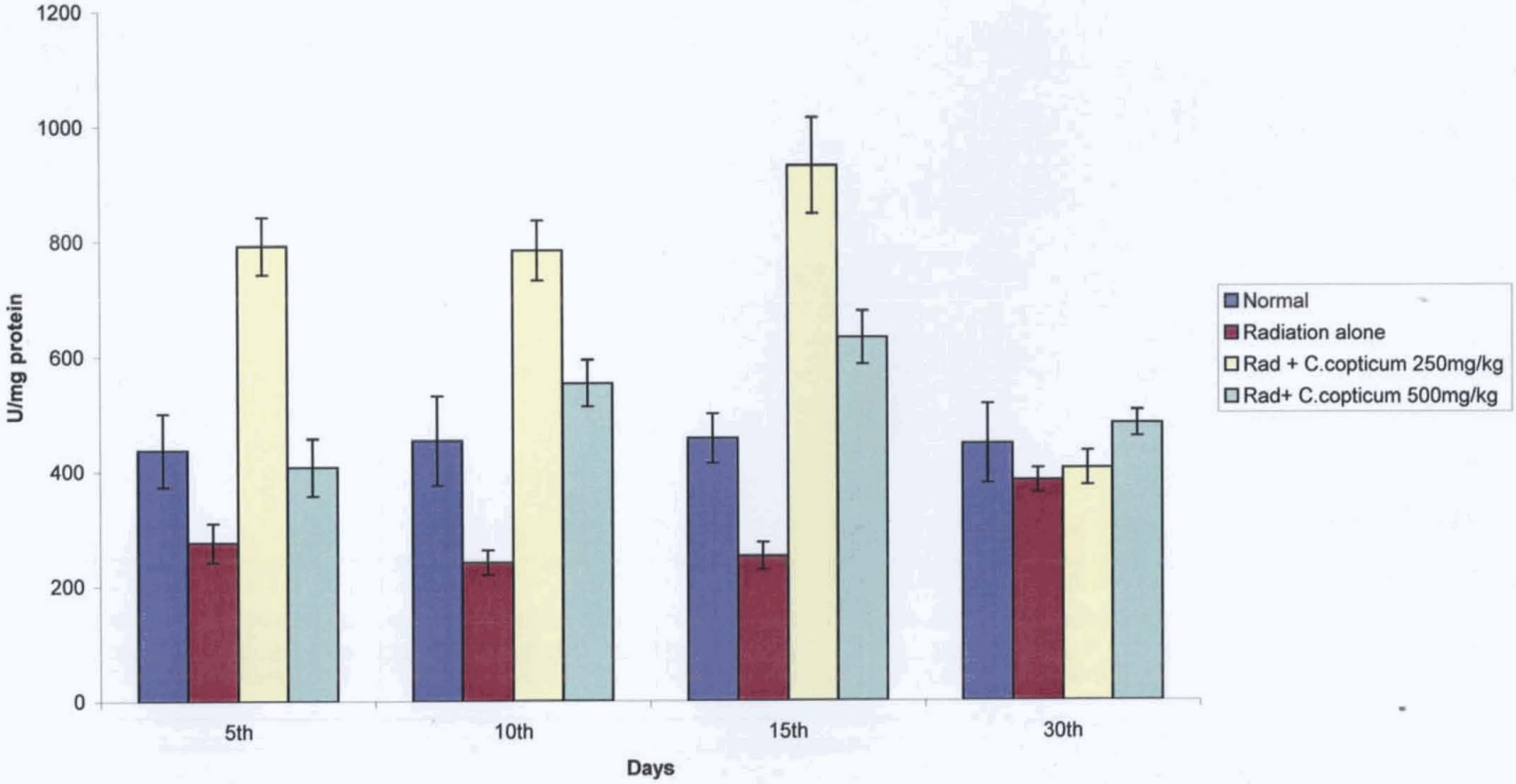


Fig No. 5.5.3 Effect of *C. copticum* on irrdiated animals (Kidney SOD level)

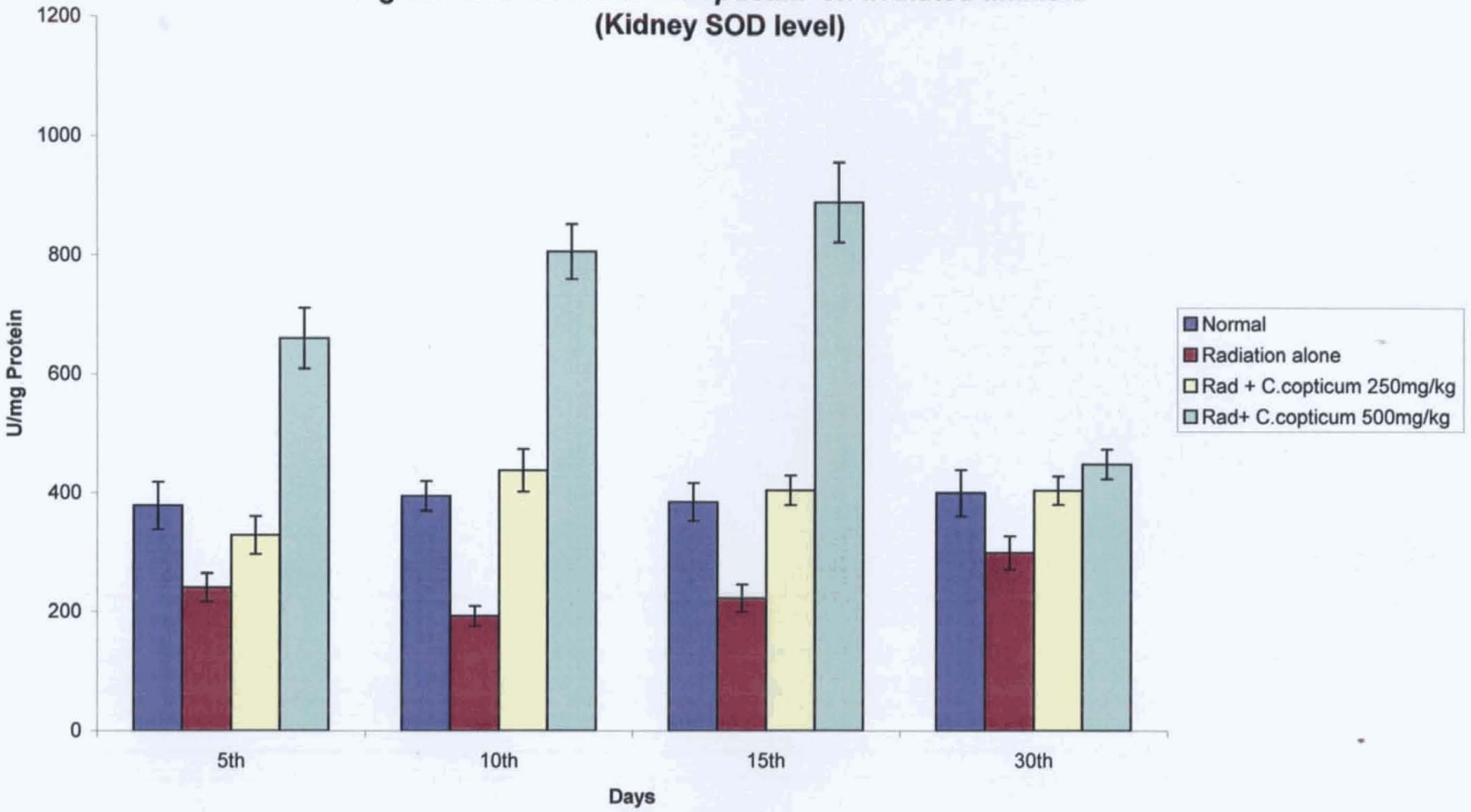


Fig No. 5.5.4 Effect of *C. copticum* on irrdiated animals (Blood Catalse level)

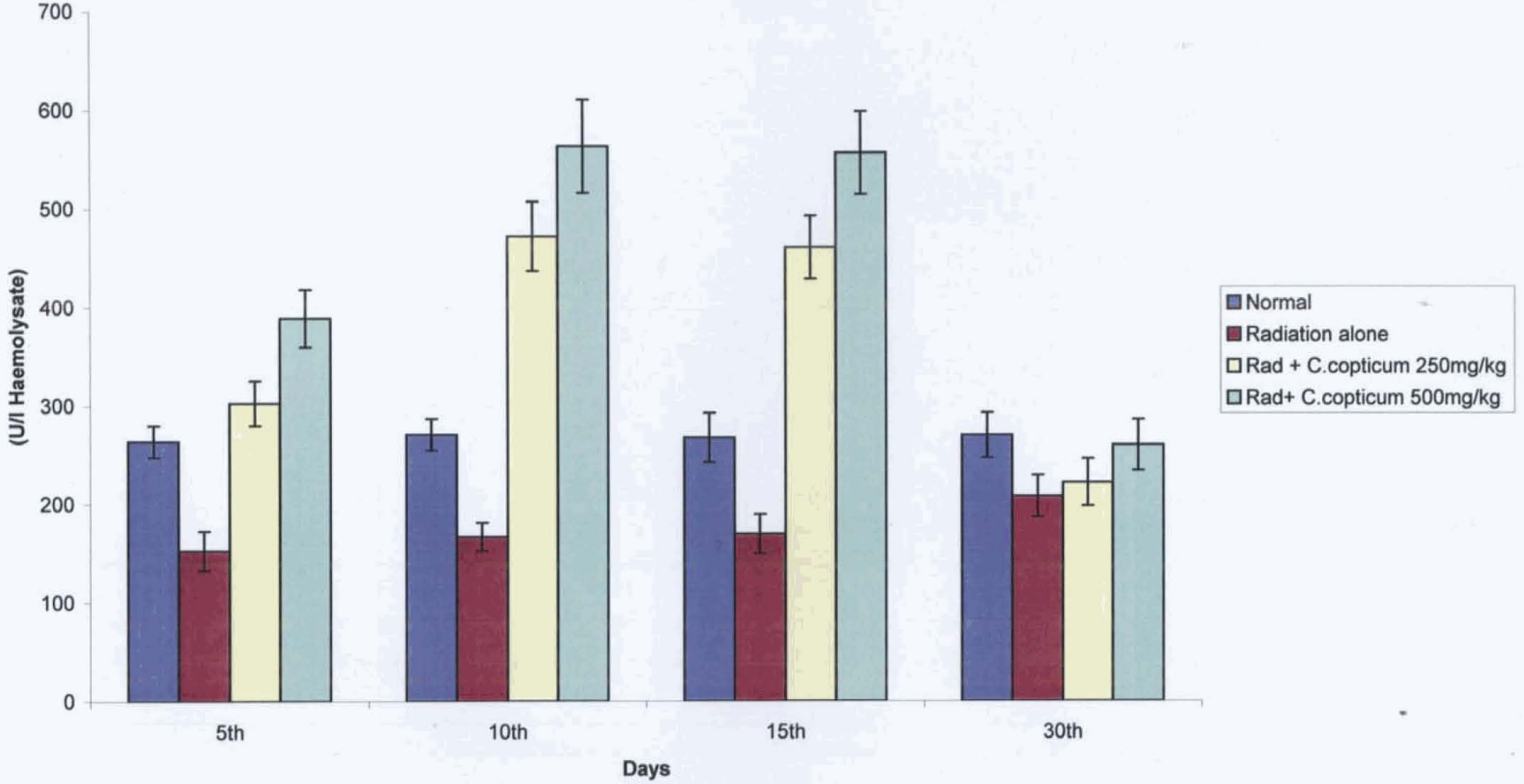


Fig No. 5.5.5 Effect of *C.copticum* on irrdiated animals (Liver Catalase level)

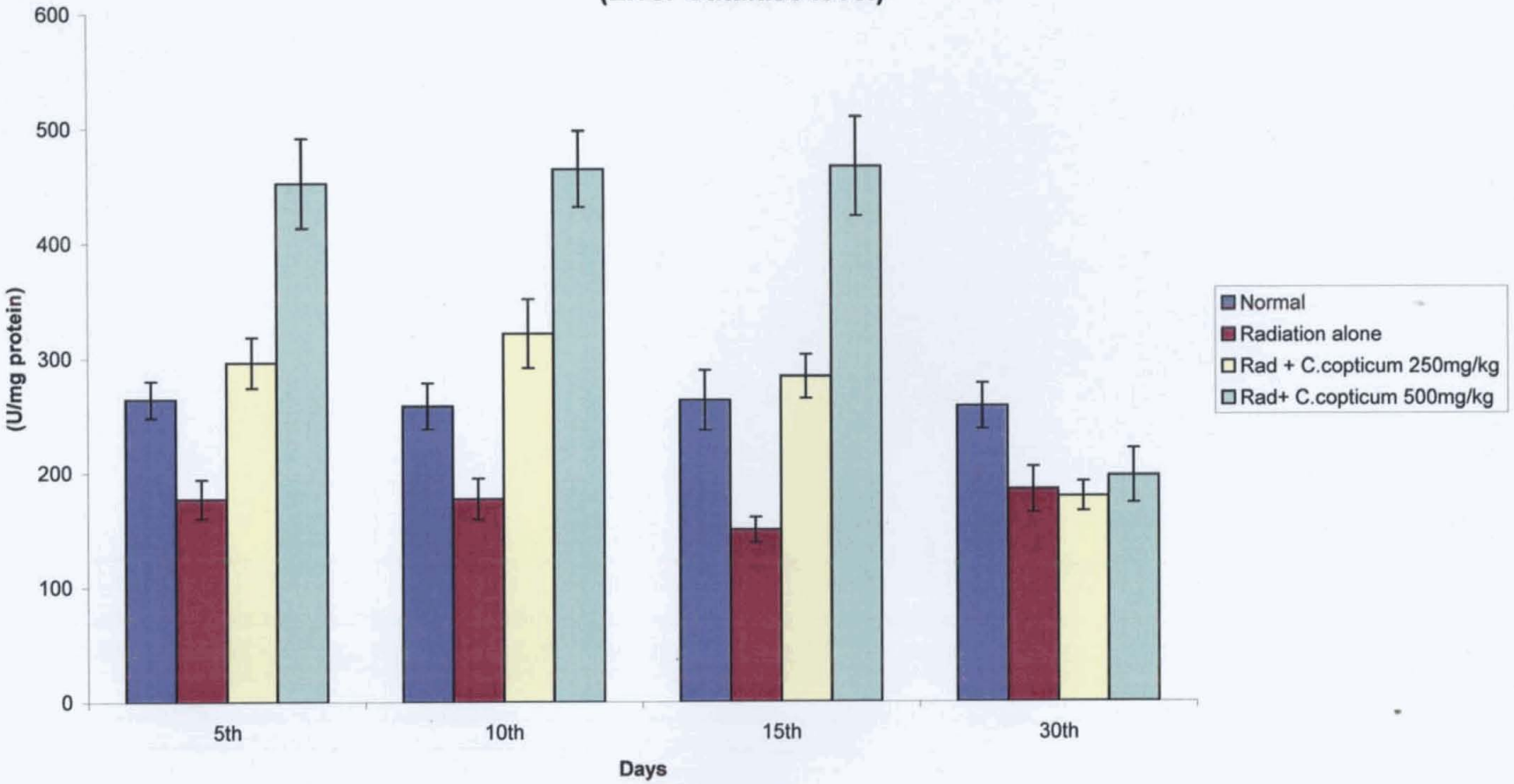


Fig No. 5.5.6 Effect of *C.copticum* on irrdiated animals (Kidney Catalase level)

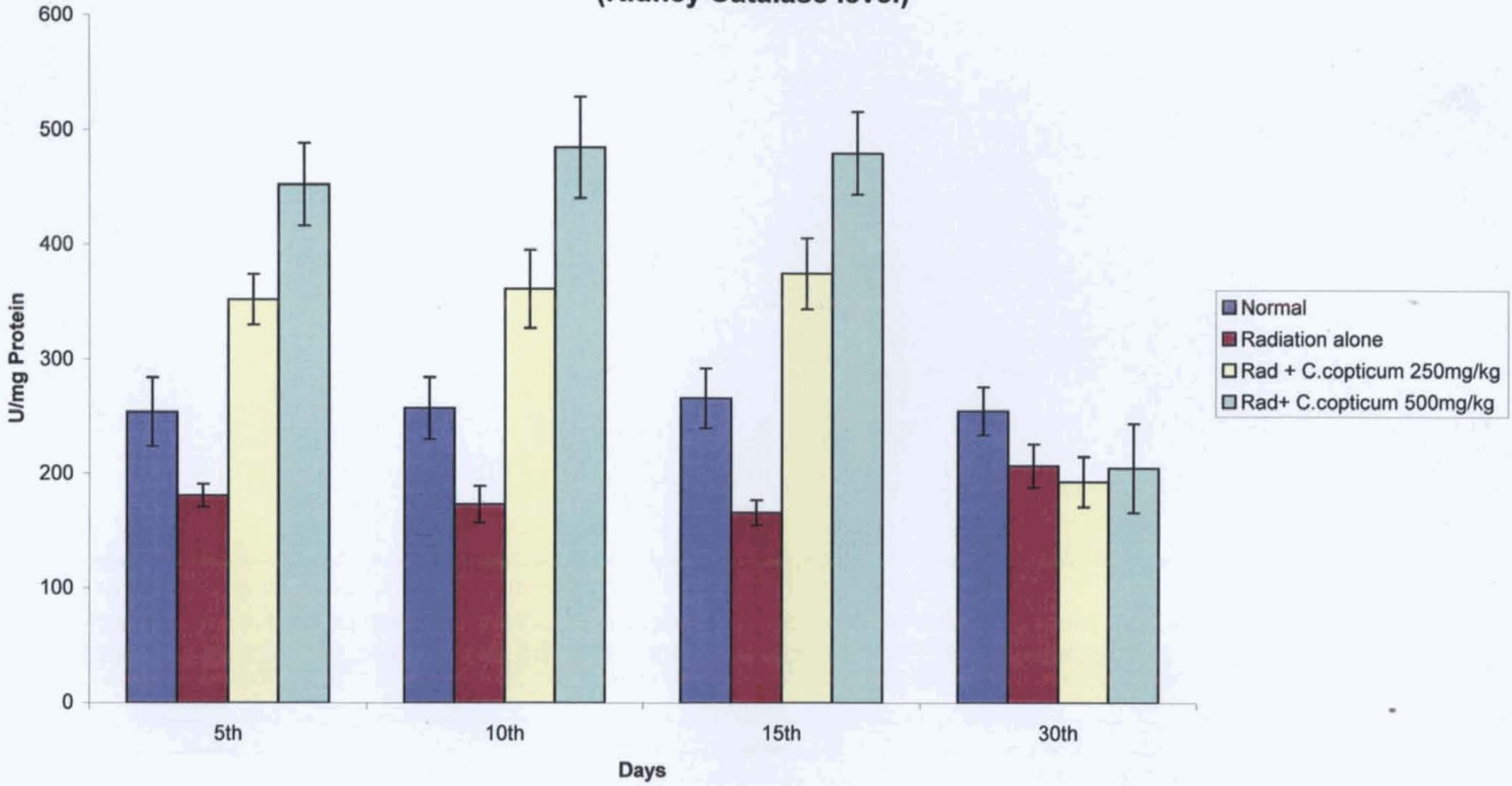


Fig No. 5.5.7 Effect of *C.copticum* on irradiated animals (Blood GSH level)

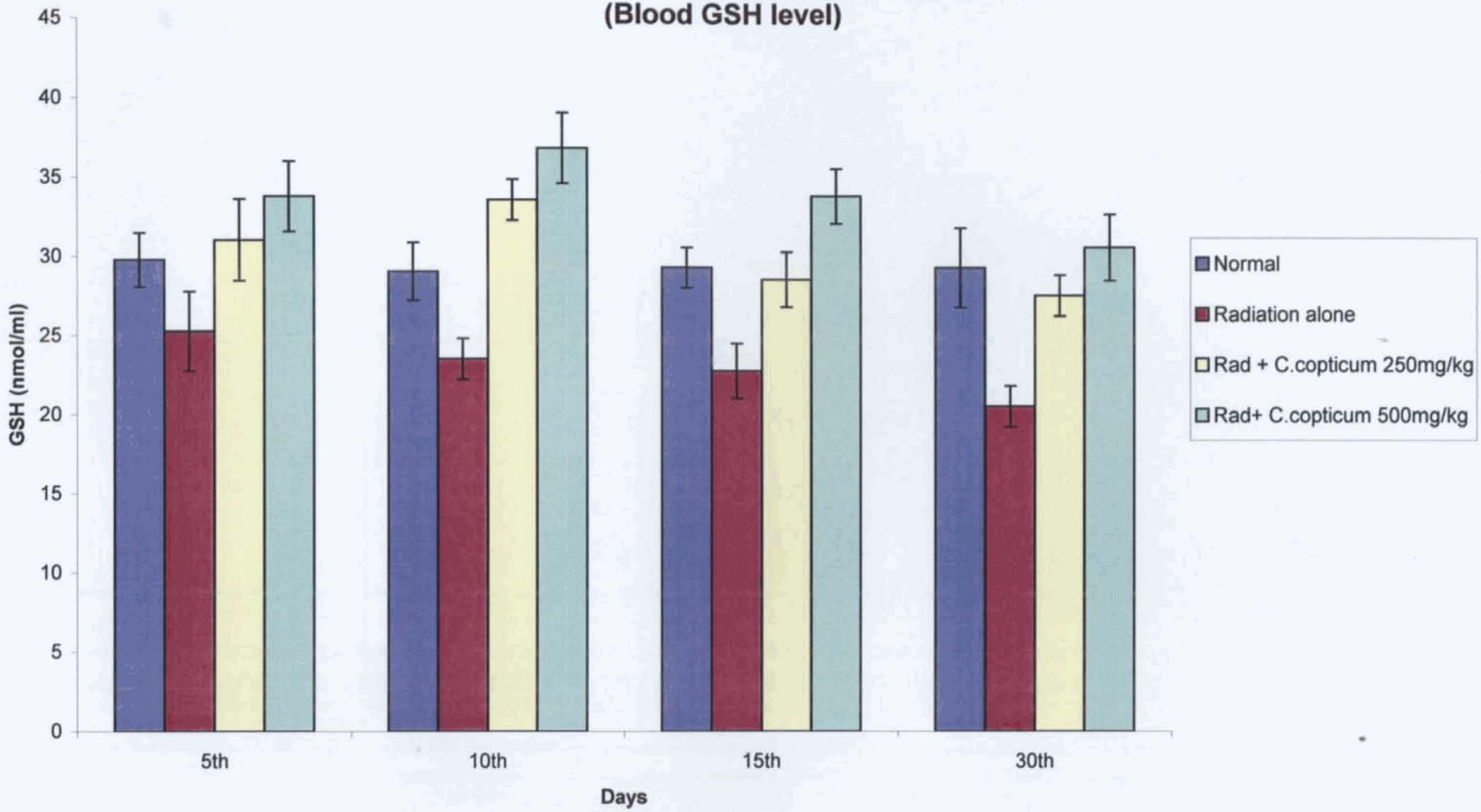


Fig No. 5.5.8 Effect of *C.copticum* on irrdiated animals (Liver GSH levels)

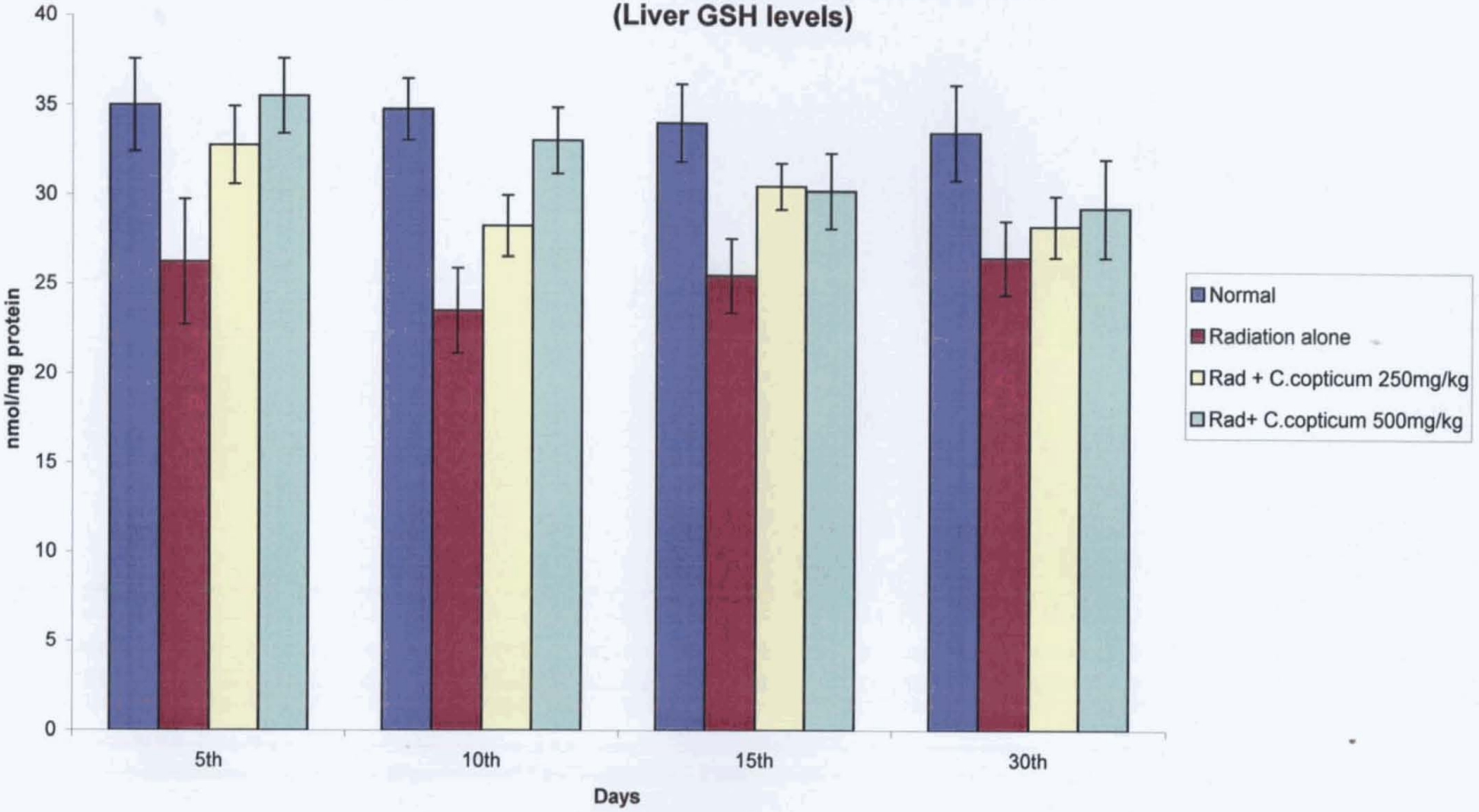
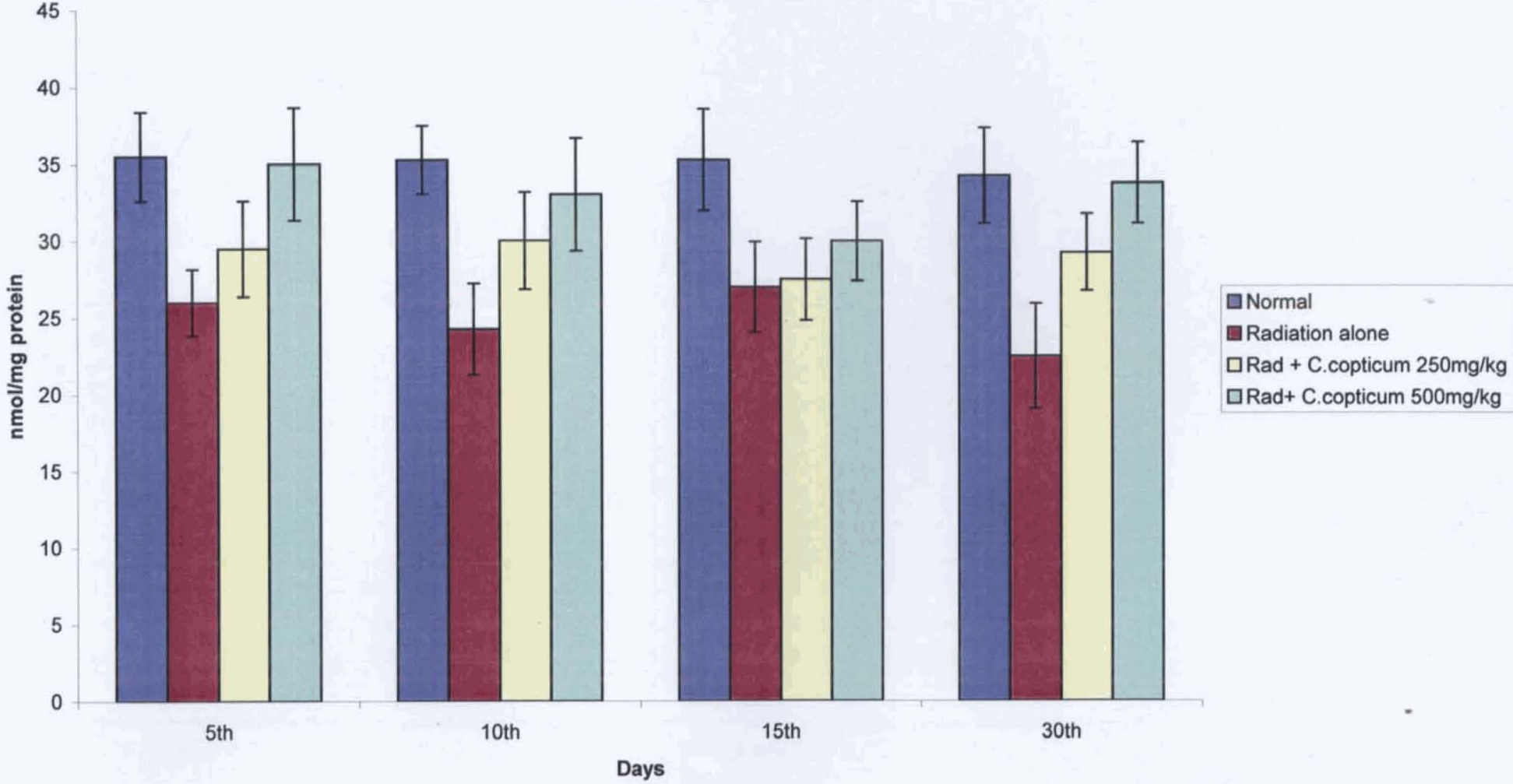


Fig No. 5.5.9 Effect of *C.copticum* on irrdiated animals (Kidney GSH levels)



5.5.10. Effect of *C.copticum* on irrdiated animals (Blood GPx level)

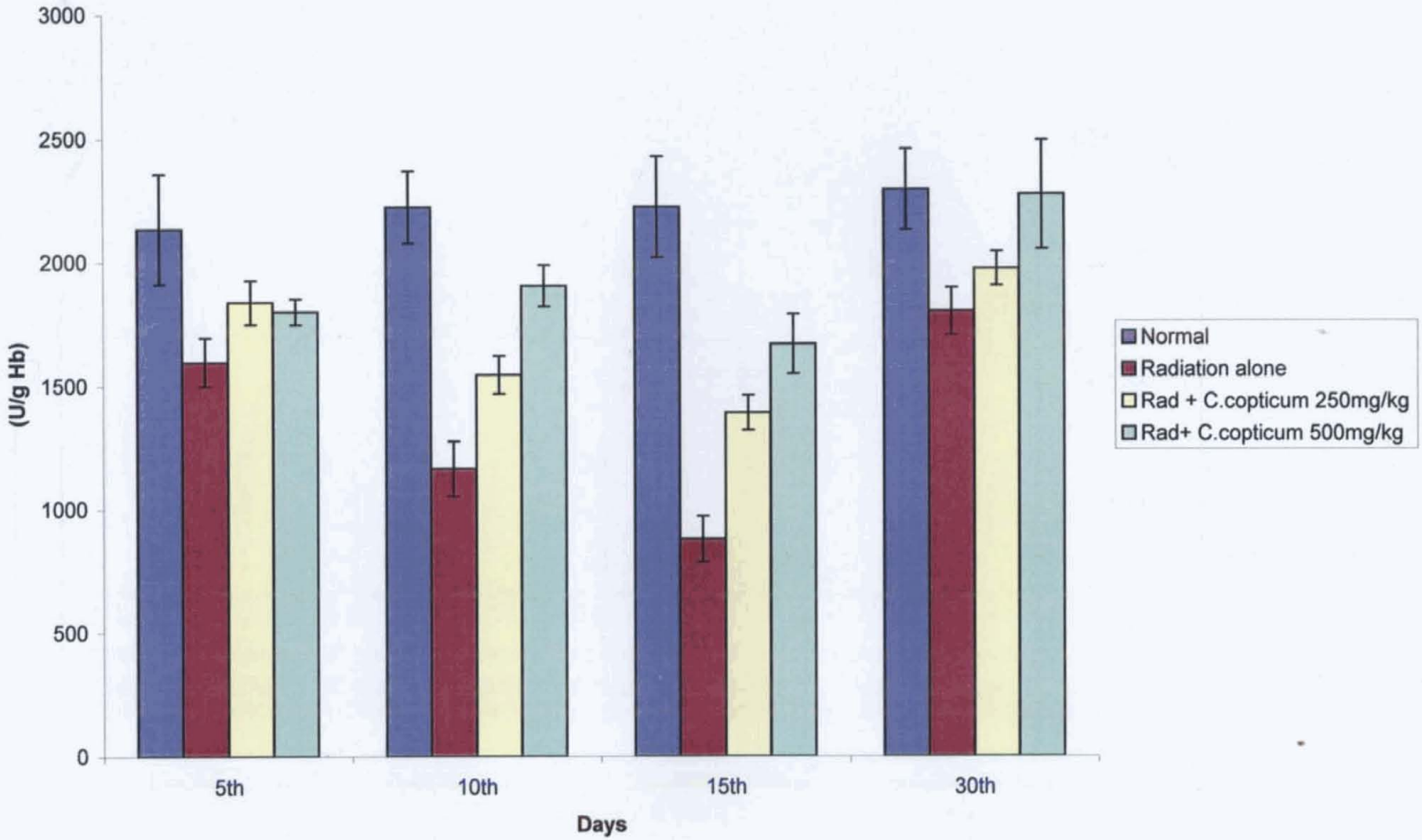
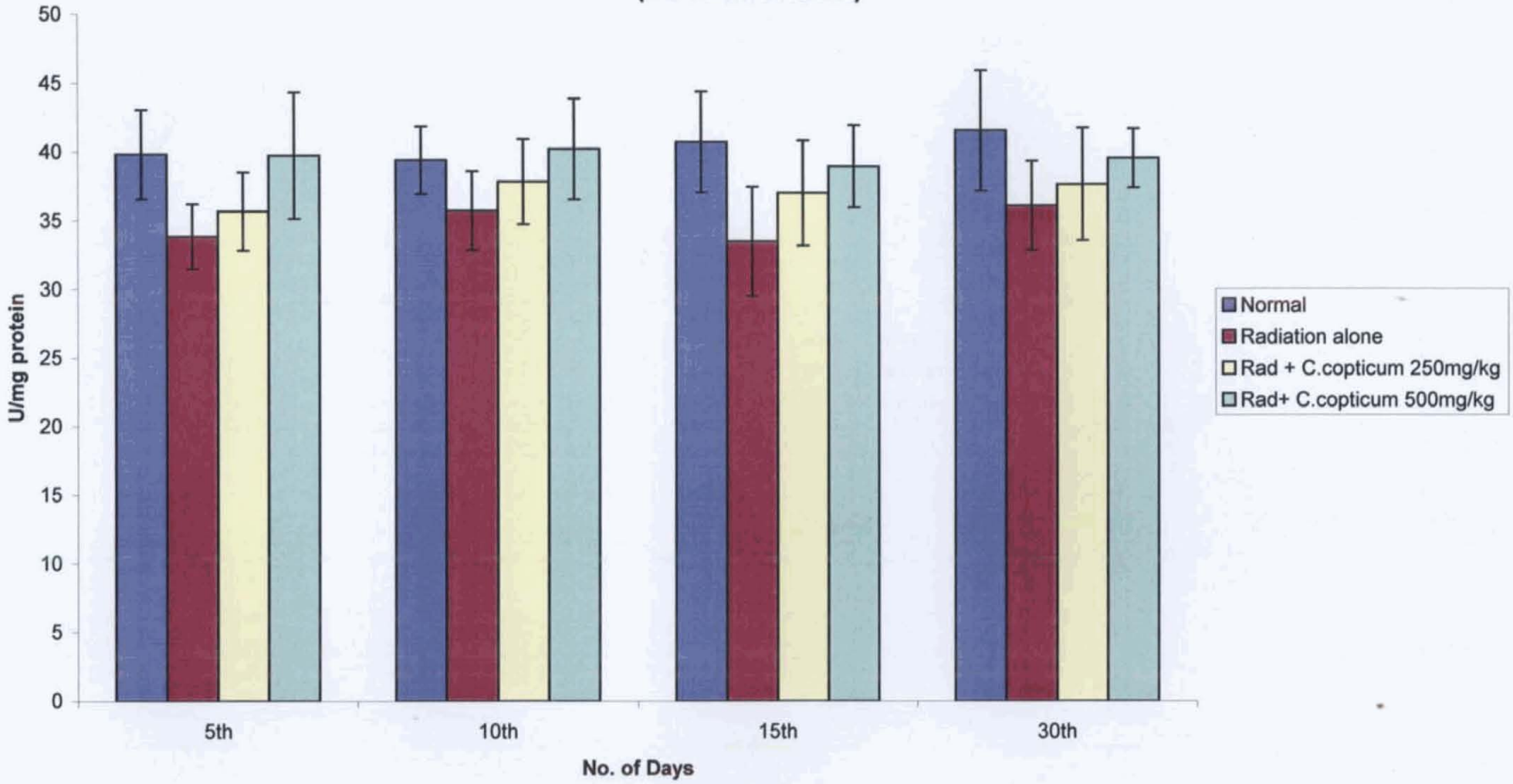


Fig No. 5.5. 11 Effect of *C. copticum* on Radiation induced toxicity (Liver GPX level)



5.5.12. Effect of *C. copticum* on Radiation induced damage (Kidney GPx level)

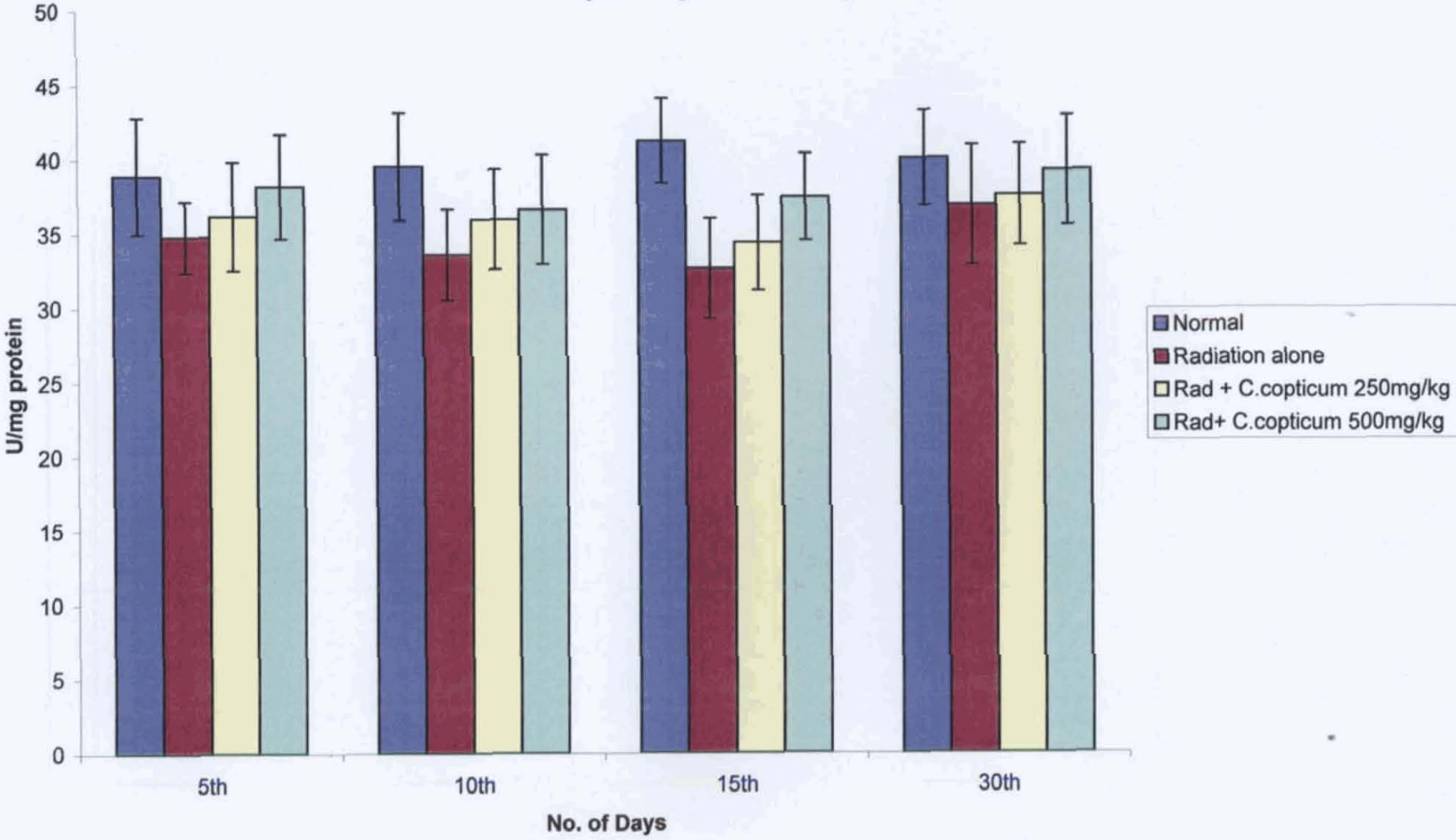


Fig No. 5.5.13 Effect of *C. copticum* on Radiation induced damage (Liver MDA level)

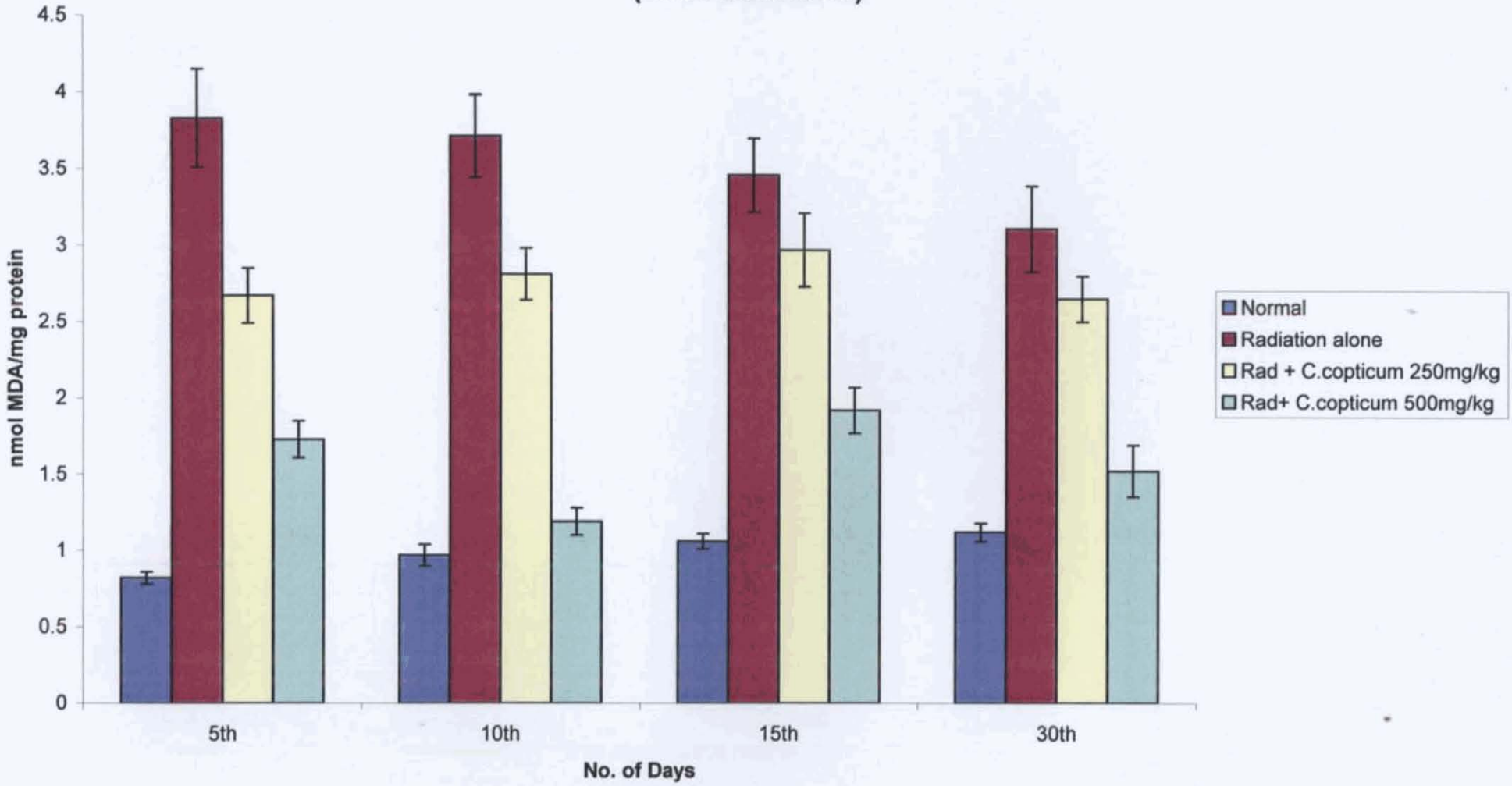


Fig No. 5.5.14 Effect of *C. copticum* on irradiated animals (Bone marrow cellularity)

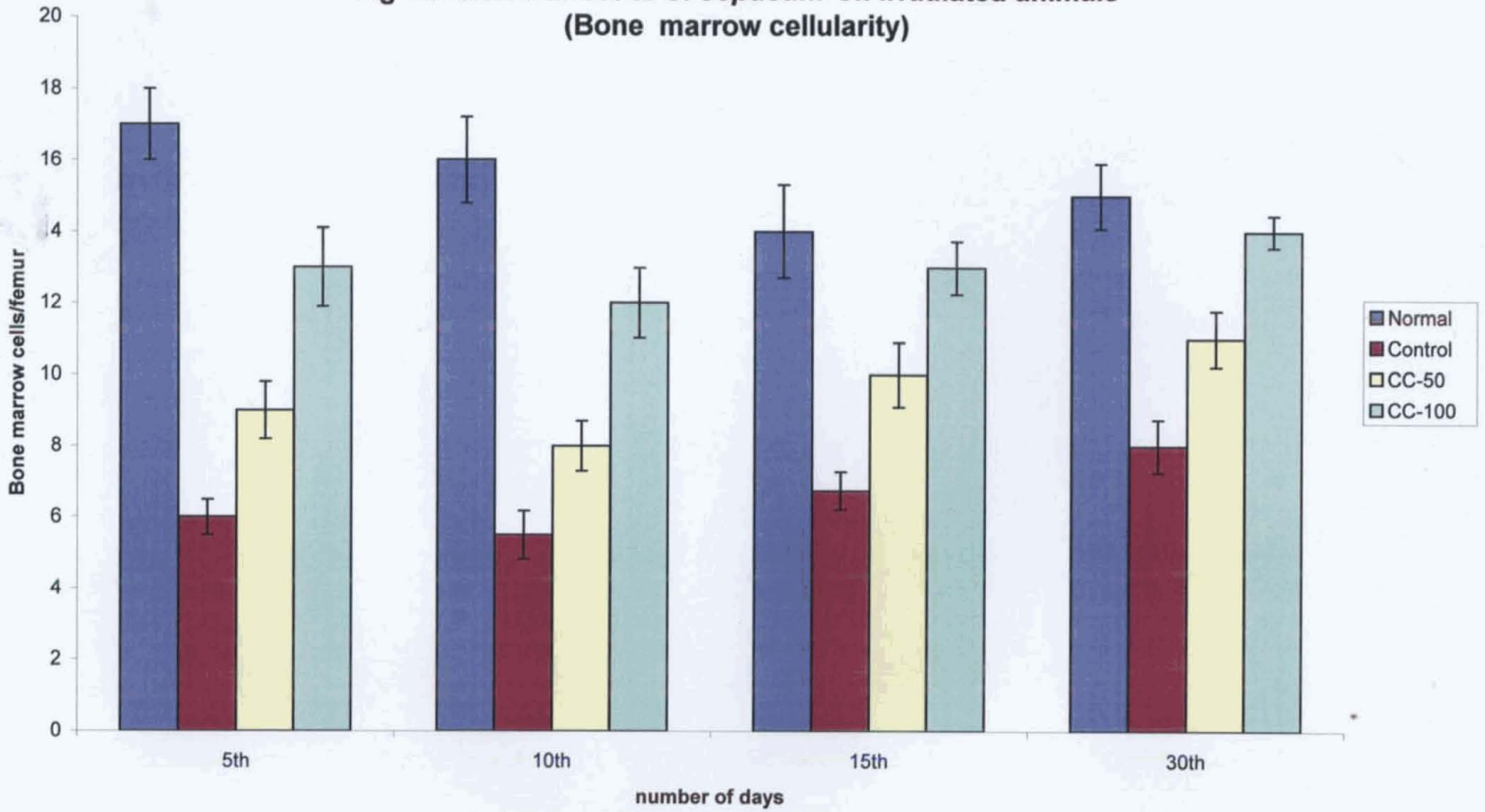
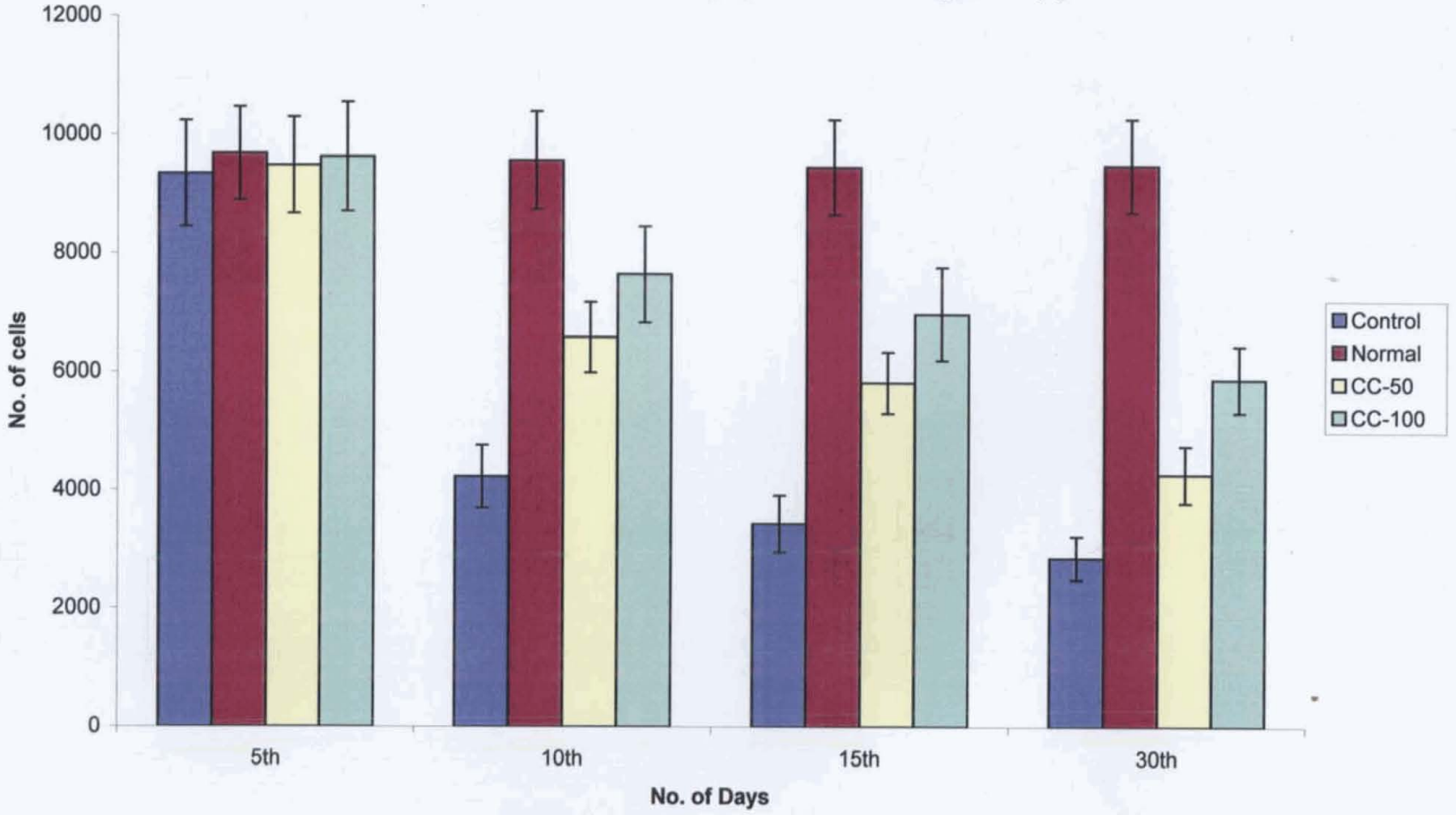


Fig No. 5.5.15 Effect of *C.copticum* on haematological parameters in gamma-radiation induced toxicity [WBC count (per ml)]



of the cell within the cell cycle, the levels of cellular antioxidant defense system and the availability of oxygen in the tissues (Weichselbaum et al, 1997).

The interaction of ionizing radiation with biological system results in the generation of many highly reactive short-lived reactive oxygen species (ROS) mainly due to the hydrolysis of water. These ROS attack cellular macromolecules like DNA, RNA, proteins, membranes etc. causes its dysfunction and damage (Moller and Wallin, 1998). ROS increased the lipid peroxidation which in turn can alter the intergrity of membrane structure leading to inactivation of membrane bound enzymes, loss of permeability of the membrane and decrease in membrane fluidity. Whole body irradiation increased the levels of lipid peroxidation both in serum and tissue. *C. copticum* treated animals showed a very low level of lipid peroxide levels comparable to normal levels. The in vivo antioxidant enzyme levels such as SOD, Catalase, GSH and GPx (Figure No. 5.5.3-5.5.14; $p<0.01$) levels are also coming closer to the normal values in *C. copticum* treated groups. One of the most important side effects of ionizing radiation is myelosuppression. In extract treated groups of animals an increase in total count and bone marrow cellularity were also observed which indicate the protective effect of *C. copticum* on radiation induced damage (Figure No.5.5.14, 5.5.15; $p<0.01$).

Chapter 6

Chemopreventive activity of Carum copticum in Azoxymethane induced Colon cancer

6.1.1 INTRODUCTION:

Colorectal cancer represents a major public health problem, especially in developed countries. Colorectal cancers rank third in frequency in men and second in women. Male incidence rates, adjusted for age and race, appears greater than female rates for both proximal and distal cancers. The essential element of the etiology of colorectal cancer is a process of genetic change in the epithelial cells of the colonic mucosa (Vogelstein et al, 1988). Epidemiologic factors have provided initial evidence about the specific factors that initiate the process of carcinogenesis in the large bowel mucosa (Winawer and Shike, 1992). Chief among the factors that can initiate colorectal cancer development are predisposition to mutagen effects, fecal mutagens, meat intake, bile acids, altered vitamin and mineral intake and fecal pH.

There is an interaction between mutagen exposure and genetic constitution. Metabolic pathways may be altered by polymorphisms in genes responsible for detoxifying mutagens (Potter, 1999). Protection from the effects of mutagen-induced DNA damage is achieved by a range of detoxification enzymes. Examples are reduced glutathione-S-transferase (GSH transferase), DT-diaphorase, and N-acetyl transferase (Gerting and Hunter, 1998).

Mutagenic compounds such as fecapentaenes, 3-ketosteroids and heterocyclic amines in the stool may be produced by the interaction of digestion and food products (Reddy et al, 1987). These compounds produce reactive molecules that may form bulky adducts to DNA. Changes in the fecal microflora indicate that changes in diet may alter mutagenic activity by altering extracellular superoxide formation (Winters et al, 1998). In addition to mutagenic compounds such as fecapentanes, the presence of other products of digestion such as 3-ketosteroids, which are products of cholesterol metabolism, may act as tumor promoters or initiators.

Armstrong and Doll (1975) described the high correlation of meat intake and mortality from colorectal cancer. Among the risk factors are the intake of red meats and the compounds that result from cooking meats at high temperatures (de Meester and Gerber, 1995). In a study of meat preparation, it was observed that the association between red meat and colorectal cancer could be due to heterocyclic amines present in cooked meat (Gerhardsson et al, 1991).

Normal bile acids that are related to the digestion of fat can induce intestinal mucosal hyperproliferation, which acts as a marker for neoplasia risk (Suzuki and Bruce, 1986). The presence of bile acids correlates with fat consumption, which is a known risk factor for colorectal cancer (Minsky et al, 1995). Bile acids have been shown to activate AP-1, a transcription factor associated with the promotion of neoplastic transformation in colonic cells (Glinghammer et al, 1999).

Calcium can alter colonic mucosal proliferation by binding fatty acids and bile acids in the stool resulting in insoluble complexes that are less likely to affect the mucosa. It can also decreases proliferation of the mucosa directly (Rozen et al, 1989). These effects of calcium may be site-specific within the colon (Cats et al, 1995). In a large study of US health professionals, the risk reduction from high calcium intake appeared modest after adjusting for confounding variables (Kearney et al, 1996). Two case-control studies suggest that any protective effect of calcium may occur only at low levels of fat intake (De et al, 1997).

In case-control studies, the use of multivitamins has been shown to reduce the risk of adenoma formation in high-risk patients (Whelan et al, 1999). In an uptake of the Nurse's Health study, Giovannueci et al (1998) found a reduced risk of colon cancer after 15 years of use of folate-containing multivitamins.

Another aspect of the interaction between the intestinal milieu and the genome of the intestinal mucosa is the fact that alkaline environments in the stool support higher concentrations of free bile acids and other potential carcinogens (McKeown-Eyssen et al, 1986). This pH may affect the solubility of bile acid and carcinogens and make them more damaging to the DNA of the intestinal mucosal cells. Epidemiologic

studies shows that higher rates of colon carcinoma are found in subjects with a higher stool pH (Mikhailowski et al, 1998).

In the present study we induced colon cancer using Azoxymethane and find out the inhibitory activity of *C.copticum* in aberrant crypt formation which is the initial stage of colon cancer.

6.1.2 MATERIALS AND METHODS

Female BALB/c mice (4weeks old) were used for the present study. The animals were divided into 4groups of 10 animals in each group. The treatment schedule is as follows

Group I – Normal administered with normal saline 0.1ml

Group II- Azoxymethane only (15mg/kg, i.p)

Group III- Azoxymethane only (15mg/kg, i.p) + *C.copticum* 250mg/kg

Group IV- Azoxymethane only (15mg/kg, i.p) + *C.copticum* 500mg/kg

Group II, III, IV were injected with Azoxymethane once (15mg/kg) in a week upto 6 weeks. The *C. copticum* extract were given orally upto 2months after the first injection of azoxymethane. On the 3rd month the animals were sacrificed blood and colon were collected. The blood is subjected to antioxidant analysis (SOD, Catalase, GSH and GPx) and colon, histopathological analysis. The sectioned were stained with H&E. The sections were examined by a Veternary pathologist at Veternary college, Mannuthy, Kerala, India.

6.1.2.1 BIOCHEMICAL ANALYSIS

6.1.2.2.1 Evaluation of antioxidants enzymes and GSH in blood

Non-coagulated (heparin) blood was used for the determination of antioxidant status. SOD (section 3.2.21), CAT (section 3.2.22), GSH (section 3.2.23), and GPx (section 3.2.24) were estimated in erythrocyte lysate. Haemoglobin was determined using Drabkin's reagent (section 3.2.25).Non-coagulated (heparin) blood was used for the determination of antioxidant status. SOD (section 3.2.22), CAT (section 3.2.23), GSH (section 3.2.24), and GPx (section 3.2.26) were estimated in erythrocyte lysate. Haemoglobin was determined using Drabkin's reagent (section 3.2.27).

6.1.3 RESULTS AND DISCUSSION

The intraperitoneal injection of AOM produced ACF in control as well as treated animals. The administration of *C.copticum* extract 250mg/kg and 500mg/kg inhibited the formation of ACF in female BALB/c mice. The table summarizes the mean number of ACF/colon, total number of aberrant crypts/colon and the mean number of aberrant crypts/focus. The mice treated with AOM showed a100% incidence of ACF. No ACF were seen in the colons of mice without AOM treatment (Group I). The number of ACF/colon was significantly decreased by *C.copticum* administration ($P<0.05$). *C.copticum* administration significantly decreased the number of aberrant crypts/colon and the number of aberrant crypts/focus in a dose dependent manner (Table No. 6.1.1), (Figure No. 6.1.1).

ACF are one of the earliest hallmarks of colon carcinogenesis (Rao et al, 1993). They are readily utilizable as intermediate end points in colon cancer prevention studies because they are easily and rapidly quantified topographically. In addition, ACF have been reported to be associated with gene alterations in rats (Stopera et al, 1992; Vivona et al, 1993; Stopera et al, 1993) and in human (Pretlow et al, 1993; Smith et al, 1994). In this study, *C. copticum* extract administration inhibited the development of ACF in a dose dependent manner. These results suggest that *C. copticum* extract administration has blocking effects on the formation of ACF and suppressing effects on the growth of ACF.

It is well known that the free radicals play a key role in the initiation, promotion and progreesion of carcinogenesis. In the present study we examined the level of SOD, Catalase, GSH and Gpx in the blood of control and the treated groups of animals which showed an increase in these antioxidant levels compared to the control group (Table No.6.1.2). Moreover, we identified the active principle as falvanoids. Flavanoids are highly antioxidants in nature. Hence it can be concluded that the inhibitory colon cancer activity of *C. copticum* may be due its antioxidant effects and due to the presence of these compounds.

Table No. 6.1.1 Effect of *C. copticum* on AOM-induced ACF in mouse colon

Groups	Incidence	No. of ACF/colon	No. of aberrant crypts/colon	No. of aberrant crypts/focus
Normal	0/6	----	----	----
AOM alone	6/6	93.4±12.6	189.5±32.8	1.63±0.16
AOM + *C.copticum* 250mg/kg	6/6	84.6±10.7*	153.7±29.6	1.48±0.12*
AOM + *C. copticum* 500mg/kg	6/6	65.2±7.4*	119.3±24.9	1.09±0.08*

P<*0.01. Values are Mean ± SD of 6 animals in each group

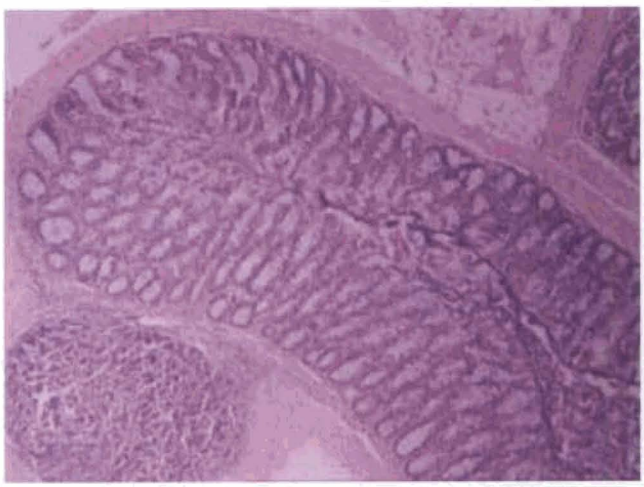
Table No. 6.1.2 Effect of *C. copticum* on AOM-induced ACF in mouse colon (Blood antioxidant levels)

	SOD U/g Hb	CAT k/g Hb	GSH mol/ml	GPX U/g Hb
Normal	1433.6 ± 62.51	85.26±7.93	3.94±0.27	4895.0±514.25
AOM alone	954.3± 52.67	62.87±4.69*	3.35±0.21*	2946.0 ±317.0*
AOM + *C.copticum* 250mg/kg	1187.0±42.74*	74.68±5.43*	3.49±0.17*	3478.0±325.75*
AOM + *C.copticum* 500mg/kg	1384.8±62.42*	78.67±4.09*	3.85±0.22*	4283.0±476.50

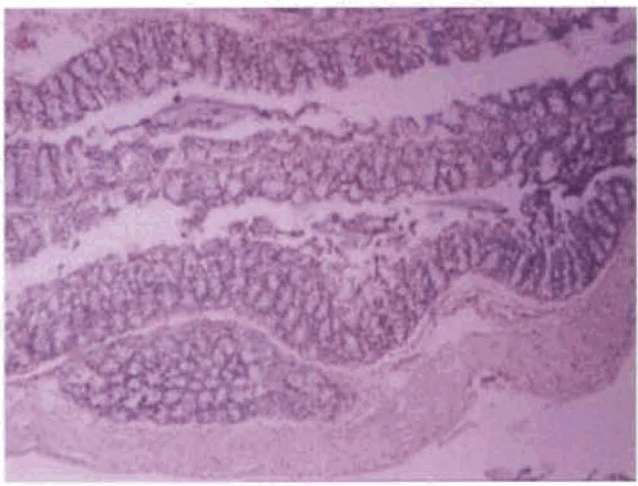
P< *0.01. Values are Mean ± SD of 6 animals in each group

Figure 6.1.1

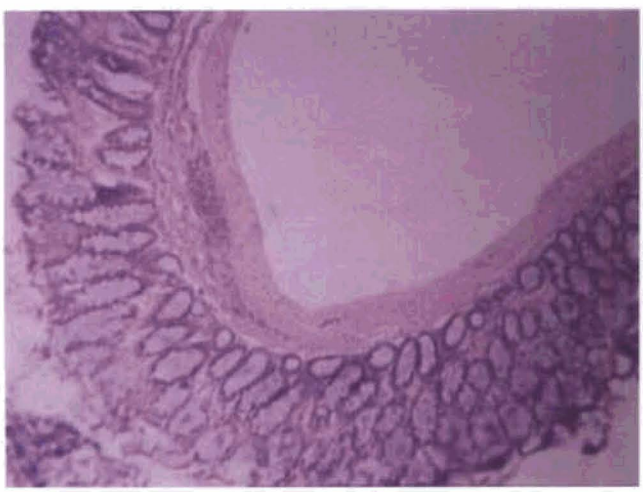
Chemopreventive effect of *C.copticum* on AOM induced colon carcinogenesis (Histoptahological analysis of the colon)



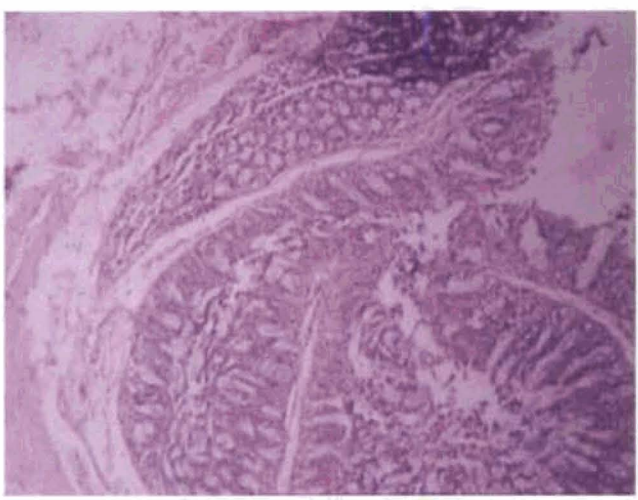
Normal



AOM alone



AOM + *C.copticum* 250mg/Kg



AOM + *C.copticum* 500mg/Kg

The inhibitory activity of tamoxifen induced Hepatocelluar carcinoma by Carum copticum

6.2.1 INTRODUCTION:

Hepatocelluar carcinoma is the most common solid- organ tumor worldwide, being responsible for more than 1million deaths annually. The difficulties in treating HCC and the high mortality associated with it are attributed to a number of factors. First, this cancer is usually associated with cirrhosis, which is not only a cause of morbidity but also limits treatment options for the cancer. Second, HCC is usually asymptomatic at early stages and has a great propensity for intravascular or intrabiliary extension, even when the primary tumor is small. As a result, the carcinoma is usually at an advanced stage when discovered. This tumor is, therefore, usually beyond curative therapy at presentation and, indeed, often began any useful therapy.

At least 1 million new cases of HCC occur yearly (Okuda et al,1993). HCC is closely associated with chronic liver injury and, therefore, geographic distribution of HCC closely mirrors that of vital hepatitis. Chemical carcinogens also have been linked to primary liver cancers. Chemicals such as nitrites, hydrocarbons, solvents, organochlorine pesticides, primary metals and polychlorinated biphenyls have been implicated in the development of HCC (Forman, 1991). Colloidal thorium dioxide (Thorotrast), which emits high level α, β and γ-radiation and was used as an angiographic agent in the 1930s, has been linked to angiosarcoma, cholangio carcinoma and HCC (Okuda et al,1993).

Of all the chemicals linked to development of HCC, the most important is ethanol. Ethanol is thought to produce HCC through the development of hepatic cirrhosis, or as a cocarcinogen with other agents such as HBV, HCV, hepatotoxins and tobacco (Saunder and Latt, 1993; Nalpas et al, 1995; Schiff, 1997; Austin et al,

1986; Trichopoulos, 1987; Naccarato and farinati, 1991) rather than through direct effect on the hepatocytes.

Aflatoxins is produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* have also been linked to HCC. These are fungi that grown on grains, peanuts and other food products and are the most common food spoilage in the tropics. These fungi produce aflatoxins designated as B1, B2, G1 and G2. aflatoxin B1 is the most hepatotoxic, and chronic exposure to these mycotoxins leads to the development of HCC (Yu, 1995).

The nonsteroidal antiestrogen Tamoxifen has become one of the most widely used drugs in the treatment of breast cancer, and concerns about its long-term safety and efficacy are being raised. Investigations in rats have suggested an association between the administration of tamoxifen and the development of hepatocellular carcinoma (Law and Tanden, 2000). Several studies suggests the long-term uses of tamoxifen in females produce HCC (Muhlemann, 1994). The present study investigated the inhibition of tamoxifen induced HCC by *Carum copticum.*

6.2.2 MATERIALS AND METHODS

8 weeks old female Sprague dowley rats were used for the study. The animals were divided into 4 groups of 6 animals in each group. The treatment schedule was as follows.

Group I – Normal (normal saline 1ml)

Group II- Tamoxifen 20mg/kg alone (oral)

Group III- Tamoxifen 20mg/kg alone (oral) + *C. copticum* 250mg/kg

Group IV- Tamoxifen 20mg/kg alone (oral) + *C. copticum* 500mg/kg

To Group II, III and IV animals were given tamoxifen upto 5months, 5days/week. The Group III and IV were administered with C. copticum extract 250mg/kg and 500 mg/kg 5days /week upto 10months. On the 14^{th} month the animals were sacrificed liver and blood was taken and subjected to biochemical and histo pathological analysis.

6.2.2.1 BIOCHEMICAL ANALYSIS

Liver and Kidney was removed and washed thoroughly in ice-cold saline and homogenate (10 %) was prepared in PBS (50 mM, pH 7.0) (section 3.2.2). A part of the homogenate was used for the estimation of reduced glutathione (GSH) (section 3.2.8) and tissue lipid peroxidation (3.2.12). The remaining homogenate was centrifuged at 10,000 rpm for 10 min in a cooling centrifuge at 4°C, after removal of the cell debris; supernatant was used for the assay of superoxide dismutase (SOD) (section 3.2.9), catalase (CAT) (section 3.2.10) and glutathione peroxidase (GPx) (section 3.2.11).

6.2.2.2 Evalutation of GOT, GPT, ALP and GGT

In serum GOT (section 3.2.14), GPT (section 3.2.15) and ALP (section 3.2.16) was estimated. Protein was determined by the method of Lowry *et al.* (section 3.2.13). The GGT was estimated by the method of Szas (section 3.2.17)

6.2.3 RESULTS AND DISCUSSION

Results of the present investigations indicate that the methanolic extract of *C. coptcum* is an effective chemopreventive agent against the Tamoxifen induced hepatocarcinogenesis. This conclusion is supported by various biological properties of the extract. The serum GGT (Table No. 6.2.1) activity was significantly elevated in the tamoxifen alone treated group of animals indicating the induction of hepatocellularcarcinoma. The elevated serum GOT, GPT, ALP (Table No.6.2.2) are indicative of hepatic damage in the tamoxifen treated animals compared to *C. coptcum* treated groups of animals. The elevated hepatic GGT activity is responsible for the increased GSH level in the control group, which is found to be decreased in the extract treated group. In addition to elevated γGT and GPx have been implicated in drug resistance (Tew, 1994). Decreased hepatic GPx and serum γGT activity in the extract treated animal group compared to control support the efficacy of the treatment.

Tamoxifen has been shown to metabolized by the microsomal mixed function oxidase (MFO) system and produce free radicals. These reactive radical interact with DNA producing mutation and oncogenesis. Studies in the hepatoma indicate disequilibria of the delicate oxidant versus antioxidant balance, which is tilted towards an oxidant side (Boitier et al., 1995). This oxidative stress might be the reason for the

Table No. 6.2.1 Effect of *C.copticum* on Tamoxifen induced Hepatocellular carcinoma (MDA and GGT level)

	Tissue MDA level nmol/mg	GGT (U/L) at 25^0C
Normal	1.20±0.10	22.576±2.95
Tamoxifen alone	3.33±0.28*	60.94±5.30*
Tamoxifen + C.copticum 250mg/kg	2.45±0.14*	47.01±2.53*
Tamoxifen + C.copticum 500mg/kg	1.81±0.12*	36.57±2.06*

P<*0.01. Values are Mean ± SD of 6 animals in each group

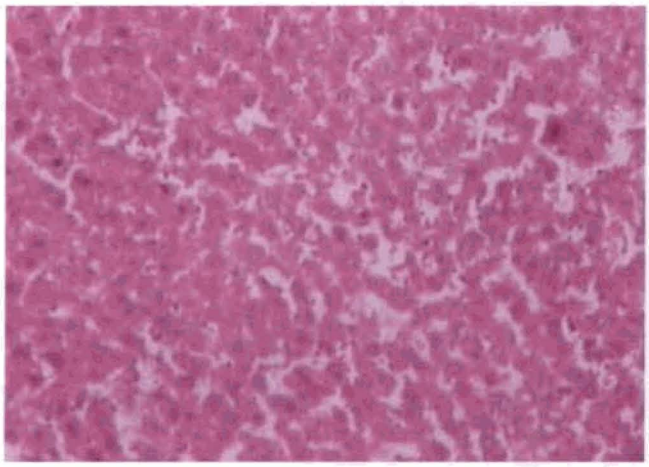
Table No. 6.2.2 Effect of *C.copticum* on Tamoxifen induced Hepatocellular carcinoma

	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	Total Protein mg/dl	Albumin mg/dl
Normal	122.50±9.54	75.67±4.32	131.67±8.07	6.06±0.45	3.55±0.38
Tamoxifen alone	717.00±60.13*	358.5±15.15*	396.83±29.10*	8.05±0.56*	2.26±0.13*
Tamoxifen + C.copticum 250mg/kg	411.67±30.73*	293.17±13.83*	250.6±23.66	7.21±0.27*	2.77±0.09*
Tamoxifen + C.copticum 500mg/kg	320.50±27.88*	211.5±17.12	202.83±15.34	6.07±0.37*	3.02±0.14*

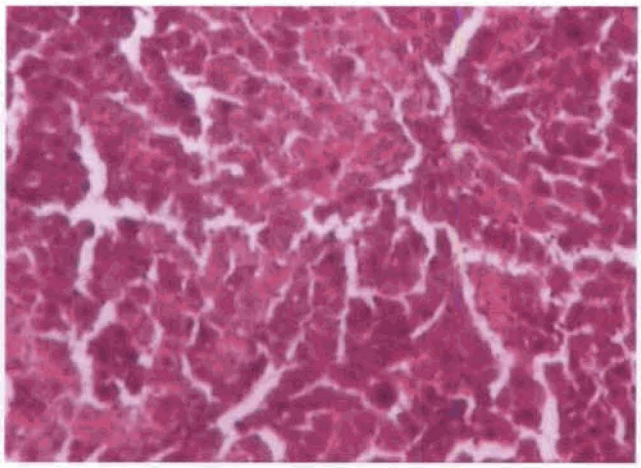
P< *0.01. Values are Mean ± SD of 6 animals in each group

Figure 6.2.1

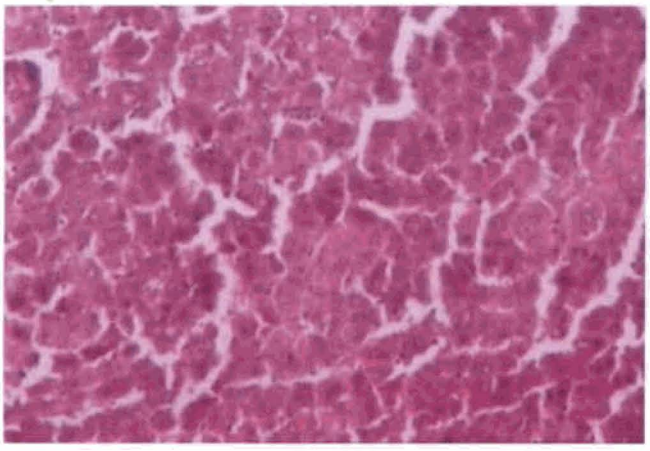
The chemopreventive effect of *C.copticum* on Tamoxifen induced HCC (Histopathological analysis of the liver)



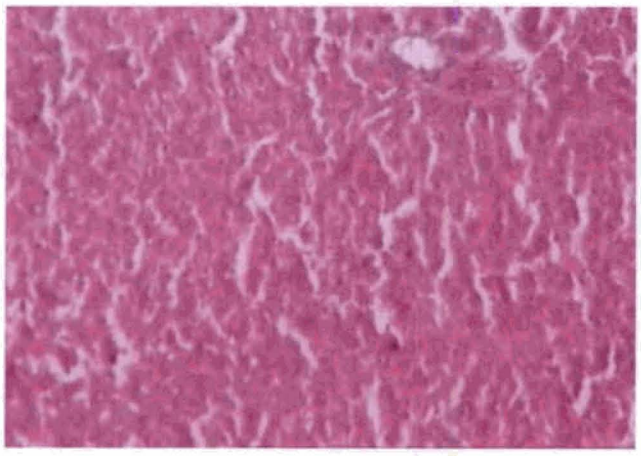
Normal



Tamoxifen alone



Tamoxifen+ *C.copticum* 250mg/Kg



Tamoxifen +*C.copticum* 500mg/Kg

elevated MDA level in the serum of tamoxifen treated animals. Lipid peroxidation can result in the formation of several toxic bye-products such as 4-hydroxynonenal and malondialdehyde that can attack the cellular targets including DNA, inducing mutagenicity and carcinogenesity (Park and Floid, 1992 and Ramal et al., 1986). The treatment of the extract significantly reduces the level of lipid peroxidation. Inhibition of lipid peroxidation may largely because of scavenging lipid peroxyl radicals. The reducing activity of the aqueous extract also partially explains the antiperoxidation activity. The observation that dietary ascorbate inhibits the carcinogenic action of several nitroso-compounds fed to animals can be attributed to its ability to reduce them to inactive forms (Halliwell and Gutteridge, 1999). The histopathological observations support the above findings. Histopathological analysis indicated that the tamoxifen alone treated liver cells were arranged mostly in solid and trabecular pattern, with cellular polymorphism, fatty infiltration, varying mitotic figures and focal necrotic changes (Figure No. 6.2.1). All these changes clearly indicated the hepatocellularcarcinoma. These pathological manifestations were decreased high to moderate level respectively in the 250 mg/kg and 500 mg/kg extract treated group of animals

Reactive oxygen species such as $O_2^{\cdot}$, $^{\cdot}OH$, H_2O_2 and $NO^{\cdot}$ participate in the initiation or promotion of cancer through their ability to cause point mutations, DNA cross-links and DNA strand breaks (Park and Floid, 1992 and Nguyen et al., 1992). Oxidants have the capacity to induce the transcription of growth competence related protooncogene C-*fos* and C-*jun* (Burdon, 1995). Induction of these immediate genes represents a prerequisite for the stimulation of the cell proliferation. A marked increase in the expression of cellular oncogenes such as C-*ras*, C-*fos*, C-*myc* and N-*myc*, involved in neoplastic transformation, has been detected in the rat hepatomas as early as the first month after diethylnitrosamine treatment (Boitier et al., 1995).

The *in vitro* radical scavenging activity of the extract partially explains its mechanism in the prevention of hepatocarcinogenesis. Reducing the nitric oxide generation in the digestive tract was found to be effective in preventing the reaction of nitrites with amines and amides to form carcinogenic nitrosamines and nitrosamides (Boone et al., 1990). The $NO^{\cdot}$ scavenging activity of *C.copticum* extract could also support the preventive role against Tamoxifen induced hepatocellularcarcinoma. Recently, neutrophil-mediated nitrosamine formation has been showed to be a

possible endogenous carcinogen, which may promote neoplasia (Grishman et al., 1992). The results of the earlier study reveal that aqueous extract of *C.copticum* possesses antimutagenic and anti-inflammatory activities that may also contribute to the exhibited anticancer activity.

Chapter 7

Isolation of the antitumor principle from Carum copticum and its Mechanism of action

7.1 INTRODUCTION:

Plants contain a variety of antineoplastic secondary metabolites. For example the Vinca alakaloids isolated from *Catharanthus roseus*, Camptothecin from *Camptotheca acuminata*, Taxol from *Taxus* species etc. These compounds kill cancer cells in different ways. Vinca alkaloids and taxol by the disruption of tubulin microtubules which is very essential for chromosomal separation, Camptothecin by inhibiting topoisomerase I, a key enzyme for DNA replication. Some of these compounds also destroys cancer cells by inducing Apoptosis. Nowadays several antineoplastic compounds have been identified such as curcumin, abrin, quercetin etc which induces apoptosis in cancerous cells. The importance of apoptosis in normal as well as in pathological situations are described in Review of Literature. Shortly, apoptosis is a process in which cells die in a controlled manner, in response to specific stimuli, following an intrinsic programme. Since then, various inducers of apoptosis and mechanisms of apoptosis have been reported. Apoptosis inducers for cancer cells are ideal for cancer chemotherapy.

The present chapter describes the identification of the active fraction from *C. copticum* and the induction of apoptosis by the active fraction in DLA and EAC cell lines.

7.2 MATERIALS AND METHODS

7.2.1 TLC examination of the 70% methanolic extract

The crude methanolic extract was loaded on the TLC plate coated with silica gel G. The extract was separated in toluene:chloroform:ethyl acetate (20:40:1)

solvent system. The compounds of different Rf values (0.1 to 1.0) were separately extracted with methanol (overnight) evaporated to dryness under vaccum and subjected to in vitro cytotoxicity and antitumor studies.

7.2.2 Preparation and application of Spray reagents for TLC (Egon Stahl, 1969)

Libermann-Burchard reagent reagent for triterpene glucoside : five ml acetic anhydride were mixed under cooling with 5ml conc. H_2SO_4. This mixture was added to 50ml ethanol with cooling, sprayed on TLC. After treatment the TLC plate was heated 10min. at 110^0C and observed in long UV light.

Aluminium chloride for falvonoids: The TLC plate was treated with 1% aluminium chloride solution in ethanol and observed under UV illumination for fluorescent spot.

Lead acetate for flavinoids: The TLC plates was treated with 25% aqueous solution of basic lead acetate and observed under UV illumination for fluorescent spot.

7.2.3 Solid tumor activity of the flavonoid fraction (FC)

The animals (female Swiss albino mice) were divided into 4 groups of 10 animals in each group. The solid tumor was induced in all groups of animals by submuscular injection of $3X10^6$ Dalton's Lymphoma Ascites Cells (DLA) in the hind limb of mice. The tumor volume and the body weight of the animals were noted. The treatment schedule is as follows

Group I –Vehicle – (0.1ml normal saline)
Group II - Cyclophosphamide (CTX) 4mg/kg
Group III - FC 5mg/kg
Group IV - FC10mg/kg

7.2.4 Ascites tumor studies of FC

The animals (Swiss albino mice) were divided into 3 groups of 10 animals in each group. The ascites tumor was induced in all groups of animals by intraperitoneal injection of $1X10^6$ Dalton's Lymphoma Ascites Cells (DLA) in the hind limb of

mice. The percent of increase in life span was noted. The treatment schedule is as follows

Group I – Vehicle (0.1ml Normal saline)
Group II- FC 5mg/kg
Group –III- FC10mg/kg

7.2.5 Trypan blue uptake and morphological assessment of Flavonoid fraction induced cell death.

Cells were seeded on 60mm petridishes at the density of 10^7 cells/well containing 5ml DMEM supplemented with 10% FCS. The cells were treated with various concentrations of test materials (1, 2, 4, 8 and 16μg/ml) and incubated at 37^0C for 24 hour. Necrosis was induced by exposing the cells to 60^0C for 15minutes which was used as control. After incubation the cells were harvested by centrifugation. The viability of cells were analysed by trypan blue exclusion method. The cell suspension was stained with Propidium iodide. Then aliquots of the cell suspension were added in a clean slide, laid the cover slip and examined under a florescent microscope. The percent of apoptotic cells were calculated in 20 fields.

7.2.6 Analysis of DNA fragmentation:

The cells were harvested and washed twice with cold PBS. After centrifugation the pellet suspended in lysis buffer (50mM Tris-HCl, 10mMEDTA, 0.5% SDS and 100μg/ml proteinase k) for 5hour at 50^0C. The lysate were extracted with phenol and chloroform: isoamylalcohol (24:1) and the DNA aqueous layer was precipitated with ethanol following the addition of sodium acetate (0.3M). The DNA was collected by centrifugation and dried under vaccum. The Dried DNA was dissolved in TE buffer and incubate with DNase free RNase (1μg/ml) for 3hour. The samples were kept at -20^0C for further use.

7.2.7 DNA electrophoresis

Isolated DNA samples were separated by agarose gel electrophoresis. Ten μg of DNA samples were mixed with loading dye containing glycerol and bromophenol blue (tracking dye) and electrophoresed with TAE buffer. The agarose gel was stained

with ethidium bromide (1μg/ml) and presence of DNA bands were viewed with transilluminator (312nm) (Daniel and Bernard, 1993).

7.3 RESULTS AND DISCUSSION

Of the 10 Rf value compounds separated from the TLC, the 0.4 Rf value fraction showed maximum cytotoxic activity. The TLC examination using the spray reagents showed that this fraction is flavonoid in nature (Table No. 7.1). The FC is cytotoxic to DLA and EAC cell lines (26μg/mland 31μg/ml respectively). We studied the antitumor activity of this fraction using DLA induced solid tumor model and EAC induced ascites tumor model. The flavonoid fraction showed high antitumor activity which is shown in (Figure No.7.2, 7.3; $p<0.01$.TableNo. 7.2, 7.3).

The present study showed that the FC fraction induced apoptosis in DLA and EAC cell lines in a dose dependent manner. The cells treated with 2μg/ml of FC showed more number of apoptotic cells compared to other concentrations. The morphological examination of the FC treated plates showed the presence of apoptotic bodies in DLA and EAC cells. The DNA fragmentation analysis of the FC treated plates showed a ladder pattern which is a characteristic feature of apoptosis. The DNA fragmentation showed that maximum at 2μ/ml of FC treated plates (Table No. 7.4, Figure No.7.4).

Apoptosis is one of the two major modes of death in mammalian cells. Apoptotic cells display DNA fragmentation at internucleosomal sites followed by altered nuclear morphology and finally loss of membrane integrity. In view of increasing evidences that a variety of chemotherapeutic agents such as cisplatin, cytarubicine, camptothecin, Etoposide etc...(Eastman, 1990). Can trigger active cell death an attempt was done whether FC can induce DNA fragmentation. The results presented here, using biochemical and morphological analysis on various cell lines may suggest that possible mode of FC induced cell death is due to the DNA cleavage. The microscopic study of treated cells were showed two important characteristics of apoptosis, chromatin condensation and apoptotic bodies. Analysis of genomic DNA from treated cells by agarose gel electrophoresis revealed a 'ladder' of DNA fragments.

Fig No. 7.1.1 Effect of FC on DLA induceed solid tumor model (Tumor volume)

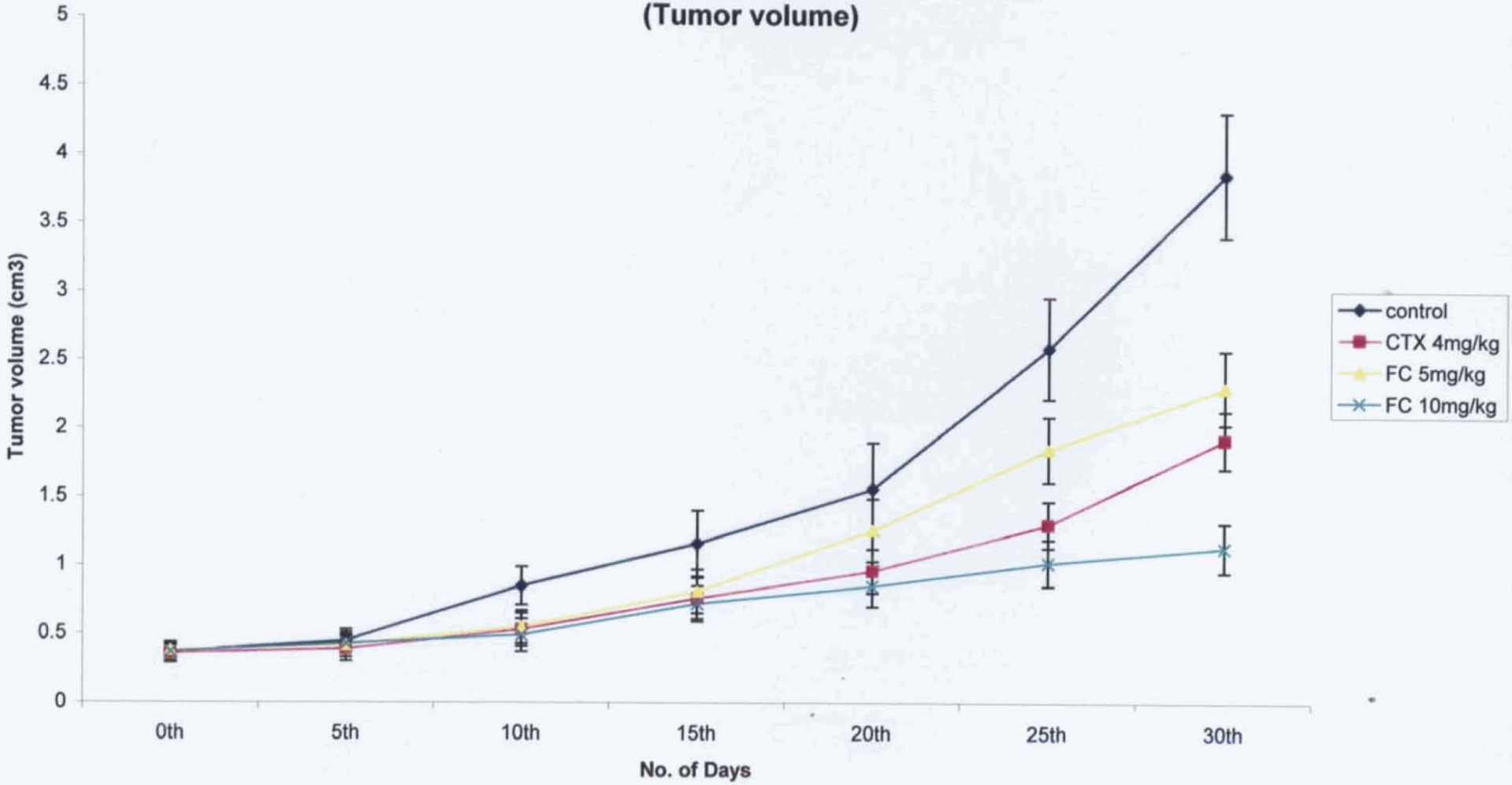
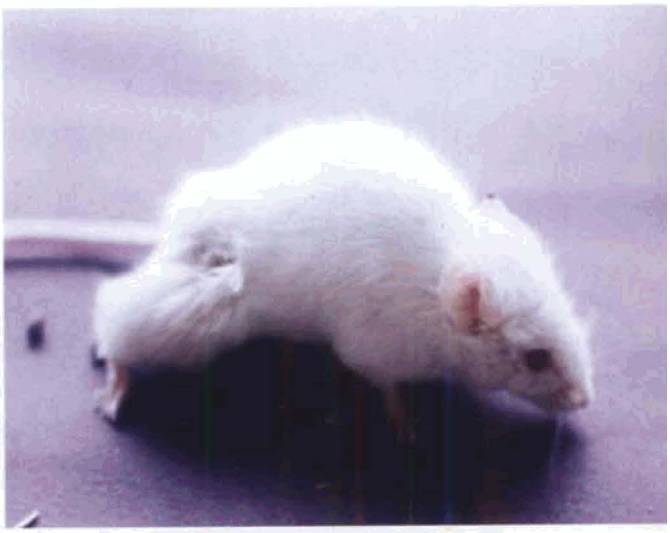


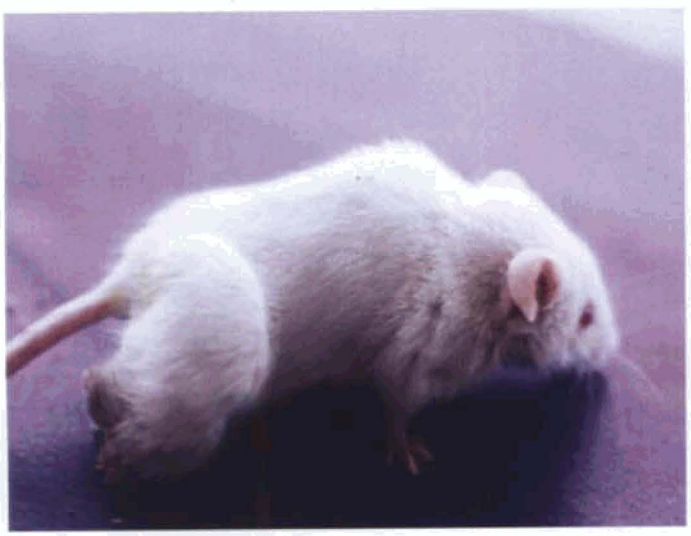
Figure 7.1.2

Effect of FC on DLA induced solid tumor

Control



CTX alone



FC 5mg/Kg

FC 10mg/Kg

Table No. 7.1 Effect of FC on DLA induced solid tumor model (Body weight)

	0th day	5th day	10th day	15th day	20th day	25th day	30th day
Control	22.57±0.81	22.81±0.82	23.32±0.78	23.79±0.84	24.13±0.85	25.94±0.90	27.28±0.91
CTX 4mg/kg	21.34±0.69*	21.63±0.68*	22.14±0.72*	22.52±0.70*	23.03±0.73*	24.13±0.72*	25.42±0.76*
***FC* 5mg/kg**	22.61±0.70*	22.93±0.68*	23.48±0.69*	23.97±0.69*	24.53±0.70*	25.15±0.73*	25.27±0.72*
FC 10mg/kg	21.94±0.74*	22.36±0.73*	22.69±0.75*	23.14±0.79*	23.47±0.81*	24.05±0.83*	24.37±0.84*

P<*0.01. Values are Mean ± SD of 10animals in each group

Table No. 7.2 Effect of FC on EAC induced Ascites tumor model

Groups	No. of Days survived	% of increase in life span
Control	17.34±1.27	
FC 5mg/kg	32.69±3.40	46.96
FC 10mg/kg	48.08±5.24*	177.28

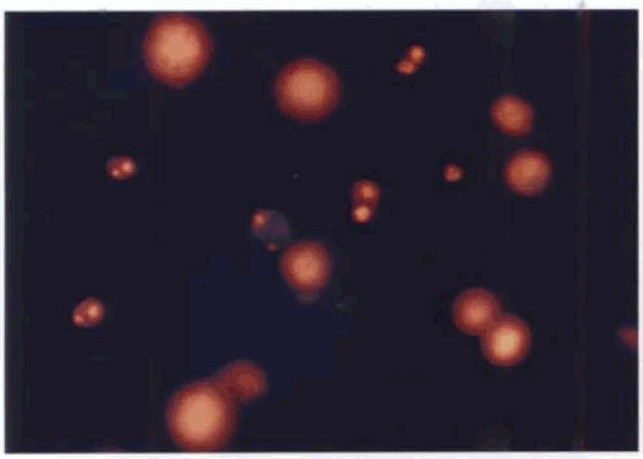
P<*0.01. Values are Mean ± SD of 10animals in each group

Table No. 7.3 FC induced Apoptosis in DLA and EAC cells (% of apoptotic cells)

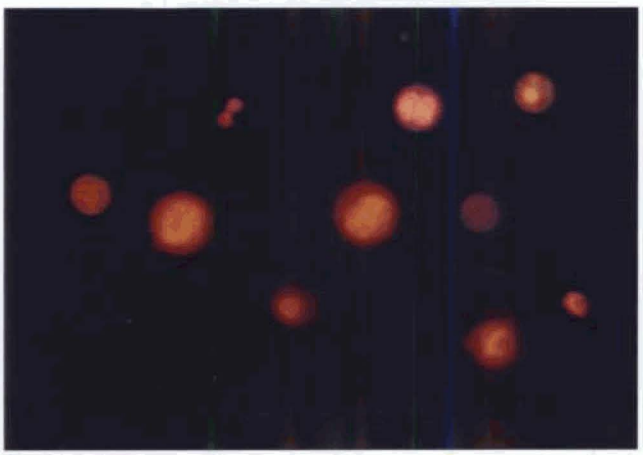
Group	DLA (% of apoptotic cells)	EAC (% of apoptotic cells)
Control (without FC)	-----	----
FC 1µg/ml	9	6
FC 2µg/ml	65	52
FC 4µg/ml	27	16
FC 8µg/ml	6	2
FC 16µg/ml	----	—

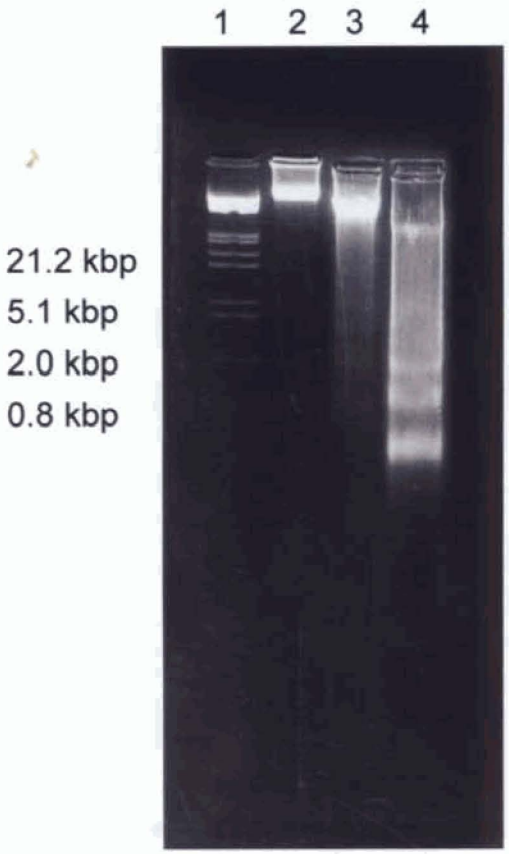
Figure 7.3

FC treated DLA cells (2μg/ml)



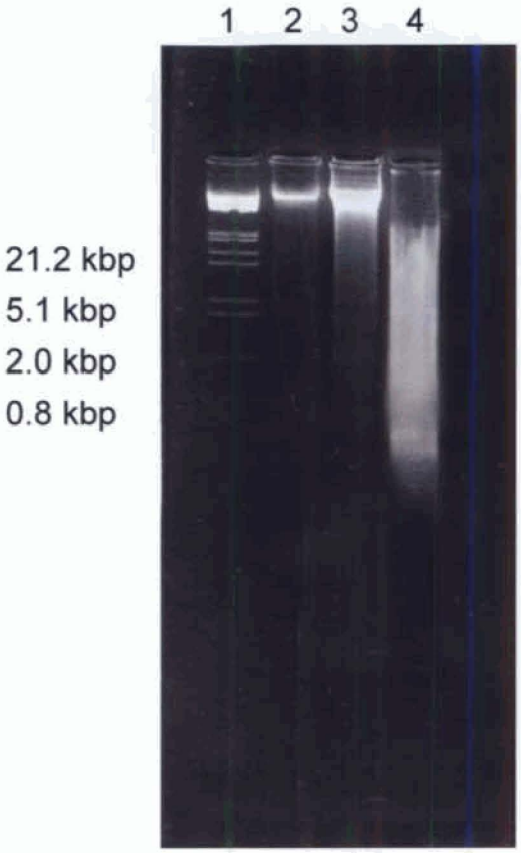
FC treated EAC cells (2μg/ml)





DNA fragmentation analysis of DLA Cells

No. 1. Marker
No. 2. Control Cells
No. 3. 2μg/ml
No. 4. 4 μg/ml



DNA fragmentation analysis of EAC Cells

No. 1. Marker
No. 2. Control Cells
No. 3. 2μg/ml
No. 4. 4 μg/ml

According to Dorota H et al, the percentage of dead cells estimated by trypan blue assay did not always correlate with the percentage of apoptotic cells. Since only at relatively later stage of apoptosis cells lose the ability to exclude cationic dyes such as, trypan blue, the dead cells estimated by this assay were most likely at advanced stages of apoptosis (Halicka and Zbigniew, 1995). Most of the Apoptotic cells were cleaved itself during the culture or mechanical processing and released the DNA content into the medium. So this makes difficult to the simultaneous precise quantification of Apoptotic cells and measurement of DNA cleavage progresses during apoptosis, and the cell also progressively lose DNA by shedding apoptotic bodies. So the time window for the detection of apoptosis, therefore, is not the same for different methods.

The cell death by apoptosis is of different occurrence and can be extensive not only in regressing but also in growing neoplasms (Searle et al, 1977; Columbano 1984). Apoptosis occurs widely in tumors is not the only mode of cell death. According to Luciana et al the cell death by apoptosis is indeed a significant process in hepatoma and that its modulation contributes substantially to cell turn over as well as to growth phase transitions (Luciana, 1993). Since then, various inducers of apoptosis and mechanisms have been reported. Various studies suggest that apoptosis inducers are ideal for cancer therapy (Eastman, 1990).

Chapter 8

The callus culture of Carum copticum and its comparison with the seed extract

8.1 INTRODUCTION:

The World Health Organization (WHO) estimated that 80% of the population of developing countries rely on traditional medicines, mostly plant drugs, for their primary health care needs. Also, modern pharmacopoeia still contain at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants. Demand for medicinal plant is increasing in both developing and developed countries due to growing recognition of natural products, being non-narcotic and having no side-effects. The continued harvestation of these plants from the natural flora lead to the extinction of these plants from the natural habitat. The plant tissue culture is a better alternative for the production of these plants and bioactive compounds without affecting the natural flora. In cultures, factory- type production of plants and bioactive compounds can be carried out throughout the year, unaffected by the season. The risk of crop failure due to natural hazards and the danger of extinction of some species due to their mass extraction from natural populations are eliminated. Moreover, some novel compounds produced in cell cultures are not produced in intact plants.

The present study was aimed at establishing the callus cultures of *Carum copticum* and the comparison of the callus extract with that of the intact plant.

8.2 MATERIALS AND METHODS:

The fertile seeds were obtained from Amala Ayurveda Hospital, Thrissur, Kerala. The seeds were soaked in Tween 20 for 5minutes and thoroughly washed with distilled water. The seeds were transferred to sterile laminar airflow hood (Kemi,

India) for processing under aseptic conditions. The seeds were then washed in 0.1% funiside (Emisan) for 3 minutes and washed in sterile distilled water 4times. Then the seeds were immersed in 0.1% $HgCl_2$ solution for 1minute and washed with strile distilled water 3times. Then the seeds were transferred to the culture flasks containing MS media supplemented with hormones.

8.2.1 Preparation of Nutrient media:

All cultures or the present study were inoculated in Murashige and Skoog medium (Murashige and Skoog, 1962). For preparing the medium, all stock solutions were taken in appropriate portions and final volume was made up by the addition of double distilled water and stock of plant growth regulators.

The prepared medium was supplemented with 3% (w/v) sucrose experimentally. After adding the growth regulators, the pH was adjusted to 5.6-5.8. Agar 0.75% (w/v) dissolved by heating was used as the gelling agent for semi solid medium and with out agar for liquid medium.

The medium containing agar and all other ingredients was dispensed into culture tubes and conical flasks, which were presterilized by autoclaving. The tubes and flasks were plugged with sterile cotton plugs. The tubes containing medium were finally sterilized by autoclaving at 121 ^{0}C and 15 lbs pressure for 20 minutes. The cultures were placed in a culture room on a 12hour dark/light photoperiod with light intensity 2000-3000 lux by cool incandescent tubes maintained at 25±1 ^{0}C and relative humidity 60-80%.

8.2.2 Culture environment

Primary explants as well as pieces of calli were cultured in media and incubated in a culture room maintained at 25^{0}C. Callus induction started under dark and calli were subcultured every four weeks to the same medium or other combinations of the media. All cultures were alternatively exposed to 12 hr photoperiod at a photon flux density 30-50uE m-2s-1 light fluorescent lamp (Philips India Ltd).

8.2.3 Estimation of relative growth rates of calli

Fresh weight of initiated calli in promising culture media were recorded at the time of initial subculture (four weeks after callus initiation). All the experiments were replicated three times. Growth of established calli was estimated as relative growth rate (RGR) over 28 days subculture period.

$$RGR = \frac{3(W_2 - W_1)}{t_2 - t_1}$$

Where W_1 = Initial callus mass at t_1 (g)

W2 = Final callus mass at t_2 (g)

t = Period (days)

$t_2 - t_1$ = 28 days

8.2.4 Superoxide radical scavenging activity (section 3.2.3).

8.2.5 Hydroxyl radical scavenging activity (section 3.2.4).

8.2.6 Nitric oxide radical scavenging activity (section 3.2.6).

8.2.7 Inhibition of in vitro tissue lipid peroxidation (section 3.2.5).

8.3 RESULT AND DISCUSSION:

We have germinated the *C. copticum* seeds in vitro and established the callus culture from the in vitro germinated plants. We used MS and Whites medium for the present study but got maximum response in MS medium compared to whites medium. So we selected MS medium for further experiments. We used 5 hormonal combinations to generate callus from in vitro grown plants. These are as follows

MS + KN2mg/l + NAA 4 mg/l

MS + KN0.5mg/l +2,4-D 2mg/l

MS + BA 4mg/l

MS + KN1mg/l+ NAA 2mg/l

MS + KN4mg/l+ NAA 2mg/l

Table No. 8.1 The relative growth rate of *C. copticum* callus in different hormone combinations

Media composition	Relative growth rate
MS + KN 2mg/l+ NAA 4mg/l	23.03±2.57
MS + KN 0.5mg/l+ 2,4-D 2mg/l	6.78±1.24
MS + BA 4mg/l	20.96±2.92
MS + KN1 mg/l + NAA 2mg/l	8.67±1.53
MS+ KN4mg/l+ NAA2mg/l	3.03±0.68

P<*0.01. Values are Mean ± SD of 20 tubes in each group

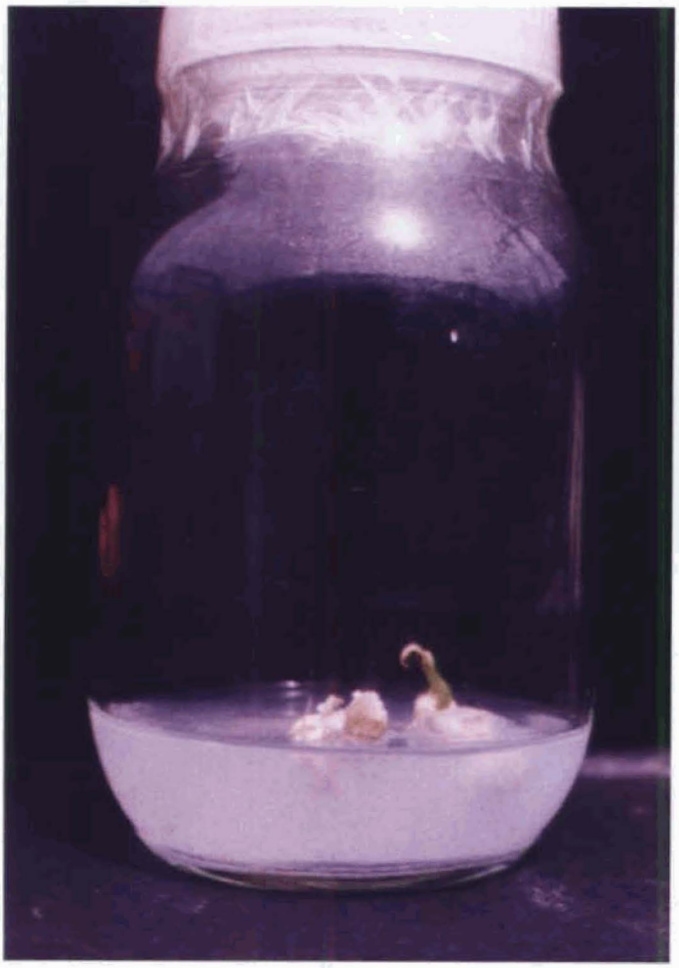
Table No. 8.2 In vitro antioxidant Status of the extract of *C. copticum* callus and the comparison with the seed extract

	Seed extract µg/ml	Callus extract µg/ml
Superoxide radical	67.13±3.4	1260.0±34.45*
Hydroxyl radical	132.40±6.98	1968.0±56.83*
Nitric oxide radical	45.97±2.86	656.45±34.60*
In vitro lipid peroxidation	148.0±8.9	2456.7±132.5*

P<*0.01. Values are Mean ± SD of triplicate in each group

Figure 8.1

In vitro generation of callus from *C.copticum* leaf



Of these 5 different hormone combinations MS + KN2mg/l + NAA 4mg/l showed maximum response with a relative growth rate of 23.03±2.57. The callus are yellow in colour and fragile in nature (Figure No. 8.1). The MS + KN4mg/l + NAA 2mg/l showed very little response compared to the other 4 hormonal combinations. That is in this plant high auxins and low kinetins is responsible for callus induction (Table No. 8.1).

We have harvested the callus, freeze dried and extracted with 70% methanol and subjected to in vitro antioxidant analysis. The results showed that the *C. copitcum* callus extract showed very low activity compared to the seed extract. The production of secondary metabolites is very low in the undifferentiated cells, ie; in callus and higher in the differentiated tissues such as leaf, stem, seeds etc. These may be reason for the low activity of the callus compared to the seed extract (Table No. 8.2).

Chapter 9

Summary and conclusion

Cancer is a major health problem worldwide and of the most important causes of morbidity and mortality in children and adults. Cancer arises from the uncontrolled proliferation and spread of clones of transformed cells. In a year presently, a little over 10 million new cases of cancer and 6.4 million deaths due to cancer have been estimated to occur globally based on the rates of the year 1990. The prevention of cancer requires knowledge of its causes. History shows that epidemiological studies have been the key to the control of a wide range of infectious diseases.

Primary prevention of cancer is one of the key approaches to the control of cancer. It includes i) avoiding exposure to known cancer causing agents, ii) enhancement of host defence mechanisms, iii) modifying lifestyle, and iv) chemoprevention.

Cancer chemoprevention means prevention or inhibition of carcinogenesis or prevention of the development of invasive cancer using synthetic or naturally occurring chemicals (Kelloff and Boone 1996; Kelloff et al, 1997; Mayne and Lippman 1997; Sporn 1991). Although still in its infancy, the new science of chemoprevention has been established as an important approach to control malignancy. For the first time, it has been shown convincingly that the use of chemopreventive agents in men and women with premalignant lesions can substantially reduce the subsequent development of truly invasive cancer. Chemoprevention is now recognized as both a clinical and basic science.

The natural products are the most consistently successful source of drug leads. The chemical novelty associated with natural products is higher than any other source. The new developments of natural products in cancer treatment include camptothecin, podophyllotoxins, taxol etc.

The plants belonging to the family Apiaceae have high medicinal properties. Some are used in Ayurvedic system of medicine. Some are reported to have anticancer activity. Some anticancer compounds were isolated from these plants including D-Limonene, Carvone etc. The important plants belonging to this family include *Hydrocotyle asiatica*, *Eryngium coeruleum*, *Apium graveolens*, *Carum carvi*, *Carum copticum*, *Daucus carota*, *Ferula asafetida*, *Foeniculum vulgare*, *Coriandum sativum* etc.

We selected four plants *Carum copticum*, *Pseucedanum vulgare*, *Coriandum sativum* and *Cuminum siminum* for the present study. These plants are used in traditional Indian medicine for the treatment of different diseases such as Stomach ulcers, liver damage, snakebite, scorpion sting etc. We prepared the 70% methanolic extract of the seeds of these plants and screened for its in vitro cytotoxic activity in DLA and EAC cell lines using Trypan blue exclusion method. The results of the cytotoxic studies revealed that the extract of *C. copticum* is more cytotoxic than other three plant extracts. Hence, we continued further studies with *C. copticum* only.

The Toxicity studies revealed that the *C. copticum* is non toxic to animals. The *C. copticum* extract treated animals didn't show any considerable change in SGOT, SGPT, ALP and creatinine levels compared to the normal group. The histopathological studies of the liver and kidney revealed that the extract treated group of animals maintained the normal architecture.

C. copticum extract treated groups of animals (50mg/kg and 100mg/kg) showed a significant reduction in DLA induced solid tumor model in a dose dependent manner. The *C. copticum* extract also showed a synergistic action when administered along with cyclophosphamide and radiation. The ascites tumor studies also revealed that administration of the extract (50mg/kg and 100mg/kg) increased the life span of the ascites tumor bearing animals in a dose dependent manner.

The TLC analysis of the methanolic extract showed that the active component may be the flavonoid fraction (FC, Rf value 0.4). The studies of the FC showed that it is highly toxic to DLA and EAC cell lines and are highly antitumor in nature.

The free radicals are implicated in the main causes of diseases such as cardiovascular diseases, cancer, ageing, diabetics, inflammation etc. We studied the in vitro free radical scavenging activity of methanolic extract of *C.copticum*. These studies revealed that the *C.copticum* methanolic extract could inhibit the formation of superoxide radical, hydroxyl radical and nitric oxide radical in vitro. The extract also prevents in vitro lipid peroxidation.

The liver protects the body from potentially injurious substances (endotoxins) absorbed from the intestinal tract, as well as the toxic byproducts of metabolism. The most important in the detoxification process is that of the microsomal drug metabolizing system of the liver. A large number of xenobiotics are reported to be potentially hepatotoxic due to the generation of free radicals, which can undergo a variety of secondary reactions. Sometimes these may lead to liver cancer. We studied the hepatoprotective activity of *C. copticum* 70% methanolic extract in CCl_4 and paracetamol induced hepatic damage. The control animals showed an increase in levels of SGOT, SGPT and ALP levels whereas the extract treated groups of animals showed a significant decrease in these levels compared to the control group. The in vivo antioxidant status such as SOD, CAT, GSH, and GPx levels are also increased in extract treated groups of animals compared to the control group.

Gastric ulceration in the stomach due to various factors is a serious health problem today. The NSAIDs and ethanol are the main causes of ulcer due to the generation of free radicals. These may sometimes lead to stomach cancer. Hence, the search is still on to find a compound that possesses antioxidant, anti-inflammatory activity to control ulceration. The present study showed that the *C. copticum* extract administration (250mg/kg and 500mg/kg) could inhibit the aspirin and ethanol induced gastric ulceration in a dose dependent manner. The extract (250mg/kg and 500mg/kg) also inhibited the carrageenan, dextran and formalin induced paw oedma in a dose dependent manner. Hence, the extract can be used as both anti-inflammatory and antiulcer agent.

The mutations are the main causes of cancer. Several compounds are mutagenic to animals and human beings. Several plant extracts and compounds showed antimutagenic activity in laboratory experiments. We investigated the

antimutagenic activity of *C. copticum* extract (1,2 and 4mg/plate) using *Salmonella typhimurium* strains (TA 98 and TA 100). We used four mutagens NaN_3, NPDA, MNNG and doxorubicin which revealed that the extract is antimutagenic to these mutations.

The radiotherapy and chemotherapy are the most commonly used treatment modalities of cancer. But these posses side effects like myelosuppression, alopecia, mental retardness etc. Hence the search is still on to find a drug which can inhibit the toxicities due to radiation and chemotherapy. We studied the radioprotective and chemoprotective activity of *C. coptium* using γ-irradiation and CTX induced toxicities. The administration of *C. copticum* extract (250mg/kg and 500mg/kg) increased the total count and bone marrow cellularity in a dose dependent manner in both irradiated animals and CTX treated animals. The in vivo antioxidant enzyme status of the extract treated groups of animals also increased compared to the control groups. This results *C. copticum* is radioprotective as well as chemoprotective.

Colorectal cancer represents a major public health problem, especially in developed countries. Colorectal cancers rank third in frequency in men and second in women. Epidemiologic factors have provided initial evidence about the specific factors that initiate the process of carcinogenesis in the large bowel mucosa. The important factors that can initiate colorectal cancer development are predisposition to mutagen effects, fecal mutagens, meat intake, bile acids, altered vitamin and mineral intake and fecal pH. We studied the colon cancer chemopreventive activity of *C. copticum* using AOM induced colon cancer in female Balb/c mice. The results of the present investigation revealed that the *C. copticum* extract (250mg/kg and 500mg/kg) significantly inhibited the formation of ACF in a dose dependent manner which is an initial stage in the development of colon cancer.

Hepatocelluar carcinoma (HCC) is the most common solid-organ tumor worldwide, being responsible for more than 1million deaths annually. HCC is closely associated with chronic liver injury and, therefore, geographic distribution of HCC closely mirrors that of vital hepatitis. Chemical carcinogens also have been linked to primary liver cancers such as nitrites, hydrocarbons, solvents, organochlorine

pesticides. The nonsteroidal antiestrogen tamoxifen has become one of the most widely used drugs in the treatment of breast cancer and concerns about its long-term safety are being raised. Investigations in rats have suggested an association between the administration of tamoxifen and the development of hepatocellular carcinoma (Law and Tanden, 2000). In view of our results we studied the hepatoprotective activity of *C. copticum* in tamoxifen induced HCC. The results of the present investigation showed that the *C. copticum* extract (250mg/kg and 500mg/kg) inhibited the formation of HCC in a dose dependent manner.

The anticancer agents possess different mechanisms to kill or prevent the development of cancerous cells. Apoptosis is one among them. We studied the apoptotic activity of FC in DLA and EAC cell lines. The morphological examination showed the presence of apoptotic bodies in FC treated plates. The DNA fragmentation analysis which is characteristic feature of apoptosis revealed that the FC treated cells showed a characteristic DNA ladder pattern. Further experiments is necessary to find out the enzyme and gene responsible for FC induced apoptosis.

The medicinal plants are exploited from the natural flora for the isolation of chemicals. This lead to the disappearance of these plants from the natural habitat. The biotechnological approach such as plant tissue culture is a viable option to produce the medicinal plants in laboratory conditions without affecting the natural flora. We have established the callus culture of *C. copticum* and compared its in vitro antioxidant activity with that of the seed extract. The callus extract showed very little activity compared to the seed extract. The secondary metabolites are produced more in the differentiated state than in the undifferentiated state. This may account for the low antioxidant activity of callus extract compared to the seed extract.

The following conclusions could be drawn from the present investigations

The *Carum copticum* metaholic extract showed significant antitumor activity in DLA induced solid tumor as well as EAC induced ascites tumor model. The extract is non toxic to the animals. The extract showed significant antioxidant, anti-inflammatory, hepatoprotective, gastroptotective, radioprotective and chemoprotective activity. The extract is antimutagenic. The extract inhibited the

formation of colon cancer and hepatocellular carcinoma in experimental animals. The active principle may be the flavonoid fraction which is cytotoxic and antitumor against DLA and EAC induced solid tumor models. It induce apoptosis in DLA and EAC cell lines. The callus extract of *C. copticum* showed very little activity compared to the seed extract. However, further investigations are necessary to isolate and characterize the active principle and to find out its mechanism of action.

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