STUDIES ON THE ANTIOXIDANT AND ANTICARCINOGENIC ACTIVITIES OF A WOOD INHABITING MACROFUNGUS, *PHELLINUS RIMOSUS* (BERK) PILAT

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Certificate

I certify that the thesis entitled " Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrofungus, *Phellinus rimosus* (Berk). Pilat." submitted to Calicut University, Calicut, in partial fulfillment of the requirement for the award of Doctor of Philosophy in Biochemistry (faculty of medicine) is an authentic account of the work carried out by Ajith. T.A. under my supervision and guidance. And no part these has been presented for the award of any other degree, fellowship or any other similar titles of any university or society.

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Declaration

I hereby declare that the thesis entitled " Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrofungus, *Phellinus rimosus* (Berk). Pilat." is bonafied record of research work done by me under the supervision of Dr. K.K. Janardhanan, Professor, Amala Cancer Research Centre and Dr. Ramadasan Kuttan, Director, Amala Cancer Research Centre, Amala Nagar, Thrissur for the award of "Doctor of Philosophy" and no part of these has been presented for any other degree, fellowship or any other titles of any university or society.

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CONTENTS

List of abbreviations

AHH	:	Aryl Hydrocarbon Hydroxylase
Ah	:	Aromatic hydrocarbon
ALP	:	Alkaline Phosphatase
AP-1	:	Activator Protein 1
APC	:	Adenomatous Polyposis Coli
2-AF	:	2-acetamidofluorene
AQ	:	Aqueous
B[*a*]P	:	Benzo[*a*]pyrene
BPDE	:	Benzo[*a*]pyrene-7,8-diol-9,10-epoxide
CCl_4	:	Carbon tetrachloride
CDNB	:	1-chloro-2,4-dinitrobenzene
CDK	:	Cyclin Dependent Kinase
COX-1	:	Cyclooxygenase-2
COX-2	:	Cyclooxygenase-1
DCC	:	Deleted in Colon Carcinoma
DLA	:	Dalton's Lymphoma Ascites
DNA	:	Deoxyribonucleic acid
DMBA	:	7,12-dimethyl benz[*a*]anthracene
DMSO	:	Dimethyl sulphoxide
DTNB	:	5,5-dithiobis-(2-nitrobenzoic acid)
EAC	:	Earlich's Ascites Carcinoma
EtOAc	:	Ethyl acetate
GGT	:	γ-Glutamyl transpeptidase
GPT	:	Glutamate pyruvate transaminase
GOT	:	Glutamate oxaloacetate transaminase
GSH	:	Reduced glutathione
GST	:	Glutathione *S*-transferase
Hb	:	Haemoglobin
HBM	:	Higher basidiomycetes
HCC	:	Hepatocellularcarcinoma
H_2O_2	:	Hydrogen peroxide
IC_{50}	:	50 % inhibiting concentration
ILS	:	Increase in life span

JNK	:	*c-jun* NH_2 terminal Kinase
KCl	:	Potassium chloride
MDA	:	Malondialdehyde
MeOH	:	Methanol
MFO	:	Mixed function oxidase
$MgCl_2$	:	Magnesium chloride
MNNG	:	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
NADPH	:	Reduced nicotinamide adininedinucleotide phosphate
NADP	:	Nicotinamide adenine dinucleotide phosphate (disodium)
NaN_3	:	Sodium azide
NBT	:	Nitroblue tetrazolium
NDEA	:	*N*-nitrosodiethylamene
NF-κB	:	Nuclear factor kappa B
$(NH_4)_2SO_4$	:	Ammonium sulphate
NPDA	:	4-nitro-*o*-phenylenediamine
NO	:	Nitric oxide radical
NSAID	:	Non-steroidal anti-inflammatory drugs
O_2^-	:	Superoxide anion radical
OFR	:	Oxygen free radical
OH	:	Hydroxyl radical
$ONOO^-$	:	Peroxynitrite
PAH's	:	Polycyclic aromatic hydrocarbons
PBS	:	Phosphate buffered saline
PGs	:	Prostaglandins
PKC	:	Protein kinase C
PMN	:	Polumorphonuclear neutrophils
P. rimosus	:	*Phellinus rimosus* (Berk.) Pilat.
RBC	:	Red blood cells
RNA	:	Ribonucleic acid
RNS	:	Reactive nitrogen species
ROI	:	Reactive oxygen intermediate
ROS	:	Reactive oxygen species
SDS	:	Sodium dodecyl sulphate
SGPT	:	Serum glutamate pyruvate transaminase

SGOT	:	Serum glutamate oxaloacetate transaminase
TBA	:	Thiobarbituric acid
TCA	:	Trichloroacetic acid
TPA	:	12-*O*-tetradecanoylphorbol-13-acetate
WBC	:	White blood cell
WT	:	Wilms' tumor

INTRODUCTION

Ajith. T.A "Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrohungus, Phellinus rimosus (Berk) Pilat. " Thesis. Amala Cancer Rcsearch Centre , University of Calicut, 2004

Chapter 1

Introduction

Although substantial progress has been made in the chemotherapeutic design and implementation, metastatic cancer is an insidious disease for which definitive treatment is rarely predictable. In spite of understanding of some of the major etiological factors, prevention is undeniably a sensible approach towards the ultimate goal of cancer control or eradication (Suffness and Douros, 1991). The number of deaths related to cancer continues to increase on an annual basis.

Several methods exist for the treatment of cancer in modern medicine, which include chemotherapy, radiotherapy and surgery. The discovery of drugs for the effective treatment of cancer is an extraordinary challenge. The drug must kill or disable a variety of subpopulations of a tumor without undue harm to normal tissue of the same origin and also to normal tissue of other types throughout the body. Natural products have been of exceptional value in drug discovery programmes and also in the mechanistic studies involving biological components of relevance in the control of cancer. Key examples include taxol, bleomycin, CC-1065, camptothecin, bryostatin and phorbol esters and forskolin (Suffness and Douros, 1991). The National Cancer Institute (NCI), United States has recently intensified its emphasis upon natural products such as plants, marine organisms and selected class of microorganisms as sources for new drug discovery. Screening of plant extracts for anticancer activity began at NCI in 1956. Among the microbial sources, fungi are currently of major interest.

The mushrooms are mainly represented as macrofungi. They are widely distributed in nature, many are edible and are highly medicinal. Attempts have been made in many parts of the world to explore the use of mushrooms and their metabolites for the treatment of a variety of human ailments. Higher Basidiomycetes mushrooms have been used in folk medicine throughout the world since ancient times (Wasser and Weis, 1999a). Many pharmaceutical substances with potent and unique properties were isolated from mushrooms and distributed worldwide. Most mushrooms derived preparations and substances find their use not as pharmaceuticals ('real' medicines) but as a novel class of dietary supplements (DS) or 'nutriceuticals' that fall very well into the concept of functional foods. A recent survey by Wasser and Weis (1999a) indicated that fungi produced a large number of antitumor agents. Medicinal mushrooms useful against cancer are known in China, Russia, Japan and Korea as well as in United States and Canada (Wasser and Weis, 1999b). Hence, the search for new antitumor and other medicinal substances from higher Basidiomycetes and the study of the medicinal value

of these edible and non-edible mushrooms have become matters of interest. Species of *Phellinus* are wood inhabiting macrofungi found growing on tree trunks. Present investigation envisages to evaluate the antioxidant and anticarcinogenic activities *Phellinus rimosus,* a macrofungus found growing on the tree trunks of jackfruit tree.

The oxidative properties of oxygen play a vital role in diverse biological functions such as utilization of nutrients, electron transport to produce ATP and the removal of xenobiotics (Hemnani and Parihar, 1998). While oxygen is essential for life, it also can provoke damaging oxidative events within cells. Oxygen, by it's transformation to more reactive forms i.e., superoxide radical (O_2), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) can nick DNA, can damage essential enzymes and structural proteins and can also provoke uncontrolled chain reactions, such as lipid peroxidation or autooxidation reactions (e.g. polymerization of catecholamines) (Bijlani, 1995 and Halliwell and Cross, 1994). There is considerable interest in the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) as mediators of tissue injury in human diseases. Lipid peroxides, produced from unsaturated fatty acids via radicals cause histotoxicity and promote the formation of additional free radicals in a chain reaction-type manner. These radical affects cytoplasmic and nuclear signal transduction pathways, nitration of tyrosine residues by $ONOO^-$ may block phosphorylation and modulate the activity of the proteins and genes that are related to cell proliferation, differentiation and apoptosis. Hence, it is proposed that ROS and RNS play a key role in human cancer development. Several lines of evidence now indicate that antioxidant may prevent or delay the onset of some types of cancer.

Several mechanisms are responsible for the protection of the cells from potential cytotoxic damages caused by the radicals. Cells have developed various enzymatic and nonenzymatic defense systems to control excited oxygen species. However, a certain fraction that escapes the cellular defense may cause permanent or transient damage to nucleic acids within the cells, leading to such events as DNA strand breakage and disruption of Ca^{++} metabolism. The *in situ* metabolic activation of chemicals results in selective toxicity to target organs. The generated reactive intermediates can initiate toxicity via binding to cellular macromolecules or via generation of ROS, which may lead to peroxidative damage of functionally crucial cellular structure such as membranes or nucleic acid. Liver and kidney are the major organs capable of metabolizing chemicals to toxic reactive metabolites are severely affected by these chemicals.

ROS can produce gross chromosomal alterations in addition to point mutations and thus could be involved in the inactivation or loss of the second wild type allele of a mutated proto-oncogene or tumor suppressor gene that can occur during tumor promotion, allowing expression of the mutated phenotype. The various agents known to cause cancer by oxidative mechanisms are prospective mutagenic agents for the germ line. In the face of the intense mutagenic pressure that drives the process of carcinogenesis, it will be necessary to use agents that either are potent antimutagens or can significantly alter patterns of gene expression. The rise in DNA base damage could be either due to increased oxidative damage and/or decreased repair activity; the increased damage to DNA in inflammation is presumably due to increased ROS/RNS production, often by the activation of phagocytes. A notable activity of tumor promoters is their ability to recruit inflammatory cells and to stimulate them to generate ROS/RNS. Over the past several years, hundred of plant extracts have been evaluated for new cancer chemopreventive agents using their potential to inhibit inflammation.

Removing ROS/RNS is probably one of the most effective defenses of a living body against diseases. Any compound, natural or synthetic, with antioxidant properties when continuously taken as components as dietary food, health spices and drugs might contribute towards the partial or total alleviation of this damage, would play a pivotal role in maintaining health. The putative therapeutic use of many traditional medicines appears to be attributed to the presence of antioxidant principles. The great variation in their magnitude as well as multitude of activities may even become more important for protective effects in situations where free-radical species are not directly involved in the disease process but may participate and or foster the secondary events. Structurally different cancer preventive agents suppress superoxide anion radical and hydrogen peroxide production by phorbol ester tumor promoter-activated human neutrophils. This inhibition of H_2O_2 generation can serve as a facile system for identifying and measuring the activity of cancer preventive agents. Such agents inhibit inflammation and oxidative damage as well as tumor promotion.

Considering the significant importance of cancer chemotherapeutic agents from natural products in chemotherapy, development of new effective agents with fewer side effects is a compelling urgency. In the present study intensive investigation on the antioxidant, anti-inflammatory, anticarcinogenic, antimutagenic, antitumor, hepatoprotective and nephroprotective activities of ethyl acetate, methanol and aqueous extracts of *Phellinus rimosus* was carried out. The findings are presented in this thesis.

Review of literature

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Chapter 2

Review of literature

2.1CANCER

The word cancer refers to a state of uncontrolled malignant growth of poorly differentiated cells. Cancer can occur essentially in any organ of the body and the cancer cells will tend to resemble their normal tissue of origin. Cancers of various organs are quite different from another and even within organs, there are many different subtypes. The natural etiology of tumors is complex and usually occurs over a long time (Suffness and Douros, 1991).

2.2 ETIOLOGY OF CANCER

Carcinogenesis may result from the activity of any one or a combination of chemical, physical, biological and genetic insults to cells. The genetic changes evidenced by virtually every possible type of mutation including transaction, transversion, deletions of various sizes, chromosomal rearrangements, gene amplification and insertional mutagenesis have been described (Harris, 1990; Schwab and Amler, 1990 and Gray, 1991). Many of these genetic changes are acquired, but some are inherited as germline mutations. Many chemical and physical carcinogens can induce one or more of a variety of mutations in cells when given chronically. These mutagens also include the hair dye amine, 1-nitropyrene from diesel exhaust, the flame retardant, tris (2,3-dibromopropyl) phosphate and several of the proteins pyrolysis products produced by cooking foods (Maron and Ames, 1983). The flow chart depicting a simplified scheme of transformation of normal cell to malignant neoplasm is given in figure 2.1.

2.2.1 Chemicals

Many human cancers are caused by environmental factors, mainly chemicals. It is now about 200 years, since the London surgeon Sir Percival Pott correctly attributed scrotal skin cancer in chimney sweeps to chronic exposure to soot. Yamagiwa and Ichikawa in 1915 reawakened interest in Pott's observation by inducing cancer in a rabbit's ear with the repeated application of coal tar. Despite the diversity of chemistries, more than 95 % of the various carcinogenic chemicals fall into one of five major categories: a) direct acting carcinogens, b) procarcinogens that require metabolic activation, c) aromatic amines, amides and azo dyes, d) natural plant and microbial products and e) others (Table 2.1).

The following pertinent observations have emerged from the study of chemical carcinogens (Kumar et al., 1992):

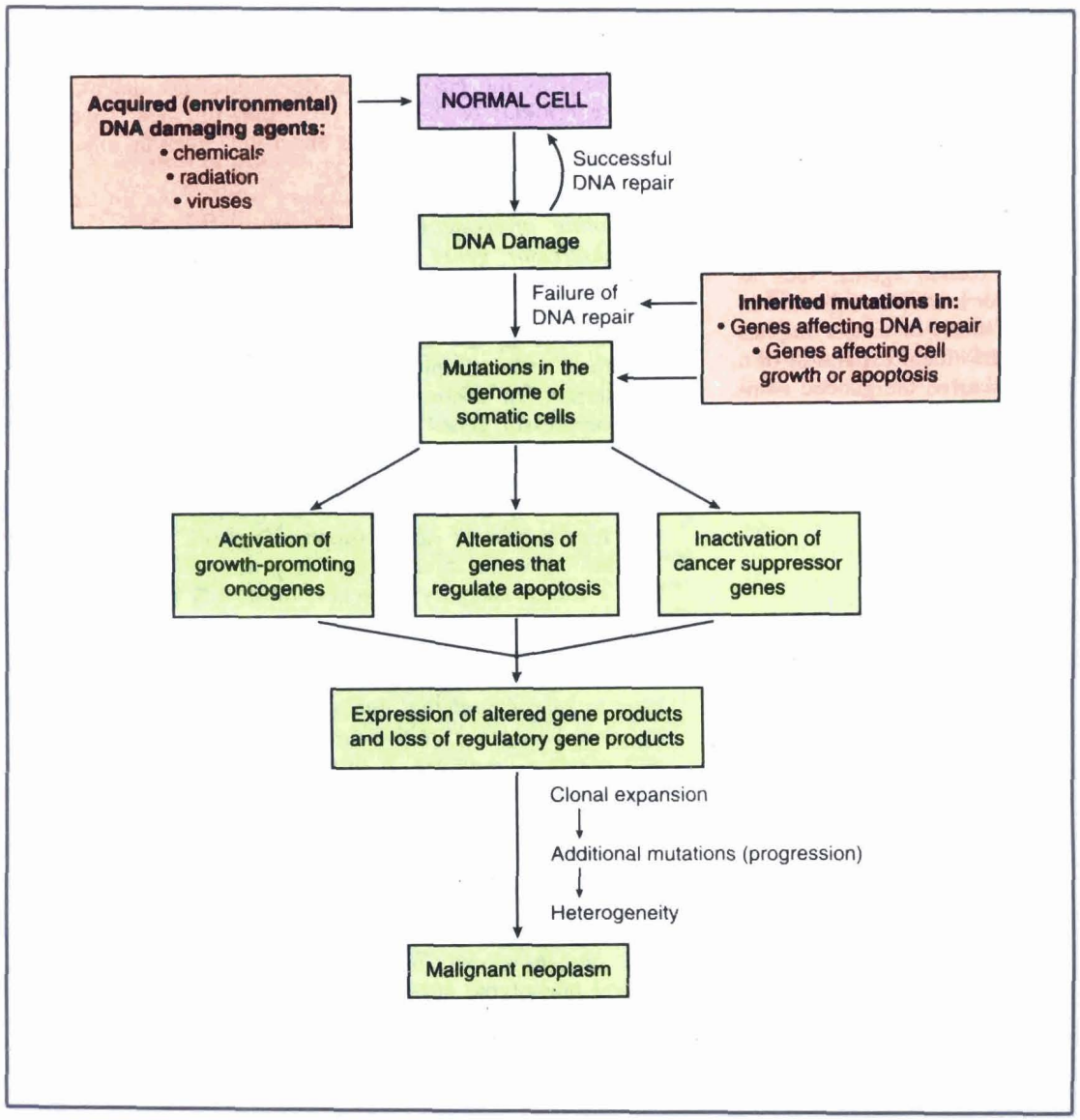


Figure 2.1. Flow chart depicting a simplified scheme of the molecular basis of cancer (Adopted from Text book of Robbins Pathologic Basis of Diseases. Cotran et al., 1999)

TABLE 2.1

MAJOR CHEMICAL CARCINOGENS

Direct-acting carcinogens

Alkylating agents

Anticancer drugs (cyclophosphamide, chlorambucil, nitrosoureas and others)

Acylating agents

1-Acetyl-imidazole

Dimethylcarbamyl chloride

Procarcinogens that require metabolic activation

Polycyclic and heterocyclic aromatic hydrocarbons

Benzo[*a*]anthracene

Benzo[*a*]pyrene

Dibenz[*a,h*]anthracene

3-Methylcholanthrene

7,12-Dimethylbenz[*a*]anthracene

Aromatic amines, amides, azo dyes

2-Naphthylamine (β-naphthylamine)

2-Acetylaminofluorene

Dimethylaminoazobenzene (butter yellow)

Natural plant and microbial products

Aflatoxin B1

Griseofulvin

Tobacco

Others

Nitrosamine and amides

Vinyl chloride, Nickel, Chromium

Insecticides, fungicides

Polychlorinated biphenyls (PCBs)

Arsenic

Asbestos

1) They are extremely diverse structure and include both natural and synthetic products.
2) Some are direct acting (require no chemical transformation to induce carcinogenicity). eg. alkylating agents (cyclophosphamide, chlorambucil, nitrosoureas etc.) The indirect acting carcinogens are referred as procarcinogens, require activation to become ultimate carcinogens. eg. benz [*a*] anthracene, benzo [*a*] pyrene, 7,12-dimethylbenz [*a*]anthracene, 3-methylcholanthrene etc.
3) All chemical carcinogens are highly reactive electrophiles that react with the electron rich atoms in RNA, DNA and proteins. The critical target most probably is DNA (Miller, 1978). In DNA nucleotides they bound with electron sharing atoms such as ring nitrogen or exocyclic oxygen atoms to form stable altered nucleotides or adducts (Hemminki, 1994).
4) The carcinogenisity of some of the chemicals is augmented by agents that by themselves have little if any cancerous activity, called as promoters.

The principle underlying the metabolic activation and inactivation of inactive carcinogens (procarcinogens) and inactive mutagens (promutagens) is by the use of enzymes referred as phase I and Phase II respectively. The metabolically activated carcinogens (ultimate carcinogens) can undergo critical or non-critical binding with the informational cellular macromolecules. The metabolic activation of procarcinogens to ultimate carcinogens and their fate are shown in figure 2.2. The production of carcinogenic or mutagenic metabolites is thus the result of a balance between activation and detoxification pathways.

2.2.1.1 Metabolic activation to electrophilic intermediates

The metabolic activation of most xenobiotics involves reactions catalyzed by phase I and phase II enzymes (Table 2.2). Among the phase I enzymes, cytochrome(s) P450-dependent mixed-function oxidase have received particular attention, as they are considered to be major catalysts of the rate limiting steps in the metabolic activation of most chemical carcinogens and mutagens. The mixed-function oxidase consists of three components (Lu, 1976): 1) reduced nicotinamide adenine dinucletide phosphate (NADPH) cytochrome-c reductase (NADPH-cytochrome-P450 reductase), 2) cytochrome P450, and 3) a phospholipid, phosphatidylcholine. NADPH-cytochrome-P450 reductase uses reducing equivalents supplied by NADPH to reduce cytochrome P450, the terminal oxidase, which binds the lipophilic substarte such as, drugs,

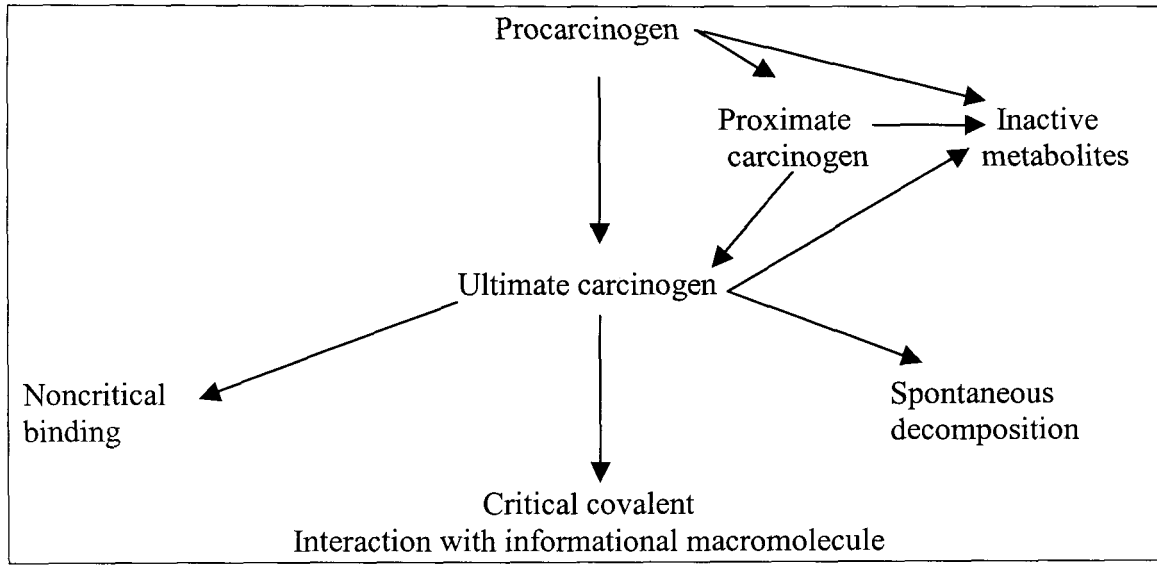


Fig 2.2-Metabolic activation of procarcinogens to ultimate carcinogens

Table 2.2– Enzymes Phase-I and phase-II involved in the metabolism of carcinogens

Phase I	Azo-nitro-reductase (M/C)
	CytochromeP450-dependent monooxygenases
	Cytochrome P450-independent oxidase (M)
	Epoxide hydratase (M/C)
	Hydrolases (M)
	Dehydrogenases (C)
Phase II	Acyl-transferases (C)
	Glucuronyl transferases (M)
	Glutathione S-transferases (C)
	Sulfotransferases (C)

M, microsomal enzymes; C, cytosolic enzymes

carcinogens and steroids. Cytochrome exists in multiple forms with different but overlapping substrate specificities (Lu and West, 1981 and Guengerich et al., 1982). Many factors that affect xenobiotic metabolism qualitatively or quantitatively do so by altering the relative amounts or activities of the various forms of cytochrome P450. Induction of enzyme molecule synthesis is frequently used to modify enzyme activities. Aroclor, Phenobarbital and 3-methylcholanthrene are inducers used most commonly for this purpose. Cytochrome P450 inducibility has been linked with carcinogenesis in humans especially smoking populations. Arylhydrocarbon hydroxylase (AHH), a catalytic activity for polycyclic aromatic hydrocarbons (PAH) associated with P450 isoform 1A1, is associated with lung cancer in Japanese population (Nakachi et al., 1991). Most of the PAH interact with a cytoplasmic steroid family receptor Ah (aromatic hydrocarbon), upon complexing with the ligand, is translocated to the nucleus and enhances the transcription of specific CYP450 isozyme.

2.2.1.2 Inactivation of chemical carcinogens

Inactivation of chemical carcinogens plays a similar important function in determining the susceptibility of toxicity. The phase II enzymes collectively add polar groups to P450 reaction products, increasing their water solubility or hydrophilicity and allowing their elimination by excretion. In liver these enzymes are present in higher levels.

2.2.2 Physical agents

Physical carcinogens, such as x-ray, γ-ray and uv-ray may cause mutation and genomic instability. In 1928, the role of uv radiation was demonstrated in skin cancer in experimental animals. Ultraviolet radiation catalyses the formation of pyrimidine cyclobutane dimmers (Beukers and Berends, 1960) and photoproducts, both of which are formed between adjacent thymine bases (Fig. 2.3a & b) and can cause GC-to-AT transition mutations in DNA, if not repaired. The formation is supported by the genetic defect in patients with xeroderma pigmentosum, characterized by deficiency of excision repair system, who have a marked susceptibility to melanoma and non-melanoma skin cancer. The biologic effects are elicited primarily with the UV region 280-320 nm (UV-B) (Perantoni, 1998). Ionizing radiations such as x rays and γ rays are linked with carcinogenesis. Due to tissue penetration of certain type of ionizing radiations, the generated oxygen free radicals are involved in the mutagenic and carcinogenic potency of these radiations. Formation of free radicals cause thirty

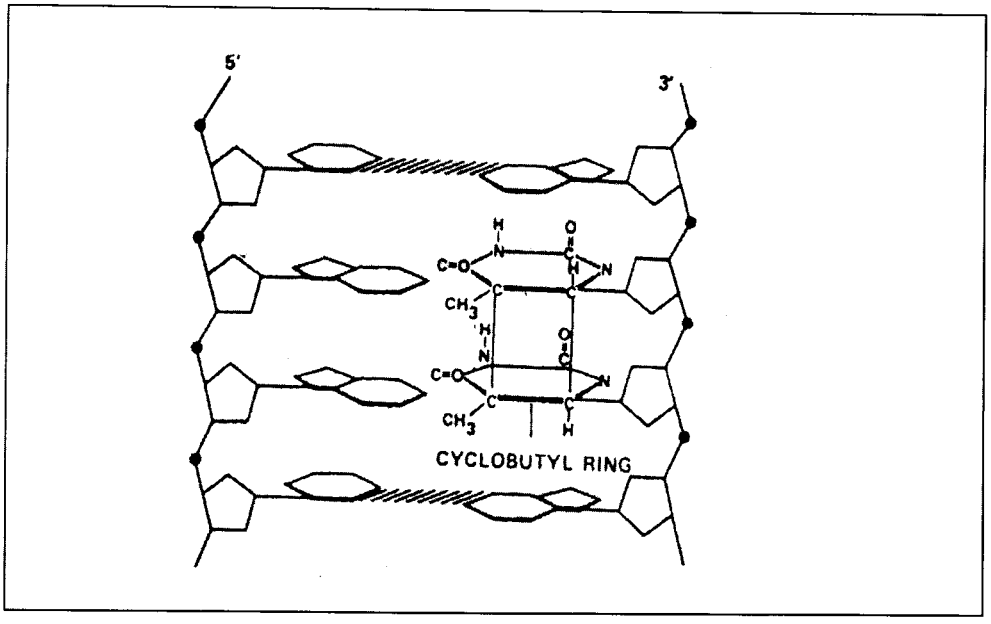


Figure 2.3a. The formation of cyclobutane dimmers induced by u.v exposure

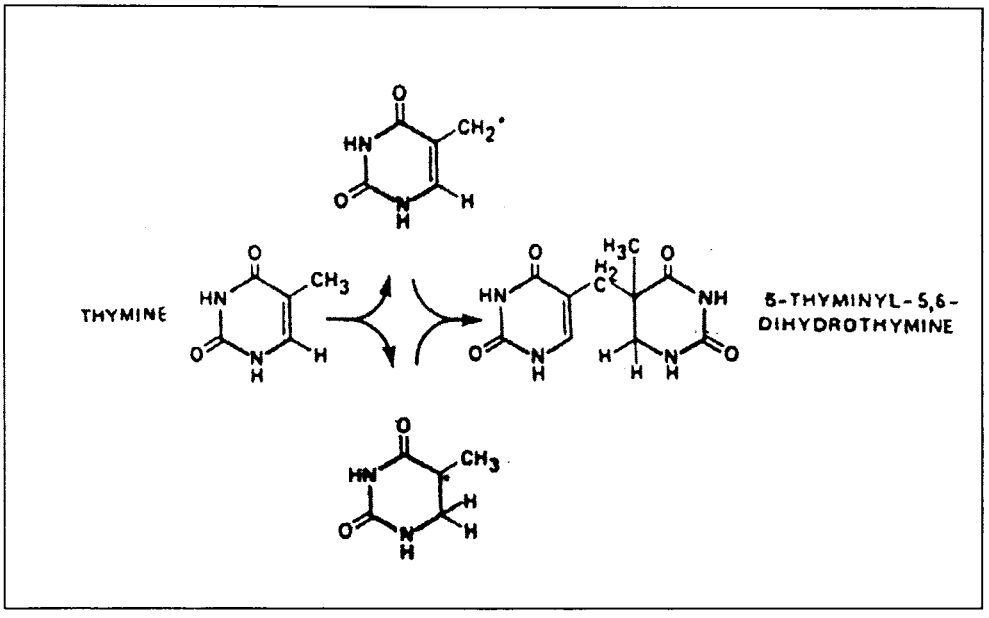


Figure 2.3b. The formation of 5-thyminyl-5,6-dihydrothymine (spore photoproduct) induced by u.v exposure

different DNA adducts as well as DNA-protein cross-links (Feig et al., 1994). The free radical generated mutations may results either from these adducts or indirectly from the radical induced alteration in the DNA polymerase.

2.2.3 Biological agents

The role of virus in the causation of cancer came from the observations made by Peyton Rous in 1908 at Denmark defining the transmissibility of avian leukemia. The first oncogenic virus, an RNA tumor virus or retrovirus was described by him in 1911as an ultrafiltrable agent could induce sarcoma in chickens. Later a large number of DNA and RNA viruses have been proved to be oncogenic in animals. Only a few viruses have been linked with human cancer. Epstein bar virus (EBV) has been implicated in the pathogenesis of Burkitt's lymphoma and nasopharyngeal cancer. Human papillomavirus (HPV) definitely cause benign sqamous papillomas particularly squamous cell carcinoma of the cervix and anogenital region. Hepatitis B virus (HBV) is closely associated with hepatocellular carcinoma. Human T- cell leukemia virus- 1 (HTLV-1) is associated with a form of T-cell leukemia/ lymphoma that is endemic in certain parts of Japan (Kumar et al., 1992).

2.3 Critical molecular targets during the stages of carcinogenesis

The process of carcinogenesis may be divided into at least three stages; initiation, promotion and progression (Table 2.3). Three major classes of genes that are likely to be the molecular targets for the development of neoplasia. These are protooncogene (Temin, 1974), cellular oncogene (Garrett, 1986) and tumor suppressor genes (Marshall, 1991 and Boyed and Barrett, 1990). Oncogenes are altered form of normal cellular genes, called protooncogenes that regulate cellular growth process such as proliferation, differentiation and programmed cell death. Their products are positive effectors of transformation. Oncogene may have a viral counter part or no viral counter part. Protooncogenes are localized in different compartments and are expressed at different stages of the cell cycle. Protooncogenes are converted into oncogenes by genetic mechanisms of mutation, gene amplification and chromosomal rearrangement (Bishop, 1989). Various types of mutations include base substitutions, deletions and insertions have been explained (Bishop, 1991). In human tumors most characterized oncogene mutations are point mutations that are frequently detected in the *ras* family of protooncogenes (Rodenhuis, 1992), K-*ras* (90% carcinomas of pancreas, 50% colon carcinomas and 30% lung adenocarcinomas), H-*ras* and N-*ras* (found in haematologic malignancies). The consequence of *ras* mutations is the constitutive activation of the

TABLE 2.3

Morphologic and Biologic characteristics of the stages of initiation, promotion and progression during carcinogenesis.

Initiation	Promotion	Progression
Irreversible with constant "stem cell" potential	Operationally reversible both at the level of gene expression and at the cell level by "remodeling" and /or apoptosis.	Irreversible Measurable or morphologically discernible alteration in cell genome's structure.
Efficacy sensitive to xenobiotic and other chemical factors.	Promoted cell population existence dependent on continued administration the promoting agent.	
Spontaneous (fortuitious) occurrence of initiated cells can be quantitated.	Efficacy sensitive to aging and dietary and hormonal. factors.	Growth of altered cells sensitive to environmental during early phase of this stage.
Requires cell division for "fixation".		
Dose- response does not exhibit a readily measurable threshold .	Dose-response exhibits measurable threshold and maximal effect dependent on dose of initiating agent.	Benign or malignant neoplasms characteristically seen.
Relative effect of initiators depends on quantitation of focal lesions following defined period of promotion.	Relative effectiveness of promoters is measured by the effectiveness when given at a constant dose rate to cause an expansion of the progeny of the initiated cell population.	"progression agents act to advance promoted cells into this stage but may not be initiating agents.

signal transducing function of the *ras* protein. Studies demonstrated that three protooncogene families-*myc* (10-20% of breast and ovarian cancers show c-*myc* amplification), e*rb* B (15-30% of breast and ovarian cancers have e*rb* B 2 (HER-2/neu) amplification) and *ras* (K-*ras* and N-*ras*) are amplified in a number of tumors. Chromosomal rearrangements are often detected in haematologic malignancies as well as in some solid tumors (Solomon et al., 1991, Rabbits, 1994). These arrangements consist mainly of chromosomal translocations and less frequently chromosomal inversions. The t (8;14) (q24;q32) translocation found in about 85 % of cases of Burkitt's lymphoma, resulted transcriptional activation of c-*myc*, which encode a nuclear protein involved in the cell proliferation (Dalla et al., 1982). In most follicular lymphomas and some large cell lymphomas, the *bcl*-2 gene (located at 18q21) is activated as a consequence of t (14;18) (q32;q21) translocations (Cleary et al., 1986). Fusion genes can be created by chromosomal rearrangements when the chromosomal breakpoints fall within the loci of two different genes. The product of such gene fusions leads to the formation of chimeric transcription factors and chimeric protein with transforming activity. Chimeric protein with transforming activity has been studied in chronic myelogenous leukemia (CML). The t (9;22) (q 34;q11) translocations in CML fuses the c-*abl* gene, normally located at 9q34, with the *bcr* gene at 22q11 (Shitvelman et al., 1985). The *bcr.abl* fusion gene encodes chimeric protein of 210 kd with increased tyrosine kinase activity (Sawyers, 1992) (Fig. 2.4). Translocations in solid tumors result in gene fusions that encode chimeric oncoproteins (Rabbits, 1994).

2.3.1Tumor suppressor genes (antioncogenes)

The tumor suppressor genes were studied in retinoblastoma. These genes encode proteins that negatively regulate the growth of cells and just as for the protooncogenes, function at a variety of levels in signal transduction and cell cycle regulation. Families predisposed to certain types of cancers often exhibit the loss of deletion of one copy of a specific chromosomal regions that contain tumor suppressor gene (heterozygous). This locus occurs in germ line, allowing it to be passed to subsequent generations. Tumorigenesis occurs with the loss of second copy (loss of heterozygocity). Non-familial cancers also arises through this mechanism, but both alleles must be mutated or lost, one allele is mutated and the other is lost or mutation of one allele can act as a dominant negative mutation to incapacitate the function of the normal allele (Knudson, 1985; Marshall, 1991 and Ponder, 1988). In such sporadic cases both mutations occur somatically within the same cell. Hence, cancer can be

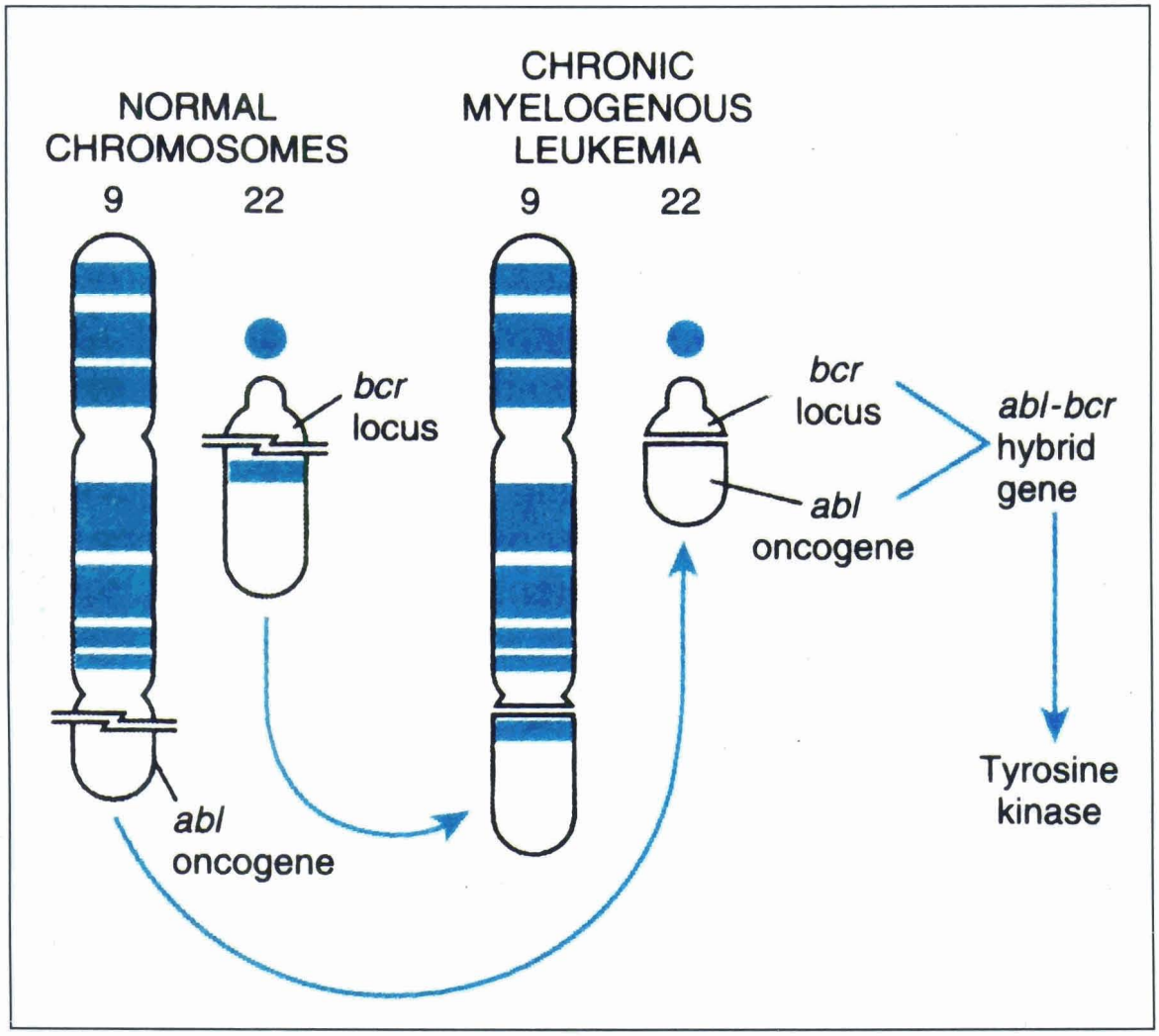


Figure 2.4. Chromosomal translocation and formation of fusion gene product im chronic myelogenous leukemia

(Adopted from Text book of Robbins Pathologic Basis of Diseases. Cotran et al., 1999)

viewed fundamentally as a genetic disease involving germline or somatic mutations of genes that control the cell cycle, apotosis and key regulators of cellular interactions and differentiation (Leong and Leong, 1998).

a) *Retinoblastoma gene (RB gene)*

The mutations required to produce retinoblastoma involve the Rb gene, located on chromosome 13q14. Both normal alleles of the Rb locus must be inactivated for the development of tumor. The RB gene encodes a 928-residue nuclear protein p105 which serve as a negative regulatory factor in cell proliferation. The nuclear protein found in the phosphorylated and unphosphorylated forms. Unphosphorylated form of p105-RB bind with E2F family of transcription factors, and inhibits the transcription of S phase gene; break the cell cycle at the G1 to S transition. Several proteins from viruses bind to pRB. The proteins are capable of inactivating pRB by sequestering pRB and releasing the E2F transcription factor like the mitogen mediated phosporylation. These proteins are EBNA-5 from the Epstein-Barr virus (Szekely et al., 1993), E7 protein from human pappiloma virus (Munger et al., 1989) adenovirus E1A protein (Whyte, 1989) and SV 40 large T antigen (De Caprio et al., 1988). Phosphorylated (inactive) or mutated form of pRB is essential for tumorigenesis. In addition to retinoblastoma, osteosarcomas, small cell lung, bladder, breast and pancreatic carcinomas have altered pRb protein to varying extents (Perantoni, 1998).

b) p53 gene

p53 gene located on chromosome 17p. Its action as a tumor suppressor gene is found only when it is found in the nucleus. DNA damaging agents (radiations and chemotherapy) or hypoxia induce *p53* gene, the accumulated wild type *p53* binds to DNA, stimulates transcription of several genes that mediate the two major effects of *p53*: cell-cycle arrest in the late G_1 phase and apoptosis. The cell cycle arrest is mediated by *p53*-dependent transcription of the CDK (cyclin dependent kinase) inhibitor $p21^{WAF1/CIP1}$(Xiong et al., 1993). This *p21* gene inhibits the cyclin/CDK complexes and thus prevents the phosphorylation of *pRB* necessary for the cell to enter S phase. *p53* also induce the transcription of *GADD45* (*G*rowth *A*rrest and *D*NA *D*amage), a protein involved in DNA repair. Successful repair of DNA allows cells to proceed with the cell cycle. If DNA repair fails, *p53* induced activation of *bax* gene promotes apoptosis. In cells with loss or mutation of *p53* gene results in cell proliferation and malignant neoplasm. The role of *p53* in maintaining the integrity of the genome is given in figure 2.5. Inactivation of normal *p53* can occur through the

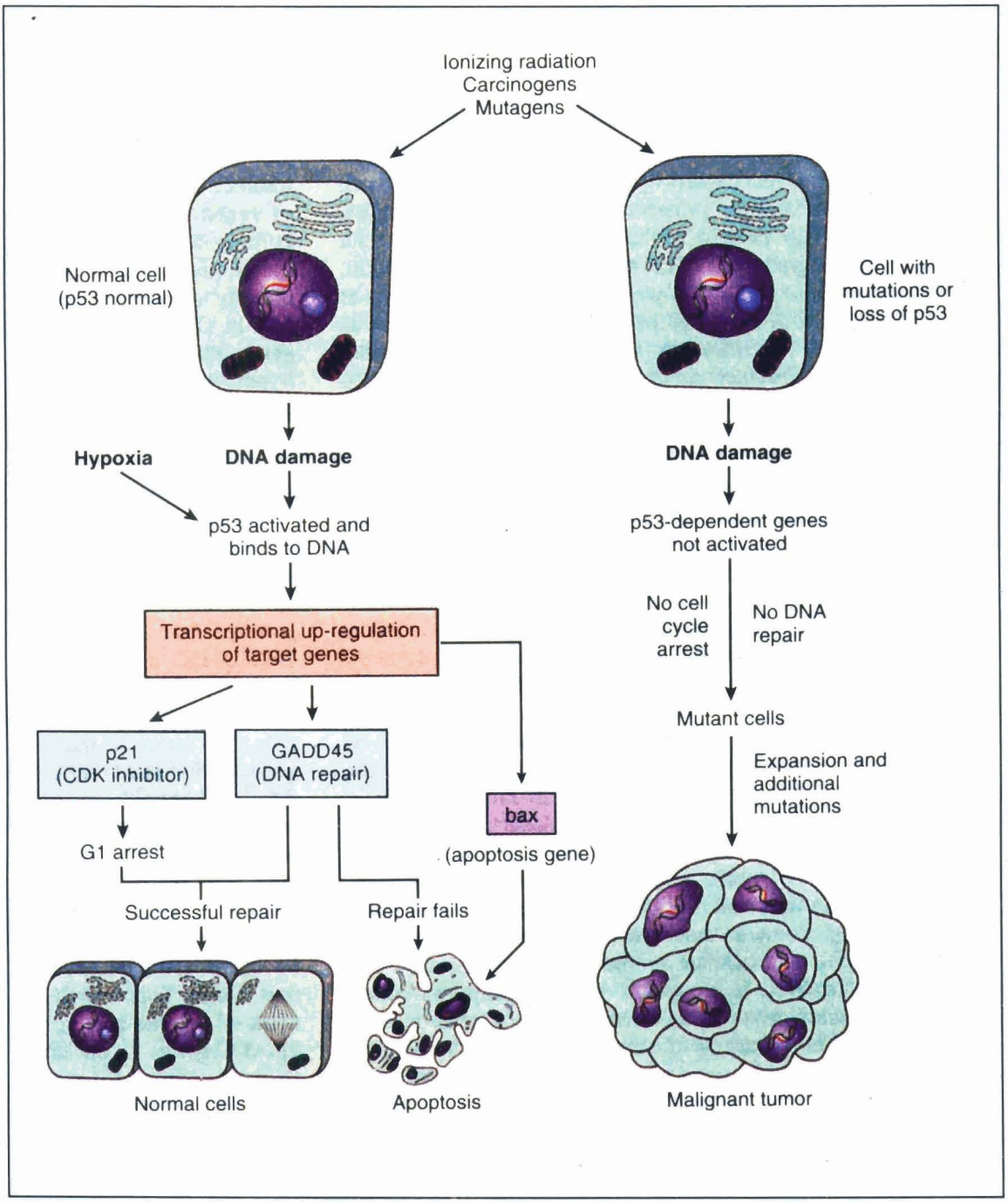


Figure 2.5. The role of p53 in maintaining the integrity of the genome
(Adopted from Text book of Robbins Pathologic Basis of Diseases. Cotran et al., 1999)

action of several virally encoded proteins, including SV 40 T antigen, adeno virus E1B and oncogenic human papillomavirus E6 protein (Lane et al., 1979; Sarnow et al., 1982 and Werness et al., 1990). Inactivation of *p53* function can also from the loss of effect of *p53* protein on gene transcription, changes in the *p53* coding sequence, posttranslational modifications such as phosphorylation. The loss of function of this nuclear protein by germ line mutations in one allele of *p53* gene is associated with the Li-Fraumeni syndrome, a familial condition characterized by high frequency of a diverse group of neoplasms. 75% to 80% of colon tumors show abnormalities at both *p53* alleles.

Mutation and inactivation of the other antioncogenes also associated with the development of tumor in humans. Mutations of the genes, *BRCA-1 and BRCA-2,* account for 80% of familial cases and are involved in breast and ovarian cancers respectively. Both are involved in the repair of DNA. Mutations in WT-1 (Wilms tumor) gene appear to be responsible for at least some cases of sporadic and familial Wilms' tumor (Haber et al., 1990). Loss of both *NF-1* (*neuroblastomatosis*) alleles in neuroblastomas would allow cell proliferation. Mutation of one allele of *Adenomatous Polyposis Coli (APC)* gene occur in familial Adenomatous Polyposis, which is an autosomal dominant condition in which patients develop innumerable neoplastic polyps that carpet the entire colon. The colorectal carcinomas contained somatically acquired mutations in *Deleted in Colon Carcinoma (DCC)* gene, including, deletions, point mutations and insertions.

In human, numerous examples of mutation in the *p53* tumor suppressor gene have been described in a variety of neoplasm, some of which appear to be related to the presumed causative agent in at least some of the specific types of neoplasm that develop (Hsu et al., 1991 and Puisieux et al., 1991). Mutational alterations in tumor suppressor genes that result in the neoplastic transformation most likely develop in the stage of progression (Goyette et al., 1992 and Bevilaequa et al., 1991). The genetic alteration of a single copy of a tumor suppressor gene may be initial event for the stage of initiation, with alteration of the other allele occurring as the determinant in the transition from the stage of promotion to that of progression. Free radicals may be one of the muatgens involved in some of the mutations observed in the genes.

2.4 FREE RADICALS

A free radical is any species, which is capable of independent existence and contain one or more unpaired electrons (Halliwell and Gutteridge, 1989). The unpaired

electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non-radical.

2.4.1Oxygen radical

The biologically relevant free radicals derived from oxygen are the superoxide anion (O_2^-), perhydroxyl radical (HO_2), hydroxyl radical ($^{\bullet}OH$) and singlet oxygen. Reactive oxygen species (ROS) such as O_2^-, H_2O_2 and $^{\bullet}OH$ have been conventionally regarded as having carcinogenic potential and have been associated with tumor promotion (Irani et al., 1997). Many of the tissue effect of O_2^- result from the secondary formation of other oxygen radicals in addition to direct reactions of O_2^- with biological targets such as lipids (Dix and Aikens, 1993), catecholamines (Macarthur et al., 2000) and DNA (Dix et al., 1996). The oxidation potential and reactivity of various ROS may be given in the order $O_2^- < H_2O_2 < {}^{\bullet}OH$ (Fridovich, 1978). The most reactive oxy-radical is $^{\bullet}OH$, formed from H_2O_2 and O_2^- by the Fenton and Haber-Wiss reaction respectively (Knight 1999).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \rightarrow \text{Fenton reaction}$$

$$O_2^- + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2 \rightarrow \text{Haber-Wiss reaction}$$

Several sources of ROS in the cells are proposed (Fig. 2. 6). They are the following.

1. From leakage of electrons on to oxygen from mitochondrial electron transport chains, microsomal cytochrome P-450 and their electron donating enzyme systems (Beal, 1997 and Fridovich, 1989). About 1-4% of the oxygen taken by the body is converted as free radicals.
2. Reactive oxygen species are produced in living cells as by products of normal metabolism of xenobiotics (Parihar et al., 1997; Stohs and Bagehi, 1995 and Wintson and Digiulio, 1991).
3. During exposure to high temperature (Parihar and Dubey, 1996), or radiation (Sen, 1995).
4. For useful purposes, ROS, e.g. O_2^-, HOCl and H_2O_2 are produced from activated phagocytes such as monocytes, neutrophils, esinophils and macrophages at the site of inflammation (Babior and Woodman, 1990 and Prakash et al., 1998). Formation of O_2^- in a respiratory burst has also been observed in the Kuffer cells of the liver.
5. The univalent reduction of O_2 forming O_2^- also occurs from other normal biochemical oxidation- reductions, both enzymatic (e.g. xanthine oxidase,

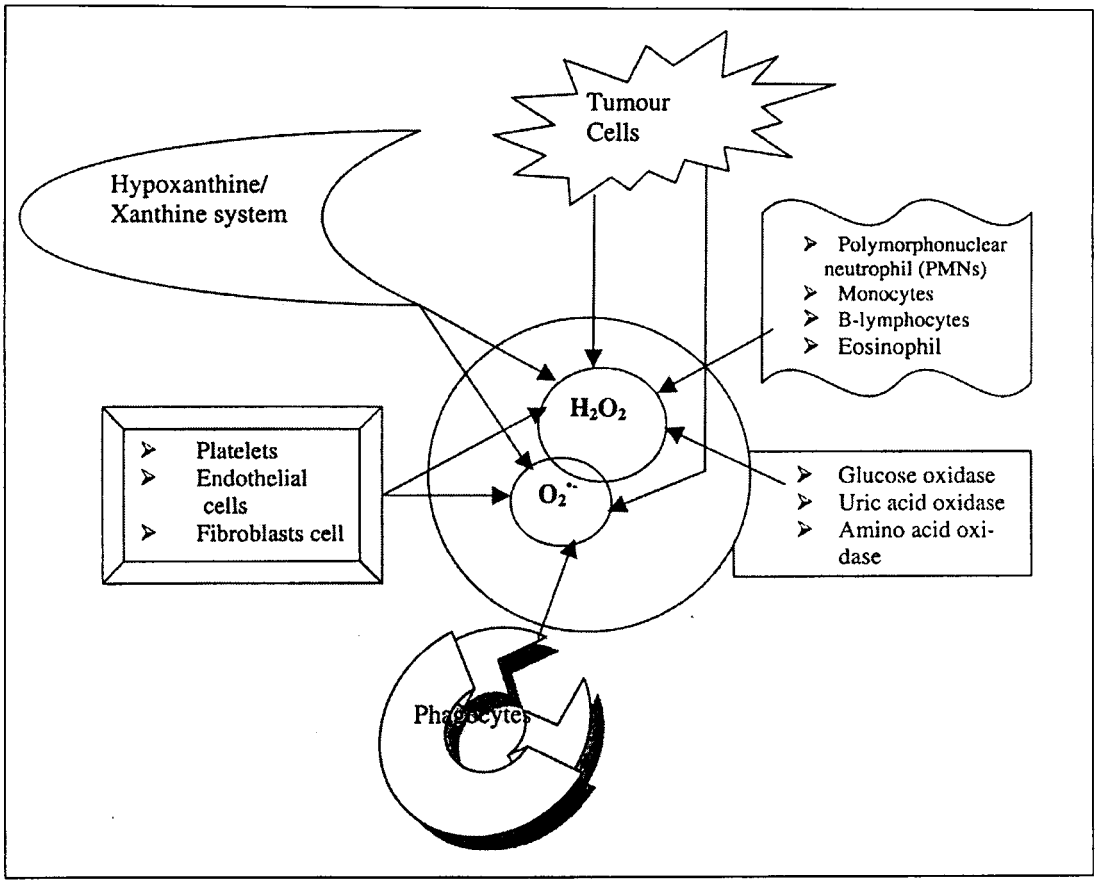


Figure 2.6. Formation of ROS from various sources
(Adopted from Ray and Husain, 2002)

aldehyde oxidase, dihydroorotate dehydrogenase and peroxidases) and non-enzymatic reactions (e.g. autooxidation of catecholamines).

6. H_2O_2 is additionally generated *in vivo* by several oxidase enzymes, viz. monoamine oxidase (MAO), tyrosine hydroxylase, L- amino acid oxidase, glycolate oxidase and urate oxidase (Coyle and Puttfarcken, 1993).
7. Cigarette smoke contains high concentrations of various free radicals. The gas phase contains about 10^{15} radicals per puff, both peroxy radicals and to a lesser extent, carbon centered radicals (Halliwell and Guttaridge, 1989).

2.4.2 Nitrogen species

Nitric oxide ($NO^{\bullet}$) is a unique signaling molecule, which was labeled as the "Molecule of the year" in 1992 (Stamler et al., 1992). Nitric oxide is synthesized from the guanidine group of L-arginine by a family of enzymes termed $NO^{\bullet}$ synthases (NOSs). Three isoforms have been described and cloned: brain NOS (b NOS or n NOS or type 1), inducible macrophage type NOS (i NOS or type 2) and endothelial NOS (eNOS or type 3). The molecular targets of $NO^{\bullet}$ in the victim cell are Cu-Fe proteins, releasing free Cu^{2+} and Fe^{2+} and generating O_2^- and highly reactive $^{\bullet}OH$, leading to oxidative injury. The lipophilic $NO^{\bullet}$ free radical gas diffuses freely through the plasma membrane to the underlying smooth muscle and activates the cytosol soluble guanyl cyclase/cyclic GMP, enhance the extrusion of calcium. The decrease in intracellular calcium concentration is responsible for the $NO^{\bullet}$ mediated vascular and nonvascular relaxation and signal transduction in the peripheral nervous system (Moncada et al., 1991and Dusting, 1995). Many inflammatory conditions are associated with production of comparatively large amounts of $NO^{\bullet}$ by i NOS. Cigarette smoke contains high concentrations of the gaseous compounds nitric oxide ($NO^{\bullet}$) and nitrogen dioxide (NO_2). In a radical addition reaction $NO^{\bullet}$ reacts with O_2^- to form deleterious peroxynitrite ($ONOO^-$) radical (Beckman et al., 1990).

$$NO^{\bullet} + O_2^- \rightarrow ONOO^-$$

$$ONOO^- + H^+ \rightarrow {}^{\bullet}OH + NO_2 + NO_2^+$$

Nitric oxide operated thorough the posttranslational modification of proteins (Matsumoto et al., 2003).

2.4.3 Damage produced by Reactive oxygen species in DNA, lipid and protein

ROS have been implicated in the pathophysiology of various clinical disorders, including ischemia, reperfusion injury, myocardial infarction, rheumatoid arthritis,

neurodegenerative, atherosclerosis, acute hypertension, hemorrhagic shock and diabetes mellitus (Hemnani and Parihar, 1998). Some tumor cells produce ROS (Szatrowski and Nathan, 1991), although the source of these products and their contribution to the transformed phenotype is not known. The formation of oxidative stress may result in damage to critical cellular macromolecules such as DNA, lipids and proteins.

2.4.3.1 Damage to DNA

DNA damage is the result of extrinsic and intrinsic process including ionizing radiation, toxic chemical ingestion, u.v, light exposure and oxygen-derived free radicals that are a normal consequences of the cellular metabolism of O_2 (Goetz et al., 1994). Several types of damage including base lesions, sugar lesions, proteins and DNA cross links, single-strand breaks and double strand breaks are produced by free radical induced reactions (Kaneko et al., 1996; Simic and Jovanovic, 1986 and Deng and Fridovich, 1989). $^{\bullet}OH$ radicals are showing three main types of reactions: hydrogen abstraction, addition and electron transfer. The reactivity of all five bases with $^{\bullet}OH$ is extremely high; where as that of deoxyribose is about 5 times lower (Buxton et al., 1988). More than 20 different products known to be formed by exposure of DNA bases to the $^{\bullet}OH$. One of the major oxidized DNA bases is 8-hydroxy-2^{1}-deoxyguanosine (Kasai et al., 1987 and Weitzman et al., 1994). Reaction of $^{\bullet}OH$ and H atom with DNA bases is characterized by addition to the double bonds of these molecules to give adduct radicals of bases. For example $^{\bullet}OH$ adds to the double bond of thyamine at C-5 (5-hydroxythymine) (56%) and C-6 (6-hydroxythymine) (35%) and abstracts hydrogen from the methyl group (9%) (Jovanovic and Simic, 1986) (Fig.2.7a). The 5-hydroxythymine intermediate leads to formation of thymine glycol (Fig.2 7b). Similarly the $^{\bullet}OH$ mediated mechanism of 8-hydroxyguanine (8-OHG) formation involves in two steps: first addition of $^{\bullet}OH$ to C-8 of guanine and secondly, the subsequent loss of hydrogen atom from the intermediate to form 8-OHG (Fig. 2.8). When free radicals react with the sugar moiety of DNA, some sugar products and intact bases are released from DNA (Dizdaroglu et al., 1975). Studies indicate that DNA is the early target for oxidative stress, which could contribute to the cascade of pathogenesis of cells including gene mutation and cancer.

2.4.3.2 Lipid peroxidation

Lipids are major target for free radical attack in part because oxygen is more soluble in hydrophobic membrane. Double bonds in polyunsaturated fatty acids (PUFA) are easily attacked. Peroxidation of the PUFA in lipid membranes severely

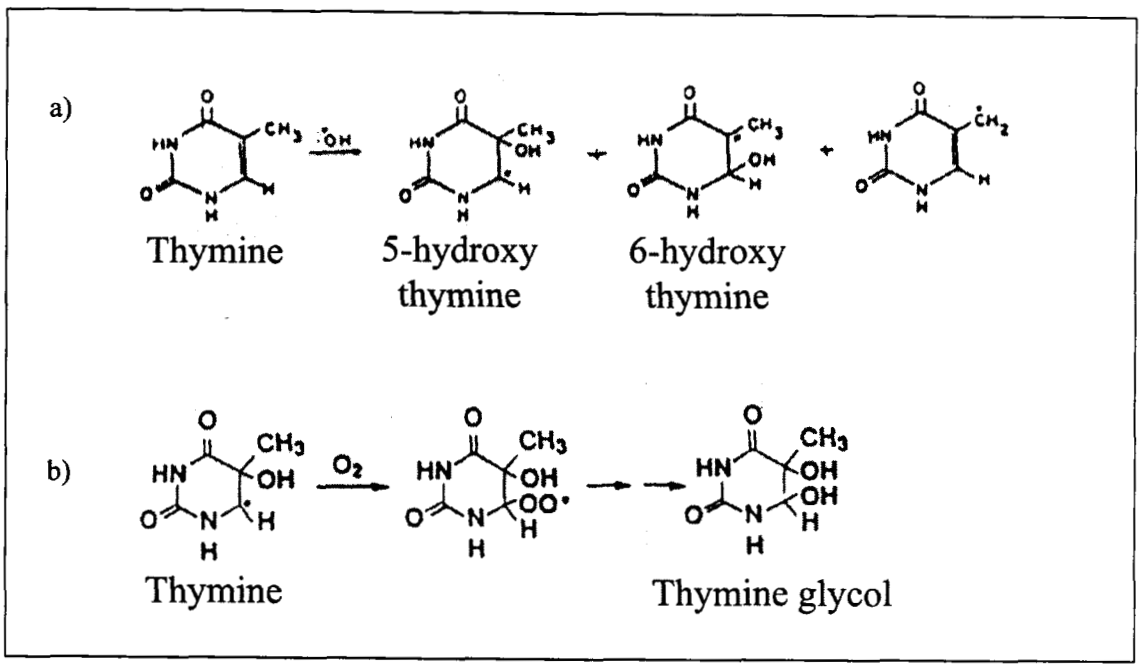


Figure 2.7. Reaction between hydroxyl radical and thymine
(Adopted from Simic, 1994)

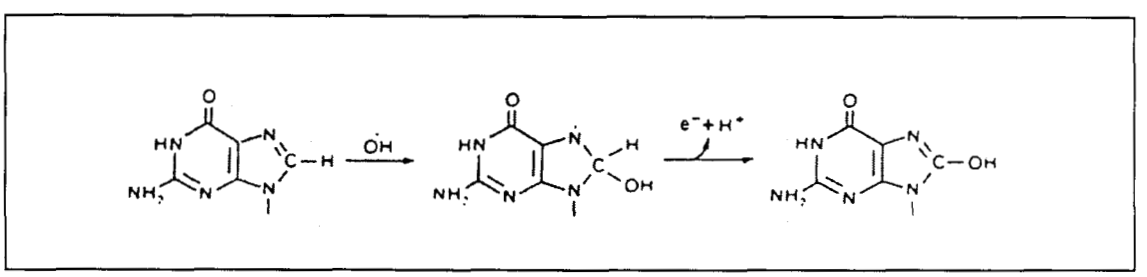


Figure 2.8. Possible mechanism to explain hydroxyl free radical mediated 8-hydroxy guanine formation

damages the cell membrane and thereby produces loss of fluidity and breakdown of the membrane secretary functions and transmembrane ionic gradient. The autooxidation can be initiated by hydroxyl radical, peroxyl radical and singlet oxygen but not by less reactive superoxide anion radical or hydrogen peroxide. The initiating free radical removes hydrogen from one of the methylene carbon of the carbon chain, a process that leaves an unpaired electron on this carbon chain and creates a lipid carbon radical. The latter undergoes molecular rearrangement to a conjugated diene, which then reacts with molecular oxygen to give a hydroperoxy radical. This radical may extract a hydrogen atom from a methylene carbon of the adjacent CH_2 groups of PUFA to form another lipid radical and lipid hydroperoxide. The hydroperoxide is a stable compound until it comes into contact with transition metal ions such as iron (Halliwell and Gutteridge, 1984). Then it produces more radicals, which in turn further initiate and propagate other chain reactions. The end products of such a lipid peroxidation are aldehydes, hydrocarbon gases and various chemical residues including malondialdehyde (MDA) and 4-hydroxynonenal (O'Brien, 1969). These degradation products can diffuse away from the site of the chain reaction and can give rise to cell edema, can influence vascular permeability, inflammation and chemotaxis.

2.4.3.3 Damage to proteins

Radical mediated damage to proteins may be initiated by electron leakage, metal-ion dependant reactions and autooxidation of lipids and sugars. ROS can interact with proteins directly, especially their sulfhydryl groups (Herington, 1986). Among the aminoacids histidine, tryptophan, methionine and tyrosine are more reactive towards ROS resulting in sulphoxides and short-lived endoperoxides that may be toxic to other cells. The consequent protein oxidation is O_2 dependent, and involves several propagating radicals, notably alkoxyl radicals. This can result in further modification of enzyme activity (Bellomo et al., 1983), damage to transport protein which lead to alterations in intracellular calcium and potassium salts that will trigger a series of changes in cells (Kerr, 1992). In selective cases alteration of protein structure and the consequent unfolding is associated with enhanced susceptibility to proteinases.

2.4.4 ROS in signal transduction and gene expression

Activation of transcription factors is an important signaling pathway for the regulation of gene transcription by ROS. ROS regulate genes via protein kinase C (PKC) activation, oxidative damage, and or ROS direct activation of transcription factors. Nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), are two

transcription factors that are modulated by ROS by direct oxidation and phosphorylation (Meyer et al., 1993). Oxidative stress also induces the immediate early protooncogenes c-*fos*, *jun*-B, c-*jun* and *jun*-D and thus increases the AP-1 transcription activity. ROS generated from UV irradiation are found to be required for the receptor tyrosine kinase activation. The mechanism for these oxidant-induced receptor activations is by the inactivation of a tyrosine phosphatase enzyme (Knebel et al., 1996). The activation of *ras*, as shown by the activation of ras GTPase activity, and down stream ERK-MAP kinase activity can be regulated by $NO^{\bullet}$ (Lander et al., 1997). The MAP kinase-signaling pathway is also regulated by ROS through inducing the *c-jun* NH2 terminal kinase (JNK) and p38 MAP kinase pathways (Medelson et al., 1996). Direct linkage between TNF-α receptor binding and NF-κB activation via a H_2O_2 intermediate has been demonstrated by several experiments. The mediation of ROS on gene transcription may also inhibit normal cell apoptosis by modulation of *myc*, *bcl-2* and *p53* expression and result in an increase in cell number.

2.4.5 Role of ROS in the cancer process

It is hypothesized that free radicals are involved in all the three stages of carcinogensis. ROS believed to mediate the activation of carcinogens through hydroperoxide dependent oxidation that can be mediated by peroxyl radicals (Trush and Kensler, 1991). This occurs with aflatoxin B1, aromatic amines and polycyclic aromatic hydrocarbon dihydrodiols. ROS or their byproduct of lipid peroxidation, MDA, can also directly react with DNA to form oxidative DNA adduct (Chaudhary et al., 1994). The MDA induced mutations include frameshifts and base-pair substitutions (Moller and Wallin, 1998). The presence of carcinogen-DNA adduct and oxidative-DNA adducts generated by chemical carcinogens suggest an interactive role of ROS in initiation. During the promotion stage ROS mediated persistent oxidative stress can contribute to abnormal gene expression, blockage of cell-to-cell communication and modification of second messenger systems, resulting in an increase in cell proliferation or a decrease in apoptosis in the initiated cell population. This results in the clonal expansion of the initiated cells to pre-neoplastic focal lesions. During the progression stage of the cancer process, ROS impart further alteration to the initiated cell population. These changes may result in abnormal enzyme activity and make the lesion resistant to normal growth control. Tumor cells continually undergo high and persistent oxidative stress, as was shown by the measurement of higher 8-OhdG levels in human carcinoma cells than in surrounding normal cells (Toyokuni et al., 1995). This

persistent oxidative stress does not appear large enough to induce cell death because tumor cells have decreased cell sensitivity to oxidative stress (Toyokuni et al., 1995 and Palozza et al., 1994). The ROS induced G to T transversion often found in *K-ras* and *H-ras* in non-melanoma skin tumors. G to T transversions has also been observed in *p53* codons in hepatocellular carcinoma and smoking related lung carcinoma. In colorectal cancer around 95 % of the mutations at *p53* hot spot codons are noted. In these tumors C to T and G to A transitions and the base pair changes are often produced by the deamination of 5-methylcytosine, which is enhanced by ROS and RNS (Wiseman and Halliwell , 1996). High antioxidants induced by the oxidative stress in cancer cells increase the chemotherapy resistance of the cells. Increased protein oxidative damage on certain protease inhibitors facilitates tumor invasion and metastasis (Toyokuni et al., 1995) (Fig. 2.9).

2.5 Role of inflammation in increased DNA damage, mutation and cancer

Inflammation can accelerate the development of cancer. Inflammatory process provides the prerequisite environment for the development of malignancy includes up regulation of mediators of the inflammatory response such as cyclooxygenase (COX-2) leading to the production of inflammatory cytokines and prostaglandins which themselves may suppress cell mediated immune responses and promote angiogenesis (O'Byrne and Dalgleish, 2001). The COX-2 is induced in inflammatory cells such as monocytes and macrophages upon stimulation by cytokines, mitogens, serum and endotoxins. Inflammation per se increases the synthesis of PGs, at least in part due to up regulation of COX-2 rather than COX-1. Inflammatory cells may also increase DNA damage by activating pro-carcinogens to DNA damaging species that may in turn induce mutation eg. neutrophils can activate aromatic amines, aflatoxins, estrogen, phenols and polycyclic aromatic hydrocarbons by ROS-dependent mechanisms (Rosin et al., 1994). At site of inflammation increased free radical activity is associated with the activation of the neutrophil NADPH oxidase and/ or the uncoupling of a variety of redox systems, including endothelial cell xanthine dehydrogenase (Winrow et al., 1993). The schistosomiasis model has been used to study the interrelationship between inflammation, oxidative DNA damage, chromosomal instability and deregulated cell proliferation (Rosin et al., 1994). Infection with *Schistosoma haematobium* produces chronic bladder inflammation and is associated with increased cancer at this site. Alterations in chromosome 11 are common

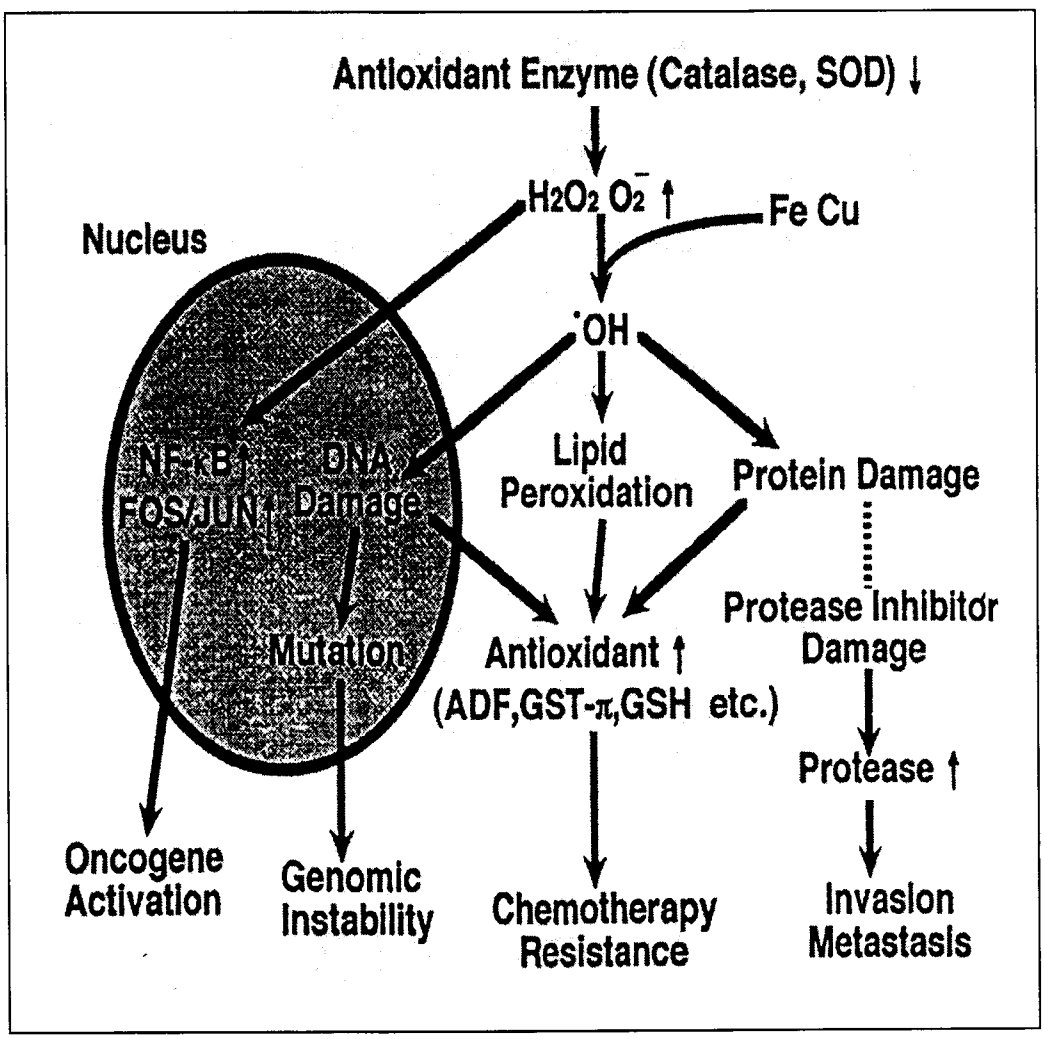


Figure 2.9. Role of reactive oxygen species in cancer
(Adopted from Toyokuni et al., 1995)

in bladder cancer (Hopman et al., 1991) and loci on this chromosome may be involved in controlling the level of chromosomal breakage caused by oxidative DNA damage. Exposure to asbestos is a major risk for mesothelioma; crocidolite (one of the most carcinogenic asbestos) induces release of ROS from neutrophils and macrophages and induced 8-OHdG levels in the DNA of promyelocytic leukemia cell line (HL-60) (Wiseman and Halliwell, 1996). Chronic inflammation of gastrointestinal tissues is a well-recognized risk factor for the development of epithelial cell-derived malignancies (Jaiswal et al., 2001). Chronic inflammation and resulting DNA damage may contribute the cancer seen in certain inflammatory bowel disease also (IBD). Chronic hepatitis is associated with the presence of inflammatory cells, presumably generating ROS and RNS. Increased levels of 8-OHdG have been detected in DNA from livers of chronic hepatitis (Shimoda et al., 1994). Free radical produced by inflammatory cells not only cause direct damage to DNA but also exert indirect effects such as deregulation of cell proliferation and apoptosis, stimulation of angiogenesis and modification of gene/protein expressions and protein activities, all of which are a critical steps toward carcinogenesis (Okada, 2002).

2.6 Protection against oxygen radicals in biological systems, antioxidants

Antioxidants have been defined by Halliwell as "any substance that when present at low concentrations with those of an oxidisable substrate significantly delays or prevents oxidation of that substrate". Antioxidants possess a variety of biological activities including the induction of drug metabolizing enzymes, inhibition of prostaglandin synthesis, inhibition of carcinogen induced mutagenesis and scavenging of free radicals (Hirose et al., 1994). For example antioxidant N- acetyl cystein has antimutagenic and chemopreventive activities in a variety of organs, such as the lung, liver, skin and colon (Flora et al., 1986; Izzotti et al., 1994). Antioxidant can alter the gene expression induced by ROS rather than their direct effect on transcription through antioxidant response elements (AREs) present in the promoters of many genes including the phase II enzyme glutathione *S*- transferase (Primiano et al., 1997). Antioxidants have potent effects on the ability of several transcription factors such as AP-1, NF-κB, Sp1 and elk-1 to bind to DNA (Winyard and Blake, 1997). They have been shown to trigger apoptosis in smooth muscle cells independent of oxidative reactions (Liu et al., 1998 and Tsai et al., 1996). Antioxidants can inhibit tumor initiation, tumor promotion and cell transformation (Steele et al., 1990). Changes in antioxidant defense enzymes such as superoxide dismutase (SOD), glutathione

peroxidase (GPx), glutathione *S*-transferase (GST) and catalase (CAT) have been widely described in cancerous cells (Cerutti et al., 1994). Evidence suggests that low molecular weight antioxidant enzymes and anti-inflammatory agents that inhibit arachidonic acid metabolism are anticarcinogenic (Kensler et al., 1983).

2.6.1 Antioxidant enzymes

The first line of defense against O_2^- and H_2O_2 mediated injury are antioxidant enzymes: SOD, GPx and CAT. The preventive role of antioxidant from the oxidative damage is given in figure 2.10.

2.6.1.1 *Superoxide dismutase (SOD)*

In 1968 the work of Mc Cord and Fridovich in the USA showed that the erythrocyte protein was able to catalytically remove the superoxide radical and thus identified as a superoxide dismutase (SOD) enzyme. They include Mn^{++} enzyme in mitochondria (SOD2) and Cu^{++}/Zn^{++} enzyme present in the cytosol (SOD1). Copper-zinc containing superoxide dismutase contain two protein subunits, each of which bears one copper ion and one zinc ion for the stability of the apoenzyme. Cyanide is an extremely powerful inhibitor of CuZnSODs. Enzymes are found in all eukaryotic cells, catalyzed the dismutation reaction of super oxide.

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

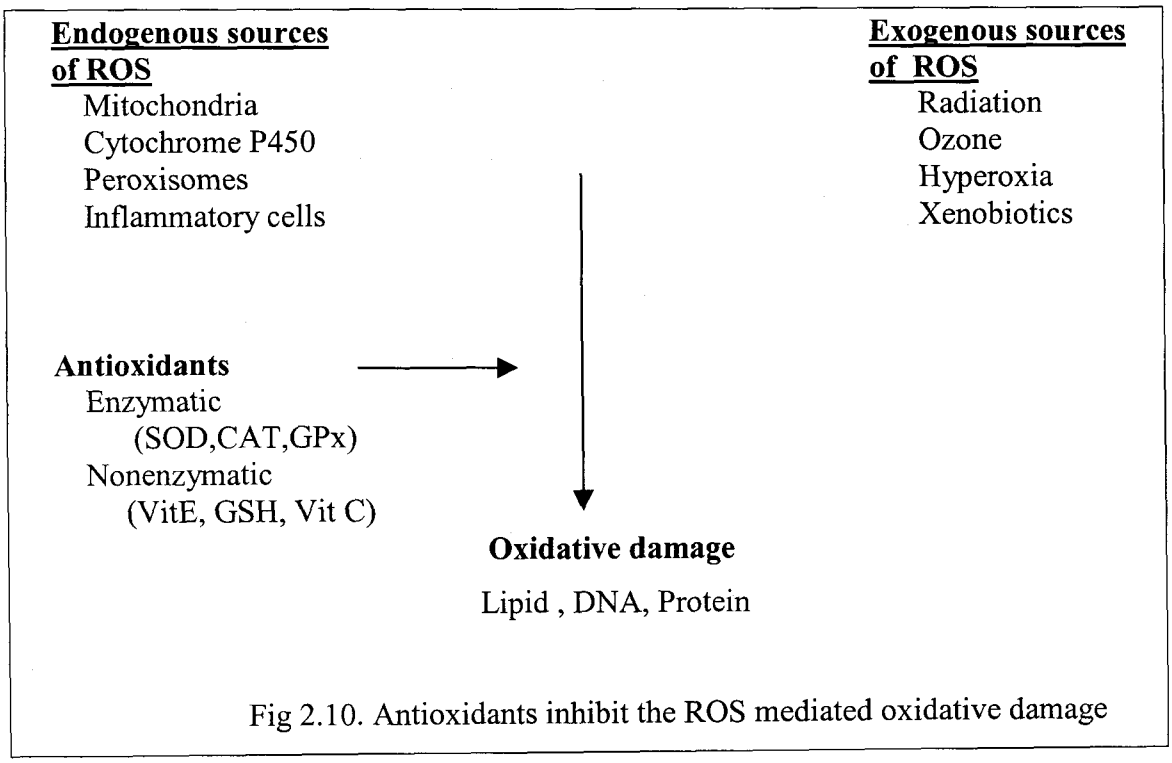


Fig 2.10. Antioxidants inhibit the ROS mediated oxidative damage

2.6.1.2 Catalase (CAT)

Most aerobic cells contain catalase activity and are largely located in peroxisomes (immobilized state), whereas it exists in a soluble state in erythrocytes (Abi, 1974). The enzyme contains 4 ferriprotoporphyrin groups per molecules. The catalase activity of tissues varies greatly; it is highest in liver and kidney and low in connective tissue.

Catalase has a double function, because it catalyses the following reactions

1) Decomposition of H_2O_2 to give H_2O and O_2.

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

2) Oxidation of H donors, for example, methanol, ethanol, formic acid, phenol with the consumption of 1 mole of peroxide.

$$ROOH + AH_2 \rightarrow H_2O + ROH + A$$

At high concentrations, H_2O_2 is removed by the enzyme catalase.

2.6.1.3 Glutathione peroxidase (GPx)

The selenoenzyme glutathione peroxidase was discovered by G. C. Mills in 1957. Glutathione peroxidase catalyses the oxidation of GSH to GSSG at the expense of hydrogen peroxide,

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

It is found at high activity in liver, moderate activity in heart, lung and brain and low activity in muscle. The enzyme is made up of 4 protein sub-units each of which contains one atom of the element selenium at its active site. At low concentrations, H_2O_2 is removed by reacting with reduced glutathione (GSH) to form oxidized glutathione (GSSG) and H_2O, catalysed by glutathione peroxidase (GPx). The enzyme resides in the cytosol and mitochondrial matrix (Mills, 1960).

2.6.2 Non-enzymatic antioxidants

2.6.2.1 Antioxidant vitamins

Antioxidant vitamins have a number of biological activities such as immune stimulation, inhibition of nitrosamine formation and an alteration of metabolic activations of carcinogens (Poppel and Van den Berg, 1997). Case control studies suggest inverse association of vitamins A, C, β-carotene and vitamin E and the risk of breast cancer (Bohlke et al., 1999).

2.6.2.1.1 Vitamin A

The naturally occurring fat-soluble preformed vitamins include the compounds retinal and its esters, retinaldehyde and retinoic acid. Vitamin A derivatives (retinoids) are potent regulators of embryogenesis, cell proliferation, epithelial cell differentiation and carcinogenesis (Easwaram et al., 1999). Experiments with laboratory animals suggested that vitamin A deficiency may enhance susceptibility to certain forms of chemical carcinogenesis (Di Giovanni, 1987).

2.6.2.1.2 Vitamin E

The fat-soluble vitamin occurs in plasma as a variety of tocopherols, of which the alpha and gamma isomers are usually the major ones. Vitamin E is thought to be an important chain-breaking antioxidant, can directly scavenge ROS and is the major lipid-soluble antioxidant present in all cellular membranes, which protects against lipid peroxidation, protect membrane polyunsaturated fatty acid (PUFA) and low density lipoprotein (LDL) (McCay, 1985). Vitamin E can directly act with a variety of oxy radicals including the peroxy radical $ROO^{\bullet}$, $CCl_3^{\bullet}$, $^{\bullet}OH$, $O_2^{\bullet}$ and siglet oxygen (Fukuzawa and Gebicki, 1983). Vitamin E donates hydrogen from the chromane ring to free radical. The tocoperoxyl radical formed can be reduced to tocopherol by interaction with reductants serving as hydrogen donors.

$$ROO^{\bullet} + \text{Vit E- OH} \rightarrow ROOH + \text{Vit E-O}^{\bullet}$$

$$\text{Vit E-O}^{\bullet} + AH^{\cdot} \rightarrow \text{VitE-OH} + A^{\bullet}$$

Because of the antioxidant properties, vitamin E neutralizes reactive oxygen species and reduces the oxidative DNA damage and mutations (Frei, 1994).

2.6.2.1.3 Vitamin C

Vitamin C (ascorbic acid) is an important water soluble antioxidant in biological fluids and an essential micronutrient required for normal metabolic functioning of the body. Vitamin C readily scavenges ROS, ozone, $ONOO^{\bullet}$, NO_2, $NO^{\bullet}$ and hypochlorous acid (Noroozi et al., 1998) and reduces oxidative damage and mutations (Frei, 1994). The most striking chemical activity of ascorbate is its ability to act as a reducing agent (electron donor). Donation of one electron by ascorbate gives the semidehydroascorbate radical, which can be further oxidized to give dehydroascorbate. Epidemiological studies have indicated an inverse association between vitamin C intake and risk of cancers. Vitamin C can act as a co-antioxidant by regenerating α-tocopherol from the α-tocopheroxyl radical produced during scavenging

of ROS. Reports suggesting that vitamin C may prevent formation of $ONOO^{\bullet}$ by reaction with O_2^{-}. And may help to release $NO^{\bullet}$ from endothelial cell.

2.6.2.2 Glutathione

Glutathione present in food and is one of the major antioxidants and antimutagens in the soluble fraction of cells. This compound protects thiol groups in protein from oxidation, functions as an intracellular redox buffer and serves as a reservoir of cysteine (Moron et al., 1979). Glutathione is one of the substrate of glutathione *S*-transferase and glutathione peroxidase. The glutathione *S*-transferase is major defenses against oxidative and alkylating carcinogens. Ascorbate (radical) is also reduced by intracellular GSH.

2.6.2.3 Flavonoids

Flavonoids are phenolic compounds, present in several plants, which inhibit lipid peroxidation and lipoxygenases *in vitro*. The polyphenolic antioxidant act either by trapping the initiating radical, propagating lipid peroxyl radicals, recycling α-tocopherol and or deactivating the excited photosentizer etc. (Tiwari, 2001). This activity is attributed to the phenolic hydroxyls, particularly in the 3,4-hydroxyl group of the (B) ring and the 2,3-double bond in the (C) ring. The activity increases with the number of OH groups in rings (A) and (B). The electron and H^{+} donating capacity of flavonoids seem to contribute to the termination of lipid peroxidation chain reaction based on their reducing power (Tiwari, 2001).

2.6.2.4. Uric acid and bilirubin

Uric acid and bilirubin are good antioxidants. Uric acid in high concentration is found to be antioxidant however, too much causes gout.

2.6.2.5 Ceruloplasmin -can acts as antioxidants in extracellular fluid.

2.6.2.6. Cysteine- is mild nonspecific antioxidants.

2.7 CANCER CHEMOTHERAPY

The word chemotherapy is coined by Paul Ehrlich refers to the treatment of metastases (De Vita, 1978). In the early 1960s Skipper and his colleagues laid down the guiding principles of present-day chemotherapy, using the rodent leukemia L 1210 as a model (Skipper et al., 1964). The objective of cancer chemotherapy is to kill cancer cells, with as little damage to normal cells. In cancerous tumors, many cells are dividing, and so most drugs used are designed to interfere with cell growth and division by blocking synthesis of DNA, RNA, or protein. Examples of such drugs include,

2.7.1 Antimetabolites

The first agent in the antifolate class of antimetbolites to find clinical application was aminopterin, a 4-NH_2 analogue of folic acid, which has been replaced by its methyl analogue, amethopterin or methotrixate. Methotrixate exert their effect through inhibition of the enzyme dihydrofolate reductase. Polyglutamate form of methotrexate directly inhibits thymidylate synthase and enzymes of denovo purine synthesis. 5-Fluorouracil (5-FU) incorporation into DNA appears to inhibit chain elongation (Schetz et al., 1986), incorporation into RNA inhibits formation of mRNA (Kanamaru et al., 1986). Cytosine arabinoside (ara-C) nucleotide acts as an inhibitor of DNA polymerase in competition with d CTP. Purine analogues, 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), as monophosphate nucleotides inhibit denovo purine synthesis. The triphosphate nucleotides of 6-MP and 6-TG incorporated into DNA and produce strand breaks (Christie et al., 1984). Adenosine analogues, 9-β-arabinofuranosyladenine (ara-A) inhibits DNA polymerase.

2.7.2 Alkylating agents

Primary cytotoxic and mutagenic effects of alkylating agents are caused by their interactions with DNA (Ludlum, 1977). Nitrogen mustard or mechlorethamine was the first alkylating agent found to produce responses in patients with lymphomas. Phosphoramide mustard and acrolein, active principle metabolites of cyclophosphamide, have respectively greater toxicity to tumor cells and alkylate DNA. Busulfan after forming carbonium ions thorough the release of methane-sulfonate group alkylates DNA (Nedkarni et al., 1959). Busulfan is primarily used for the treatment of chronic granulocytic leukemia. Nitrosoureas in aqueous solution yields two reactive intermediates, a chloroethyldiazohydroxide (II) and an isocyanate group (III). The chloroethyldiazohydroxide (II) decomposes further to a reactive chloroethylcarbonium ion (IV) that forms a single strand adduct with DNA (Ewig et al., 1977). The activated complexes of cisplatin in aqueous solution interact with nucleophilic sites on DNA, RNA and protein to form bifunctional covalent link analogues to alkylating agents. The favored positions of attack are the N_7 of guanine and N_3 of cytosine (Scovell and O'Connor, 1977).

2.7.3 Antitumor drugs of plant origin

Among the plant products the most important have been the vinca alkaloids, vincristine and vinblastine, which are derived from ornamental shrub *Vinca rosea* and the epipodophyllotoxins, VM-26 and VP-16, derived by modification of a product of

the mandrake plant. The vinca alkaloids possess cytotoxic activity by virtue of their binding to tubulin. Through their high-affinity binding to tubulin, the vinca alkaloids inhibit the assembly of microtubules and lead to the dissolution of the mitotic spindle (Owellen et al., 1976). A new spindle poison taxol acts by promoting the assembly of microtubules (Horwitz et al., 1986). VM-26 and VP-16 produces single strand and double strand breaks in DNA.

2.7.4 Antitumor antibiotics-Microbes in the development of anticancer drugs

Although the search for new anticancer drugs through rational chemical synthesis of antimetabolites has yielded useful compounds, many of the antitumor agents are natural products of fungi, plants and marine animals, and these sources are likely to be the primary resources for compounds of the future. A number of antitumor antibiotics occur naturally and can be harvested after microbiological fermentation. The antitumor antibiotics can be grouped under several distinct chemical types. These include the anthracyclins and other quinone-containing drugs such as mitomycins, streptonigrin, daunomycin and doxorubicin (adriamycin); metal chelators such as tallysomycin and bleomycin; the protein antitumor antibiotics such as macromomycin and neocarcinostatin and the aureolic-acid based antibiotics such as mithramycin, chromomycin and olivomycins.

2.7.4.1 Bleomycin

Bleomycin is one of a family of antibiotic polypeptides isolated from the fungus *Streptomyces verticullus* possess both antitumor and antimicrobial activity. The primary action of bleomycin is through the generation of active oxygen intermediates such as superoxide or hydroxyl radicals produce single-strand and double strand breaks in DNA (Takeshita et al., 1978). Bleomycins are active against several human cancers including Hodgkin's disease and cancer of the testes.

2.7.4.2 Anthracyclines

The first anthracyclines in clinical use, daunomycin and doxorubicin are antibiotics produced from the *streptomyces* species. Daunomycin and doxorubicin are known to intercalate DNA, chelate transition metal ions, such as iron or copper and engage in oxidation-reduction reactions (Handa and Sato, 1975 and Pigram et al., 1972).

2.7.4.3 Mitomycin C

Mitomycin C isolated from *Streptomyces caespitosus* is used in the palliative treatment of number of advanced human cancers. The activated mitomycin-C alkylates

and cross links DNA at the N_6 atom of adenine and the O_6 and N_2 atoms of guanine of DNA (Dorr et al., 1985). Mitomycin also induces lipid peroxidation through intermediate of oxygen radicals (Nakano et al., 1984).

2.7.4.4 Actinomycin D

Actinomycin D is a peptide antibiotic isolated from *streptomyces* species, effective in the treatment of Wilms tumor, Ewings srcoma, embryonal rhabdomyosarcoma and gestational choriocarcinoma (Frei, 1974). The effect is mediated through the intercalation of polypeptide chain along the minor grove of DNA; block the DNA and RNA synthesis.

2.7.4.5 Mithramycin

Mithamycin is isolated from *Streptomyces plicatus.* Mithramycin has antitumor activity against testicular carcinoma but also has a specific hypocalcaemic effect that is valuable in the treatment of malignant hypercalcaemia (Kennedy, 1972).

Most of the commonly used antineoplastic agents produce immediate toxicity in organs composed of self-renewing cell populations such as bone marrow, skin and gastrointestinal tract epithelium. Pancytopenia, stomatitis, alopecia and nausea and vomiting occur frequently but generally are not prolonged or irreversible once chemotherapy is completed. Some antitumor agents are associated with more delayed toxic effects, such as cardiomyopathy of doxorubicin or the pulmonary fibrosis associated with bleomycin, which become clinically apparent even after chemotherapy, previously unrecognized toxic effects are becoming manifest. Important among these late effect is infertility (Byrne et al., 1987).

2.7.5 Chemoprevention

Chemoprevention represents a new intervention strategy to control some types of carcinogenesis and may be defined as the use of specific natural or synthetic chemical agents which may enhance intrinsic physiological mechanisms that protect the organism against the development and progression of mutant clones of malignant cells (Sporn, 1993). Chemoprevention appears particularly indicated and helpful in subjects at high risk for cancer development. The ideal chemopreventive agent is expected to have low toxicity, with no untoward or toxic effects and high efficacy. Moreover, these compounds should be characterized by known mechanism of action, low cost and oral administration. Nearly 2000 natural and synthetic chemicals that have shown cancer chemopreventive activity in animal models are now tested in various stages of clinical trials to prevent specific cancer sites in human (Lippman et al., 1994). Agents for

chemoprevention-fall into two principle categories 1) agents prevent the mutagenic initiation of the carcinogenic process (blocking agents) 2) agents prevent the further promotion or progression of lesions that have already been established (suppressing agents) (Wattenberg, 1985). Mechanism of action of chemopreventive agents is given in figure 2.11. Compounds currently being tested for chemopreventive activity in animals models are **a) Oltipraz-** is an antioxidant catalyses the inactivation of electrophilic carcinogenic compounds, that are formed by the activation of cytochrome P-450 mixed function oxidase, by increase cellular GSH level and inducing the GST. **b) Ellagic** acid-is related to coumarins, a subclass of lactones, found in a wide variety of fruits and vegetables in the human diet. Ellagic acid has been shown to inhibit carcinogen-induced neoplasia in mouse skin, rat mammary gland and mouse fore stomach **c) Benzylisothiocyanate**–one of the major isothiocyanate compounds found in cruciferous plants. Reported to inhibit DMBA-induced mammary tumor formation in rats and benzo[*a*]pyrene induced tumor formation in the fore stomach of mouse. **d) Indole-3-carbinol-** is one of the major indole components of cruciferous plants. It increases the speed of detoxification of procarcinogens by augmenting their solubilization and elimination. **e) Difluromethylornithine**- is an irreversible inhibitor of ornithine decarboxylase and early enzymes in the synthesis of active polyamines. **f) Resveratrol**- phytoalexin isolated from red grapes and vegetables reported to have antioxidant, anti-inflammatory, antiproliferative and anticancinogenic activity. **g) Non-steroidal anti-inflammatory drugs (NSAID)**- a large number of anti-inflammatory agents have been shown potent chemoprevetive activity in many test systems. Among the NSAIDs that have been studied are aspirin, ibuprofen, sulindac, indomethacin and piroxicam. All these molecules are cyclooxygenase inhibitors that block prostaglandin synthesis and they are in widespread clinical use for the chronic treatment of various inflammatory diseases. The inhibitors of prostaglandin synthesis have been shown to be active in a multiplicity of animal models for the suppression of carcinogenesis, with particular efficacy in preventing experimental colon carcinogenesis. **h) Vitamins-** Among the compounds with potential chemopreventive efficacy some vitamins and particularly retinoids, carotenoids ascorbic acid and tocopherol are the most studied class of drugs that are a promising tool in the field of primary prevention of some cancers (Giacosa et al., 1997). It has been suggested that some antioxidant vitamins could reduce cancer risk by 20-30% (Hennekens, 1994), inactivating the action of free radicals and preventing carcinogen formation from inactive precursors. **i) Curcumin**- a

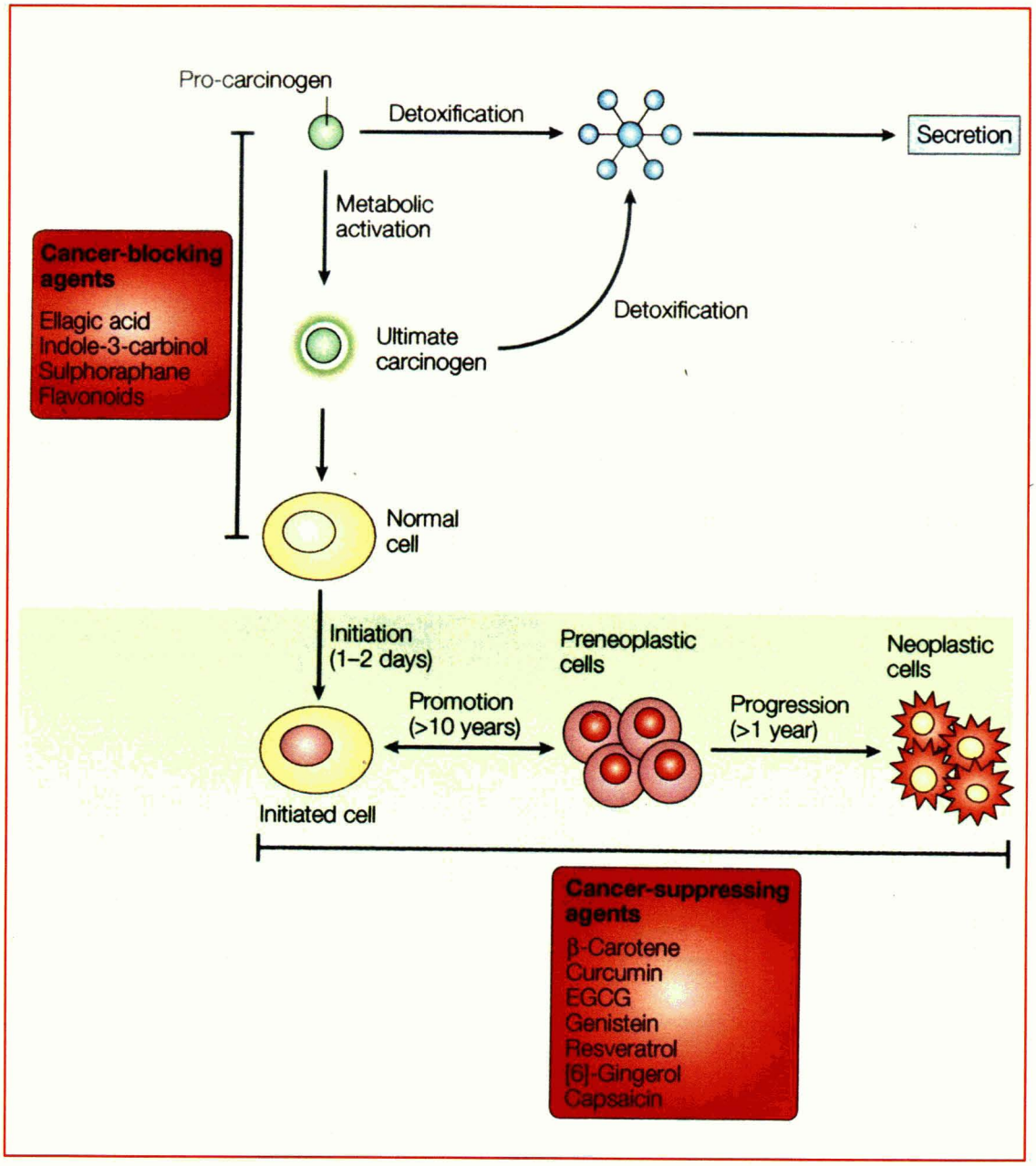


Figure 2.11. Mechanism of action of chemopreventive agents
(Adopted from Surh, 2003)

major yellow pigment in turmeric, obtained from rhizome of plant *Curcuma longa* is known to have antioxidant (Anto et al., 1995), *in vitro* antimutagenic (Anto et al., 1996), anticancer effect in animals (Huang et al., 1988) and cell cultures (Kuttan et al., 1985). Chemically induced skin, oral, forestomach, duodenal and colon carcinogenesis also suppressed by the curcumin (Rao et al., 1995).

Some of the compounds being tested in clinical trials included Vitamins A , C, E, calcium, β-carotene, retinol, 11-cis-retinoic acid, trans-retinoic acid and 4-hydroxyphenylretinamide, (Boone et al., 1990). Results of these trials as the preventive role are contradictory. Various primary and secondary intervention trials currently under way assess the role of some vitamins in diseases prevention. Among these role of antioxidant activity of vitamins in preventing cancer and cardiovascular disease are of particular attention. Future trials of antioxidant are needed that act in early phases of carcinogenesis and particular attention must be paid to possible side effects and safety of the agents and on optimal doses in long-term administration.

2.8 MEDICINAL PROPERTIES OF MUSHROOMS

Fungi of various types normally inhabit the different parts of a plant body such as roots, stem and leaves. Some of these may be harmless while others may be weak or dangerous pathogens. A Higher Basidiomycetes mushrooms (HBM) are historically and economically highly praised for their nutritional value and acceptability, as well as their pharmacological properties. They include species from the Basidiomycetes class that have macroscopic fruit bodies large enough to be seen by the naked eye, and usually picked by hand. HBM contain approximately 10,000 species from 55 genera and 80 families (Wasser and Weis, 1999a).

The characteristic features of higher Basidiomycetes could be divided into terrestrial or hypogeous, lignicolous or saprobic, mycorrhizal or pathogeneic and edible, medicinal, hallucinogenic and poisonous mushrooms (Miles and Chang, 1997). Among the medicinal mushrooms, poisonous and some inedible had a very prominent place in the folklore, represent a major and as yet largely untapped source of powerful new pharmaceutical products. Edible ones are known to possess all essential aminoacids, minerals, vitamins etc. in adequate quantities and low sugars (Chang and Miles, 1982). Among the 10,000 species known, 2000 are safe and about 300 have significant pharmacological properties (Wasser et al., 2000). Most of the traditional

knowledge about medicinal properties comes from the Far East (China, Japan, Korea, Russia). Anticancer drugs isolated from mushrooms such as *Lentinus edodus, Coriolus versicolor* and *Schizophyllum commune* are sold in Japan (Jong and Birmingham, 1992) (Table 2.3).

Table 2.3- Anticancer drugs from mushrooms sold in Japan

Name	Krestin	Lentinan	Schizophyllan
Source	*Coriolus versicolor*	*Lentinus edodes*	*Schizophyllum commune*
Active principle	Protein bound polysaccharide	β-1,3-D-glucans	β-1,3-D-glucans
Route	Oral	Injection	Injection
Type of cancer	Gastrointestinal, Breast, lung	Stomach	Cervical

(Adopted from Jong and Birmingham, 1992)

Medicines from poisonous mushrooms have been considered in China as 'poison as an antidote for poison' (Yang and Jong, 1987). Various preparations are still used as medicine eg. *Amanita muscaria* used therapeutically as a powder, tincture for swollen glands, nervous troubles and epilepsy etc. A lotion made out of this can be used externally and internally for the ailments of heart and inflammation of eye. *Amanita phalloids* is used against cholera and intermittent fever. Psilocybin and Psilocin are two other drugs extracted from *Psilocybe mexicana* used to treat mental disorders (Bahl, 1987).

Significant pharmacological effects or physiological properties of mushrooms are bioregulation (immunological enhancement), maintenance of homeostasis, regulation of biorhythm, cure of various diseases and prevention and improvement of life threatening diseases such as cancer, cerebral stroke and heart diseases. It is also confirmed that mushrooms have effective substances for antifungal, anti-inflammatory, antitumor, antiviral, antibacterial, hepatoprotective, antidiabetic, hypolipedemic, antithrombotic, hypotensive and other applications (Wasser and Weis, 1999a). Medicinal properties of some of the commercially/non-commercially cultivated mushrooms are given in table 2.4. Recently extracts from fruiting bodies and mycelia of *Ganoderma* species found to possess *in vitro* antioxidant activity (Jones and Janardhanan, 2000; Lakshmi et al., 2003 and Mau et al., 2002) and antimutagenic

Table 4. Medicinal properties of mushrooms

No	Medicinal properties	Mushrooms
1	Antibacterial	*Schizophyllum commune, Grifola frondosa, Fomus fomentarius, Trametes versicolor, Piptoporus betulinus, Ganoderma lucidum, Ganoderma applanatum, Pleurotus ostreatus*
2	Antifungal	*Grifola frondosa, Piptoporus betulinus, Falmulina velutipes, Armilleriella mellea, Agrocybe aegerita*
3	Antiviral	*Grifola frondosa, Trametes versicolor, Ganoderma lucidum, Ganoderma applanatum, Pleurotus ostreatus, Falmulina velutipes, Lentinus edodus*
4	Anti-inflammatory	*Schizophyllum commune, Ganoderma lucidum, Lentinus edodus, Falmulina velutipes*
5	Blood pressure regulation	*Grifola frondosa, Ganoderma lucidum, Lentinus edodus, Armilleriella mellea, Auricularia auricula*
6	Hypolipedemic	*Lentinus edodus, Pleurotus ostreatus, Agrocybe aegerita, Volvariella volvaceae, Pleurotus pulmonaris*
7	Antidiabetic	*Grifola frondosa, Lentinus edodus, Tremella fuciformis*
8	Immunomodulating	*Tremella fuciformis, Lentinus edodus, Falmulina velutipes, Ganoderma lucidum, Ganoderma applanatum, Grifola frondosa, Schizophyllum commune*
9	Kidney tonic	*Trametes versicolor, Ganoderma lucidum, Lentinus edodus, Agaricus bisporus*
10	Hepatoprotective	*Schizophyllum commune, Grifola frondosa, Trametes versicolor, Ganoderma lucidum, Lentinus edodus*
11	Nerve tonic	*Ganoderma lucidum, Pleurotus ostreatus, Armilleriella mellea*
12	Chronic bronchitis	*Ganoderma lucidum, Grifola frondosa, Tremella fuciformis*

activities (Lakshmi et al., 2003). Oyster mushrooms (species of genus *Pleurotus*) are highly edible and nutritious, rank second among the commercially cultivated mushrooms in the world (Chang, 1999), found to possess antioxidant, anti-inflammatory and antitumor activities (Jose and Janardhanan, 2000 and Jose et al., 2002).

2.9 ANTITUMOR ACTIVITY OF MUSHROOMS

As the great threat to human life by neoplastic diseases continues to increase, the pursuit of anti-tumor drugs takes on a compelling urgency. Attempts have been made in many parts of the world to explore the use of mushrooms and their metabolites for the treatment of a variety of human ailments (Jong and Birmingham, 1992). The most significant medicinal effect of mushrooms and their metabolites that attracted the attention of the public is their antitumor property. Lucas and his collaborators first demonstrated the antitumor activity of the higher Basidiomycetes in 1957. Selected mushrooms of higher Bsidiomycetes origin are effective against cancer of the stomach, esophagus, lungs, etc (Yang and Jong, 1989). However, the components responsible for such activity have not yet been completely identified (Wasser and Weis, 1999a).

According to Hata, over 300 antitumor substances had been isolated from microbial origin. Among the 220 antitumor substances from fungi, 208 were obtained from fungi imperfecti, 109 from Basidiomycetes and 34 from Ascomycetes (Hata, 1977). Methanolic extract taken from the fruiting bodies of *Pleurotus florida* (Jose and Janardhanan, 2000), *Pleurotus pulmonarius* (Fr.) Quel. (Jose et al., 2002) and *Ganoderma lucidum* (Curt.: Fr.) P. Karst. Reshi (Jones and Janardhanan, 2000) occurring in South India possess antitumor activity against the Ehrlich's ascites carcinoma (EAC) cell line induced solid tumor model in mice. Fermentation products, polysaccharides, novel phenols and terpenes were isolated from mushrooms and found to be effective against various tumor models in animals.

2.9.1 Fermentation products

Gregory et al., screened more than 7000 cultures of Basidiomycetes for antitumor activity against three rodent tumor systems. Strains representing 20 genera produced materials in fermentation cultures that have inhibitory effects on sarcoma, mammary adenocarcinoma 755, or leukemia L-1210 *in vivo* (Gregory et al., 1966). These results were later confirmed and the cultures were identified as *Coprinus nycthemerus, Corticium rolfsii, Irpex consors, Irpex flavus* etc (Espenshade and Griffith, 1966).

2.9.2 Antitumor polysaccharides

Analysis of structural features of the antitumor extract shows that all compounds responsible for antitumor activity in mushrooms were higher molecular weight glucans, composed of glucose units linked in 1-3 and 1-6 linkages (Mascarenhas, 1994). Antitumor polysaccharides have been obtained from a number of Basidiomycetes, including *Coriolus consors, Coriolus versiocolor, Corficium centrifugum, Cerpidotus sp., Flammulina velutipes, Ganoderma applantum, Phellinus lintus, Pholiota nemeko, Pleurotus ostreatus, Poria cocos, Schizophyllum commune* and *Tricholoma aggregatum* (Ikekawa et al., 1982). These polysaccharides, their derivatives and partially hydrolyzed products are prepared from culture filtrates or by extracting the fruiting bodies, sclerotia and mycelia of the fungi with hot water or an alkaline aqueous solution.

An acidic polysaccharide fraction with antitumor activity has been isolated from *Pleurotus ostreatus* (Yoshioka et al., 1975). This antitumor-active component consists of a skeleton of β(1,3)-linked glucose residues having branches of galactose and mannose residues, and also contains acidic sugars. The protein bound polysaccharides, which have an excellent antitumor activity not only by intraperitoneal administration but also by oral administration. They have been contemplated for use against a wide variety of mammalian cancers, including mammary cancer, gastrointestinal cancers, such as those of the esophagus, stomach and large colon; lung cancer and brain tumors. Some of the antitumor polysaccharide isolated from mushrooms is given in table 2.5.

Table 2.5. Antitumor polysaccharides from mushrooms.

Glucan	Source of fungus	Linkages
Lentinan	*Lentinus edodes*	(1-3)β D
Schizophyllan	*Schizophyllum commune*	(1-6) β D
Pachymaran	*Poria cocus*	(1-3) β D
Auricularia glucan	*Auricularia auricula*	(1-3) β D
Ganoderma glucan	*Ganoderma lucidum*	(1-3) β D
Agrocybe	*Agrocybe cylindrica*	(1-3) β D
Volvariella glucan	*Volvariella volvacea*	(1-3) β D

Adapted from Mascarenhas, 1994.

Lentinan, extracted from an edible mushroom, *Lentinus edodes*, shows significant inhibition of the growth of subcutaneously implanted sarcoma 180 in laboratory animals. Its antitumor activity is elicited by the stimulation of host-mediated responses. The water-soluble polysaccharide GL-1 is isolated from the fruiting bodies of *Ganoderma lucidum*, strongly inhibits the growth of the sarcoma 180 solid type tumor. The essential structure for the antitumor activity is a branched glucan involving (1,3)-β, (1,4)- β and (1,6)- β linkages. The polysaccharide PS-K has been extracted by Hirase et al. from *Coriolus versicolor*, found to be effective against sarcoma 180 when administered orally (Hirase et al., 1970).

2.9.3 Dietary fiber

Mushrooms contain dietary fibers belonging to β-glucans, chitin and heteropolysccharides. They may work effectively to prevent cancer of the colon and rectum (Mizuno, 1996).

2.9.4 Coriolins

Coriolin, coriolin B and coriolin C are tricyclic antibiotics produced from *Coriolus consors* under submerged conditions. Coriolins are active against sarcoma cells, inhibitis the growth of Ehrlich ascites sarcoma and leukemia L-1210 cells. Diketocoriolin B is an oxidized product of coriolin B and has a sesquiterpene skeleton, is effective against Ehrlich carcinoma and leukemia L-1210 in mice (Nishimora et al., 1980).

2.9.5 Terpenoids

About 100 different triterpenoids can be found in fruiting bodies and mycelia of *Ganoderma lucidum* and *Ganoderma applanatum*. These include highly oxidized lanostanine-type triterpinoids, such as ganoderic acids. Some of them reported to inhibit growth of hepatoma cells *in vitro* (Lindequist, 1995). Illudins M and S, are two terpene compounds with antitumor activities isolated from *Clitocybe illudens* (Anchel et al., 1950).

2.9.6 Lectins

The multifarious potentially exploitable activities of some lectins isolated from *Agaricus bisporous, Ganoderma lucidum, Flammulina velutipes and Volvariella volvacea* possess mitogenic, immunomodulating, antiproliferative, antitumor, vasorelaxing and hypotensive activities (Wang et al., 1998).

2.9.7 Merulinic acids

Merulinic acids A, B and C isolated from the fruiting bodies of *Merulius tremellosus* and *Phlebia radiata* have been shown to inhibit Ehrlich carcinoma ascites cells (Giannetti et al., 1978).

2.10 *PHELLINUS* SPECIES AND THEIR MEDICINAL PROPERTIES

Phellinus is a large and widely distributed genus of the family Hymenochetaceae. (Donk) under the class, Basidiomycetes. The species are mostly confined to the plains/tropical forests. Environmental factors such as temperature, humidity, light and host trees are very important for development of basidiocarps. The dominant and most frequently found species are *P. senex, P. rimosus, P. badius, P. fastuosus, P. adamantinus, P. caryophylli* and *P. durrissimus* (Sharma, 1995). About 18 species are found to occur in Kerala, most of them are wood inhabiting (Leelavathy and Ganesh, 2000). *Phellinus rimosus* is a wood rotting macro fungus, found growing on jackfruit tree trunks in Kerala (Fig. 2.12). In Kerala, it is common on living Moraceae members. It causes white pocket rot initially but later the heartwood is transformed into a white spongy mass.

No significant information is available on the medicinal properties of *Phellinus* species occurring in India. However, some of the species are extensively studied in China, Japan and Korea. *Phellinus lintus* has been considered to be a traditional Chinese medicine (Ying et al., 1987) and it was reported that water-soluble extracts of *Phellinus lintus* inhibit the growth of sarcoma 180 implanted subcutaneously in mice. Several studies on the activities of polysaccharides from *P. lintus* were carried out (Kim et al., 1994 and Lee et al., 1995). Recent studies showed that hot water extract of *P. lintus* stimulated the catalytic activity of catalase in the liver and brain (Jinseu et al., 2001), polysaccharides isolated from *P. lintus* inhibited the tumor growth and metastasis (Han et al., 1999) and stimulated the humeral and cell mediated immunity in mice (Kim et al., 1996). Ameliorative effects of *P. linteus* for digestive system cancers such as stomach, digestive tract, duodenal, colon, rectal cancer and liver cancer were discovered (Mizuno, 2000). In addition to anticancer effects, there are many other health-promoting effects that seem to originate from an enhancement of natural healing ability, chronic gastritis, constipation, diabetes, high blood pressure, low blood pressure, arteriosclerosis, cystitis, nephritis, prostatomegaly, atopic dermatitis and sexual dysfunction (Mizuno, 2000). Recently 70 % ethanolic extract of fruit bodies of *P. lintus* was reported to possess *in vitro* anti-angiogenic, antioxidant and xanthine oxidase inhibition activities (Song et al., 2003). Aqueous extract of *P. rhabarbarinus*

Figure 2.12. *Phellinus rimosus* growing on tree trunk

found to possess cytotoxicity and inhibitory activity against human immunodeficiency virus type-1 in *in vitro* culture system (Walder et al., 1995). Polysaccharides of *P. igniarius* and *P. robustus* were also reported to possess antitumor activities (Naruse et al., 1974). No biomedical properties of *Phellinus rimosus* have yet been reported, except its use in tribal medicine for the treatment of some ailments (Leelavathy and Ganesh, 2000).

Materials and Methods

Ajith. T.A "Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrohungus, Phellinus rimosus (Berk) Pilat. " Thesis. Amala Cancer Rcsearch Centre , University of Calicut, 2004

Chapter 3

Materials and Methods

3.1 MATERIALS

3.1.1 Animals

Albino mice and rats were purchased from Small animal breeding center, Kerala Agricultural University, Mannuthy, Thrissur and were kept for a week under environmentally controlled conditions with free access to standard food (Lipton, India) and water.

3.1.2 Cell lines

Ehrlich's ascites carcinoma (EAC) and Daltons lymphoma ascites (DLA) cell lines were obtained from Cancer Institute, Adayar, Chennai. The cells were maintained in mice by intraperitonial inoculation of $1x10^6$ viable cells.

3.1.3 Bacterial strains

Salmonella typhimurium strains TA 98, TA 100, TA 102 and TA 1535 were originally obtained from Prof. B. N. Ames, University of California, Berkely, USA. The strains were subcultured in nutrient broth for 12 h and stored at –70°C as frozen permanents in the presence of dimethyl sulphoxide (9%). Thawed frozen permanent (40 µl) was used to inoculate for the fresh overnight culture of each strain in nutrient broth (5 ml). The inoculated nutrient broth was incubated overnight at 37°C and used for the antimutagenic assay.

3.1.4 Chemicals

Carbon tetrachloride (CCl_4), aniline, phenol, hydrogen peroxide (H_2O_2), chloroform ($CHCl_3$), dimethylsulphoxide (DMSO), formaldehyde, sodium nitroprusside, sodium nitrite and thiobarbituric acid were purchased from Merck, India Ltd, Mumbai. Nicotinamide- adenine dinucleotide phosphate (disodium) (NADP), glucose-6-phosphate, D-biotin, L-histidine, agar-agar, sodium azide (NaN_3), reduced glutathione (GSH), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), diacetylmonoxime (DAM), nitroblue tetrazolium (NBT), reduced nicotinamide adiniedinucleotide phosphate tetrasodium (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), riboflavin and sulphanilamide from Sisco Research Laborateries Pvt. Ltd, Mumbai. Cisplatin was purchased from Dabur India Ltd, New Delhi.

7,12-dimethylbenz[*a*]anthracene (DMBA), *N*-nitrosodiethylamine (NDEA), *N*-methyl-*N* ′ -nitro-*N*-nitrosoguanidine (MNNG), 4-nitro-*o*-phenylenediamine (NPDA) and benzo[*a*]pyrene (B[*a*]P) and carrageenan were purchased from Sigma, St. Loius, USA.

All other chemicals and reagents used were analytical reagent grade.

3.1.5 Instruments

The following instruments were used for the study

Spectrophotometer	: Elico SL 159 and Elico SL 177
Research Microscope	: Meiji, Japan and Labex, india
Deep freezer (–70 and –20°C)	: Remi and Tropicana
Cold Lab	: LKB, Bromma
Cooling centrifuge	: Remi, Chennai
Electronic balance	: Contech, instrument compony, Mumbai
	: Casbee, CAS weighing India, Guraigon
pH meter	: Elico IL 120
Lyophilyser	: Labconco, Labconco corporation, Missouri

3.2 METHODS

3.2.1 Preparation of the extract

Sporocarps of *P. rimosus* growing on the jackfruit tree trunks were collected from the out skirts of Thrissur, Kerala. The specimen was identified and voucher specimen was deposited in the Herbarium of Centre for Advanced Studies in Botany, University of Madras, Chennai, India (HERB MUBL 3171).

The sporocarps were cut into small pieces, dried at 40-50°C for 48 h and powdered. Two hundred gram of the powdered materials was extracted with petroleum ether. The defatted material was extracted with ethyl acetate and then with methanol for 8-10 h using Soxhlet apparatus (Suffness and Douros, 1979). The solvents were completely evaporated at 40°C using a rotary vacuum evaporator. The residues were designated as ethyl acetate (EtOAc) extract (2.5 g) and methanol extract (MeOH) (4 g). One hundred gram of material after solvent extraction was suspended in 1litre distilled water and boiled for 1h at 90-95°C. The supernatant removed and the extraction was repeated once again. The supernatants thus obtained were combined and filtered through Whatman No. 1 filter paper, the filtrate was concentrated at low temperature and finally the concentrate was lyophilized. The residue (3.5 g) was designated as aqueous extract (AQ).

The ethyl acetate and methanol extracts were presolubilised in DMSO for assaying the *in vitro* activity. For the animal experiments, these extracts were dissolved in minimum volume of ethanol and diluted with distilled water to form a uniform suspension. The ethanol remaining in the solution was allowed to evaporate at 40-45°C.

Aqueous extract was dissolved in distilled water for the *in vitro* and *in vivo* experiments.

3.2.2 Preparation of tissue homogenates (liver, brain and kidneys)

Animals were sacrificed. Liver, brain and kidneys 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. 10 % 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 and conjugated diene. 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, catalase, glutathione peroxidase, glutathione-*S*-transferase, and malondialdehyde.

3.2.3 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.

3.2.4 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.

3.2.5 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

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

3.2.6 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.

3.2.7 Determination of tissue glutathione S-transferase (GST) activity

Glutathione-*S*-transferase activity was determined according to the method of Habig et al., (1974).

Principle

The activity was determined by the rate of increase in conjugate formation between reduced glutathione and CDNB. The conjugate absorbs at 340 nm.

Procedure

The reaction mixture contained 0.05 ml of 60 mM GSH, 0.05 ml of 60 mM CDNB (in ethanol), 0.01 ml of the tissue homogenate and sodium phosphate buffer (0.1 M, pH 6.5) in total volume of 3 ml. The reaction was started by the addition of sample and the extinction was measured by 1 min interval for 3 min at 340 nm. The reference cuvette contained the complete assay mixture minus the cytosol. The activity of GST

was calculated using the mmolar extinction coefficient of CDNB-GSH conjugate (9.6 $mM^{-1}cm^{-1}$) and is expressed as μmol of CDNB-GSH conjugate formed/ min/ mg protein.

μmol of CDNB-GSH conjugate-

formed/ min/ mg protein $= \frac{\Delta A/min \times 1000 \times 3}{9.6 \times mg\ protein\ in\ sample}$

3.2.8 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.

3.2.9 Determination of tissue conjugated diene

Conjugated diene in the tissue was determined according to the method of John and Steven (1978).

Principle

Tissue membrane lipids were extracted using $CHCl_3$: CH_3OH (2:1), the extracted lipid content was measured at 233 nm as an increase in absorbance.

Procedure

0.5 ml of tissue homogenate treated with 5 ml of $CHCl_3$: CH_3OH (2:1) the mixture was centrifuged at 2000 rpm for 5 min. 2 ml of the lower layer was taken and evaporated, the lipid residue was dissolved in 1 ml of cyclohexane and read at 233 nm against cyclohaxane in the reference cuvette. The conjugated diene was calculated

using the molar extension coefficient 2.52 x 10^4 $M^{-1}cm^{-1}$ and expressed as mM/100 g tissue.

$$\text{Conjugated diene} = \frac{\text{E at 233 nm x vol. of system x total vol. of homogenate x } 10^5}{2.52 \times 10^4 \text{ x vol. of lower layer taken x wt. of tissue}}$$

3.2.10 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).

3.2.11 Determination of tissue Aniline hydroxylase activity

Aniline hydroxylase was assayed according to the method of Bourrie et al., (1996) using aniline as substrate.

Principle

Aniline hydroxylase catalyses the hydroxylation of aniline to p-aminophenol in the presence of reduced nicotinamide adinine dinucleotide phosphate. The activity of aniline hydroxylase was determined by measuring the quantity of p-aminophenol formed. P-aminophenol is allowed to react with phenol in alkaline medium to form the blue colored product phenol-indophenol complex, which can be measured at 630 nm.

Procedure

1 ml of the cytosolic fraction was incubated with 0.1 ml 150 mM aniline (in methanol), 0.1 ml of 0.5 mM NADPH and 0.1 mM phosphate buffer in a total volume of 1.5 ml at 37 °C for 20 min. After the incubation 20% of TCA was added to stop the reaction. The contents were mixed and centrifuged at 2000 rpm. To 1 ml of the

supernatant, 0.5 ml of 10% Na_2CO_3 and 1 ml of 2% phenol (in 0.2 N NaOH) was added and placed in an incubator at 37°C for 30 min. The absorbance of the color formed was read in a spectrophotometer at 630 nm against the reagent blank. The amount of p-aminophenol formed was calculated using the millimolar extinction coefficient of 284 cm^{-1}. The specific activity of aniline hydroxylase was expressed as nanomol of p-aminophenol formed/mg protein/min.

$$\mu\text{mol of p-aminophenol formed/mg protein/min} = \frac{\text{O.D at 630 nm x 1000}}{\text{284 x mg protein x 20}}$$

3.2.12 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.

3.2.13 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.

3.2.14 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.

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

3.2.15 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.

Serum GGT (U/l) = (ΔA/min) x 1158

3.2.16 Determination of serum urea

Serum urea was determined according to the method of Marsh et al., as described in Text book of Clinical Biochemistry, Varley (1980).

Principle

Urea on heating with diacetylmonoxime under acidic condition condenses with diacetyl to form a pink colored diazine complex. The reaction was catalyzed by thiosemicarbazide and Fe^{3+} ions. The absorbance of the complex was measured at 525 nm.

Procedure

Reagents used were from Span diagnostic kit. 5 ml of diluted urea reagent (1:5 with distilled water) were mixed with 0.02 ml serum and 0.5 ml of diacetylmonoxime. Mixed well and kept in boiling water bath for 10 min. Cooled and absorbance was measured at 525 nm against reagent blank. A standard solution of urea (30 mg%) was treated in the same way.

$$\text{Serum urea (mg/dl)} = \frac{O.D_T \times 30}{O.Ds}$$

3.2.17 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.

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

3.2.18 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.

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

3.2.19 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.

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

3.2.20 Determination of Plasma fibrinogen

Plasma fibrinogen was determined by the method of King and Wooten as described in Text book of Clinical Biochemistry, Varley (1980).

Principle

0.2 ml of plasma was treated with 3.8 ml of 12.5 % sodium sulphite. The optical density was measured at 680 nm against the reagent blank. Standard calibration curve was prepared using serum of known protein concentration. 0.5 ml of various dilution of normal serum was mixed with 3.5 sulphosalicylic acid (3g of sulphosalicylic acid in 7% sodium sulphate solution). After 5 min. the absorbance was read at 660 nm against reagent blank.

Calculation

$$\text{Plasma fibrinogen (mg/dl)} = \frac{O.D_T \times 0.2 \times 100}{O.Ds \times Conc.\ Std}$$

3.2.21 Determination of serum lipid peroxidation

Serum lipid peroxidation was determined by Ohkawa *et al.*, (1979) after precipitating the protein according to the method of Satoh (1987).

Principle

Lipids were isolated by precipitating them with serum protein using 0.02 % trichloroacetic acid. The level of lipid peroxidation was measured as malondialdehyde by reacting with TBA in acetic acid solution. The reaction product was assayed by measuring absorption at 532 nm.

Procedure

To 0.5 ml serum, 2.5 ml of 0.02 % TCA was added and the tube is left to stand for 10 min at room temperature. After centrifugation at 3500 rev./min for 10 min, the precipitate was washed once with 0.05 M H_2SO_4. The precipitate was suspended in 1 ml distilled water and estimated the TBARS by procedure given under tissue lipid peroxidation determination (section 3.2.8). The result was expressed as nmol/ml of serum.

3.2.22 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.

3.2.23 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 1^{st} order reaction.

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

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

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

3.2.24 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.

3.2.25 Determination of glutathione S-transferase (GST) activity in blood

Glutathione-*S*-transferase activity was determined according to the method of Habig et al., (1974).

Principle

The activity was determined by the rate of increase in conjugate formation between reduced glutathione and CDNB. The conjugate absorbs at 340 nm.

Procedure

Erythrocyte sediment was prepared from the heparinised blood and washed 3 times with isotonic saline. Prepare a haemolysate containing approximately by the addition of 4 parts by volume of distilled water. 10 µl of haemolysate for the determination of GST as described under the GST estimation in tissue (section-3.2.7). The result was expressed as mmol of CDNB conjugate formed/min/g Hb.

3.2.26 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.

3.2.27 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.

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

3.2.28 Determination of total red blood cell (RBC) count

Total RBC count was determined haemocytometer as described in the textbook of practical Physiology, Chaudhari (2000 a).

Principle

Whole blood was diluted appropriately using an isotonic diluent to avoid lysis of red cells. The number of cells in a known volume and known dilution was counted using a counting chamber.

Procedure

Added 0.02 ml blood to 3.98 ml of diluting fluid. Charged the neubauer chamber with well-mixed diluted blood. Counted the total number of red cells in the small square in the central ruled area of neubauer counting chamber using 40x objective of the microscope.

Total RBC count = Number of cells counted x 10,000 count/mm^3

3.2.29 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

3.2.30 Determination of antimutagenic activity

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

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.

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.

3.2. 31Confirming genotype of Salmonella typhimurium strains

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

3.2.31.1 Histidine requirement

Histidine requirement of the tester strains TA 98, TA 100, TA 102 and TA 1535 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.

3.2.31.2 rfa mutation

Strains having *rfa* mutation was tested using crystal violet sensitivity test. 0.1 ml of each tester strains TA 98, TA 100, TA 102 and TA 1535 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.

3.2.31.3 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. TA 1535 strain was streaked on a separate plate. 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, TA 100 and TA 102 were irradiated for 8 sec. and TA 1535 for 6 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 102 was used as the control strain.

TA 98, TA 100 and TA 1535 showed growth only on the un-irradiated side of the plate which indicated the absence of excision repair enzymes. TA102 strain with excision repair enzymes showed growth on both sides of the plate.

3.2.31.4 R-factor

R-factor was tested by ampicillin resistance on ampicillin plate. R-factor of the tester strains TA 98, TA 100, and TA 102 was confirmed by streaking across the surface of ampicillin plate. TA 1535 was used as the control strain. The plates were incubated overnight at 37°C and examined for growth.

TA 98, TA 100 and TA 102 strains showed growth in the ampicillin plate indicated the ampicillin resistance. Where as the TA 1535, a non-R factor strains showed no growth.

3.2.32 Preparation of rat liver microsomal fraction (S9)

Male Spraque Dawly rat (200 g) was treated with sodium phenobarbitone (0.1 %) in drinking water for 4 days (Moron and Ames, 1983). After an overnight fasting, animal was killed by decapitation, liver removed and washed several times in 0.15 M chilled KCl. Homogenate was prepared aseptically in 0.15 M KCl (3 ml/g wet liver). The homogenate was centrifuged in a cooling centrifuge at 8,600 rpm for 10 min at 4°C. The supernatant was used as the S9 fraction.

3.2.33 Preparation of S9 mix

5 ml of the S9 mix was prepared by adding sterile reagents in the following order, 1.675 ml of sterile distilled water, 2.5 ml of 0.2 M sodium phosphate buffer (pH 7.4), 0.2 ml of 0.1 M NADP, 0.025 ml of 1 M glucose-6-phosphate, 0.1 ml of $MgCl_2$-KCl solution (1.65 M KCl +0.4 M $MgCl_2$) and 0.5 ml of rat liver S9.

3.2.3 Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) using MSTAT-C, soft ware package, UK. If found significant pair wise comparison of ethyl acetate, methanol and aqueous extract treated groups with the control group was done by Dunnett's-*t* test (Cochran and Cox, 1957).

Critical difference (c.d) = $t \times \sqrt{2 \times EMS / r}$

Where t is the Dunnett's *t* value for $P<0.01$ or $P<0.05$, EMS is the error mean square value and r is the number of observations/group. Value is found to be significant if the difference between average value of the control group and the treated group was greater than the c.d.

For comparison of normal with control group critical difference (lsd) was used. $P< 0.05$ was found to be significant.

Antioxidant activity of P. rimosus

Ajith. T.A “Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrohungus, Phellinus rimosus (Berk) Pilat. ” Thesis. Amala Cancer Rcsearch Centre , University of Calicut, 2004

Chapter 4

Antioxidant activity of *Phellinus rimosus*

4.1 Introduction

Molecular oxygen, while providing an efficient energy production from ingested food, results in free radical and peroxide by-products, which have high intrinsic toxicity. Numerous physiological processes in living organisms occasionally produce free radicals, reactive nitrogen species (RNS) and reactive oxygen species (ROS) as by-products. Their broad ranges of effects in biological systems have drawn the attention of many experimental works. Although humans and other organisms possess antioxidant defense and repair systems to protect against oxidative damage, these systems are insufficient under certain conditions to totally prevent the damage (Simic, 1988). Hence, the oxygen free radicals dependent damage to protein, DNA and other biomolecules accumulates during the life time of organisms.

Alcohol abuse and alcoholism represent one of the major health, social and economic issues facing the world especially in the developing countries. Morphological, biochemical and molecular studies undertaken in recent years both in experimental animals and in man have shown that oxidative stress plays an important role associated with alcohol abuse (Mohammed, 2002). Liver is among the organs most susceptible to the toxic effects of ethanol. The highest degree of oxidative damage also occurs in the organs like brain, heart, kidney and skeleton muscle since these organs are composed primarily of post mitotic cells (Venkataramanujan, 2002).

The phorbol esters present in the Euphobiacea are another potent inducer of free radical and associated with promotion of skin carcinogenesis. 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) is a skin tumor promotor isolated from seed oil of *Croton tiglium* and has been extensively studied in DMBA-induced mouse skin tumor models. The experimental evidences strongly suggested the role of free radical mediated reactions in phorbol ester promoted papilloma on mouse skin (Copeland, 1983). The application of croton oil causes a reduction of reactive oxygen intermediates (ROI) detoxifying enzyme activity in the epidermal cells (Murakami et al., 2000). Hence, compounds that inhibit or scavenge the ROS/RNS are of great interest as possible protective agent to help human body from the oxidative damage.

Many synthetic antioxidant components exhibited toxic or mutagenic effects that have directed the attention to the naturally occurring antioxidants in recent years. A multitude of natural antioxidants have already been isolated from different plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs. Although research was focused on the therapeutic effects of these medicinal

mushrooms, no significant information is available on their antioxidant properties. The *in vitro* antioxidant activity of ethyl acetate, methanol and aqueous extracts of *Phellinus rimosus* and the protective effect of ethyl acetate extract against alcohol and croton oil induced lipid peroxidation in mice were evaluated. The findings are presented in this chapter.

4.2 Materials and Methods

4.2.1.Preparation of extracts

Ethyl acetate, methanol and aqueous extracts of *P. rimosus* were prepared as described in the section 3.2.1.

4.2.2.Animals

Male and female Swiss albino mice weighing 30 ± 2 g were used for the study.

4.2.3. Determination of in vitro antioxidant activity

4.2.3.1 Superoxide scavenging activity

Assay is based on the ability of the extract to inhibit or scavenge the super oxide radical generated from the photoreduction of riboflavin according to the method of Mc Cord and Fridovich (1969). The reaction mixture contained, EDTA (6 mM) contained 3 µg NaCN; riboflavin (2 µM); NBT (50 µM); KH_2PO_4-Na_2HPO_4 buffer (67 mM, pH 7.8) and various concentrations of the extract in a final volume of 3 ml. The tubes were illuminated under the incandescent lamp for 15 min. The optical density (O.D) at 560 nm was measured before and after illumination against distilled water. The inhibition of the superoxide radical generation was determined by comparing the absorbance values of the control with that of treated. Quercetin was used as standard.

4.2.3.2 Inhibition of lipid peroxidation

Lipid peroxidation was induced by Fe^{2+}-ascorbate system (Bishayee and Balasubramanian, 1979) in the rat liver homogenate in the presence and absence of extracts to form thiobarbituric acid reacting substance (TBARS). TBARS is measured according to the method of Ohkawa et al (1979). The reaction mixture contained 0.1 ml of rat liver homogenate (25 %, w/v) in Tris-HCl buffer (20 mM, pH 7); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4$. $6H_2O$ (0.16 mM); ascorbate (0.06 mM) and various concentrations of the extracts of *P. rimosus* in a final volume of 0.5 ml. The reaction mixture was incubated for 1 h at 37°C. After 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 distilled water

and then kept in a water bath at 95-100°C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1,v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The organic layer was removed and its absorbance at 532 nm was measured against n-butanol-pyridine mixture. Inhibition of lipid peroxidation was determined by comparing the optical density of the treated with that of control. Catechin was used as standard.

4.2.3.3 Assay of hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was determined by studying the competition between deoxyribose and the extracts of *P. rimosus* for the hydroxyl radicals generated from Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction). The hydroxyl radical attack deoxyribose, which eventually results in a thiobarbituric acid reacting substance (TBARS). The TBARS thus formed was measured (Ohkawa et al., 1979). The reaction mixture contained deoxyribose (2.8 mM); $FeCl_3$ (0.1mM); K_2HPO_4-KOH buffer (20 mM, pH 7.4); EDTA (0.1mM); H_2O_2 (1.0 mM); ascorbic acid (0.1mM) and various concentrations of the extracts of *P. rimosus* in a final volume of 1 ml. The reaction mixture was incubated at 30°C for 60 min. The TBARS formed was estimated by thiobarbituric acid method of Ohkawa et al (1979). The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treated. Catechin was used as standard.

4.2.3.4 Assay of nitric oxide scavenging activity

The nitric oxide scavenging activity was measured according to the method of Sreejayan and Rao (1997). Immediately before the experiment, 10 mM stock solution of sodium nitroprusside was prepared in PBS (pH 7.4). Various concentrations of the *P. rimosus* extracts and sodium nitroprusside (1mM) in a final volume of 3 ml were incubated at 25°C for 150 min. After incubation, 2.5 ml of the reaction solution was removed and mixed with 0.5 ml of Griess reagent (1 % sulphanilamide, 2 % orthophosphoric acid and 0.1 % naphthylethylenediamene dihydrochloride). The absorbance of the chromophore was read immediately at 546 nm against reagent blank. The nitric oxide scavenging activity was determined by comparing the absorbance of control with that of treated. Quercetin was used as the reference drug.

Production of nitrite from solution of 1mM sodium nitroprusside solution incubated in the presence and absence of *P. rimosus* extracts at various time intervals

(50, 100 and 150 min) were also studied and referred to the absorbance of standard solutions of sodium nitrite (0.1μM-5 μM) treated similarly with Griess reagent.

4.2.3.5. Determination of reducing activity

The reducing activity of extracts of *P. rimosus* was determined according to the method of Oyaizu (1986) with modification. Each extract (0.1–200 μg) was mixed with 200 mM sodium phosphate buffer (pH 6.6) and 1.25 ml of 1% potassium ferricyanide in a total volume of 2.5 ml and the mixture was incubated at 50° C for 20 min. After the incubation 1.25 ml of 10 % trichloroacetic acid (w/v) was added. 2.5 ml of the mixture was mixed with 2.5 ml of deionized water and 0.5 ml of freshly prepared 0.1 % $FeCl_3$. The absorbance was measured at 700 nm against reagent blank. The higher absorbance indicates a higher reducing power.

4.2.3 Determination of lipid peroxidation inhibiting activity of P. rimosus induced by alcohol

Male Swiss albino mice (30 ± 2 g) were divided into three groups of six animals each. Group I treated with vehicle was kept as normal. Group II treated with absolute ethanol 0.1 ml orally for 2 days was kept as control. Group III treated with ethyl acetate extract of *P. rimosus* 50 mg/kg body wt. orally 1 h before each administration of alcohol. 24 h after the second dose of alcohol administration, animals were sacrificed with anesthesia. Liver, kidney and brain were removed and was made homogenate as described in the section 3.2.2. Lipid peroxidation was determined by estimating the level of malondialdehyde (MDA) and conjugate diene in the homogenate as described in the section 3.2.8 and 3.2.9.

4.2.4 Determination of lipid peroxidation inhibiting activity of P. rimosus induced by croton oil

Croton oil was isolated from the croton seed *Croton tiglium* according to the method of Berenblum (1941). Briefly, 500 g of dried croton seed was macerated with 250 ml of petroleum ether for 72 h with intermitted shaking. The extract was decanted and the process was repeated once again. The extract was collected together and evaporated at low temperature (40° C). The croton oil residue thus obtained was employed for the experiment.

Female Swiss albino mice (30 ± 2 g) were shaved on their dorsal side using surgical clippers, 2 days before the experiment. Animals with complete hair growth arrest were grouped into 5 groups of six animals each and treated as follows; ethyl

acetate extract of *P. rimosus* (10 and 20 mg in 0.1 ml of acetone) was applied topically to the shaved area of the dorsal skin 30 min before each application of croton oil (0.1 ml of 50% croton oil in acetone, v/v) (Lakshmi et al., 2003). After 24 h, the extract and croton oil treatment was repeated on the same area. Group treated with 0.1 ml of croton oil in 0.1 ml acetone was kept as control. Diclofenac 10 mg in 0.1 ml acetone applied topically was used as the standard reference drug. Group treated with 0.1 ml of vehicle (acetone) was maintained as normal. 1h after the second treatment of croton oil, animals were sacrificed and the skin was removed. The skin was minced in 20 mM Tris-HCl buffer (pH 7) and a 10% homogenate was prepared. The MDA level was measured as described in the section 3.2.8.

4.3 Results

4.3.1.In vitro antioxidant activity

4.3.1.1 Superoxide radical scavenging activity

The extracts of *P. rimosus* were found to be a scavenger of superoxide generated by photoreduction of riboflavin (Fig. 4.1). The extracts showed significant superoxide inhibiting activity. The concentration of ethyl acetate, methanol and aqueous extracts of *P. rimosus* required to scavenge 50 % superoxide anion generated (IC_{50}) were found to be 22 ± 1, 25.3 ± 1.2 and 126 ± 5.1μg/ml respectively (Table1). The IC_{50} of quercetin was 3.7 ± 0.16 μg/ml.

4.3.1.2 Hydroxyl radical scavenging activity

The degradation of deoxyribose to TBARS by hydroxyl radical generated from Fe^{3+}-ascorbate-EDTA-H_2O_2 system was markedly decreased by the extracts of *P. rimosus* (Fig. 4.2). The IC_{50} of ethyl acetate, methanol and aqueous extracts of *P. rimosus* required to scavenge the generated hydroxyl radical were found to be 68 ± 4.1, 93 ± 10.3 and 71 ± 4.7μg/ml respectively (Table 4.1). The IC_{50} of catechin was 850 ± 20.0 μg/ml

4.3.1.3 Inhibition of lipid peroxidation

Extracts of *P. rimosus* were effective in inhibiting the lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate (Fig. 4.3). The generation of malondialdehyde (MDA) and related substances that reacted with thiobarbituric acid was found to be inhibited by the extracts. This indicated the significant lipid peroxidation inhibiting activity of the extracts. The IC_{50} of ethyl acetate, methanol and aqueous extracts of *P. rimosus* required to inhibit the lipid peroxidation were found to

be 162 ± 7, 282 ± 12.8 and 318 ± 2.4 μg/ml respectively (Table 1). The IC_{50} of catechin was 418 ± 28.6 μg/ml

4.3.1.4 Nitric oxide radical scavenging activity

The extracts of *P. rimosus* inhibited the nitric oxide released from the sodium nitroprosside in a dose dependent manner (Fig. 4.4). Incubation of extracts of *P. rimosus* (IC_{50} concentration) with solutions of sodium nitroprusside in phosphate buffered saline at 25°C for various time intervals (50, 100 and 150 min) resulted in linear time-dependent reduction of nitrite production (Fig. 4. 5). The IC_{50} of ethyl acetate, methanol and aqueous extracts of *P. rimosus required* to scavenge the generated nitric oxide were found to be 438 ± 21.6, 126.7 ± 12.6 and 31 ± 4.5 μg/ml respectively (Table 4.1). The extracts of *P. rimosus* did not change the absorbance of sodium nitrite upon treatment with Griess reagent. The IC_{50} of quercetin was 166.6 ± 6.6 μg/ml.

4.3.1.5 Reducing activity

The reducing activity of extracts of *P. rimosus* was found in a concentration dependent manner. The reducing activity increases with increased concentration of the extract (Fig. 4. 6).

4.3.2. Inhibition of lipid peroxidation induced by ethanol

The effect of ethyl acetate extract of *P. rimosus* on ethanol induced lipid peroxidation is given in table 4. 2 and 4. 3. Ethanol ingestion increased ($P<0.01$) the malondialdehyde and conjugated diene level at kidney, liver and brain in the control group of animals compared to the normal group of animals. Ethyl acetate extract of *P. rimosus* significantly inhibited ($P<0.01$) the ethanol induced lipid peroxidation in the liver, kidney and brain. The conjugate diene level in liver, kidney and brain was inhibited 75, 83 and 74 % respectively in the extract treated group of animals. The level of malondialdehyde also decreased significantly in the extract treated group. The level was inhibited 69, 68.8 and 70.5 % in the liver, kidney and brain respectively of animals treated with extract.

4.3.3.Inhibition of lipid peroxidation induced by croton oil

The effect of ethyl acetate extract of *P. rimosus* against croton oil induced lipid peroxidation is given in the table 4. 4. Application of croton oil significantly ($P<0.01$) induced the lipid peroxidation on mouse skin compared to normal group of animals. The croton oil induced peroxidation was inhibited ($P<0.01$) by the extract in a

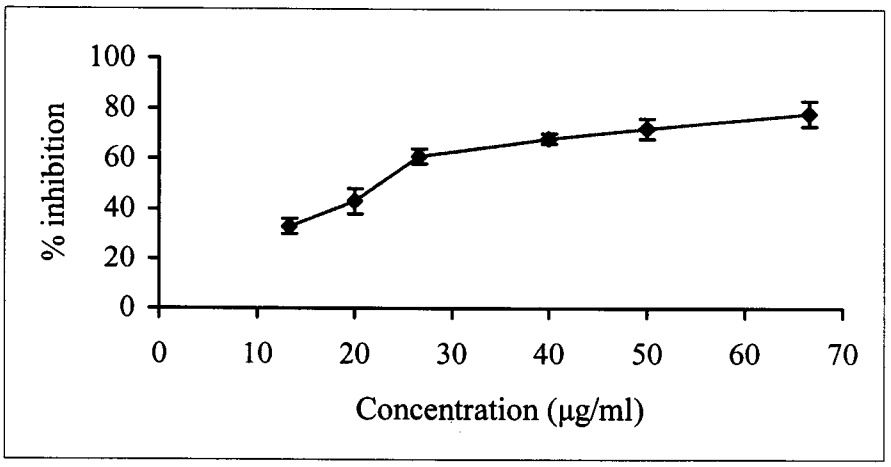
concentration dependent manner with maximum activity at 20 mg. The extract at 20 mg inhibited the peroxidation 79.6 % compared to the control. The extract at 10 mg/skin inhibited 54.3 % lipid peroxidation induced by croton oil.

Table 4.1. *In vitro* antioxidant activity of extracts of *P. rimosus*

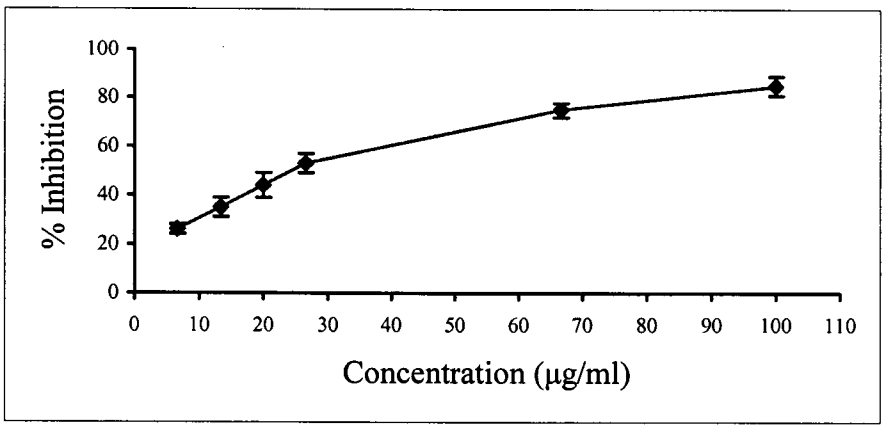
Activities	Extracts (IC_{50}) (µg/ml)			Standards	
	Ethyl acetate	Methanol	Aqueous	Catechin	Quercetin
Super oxide scavenging	22.0 ± 1.0	25.30 ± 1.20	126.0 ± 5.1	---	3.70 ± 0.16
Nitric oxide scavenging	438 ± 21.6	126.7 ± 12.6	31.0 ± 4.5	---	166.6 ± 6.6
Hydroxyl radical scavenging	68.0 ± 4.1	93.0 ± 10.3	71.0 ± 4.7	850 ± 20.0	---
Lipid peroxidation inhibiting	162 ± 7.0	282 ± 12.8	318 ± 2.4	418 ± 28.6	---

Values are mean ± S.D, n=3

a)



b)



c)

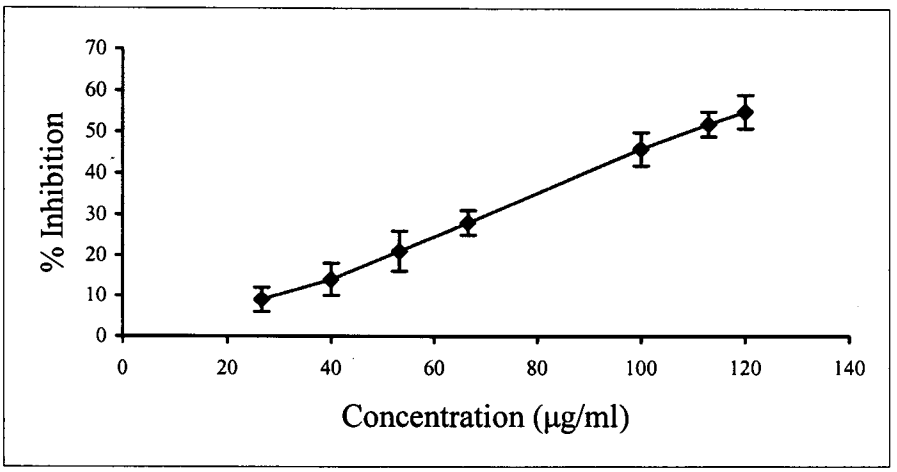
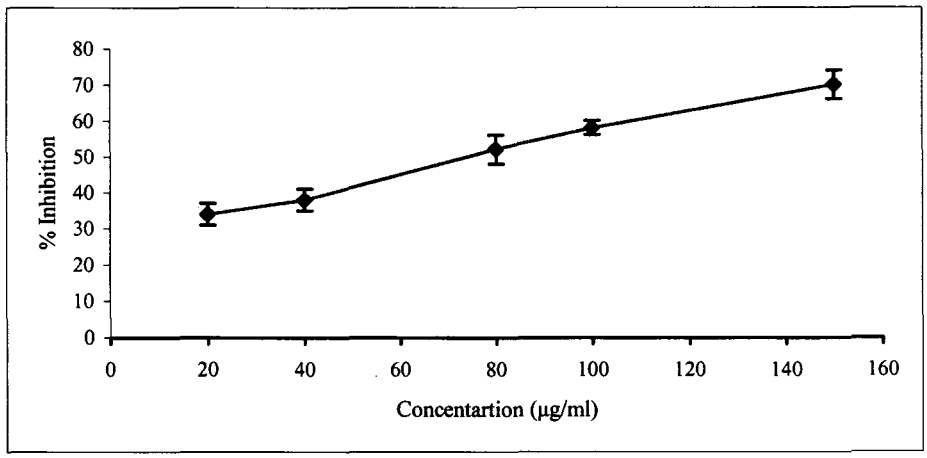


Figure 4.1-*In vitro* superoxide scavenging activity of a) ethyl acetate, b) methanol and c) aqueous extract of *P. rimosus* Values are mean ± S.D, n=3

a)



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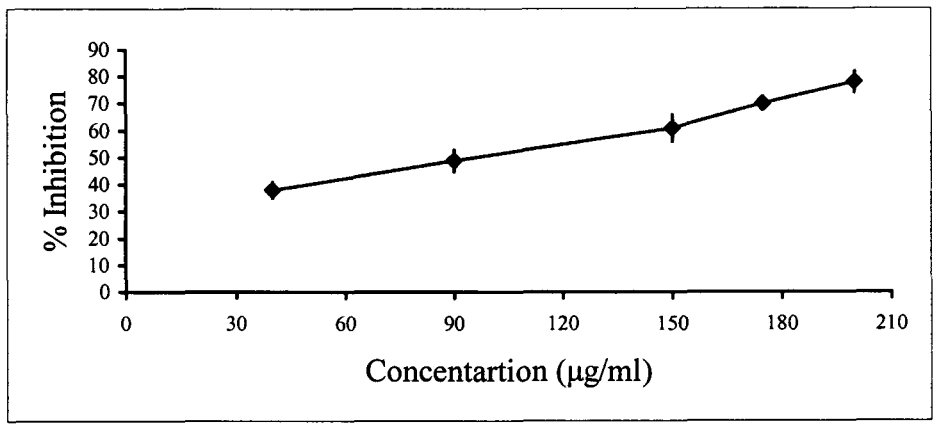

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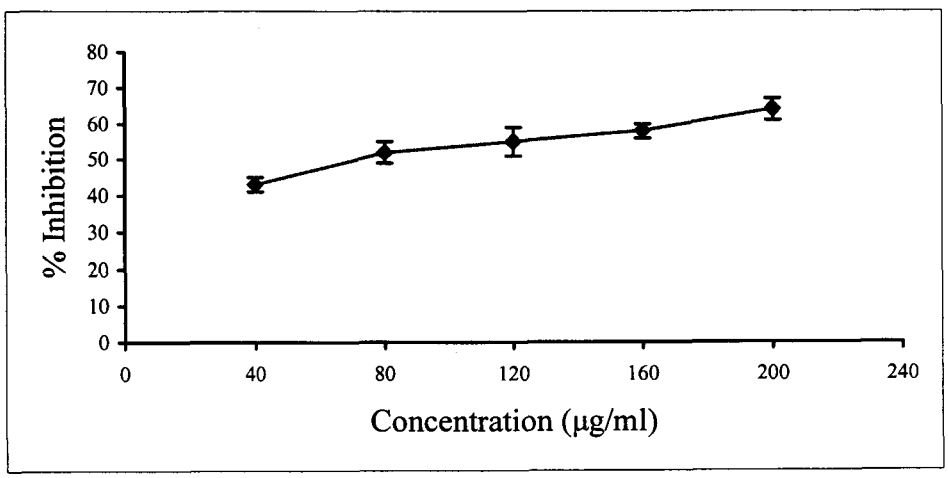
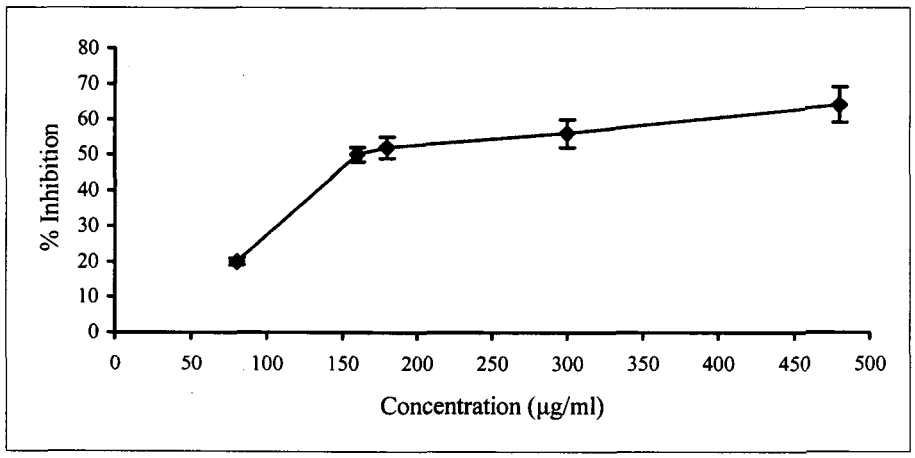
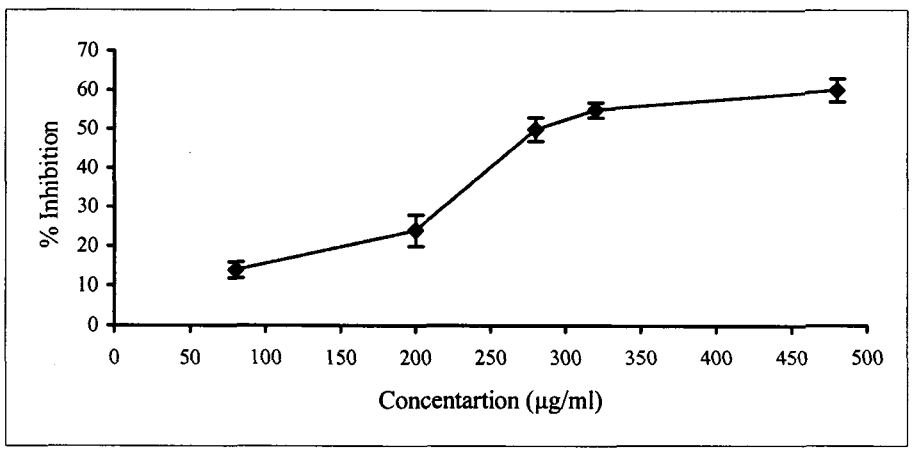


Figure 4. 2- *In vitro* hydroxyl radical scavenging activity of a) ethyl acetate, b) methanol and c) aqueous extract of *P. rimosus* Values are mean ± S.D, n=3

a)



b)



c)

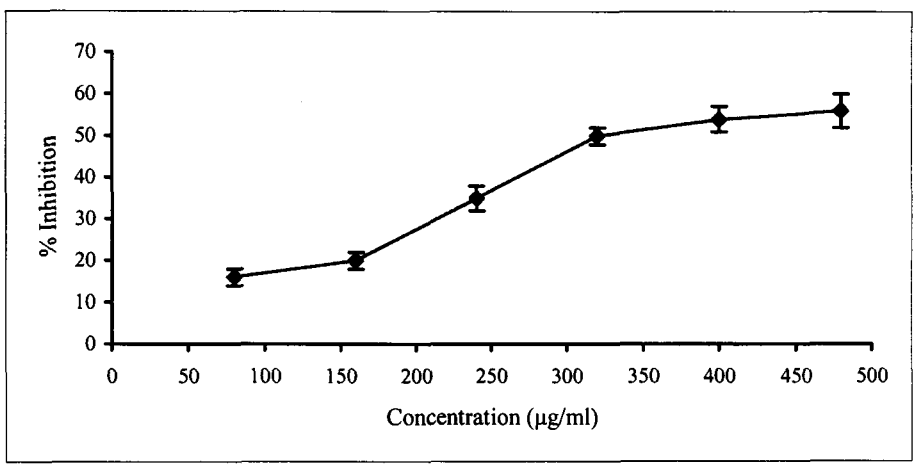


Figure 4. 3- *In vitro* lipid peroxidation inhibiting activity of a) ethyl acetate, b) methanol and c) aqueous extract of *P. rimosus* Values are mean ± S.D, n=3

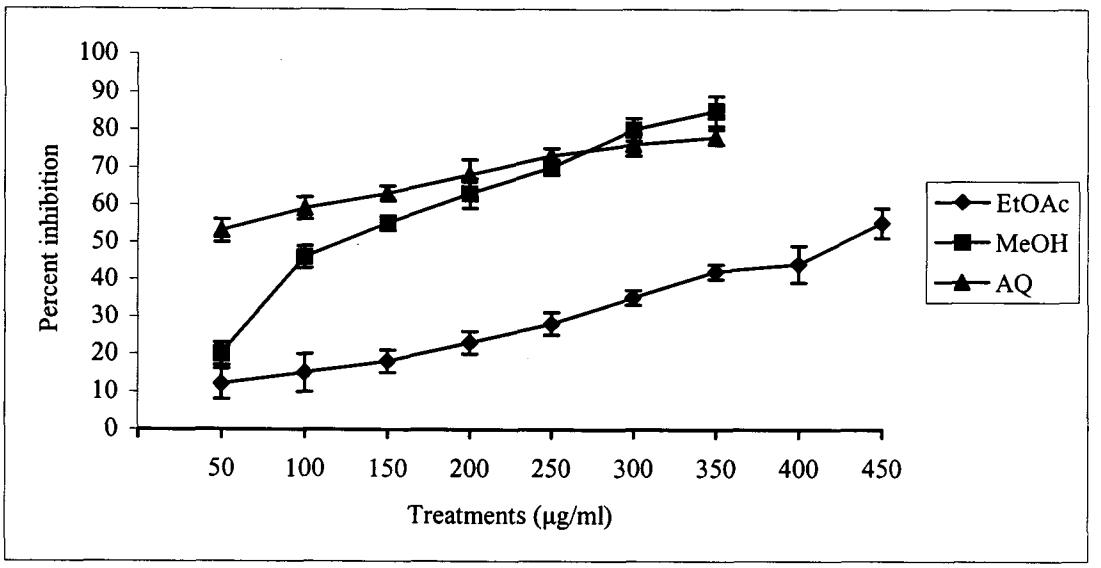


Figure 4. 4. *In vitro* nitric oxide scavenging activity of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus*. Values are mean ± S.D, n=3

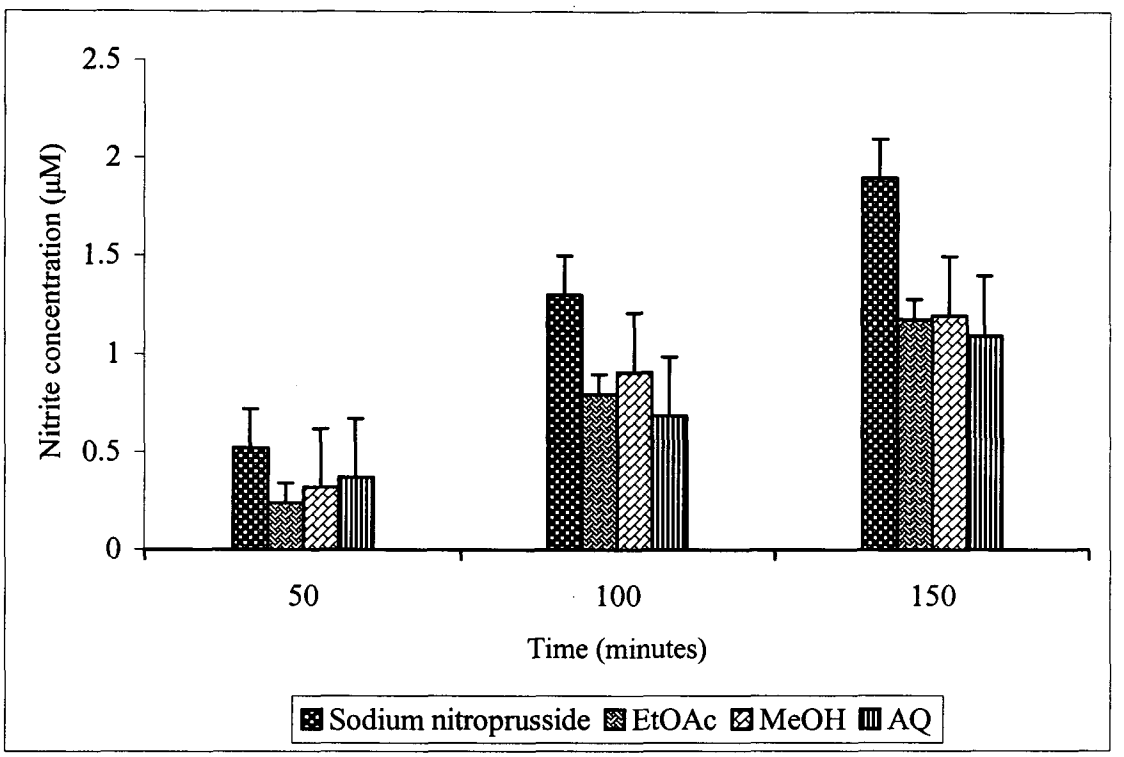


Figure 4. 5- Production of nitrite from sodium nitroprusside (1 mM) in the presence and absence of IC 50 concentration of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* at various time interwell. Values are mean ± S.D, n=3

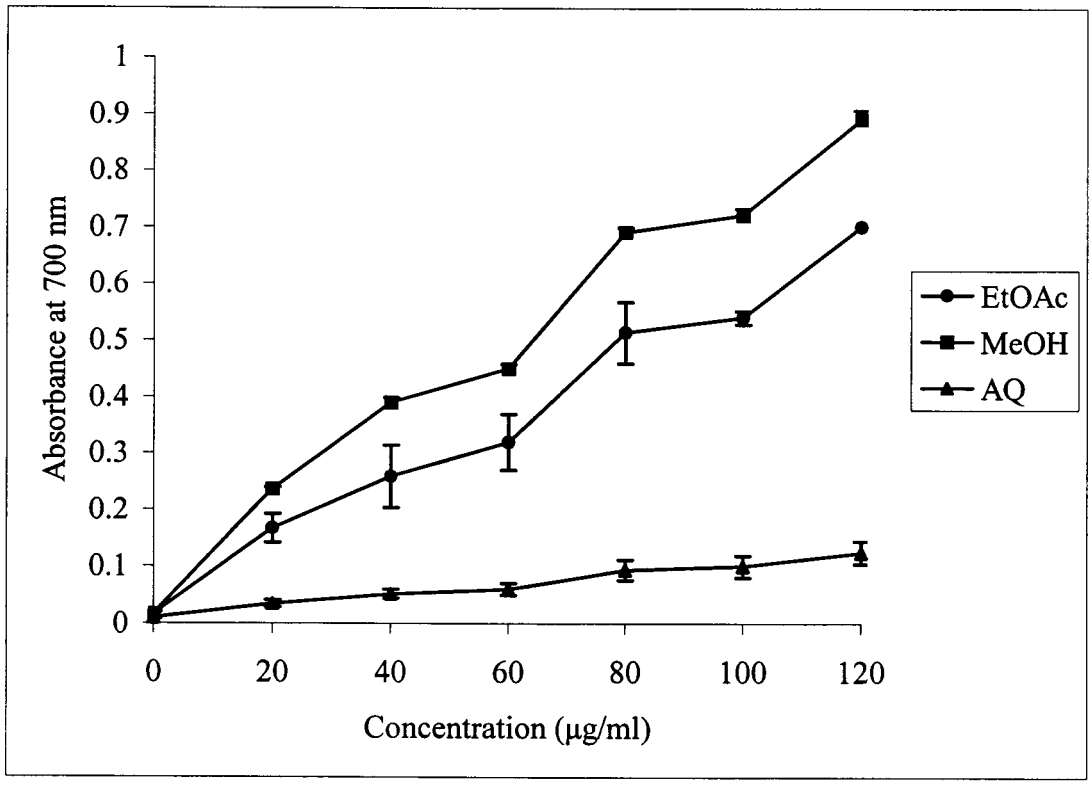


Figure 4. 6- Reducing activity of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus*. Values are mean ± S.D, n=3

Table 4. 2- Effect of ethyl acetate extract of *P. rimosus* (EtOAc) on alcohol induced conjugate diene level in the liver, kidney and brain in mice

Groups	Treatments	Liver (mM/100 g)	Kidney (mM/100 g)	Brain (mM/100 g)
Normal	Vehicle	25.0 ± 2.1	51.0 ± 4.3	35.7 ± 3.4
Control	Alcohol	46.1 ± 3.3*	74.8 ± 5.6*	47.7 ± 2.8*
EtOAc	50 mg/kg	30.3 ± 1.3[a]	55.0 ± 4.0[a]	38.8 ± 3.0[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*- test) significantly different from control.

Table 4. 3- Effect of ethyl acetate extract of *P. rimosus* (EtOAc) on alcohol induced lipidperoxidation (MDA) level in the liver, kidney and brain of mice.

Groups	Treatments	Liver (nmol/mg protein)	Kidney (nmol/mg protein)	Brain (nmol/mg protein)
Normal	Vehicle	0.77 ± 0.11	1.36 ± 0.21	0.86 ± 0.11
Control	Alcohol	2.03 ± 0.15*	3.38 ± 0.64*	1.64 ± 0.20*
EtOAc	50 mg/kg	1.16 ± 0.15[a]	1.99 ± 0.26[a]	1.09 ± 0.14[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*- test) significantly different from control.

Table 4. 4-Effect of ethyl acetate extract of *P rimosus* (EtOAc) on croton oil induced lipid peroxidation on mouse skin.

Groups	Treatments (mg)	MDA (nmol/mg protein)
Normal	Vehicle	0.57 ± 0.04
Control	Croton oil	1.60 ± 0.18*
EtOAc	10	1.04 ± 0.12[a]
	20	0.78 ± 0.08[a]
Diclofenac	10	0.92 ± 0.20[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*- test) significantly different from control.

4.4 Discussion

The experimental results indicate that the *in vitro* free radical scavenging activity of *P. rimosus* extract is in a concentration dependent manner. The exact mechanism of radical scavenging activity of extract of *P. rimosus* is unknown. The superoxide scavenging activity of the extracts may be due to the direct scavenging of superoxide anion generated from the photoillumination of riboflavin. The effect may also mediate by trapping the electron released from EDTA for the generation of superoxide anion or by reducing superoxide anion to a non- radical. The removal of oxygen from the reaction mixture can also be not ignored. The hydroxyl radical scavenging and lipid peroxidation inhibiting activity might also be mediated through the ability of the extracts in scavenging the generated radicals or through the reductive efficiency of the extracts. The increased absorbance in the reducing activity experiment is due to the increased complex, ferricferrocyanide, formation between potassium ferrocyanide and $FeCl_3$. The reducing power of extracts is due to their hydrogen-donating ability.

In addition to reactive oxygen species (ROS), nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Sreejayan and Rao, 1997). The expression or over stimulation of inducible enzyme, nitric oxide synthase (i NOS), leads to the production of large quantities of $NO^{\bullet}$ which, is implicated in the cytotoxic effects observed in various disorders like AIDS, cancer, Alzheimer's, arthritis etc (Shinde et al., 2000). Nitric oxide or reactive nitrogen species, formed during its reaction with oxygen, such as nitrate and nitrite are also very reactive. These compounds alter the structure and function of many cellular components. The extracts of *P. rimosus* significantly scavenge $NO^{\bullet}$ released from sodium nitroprusside. Incubating the extracts at their IC_{50} concentration with sodium nitroprusside shows a time dependent inhibition of $NO^{\bullet}$. The exact mechanism of inhibition of the nitrite formed during the reaction of $NO^{\bullet}$ with O_2 is unknown. The inhibition may be a result of direct scavenging of nitric oxide by *P. rimosus* extracts or may be a consequence of the reaction of the extracts with other oxides of nitrogen i.e. NO_2 and $OONO^-$, which are possible intermediates in the oxidation of $NO^{\bullet}$ to nitrite. The extracts of *P. rimosus* did not change the absorbance of sodium nitrite upon treatment with Greiss reagent. This indicates that the extracts do not interfere with nitrite detection assay. Among the extracts that scavenge $NO^{\bullet}$, aqueous extract showed maximum activity.

The *in vitro* radical scavenging activity, mainly the superoxide scavenging and lipid peroxidation inhibiting activities, of ethyl acetate extract was higher than the methanol and aqueous extracts. Hence this extract was employed for the antiperoxidative activity experiment in animals. Active oxygen species and free radicals are involved in the peroxidation of membrane lipid. Application of TPA to skin results in rapid accumulation of inflammatory cells such as neutrophils and macrophages (Lewis and Adams, 1987). Appropriately stimulated polymorphonuclear neutrophils (PNM) and monocytes produce large amount of reactive oxygen intermediate (ROI) (up to 1.5 nmol of H_2O_2/ 10^4 cells/ l) in a respiratory burst pattern (Szatrowski and Nathan, 1991). The double application of croton oil to mouse skin has been shown to stimulate the release of reactive oxygen species (ROS)(Ji and Marnett, 1992). The free radicals thus generated might be responsible for the peroxidation of lipid in the skin epidermis. Application of ethyl acetate extract of *P. rimosus* prior to the croton oil treatment reduced the lipid peroxidation.

Acute and chronic ingestion of ethanol causes a variety of changes in men. In acute alcohol intoxication, liver microsomal metabolism of ethanol is accompanied by hydroxyl radical generation from the cytochrome P-450 system. Much of the ethanol toxicity in liver might be due to acetaldehyde. Aldehyde oxidase in liver is known to produce superoxide. Further, CYP 2E1 and xanthine oxidase can also oxidize acetaldehyde, producing superoxide and hydrogen peroxide (Halliwell et al., 2000). The generated hydroxyl radical can initiate lipid peroxidation as a self-perpetuating chain reaction. Increased lipid peroxidation can also be due to depletion of hepatocyte lipid peroxidation defenses. Alcohol is absorbed unaltered from stomach and small intestine (Bergenns and Goldberg, 1940). It passes readily through the various body membranes and finally gets distributed throughout the body water. Though the brain contains very low level of alcohol dehydrogenase, CYP 2E1 of various regions of the brain may be an important route of alcohol metabolism. Ethyl acetate extract of *P. rimosus* significantly inhibited the ethanol induced conjugate diene and malondialdehyde formation in liver, kidney and brain. This conclusion is supported by the *in vitro* lipid peroxidation inhibiting activity of ethyl acetate extract against Fe^{2+} induced lipid peroxidation in the rat liver homogenate and the inhibition of croton oil induced lipid peroxidation on mice skin. The effect is mediated through the direct scavenging of the initiator radicals of lipid peroxidation such as hydroxyl radical,

peroxyl radical, alkoxyl radical or alkyl radical generated from administration of ethanol. The significant peroxidation inhibiting activity is also related to the hydrogen (electron) donating capability of the extract that can stabilize the peroxyl radical to stop the propagation steps of the peroxidation pathway. The inhibition of radical initiating enzymes, alcohol induced microsomal CYP-450 and phorbol ester induced NOS in skin, by the extract might also be contributed to the exhibited antiperoxidation activity.

The possible interference of DMSO used as solubilizer of the extract for *in vitro* antioxidant assays was also evaluated. The results suggest that DMSO does not act as an antioxidant at the given concentration, and there for could be employed to solubilize the extracts. Numerous plant constituents have been reported to show free radical scavenging or antioxidant activity. Flavonoids and other phenolic compounds (proanthocyanidins, rosmarinic acid, hydroxycinnamic derivatives, catechines, quercetin etc.) of plant origin have demonstrated as scavengers and inhibitors of lipid peroxidation (Shahidi et al., 1992). Phenols can directly scavenge ROS such as hydroxyl radical and nitroperoxyl radical (Halliwell and Gutteridge, 1999). The free radical scavenging activities of these phytochemicals are due to their phenolic hydroxyl moieties. The preliminary chemical examination of *P. rimosus* extracts shows the presence of flavonoids and polyphenols. These compounds might be responsible for *in vitro* antioxidant and antiperoxidative activities in animals.

Anti-inflammatory activity of P. rimosus

Ajith. T.A “Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrohungus, Phellinus rimosus (Berk) Pilat. ” Thesis. Amala Cancer Research Centre , University of Calicut, 2004

Chapter 5

Anti-inflammatory activity of *Phellinus rimosus*

5.1 Introduction

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.

Epidemiological studies have shown that chronic intake of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the incidence of colon, prostate, lung and breast cancer (Schreinemachers et al., 1996 and Harris et al., 1996). However, NSAIDs such as aspirin, indomethacin, ibuprofen etc., which are commonly used in the treatment of rheumatoid arthritis and many other acute and chronic inflammatory conditions, cause gastric mucosal damage and nephrotoxicity (Ivey, 1988 and Murray and Brater, 1993). For chronic diseases such as osteoarthritis and rheumatoid arthritis, life long dependency on drugs is necessary. There fore, the search for an ideal anti-inflammatory drug, which is safe and effective, is still continuing.

In searching for new anti-inflammatory agents over the past several years, hundreds of plant extracts have been evaluated for their potential to inhibit chronic inflammatory conditions. Mushrooms are widely distributed in the world and some of them have been used in traditional Chinese medicine as anti-inflammatory and analgesic agents (Koyama et al., 1997). The anti-inflammatory activity of ethyl acetate,

methanol and aqueous extracts of *P. rimosus* was evaluated and the results are presented in this chapter.

5.2 Materials and Methods

5.2.1 Preparations of extracts

Extracts of *P. rimosus* were prepared as described in the section 3.2.1.

5.2.2 Animals

Male and female Swiss albino mice weighing 30 ± 2 g were used for the study.

5.2.3 Determination of anti-inflammatory activity

Anti-inflammatory activity of extracts of *P. rimosus* was determined by carrageenan and dextran induced acute and formalin induced chronic mouse paw edema.

5.2.3.1 Carrageenan induced paw edema

Male Swiss albino mice were divided into eight groups of six animals each. The treatment schedule is described as follows.

Group I	Vehicle
Group II	Ethyl acetate extract 50 mg/kg body wt. (p.o)
Group III	Ethyl acetate extract 100 mg/kg body wt. (p.o)
Group IV	Methanol extract 50 mg/kg body wt. (p.o)
Group V	Methanol extract 100 mg/kg body wt. (p.o)
Group VI	Aqueous extract 50 mg/kg body wt. (p.o)
Group VII	Aqueous extract 100 mg/kg body wt. (p.o)
Group VIII	Diclofenac 25 mg/kg body wt. (p.o)

In all groups the inflammation was induced by single sub-plantar injection of 0.02 ml of freshly prepared 1% carrageenan in normal saline (Sheena et al., 2002). Group treated with vehicle was kept as control. The groups received extracts of *P. rimosus* at concentration of 50 and 100 mg/kg body wt. orally 1 h before the carrageenan injection. The paw thickness was measured using vernier calipers before and 3 h after carrageenan injection. Increase in paw thickness as a measure of inflammatory edema was calculated using the formula Pt-Po, where, Po initial paw thickness at time to and Pt is the thickness at time t (3 h). Percent inhibition of inflammation was calculated by the formula (1-PT/PC) X100, where PT is the increase in paw thickness of the treated and PC is that of control. Diclofenac was used as the reference drug.

5.2.3.2 Dextran induced paw edema

Experimental procedure was same as described above except that single dose of 0.02 ml of dextran (1%) was used as the inducer of inflammation (Sheena et al., 2002).

5.2.3.3 Formalin induced paw edema

Experimental procedure was same as described before except that single dose of 0.02 ml of formalin (2%) was used as the inflammatory inducer (Sheena et al., 2002). The extract was administered once daily for 6 consecutive days.

5.2.3.4 Effect of anti-inflammatory activity against croton oil induced skin inflammation

Croton oil was isolated from the croton seed *Croton tiglium* as described in section 4.2.4. Back of each female Swiss albino mouse was shaved using surgical clippers 2 days before the experiment. Animals with complete hair growth arrest were grouped into 5 groups of six animals each and treated as follows;

Group I	Normal
Group II	Acetone (vehicle control)
Group III	Croton oil (0.1 ml of 50% croton oil in acetone, v/v)
Group IV	Ethyl acetate extract 10 mg + croton oil
Group V	Ethyl acetate extract 20 mg + croton oil
Group VI	Diclofenac 10 mg + croton oil

Group treated with out any treatments was kept as normal. Ethyl acetate extract of *P. rimosus* (10 and 20 mg in 0.1 ml of acetone) was applied topically to the shaved area of the dorsal skin 30 min before each application of croton oil (0.1 ml of 50% croton oil in acetone, v/v) (Lakshmi et al., 2003). After 24 h, the extract and croton oil treatment was repeated on the same area. Group treated with 0.1 ml of croton oil in 0.1 ml acetone was kept as control. Diclofenac was used as the standard reference drug. A group applied with 0.1 ml of acetone was included to determine the effect of vehicle. One hour after the second treatment of croton oil animals were sacrificed and the skin was removed. The skin punches were obtained with a 8 mm diameter cork borer. The skin punches were weighed in an analytical balance; the percent inhibition was calculated using the formula,

% Inhibition = [1- (punch wt. of treated-punch wt. of normal)/(punch wt. of control-punch wt. of normal)] x 100.

5.2.3.4.1 Histopathological examination

Excised skin was fixed in 10 % formalin and then embedded in paraffin. Microtome sections (3 μm) were prepared from each skin and stained with hematoxilin-eosin. The sections were assessed for inflammation under a microscope.

5.3 Results

5.3.1 Anti-inflammatory activity against carrageenan induced paw edema

Anti-inflammatory activity of extracts of *P. rimosus* is given in table 5.1. The paw thickness was increased maximum 3 h after the carrageenan injection. All the three extracts were effective in inhibiting the carrageenan induced edema. Treatment of extracts of *P. rimosus* before the carrageenan injection significantly ($P<0.01$) reduced the paw edema. The increase in paw thickness in the ethyl acetate, methanol and aqueous extract treated groups (100 mg/kg body wt) after 3 h of carrageenan injection were 0.071 ± 0.008, 0.117 ± 0.007 and 0.092 ± 0.004 cm respectively. The increase in paw thickness in the control group was 0.166 ± 0.015 cm. Ethyl acetate, methanol and aqueous extracts at 100 mg/kg body wt concentration inhibited edema formation by 57.2, 29.5 and 44.5 % respectively. The standard reference drug, diclofenac, inhibited the edema 53.6 %.

5.3.2 Anti-inflammatory activity against dextran induced paw edema

Anti-inflammatory activity of extracts of *P. rimosus* against dextran induced paw edema was given in the table 5.2. The increase in paw thickness after 3 h of dextran injection was maximum in the control group of animals (0.143 ± 0.007cm). Treatment of extracts of *P. rimosus* before the dextran injection significantly ($P<0.01$) reduced the paw edema. The increase in paw thickness after 3 h of dextran injection in the ethyl acetate, methanol and aqueous extracts (100 mg/kg body wt) treated group were 0.071 ± 0.005, 0.083 ± 0.015 and 0.078 ± 0.010 cm respectively. The percent inhibition of the paw edema in the ethyl acetate, methanol and aqueous extract treated (100 mg/kg body wt) groups were 50.3, 41.9 and 45.4 % respectively. The standard reference drug, diclofenac, showed 0.072 ± 0.010 cm increase in paw thickness and 49.6 % inhibition after the dextran injection

5.3.3 Anti-inflammatory activity against formalin induced paw edema

Anti-inflammatory activity of extracts of *P. rimosus* against formalin induced paw edema was given in the table 5.3. Formalin induced a significant increase in paw thickness in the control group of animals on 6th day after the injection. Treatment of extracts of *P. rimosus* before the formalin injection significantly ($P<0.01$) reduced the

paw edema. The increase in paw thickness 6 days after formalin injection in the ethyl acetate, methanol and aqueous extract treated (100 mg/kg body wt) group of animals were 0.103 ± 0.012, 0.154 ± 0.018 and 0.126 ± 0.010 cm respectively. The percent inhibition of edema was 56.7, 35.3 and 47.0% in the ethyl acetate, methanol and aqueous extract treated group of animals respectively. The standard reference drug showed 0.141± 0.010 cm increase of paw thickness and 40.7% of inhibition of paw edema.

5.3.4 Anti-inflammatory activity against croton oil applied skin inflammation

Effect of anti-inflammatory activity against croton oil applied skin inflammation was given in table 5.4. Applications of ethyl acetate extract of *P. rimosus* 30 min before the croton oil application significantly inhibited ($P<0.01$) the croton oil induced skin inflammatory edema in a dose dependent manner. The double treatment of croton oil alone induced skin inflammation as evident from the skin punch weight of 70.8 ± 2.4 mg/punch in the control group of animals. Application of extract of *P. rimosus*, 20 mg, prior to the croton oil application inhibited 79.8% the inflammatory edema compared to the control group of animals. The skin punch weight of diclofenac (reference drug) plus croton oil treated group of animals was 44.8 ± 1.4 mg/punch and the percent of inhibition was 84.6 mg/punch. Treatment of acetone on mouse skin did not produce any change in the punch weight compared to the normal punch wt. (39.5 ± 3.1 mg/punch).

5.3.5 Histopathological evaluation

Histopathological examination of the skin of croton oil alone applied group of animals showed epidermal thickening, severe lymphocytes infiltration and marked edema in the sub-epidermis. The changes were markedly reduced in the extract or reference drug (diclofenac) plus croton oil applied group (Fig. 5.1).

Table 5.1. Effect of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* on carrageenan induced paw edema in mice.

Groups/ treatment (mg/kg)	Initial paw thickness (cm)	Paw thickness after 3 h (cm)	Increase in paw thickness (cm)	Percent inhibition
Control	0.157 ± 0.004	0.323 ± 0.013	0.166 ± 0.015	---
EtOAc				
50	0.166 ± 0.003	0.268 ± 0.011	0.106 ± 0.015[a]	36.1
100	0.162 ± 0.008	0.233 ± 0.008	0.071 ± 0.008[a]	57.2
MeOH				
50	0.172 ± 0.010	0.320 ± 0.018	0.149 ± 0.022[b]	10.2
100	0.158 ± 0.003	0.275 ± 0.009	0.117 ± 0.007[a]	29.5
AQ				
50	0.161 ± 0.002	0.270 ± 0.007	0.109 ± 0.006[a]	34.3
100	0.166 ± 0.003	0.258 ± 0.005	0.092 ± 0.004[a]	44.5
Diclofenac				
25	0.166 ± 0.004	0.243 ± 0.004	0.077 ± 0.005[a]	53.6

Values are mean ± SD, n= 6 animals

[a]$P<0.01$ and [b]$P<0.05$ (Dunnett's *t*- test) significantly different from control group.

Table 5.2. Effect of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* on dextran induced paw edema in mice.

Groups/ treatment (mg/kg)	Initial paw thickness (cm)	Paw thickness after 3 h (cm)	Increase in paw thickness (cm)	Percent inhibition
Control	0.171 ± 0.003	0.314 ± 0.009	0.143 ± 0.007	---
EtOAc				
50	0.164 ± 0.007	0.255 ± 0.008	0.091 ± 0.001[a]	36.3
100	0.164 ± 0.005	0.234 ± 0.005	0.071 ± 0.005[a]	50.3
MeOH				
50	0.161 ± 0.004	0.267 ± 0.006	0.105 ± 0.009[a]	26.5
100	0.166 ± 0.007	0.247 ± 0.010	0.083 ± 0.015[a]	41.9
AQ				
50	0.167 ± 0.007	0.273 ± 0.005	0.106 ± 0.010[a]	25.8
100	0.158 ± 0.006	0.237 ± 0.008	0.078 ± 0.010[a]	45.4
Diclofenac				
25	0.168 ± 0.005	0.240 ± 0.008	0.072 ± 0.010[a]	49.6

Values are mean ± SD, n= 6 animals

[a] $P < 0.01$(Dunnett's *t*- test) significantly different from control group

Table 5.3. Effect of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* on formalin induced paw edema in mice.

Groups/ treatment (mg/kg)	Initial paw thickness (cm)	Paw thickness after 6 day (cm)	Increase in paw thickness (cm)	Percent inhibition
Control	0.168 ± 0.006	0.406 ± 0.014	0.238 ± 0.012	---
EtOAc				
50	0.163 ± 0.006	0.330 ± 0.010	0.157 ± 0.023[a]	34.0
100	0.160 ± 0.007	0.263 ± 0.008	0.103 ± 0.012[a]	56.7
MeOH				
50	0.156 ± 0.007	0.343 ± 0.012	0.186 ± 0.010[a]	21.8
100	0.160 ± 0.007	0.314 ± 0.015	0.154 ± 0.018[a]	35.3
AQ				
50	0.155 ± 0.005	0.328 ± 0.012	0.173 ± 0.014[a]	27.3
100	0.165 ± 0.007	0.291 ± 0.006	0.126 ± 0.010[a]	47.0
Diclofenac				
25	0.164 ± 0.008	0.305 ± 0.009	0.141 ± 0.010[a]	40.7

Values are mean ± SD, n= 6 animals

[a]$P<0.01$(Dunnett's *t*- test) significantly different from control group

Table 5.4. Effect of ethyl acetate (EtOAc) extract of *P. rimosus* on croton oil applied skin inflammation in mice.

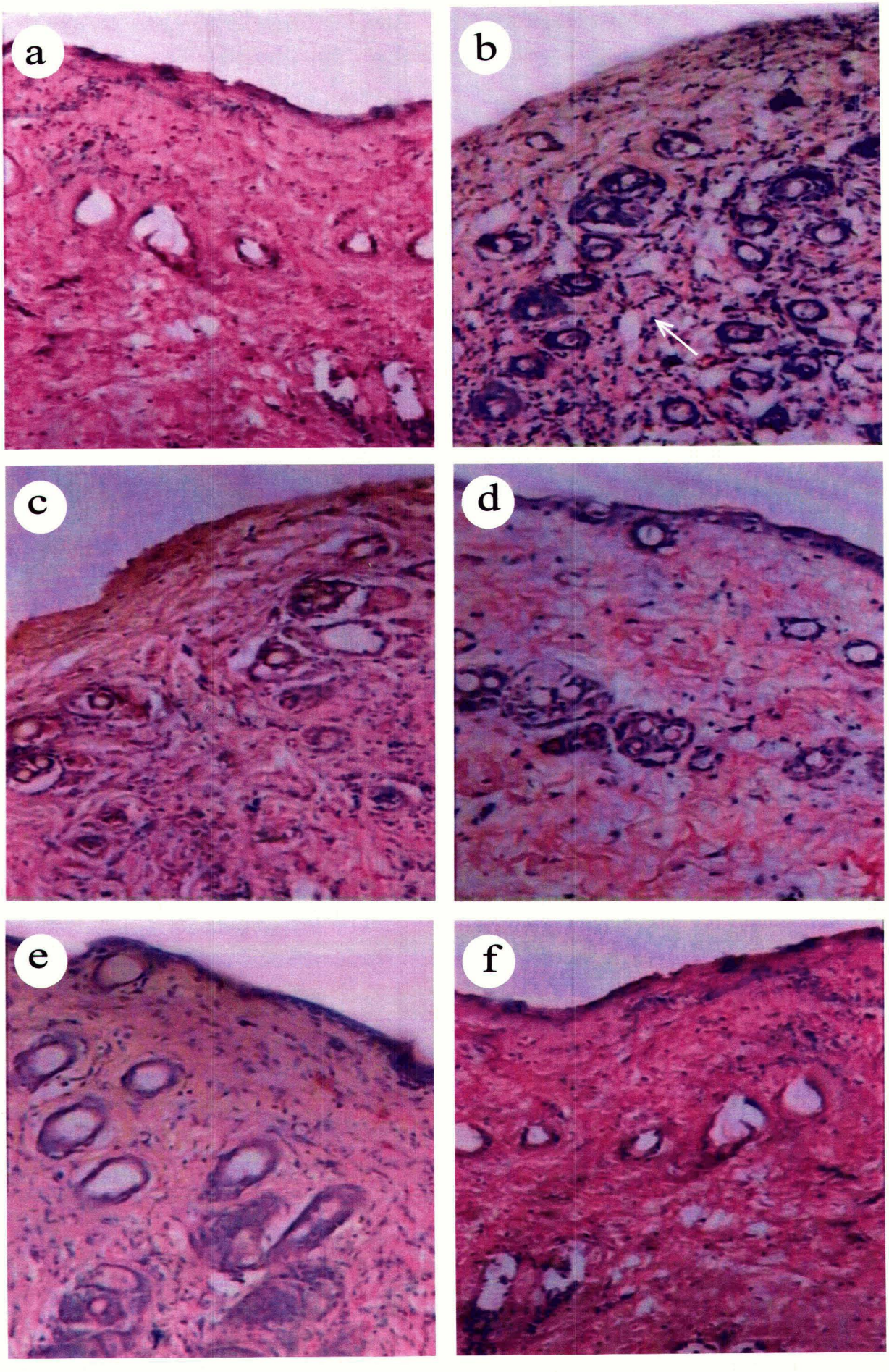
Groups	Treatments (mg)	Skin punch wt. (mg/punch)	% inhibition
Normal	--	40.1 ± 2.3	--
Vehicle control	Acetone	39.5 ± 3.1^{NS}	--
Control	Croton oil	70.8 ± 2.4*	--
EtOAc	10	54.6 ± 1.7^{a}	52.7
,,	20	46.3 ± 1.5^{a}	79.8
Diclofenac	10	44.8 ± 1.4^{a}	84.6

Values are mean ± S.D, n=6

* $P<0.01$ (lsd) significantly and NS non-significantly different from normal group.

$^{a}P<0.01$ (Dunnett's *t*- test) significantly different from control group.

Figure 5.1- Anti-inflammatory activity of ethyl acetate extract (EtOAc) of *P. rimosus* against croton oil induced inflammation on mice skin. Skin sections stained with H&E. a) Normal; b) croton oil; c) EtOAc (10 mg) + croton oil; d) EtOAc (20 mg) + croton oil; e) diclofenac (10 mg) + croton oil and f) acetone. Magnification x 20.
(Arrow mark indicated lymphocytes infiltration)


a
b
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d
e
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5.4 Discussion

Results of the study reveal that all the three extracts of *P. rimosus* inhibited the inflammation induced by carrageenan, dextran and formalin in a dose dependent manner. More over, the ethyl acetate extract inhibited the croton oil induced mouse skin inflammation.

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 *P. rimosus* 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 *P. rimosus* 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 *P. rimosus* extracts in the treatment of inflammation associated with arthritis. However, the inhibitory effect of ethyl acetate extract of *P. rimosus* is higher than the other extracts.

There is a reciprocal relationship between antioxidants and inflammation. Since the anti-inflammatory activity in the mice paw edema models showed highest activity

for ethyl acetate extract, it was selected for the croton oil induced skin inflammation model in mice. Ethyl acetate extract of *P. rimosus* inhibited croton oil induced inflammation in a dose dependent manner. The double applications of phorbol ester trigger ROS production in mouse skin, suggested that each application induces two distinguishable biochemical events namely priming and activation (Murakami et al., 2000). Priming is characterised as recruitment of inflammatory cells such as neutrophils by chemotactic factors to inflammatory regions resulting in edema formation and the latter is the induction of oxidative stress produced by the activation of neutrophils or other oxidant producing cells, including keratinocytes. The exact mechanism of the anti-inflammatory activity of the ethyl acetate extract of *P. rimosus* is not known. Application of ethyl acetate extract before the croton oil treatment was found to inhibit the activation event and the consequent lipid peroxidation on mice skin (chapter 4). Topical application of TPA on mice led to edema formation also by enhancing COX-2 protein expression. This conclusion indicates that arachidonic acid metabolites or eicasanoids released from the COX-2 activity is capable of eliciting inflammation. Application of ethyl acetate extract may inhibit the croton oil induced recruitment of inflammatory mediators mainly the arachidonic acid metabolites. The effect may also be mediated by inhibiting the activity of membrane phospholipase A2 that is important for the hydrolysis and release of arachidonic acid from the membrane phospholipids.

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 extracts of *P. rimosus* showed the significant superoxide radical scavenging activity. Hence, the extracts can directly scavenge the superoxide anion that might inhibit the recruitment of PMNs and inflammation. The scavenging activity of ethyl acetate extract of *P. rimosus* was higher than the methanol and aqueous extracts, explains the higher anti-inflammatory activity of ethyl acetate extract. 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 *P. rimosus,* is a dual inhibitor of both O_2^- and $NO^\bullet$ radical, reduce the inflammation.

Several investigators have described inhibition of histamine from mast cells (Amella 1985) and lipooxygenase activity by certain flavonoids (Yoshimoto et al., 1983). Polyphenols and flavonoids have been found to possess anti-inflammatory activity (Di Carlo et al., 1979). The experimental findings suggest that the presence of polyphenols and flavonoids in the ethyl acetate and methanol extracts of *P. rimosus* might be responsible for their anti-inflammatory activity. The anti-inflammatory activity of aqueous extract may be mediated by the polysaccharide present in the extract.

Hepato-renal protection of P. rimosus

Ajith. T.A “Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrohungus, Phellinus rimosus (Berk) Pilat. ” Thesis. Amala Cancer Research Centre , University of Calicut, 2004

Chapter 6

Hepato-renal protection of *Phellinus rimosus*

6.1 Introduction

Exposure to drugs and chemicals often induce toxicity to living organisms. Factors determine the toxicity include the pharmacokinetics of the compound, the metabolic fate of the compound and the target organs ability to respond to the toxic insult. Many organs are capable of metabolizing chemicals to toxic reactive intermediates. During the last decade, considerable attention has been focused on the involvement of oxygen free radical (OFR) in various diseases. Active oxygen molecules such as superoxide and hydroxyl radicals have been demonstrated to play important role in the inflammation process produced by ethanol, carbon tetrachloride or carrageenan (Yoshikawa et al., 1983; Halliwell and Parihar, 1984 and Yudha et al., 1991). Despite the presence of strong antioxidant defense mechanism to counteract the OFR and to minimize the plausible oxidative damage, OFR dependent damage to DNA and other biomolecules accumulate during the lifetime of organisms. Liver and kidney are the two major organs exposed to these toxic reactive intermediates and free radicals.

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. 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). The protection from hepatic diseases in experimental animals has been achieved by administering the chemopreventive agents that modulate the metabolic processing of xenobiotics include phenolic antioxidants, indoles, isothiocynates, coumarins, flavonones, allylsulfides etc. (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 kidneys are, dynamic organs and represent the major control system maintaining body homeostasis, affected by many chemicals and drugs. A number of cancer chemotherapeutic agents either natural products or synthetics has been developed during the recent years. Most of the synthetic compounds are found to be harmful to the host systems. *Cis*-Diamminedichloroplatinum (II) (Cisplatin) is extensively used clinically for the management of oncological disorders particularly for the ovary, testis, bladder, head and neck (Hamers et al., 1991). Although higher doses of cisplatin are more efficacious for the suppression of cancer, high dose therapy manifests irreversible renal dysfunction, ototoxicity and other toxicities (Bodenner et al., 1986; Gandara et al., 1991; Hamers et al., 1993; Wolfgang et al., 1994 and Rybak et al., 1995). A number of therapeutic agents have been evaluated experimentally and clinically against cisplatin induced nephrotoxicity. The protective agents that ameliorated the nephrotoxicity must neutralize the cytotoxic effects of cisplatin without interfering the antitumor activity.

Several phytochemicals pharmacological attributes have been reported to scavenge free radicals. Administration of such antioxidants decreases CCl_4 toxicity to liver and cisplatin-induced toxicity to kidney in animals. In the last 15 to 20 years, medicinal mushrooms have been subject to intensive investigation in several laboratories for their therapeutic use. They are found to be beneficial for a wide variety of hepatic disorders, including hepatitis. Hepatoprotective effect of extracts of the mushrooms, *Grifola frondosa, Dendropolyporus umbellatus*, *Shizophyllam commune*, *Ganoderma lucidum* and *Tremella fuciformis* (Lin and Wu, 1988 and Wasser and Weis, 1997a) have been documented. However, little or no information is available on their nephroprotective activity. Among the extracts of *P. rimosus* tested for their antioxidant activity, ethyl acetate extract was found to possess significantly high antioxidant activity. Hence, the ethyl acetate extract was selected for studying the hepato-renal protective activity after exposed to hepatotoxic and nephrotoxic agents. The findings are reported in this chapter.

6.2 Materials and Methods

6.2.1 Animals

Male Swiss albino mice of 6-8 weeks of age weighing 25 ± 2 g were selected from our mouse colony were employed for nephrotoxicity studies. Male Wistar and Sprague Dawley rats of 10 weeks old (180 ± 20 g) were used for the acute and chronic hepatotoxicity study.

6.2.2 Preparation of the ethyl acetate extract

Ethyl acetate extract of *P. rimosus* was prepared as described in the section 3.2.1.

6.2.3 Determination of hepatotprotective activity

Hepatoprotetctive activity was determined using CCl_4 induced acute and chronic hepatotoxicity in rat models.

6.2.3.1 Determination of hepatotprotective activity of the extract against CCl_4 induced acute toxicity

Hepatoprotective activity was determined by the method of Lin et al., (1992), with some modifications. Animals were divided into six groups of six animals each and treated as follows

Group I	Vehicle
Group II	CCl_4 in paraffin oil (1:1 v/v, 1.5 ml/kg body wt, i.p)
Group III	Ethyl acetate extract of *P. rimosus* 10 mg/kg body wt + CCl_4
Group IV	Ethyl acetate extract of *P. rimosus* 25 mg/kg body wt + CCl_4
Group V	Ethyl acetate extract of *P. rimosus* 50 mg/kg body wt + CCl_4
Group VI	Quercetin 25 mg/kg body wt. + CCl_4

Animals in the group II, III, IV, V and VI were treated with a single dose of CCl_4. Group III, IV and V were treated orally with ethyl acetate extract of *P. rimosus* 10, 25 and 50 mg/kg body wt. and group VI was treated orally with reference drug, quercetin, one hour before CCl_4 administration. The extract and reference drug were administered at 24, 48 and 72 h after the CCl_4 injection. The group treated with vehicle was kept as normal. The group II treated with CCl_4 alone was kept as control. Seventy-two hour after the CCl_4 injection, animals were sacrificed. Blood was collected by heart puncture of each animal. Serum was used for the determination of glutamate oxaloacetate transaminase (GOT) (section 3.2.12), glutamate pyruvate transaminase (GPT) (section 3.2.13) and alkaline phosphatase (ALP) (section 3.2.14).

6.2.3.2 *Determination of hepatoprotective activity of the extract against CCl_4 induced chronic toxicity*

Hepatoprotective activity against the CCl_4 induced chronic toxicity was determined by the method of Jose and Kuttan (2000), with some modifications. The animals were divided into four groups of six animals each and treated as follows

Group I	Vehicle
Group II	CCl_4 in paraffin oil (1:5 v/v, 1.5 ml/kg body wt, i.p)

Group III Ethyl acetate extract of *P. rimosus* 25 mg/kg body wt + CCl_4

Group IV Ethyl acetate extract of *P. rimosus* 50 mg/kg body wt + CCl_4

Animals in the group II, III and IV were treated with CCl_4 three times in a week for 5 weeks (total 15 doses). Group III and IV were treated orally with ethyl acetate extract of *P. rimosus* 25 and 50 mg/kg body wt. one hour before each CCl_4 administration. Group treated with vehicle was kept as normal. Group II treated with CCl_4 alone was kept as untreated control. Twenty-four after the last dose of CCl_4 injection, animals were sacrificed. Coagulated and non-coagulated (heparin) blood were collected by heart puncture of each animal. Serum was used for the determination of glutamate oxaloacetate transaminase (GOT) (section 3.2.12), glutamate pyruvate transaminase (GPT) (section 3.2.13), and alkaline phosphatase (ALP) (section 3.2.14). Serum lipid peroxidation (malondialdehyde) was estimated after precipitating the total protein as described in the section 3.2.21. Liver was removed for the determination of antioxidants, aniline hydroxylase (section 3.2.11), lipid peroxidation (malondialdehyde) (section 3.2.8) and also for the histopathological analysis.

6.2.3.2.1 Evaluation of antioxidants enzymes and GSH in liver

Liver was removed and washed thoroughly in ice-cold saline and homogenate (10 %) was prepared in PBS (50 mM, pH 7) (section 3.2.2). A part of the homogenate was used for the estimation of reduced glutathione (GSH) (section 3.2.3). 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 2.2.4), catalase (CAT) (section 3.2.5), glutathione peroxidase (GPx) (section 3.2.6), and glutathione *S*-transferase (GST) (section 3.2.7). Protein was determined by the method of Lowry *et al.* (section 3.2.10)

6.2.3.2.2 Evaluation of antioxidants enzymes and GSH in blood

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

6.2.3.3 Histopathological examination

Portions of the liver from both the experiments were fixed in 10 % formalin and then embedded in paraffin. Microtome sections 5 μm thicknesses were prepared from each liver samples and stained with hematoxilin-eosin. The sections were examined for

the pathological findings of hepatotoxicity such as necrosis, fatty infiltration, fibrosis, lymphocyte infiltration etc.

6.2.4 Determination of nephroprotective activity

6.2.4.1 Determination of nephroprotective activity against cisplatin induced nephrotoxicity.

Nephroprotective activity was determined by the method of Somani et al., (2000) with some modifications. Animals were divided into 4 groups of 6 animals each and treated as follows.

Group 1	Vehicle
Group II	Cisplatin (16 mg/kg body wt., i.p,) as a single dose.
Group III	Ethyl acetate extract 25 mg/kg body wt. + cisplatin
Group IV	Ethyl acetate extract 50 mg/kg body wt. + cisplatin

Group treated with vehicle was kept as normal. The single dose of extract was treated orally 1h before cisplatin (16 mg/kg body wt., i.p,) injection. Mice in all groups were sacrificed 72 h after the cisplatin injection. The blood was collected directly from the heart; serum was separated for creatinine and urea analysis. The kidneys were dissected and stored at -70^0 until analyses were completed. The kidneys were homogenized in 50 mM phosphate buffer (pH 7.0) to give a 10% homogenate (w/v) (section 3.2.2).

Serum creatinine and urea were estimated as described in the section (3.2.16 and 3.2.17). Tissue homogenate was used for assay of GSH (section 3.2.3), SOD (section 3.2.4), CAT (section 3.2.5), GPx (section 3.2.6), MDA (section 3.2.8) and protein as described in the section 3.2.10.

6.2.4.2 Determination of antitumor efficacy of cisplatin in the presence of ethyl acetate extract.

Animals were injected with $1x10^6$ viable DLA cells in phosphate buffered saline were divided into 3 groups of 6 animals each. After 24 h of tumor implantation, animals were treated as follows.

Group 1	Vehicle (untreated control)
Group II	Cisplatin (3 mg/kg body weight, i.p)
Group III	Ethyl acetate extract (50 mg/kg body weight, p.o) administered 1h before cisplatin injection (3 mg/kg body weight, i.p)

The treatments continued daily for 10 consecutive days (Sheena et al., 2003). At the end of 5^{th} week, the animals were sacrificed, extirpated the tumor and weighed. The

percent inhibition was calculated using the formula (1-B/A) 100 where, A is average tumor weight of the control group and B that of the treated group.

6.2.4.3 Histopathological examination

A portion of the kidney was fixed in 10 % formalin and then embedded in paraffin. Microtome sections 5 μm thickness was prepared and stained with hematoxilin-eosin. The sections were examined for the pathological findings of nephrotoxicity such as necrosis, protein ultrafiltrate in the proximal convoluted tubules, lymphocyte infiltration etc.

6.3 Result

6.3.1 Hepatoprotective activity of the extract against acute CCl_4 toxicity

The activities of SGPT, SGOT and ALP of animals seventy-two hours after the acute intoxication of CCl_4/paraffin oil are summarized in table 6.1. The results indicated the activity of SGOT, SGPT and ALP were significantly enhanced ($P<0.01$) by CCl_4/ paraffin oil injection. The treatment of ethyl acetate extract of *P. rimosus* at 10, 25 and 50 mg/kg resulted in significant decrease ($P<0.01$) of SGOT and SGPT activity. However the effect was maximum at 50 mg/kg treatment. The activity of serum ALP in the extract treated groups was also significantly reduced ($P<0.01$) compared to the CCl_4- treated control group.

6.3.2 Hepatoprotective activity of ethyl acetate extract against chronic CCl_4 toxicity

Chronic exposure to CCl_4 significantly elevated ($P<0.01$) the serum GOT and GPT activities compared to the normal group of animals (Table 6.2). The SGPT and SGOT activities in the CCl_4 alone injected animal were 959.8 ± 25.6 and 231.1 ± 21.5 IU/l respectively. The activity was significantly lowered ($P<0.01$) in the groups treated by the ethyl acetate extract of *P. rimosus* in a dose dependent manner. The inhibition of activity of the serum transaminases (SGPT and SGOT) was 85 and 90 % respectively in the 50 mg/kg body wt extract treated group of animals compared to the control group of animals. Similarly the serum ALP activity was elevated significantly in the CCl_4 treated control group of animals. The inhibition of ALP activity by the extract (50 mg/kg) was 88 % with respect to the control group of animals. The serum MDA level was also elevated in the CCl_4 alone injected group of animals. The level was reduced significantly ($P<0.01$) in the extract plus CCl_4 treated groups of animals (Fig. 6.2).

6.3.2.1 Antioxidant status in liver

The activities of hepatic SOD, CAT and GPx, were decreased significantly ($P<0.01$) in the CCl_4 alone treated group of animals compared to the normal animals

(Table 6.3). The activity of SOD in the CCl_4 injected animals was 14.5 ± 2.7 U/mg protein. Treatment of extract (50 mg/kg body wt.) prior to the CCl_4 challenge enhanced the activity to 19.1 ± 0.8 U/mg protein ($P<0.01$). The activities of CAT and GPx in the CCl_4 alone treated animals were 46.3 ± 4.8 U/mg protein and 16.9 ± 0.8 U/mg protein respectively. The activity was restored to normal in the extract (50 mg/kg) treated groups.

The level of hepatic GSH was also elevated significantly ($P<0.01$) in the extract treated animals prior to the CCl_4 challenge compared to the control group (7.94 ± 0.065 nmol/mg protein) of animal (Table 6.4). The concentration at 50 mg/kg treated group was 11.38 ± 0.43 nmol/mg protein.

The activity of aniline hydroxylase was significantly elevated in the CCl_4 alone treated group (Fig. 6.3). Administration of extract prior to CCl_4 injection inhibited the activation of aniline hydroxylase enzyme. The activity of aniline hydroxylase in the extract (50 mg/kg) plus CCl_4 treated group of animals was 0.356 ± 0.020 and 0.204 ± 0.007 n mol of p-aminophenol formed/min/mg protein respectively.

The activity of hepatic GST was significantly ($P<0.01$) elevated in the extract treated animals, 1149.4 ± 44.0 and 1407 ± 89.1 μ mol of CDNB conjugate formed/min/mg protein respectively for 25 and 50 mg/kg body wt. treated group of animals. The GST activity in the CCl_4 alone treated animals was not found to be significantly different from the normal group of animals (Table 6.5).

The lipid peroxidation level was enhanced significantly ($P<0.01$) in the control group of animals compared to the normal group of animals. The level of hepatic MDA was 1.81 ± 0.26 nmol/mg protein in the liver of CCl_4 alone treated animals (Fig. 6.2). The extract (50 mg/kg body wt) treatment prior to CCl_4 challenge reduced the MDA level to 0.52 ± 0.03 nmol/mg protein.

6.3.2.2 Antioxidant status in blood

The activities of SOD, CAT and GPx in erythrocytes were lowered ($P<0.01$) in the CCl_4 injected group compared to the normal group of animals (Table 6.6). The activities of all the antioxidant enzymes were significantly ($P<0.01$) restored to normal level in animals administered with extract (50 mg/kg) prior to CCl_4 treatment. The SOD activity in the CCl_4 plus extract (50 mg/kg body wt) treated group was 1481.6 ± 63.2 U/g Hb. Similarly the activity of catalase in the CCl_4 alone injected animals was 66.8 ± 5.7 k/g Hb and in the extract (50 mg/kg body wt) plus CCl_4 treated animals was 95.6 ± 3.6 k/g Hb. However, the activity of erythrocyte GST in the CCl_4 alone treated group

was not significantly different from the normal group of animals (Table 6.5). The treatment of extract (50 mg/kg) prior to the CCl_4 challenge significantly ($P<0.01$) enhanced the erythrocyte glutathione antioxidant system such as GPx, GST and GSH. The level of erythrocyte GSH was significantly lowered ($P<0.05$) in the CCl_4 alone treated group (Table 6.4). The activity of GPx, GST and level of GSH in the extract (50 mg/kg body wt) treated animals was 5102.4 ± 803.1 U/g Hb, 20.1 ± 1.5 mmol of CDNB conjugate formed/min/g Hb and 3.87 ± 0.09 μ mol/ml respectively.

6.3.3 Histopathological examinations

Histopathological examination of the liver challenged with acute doses of CCl_4 showed centrilobular necrosis, inflammatory infiltration of lymphocytes and fatty changes. The liver sections of rats treated with the extract (25 and 50 mg/kg body wt) plus CCl_4 showed well-preserved architecture (Fig. 6.1), where as liver challenged with chronic doses of CCl_4 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 (25 and 50 mg/kg body wt.) treated animals (Fig. 6.4).

6.3.4 Nephroprotective activityof the extract

Serum creatinine and urea levels were significantly elevated ($P<0.01$) in cisplatin treated group compared to the normal group (Table 6.7). The elevation of serum creatinine and urea were 9 and 5 fold respectively. Treatments of ethyl acetate extract of *P. rimosus* significantly lowered the elevated levels of serum creatinine and urea ($P<0.01$). The levels of serum creatinine and urea were close to that of the normal in the 50 mg/kg body wt. extract treated group.

Renal SOD activity was decreased significantly ($P<0.01$) in the cisplatin treated group compared to the normal group. The extract administration restored the level of SOD in a dose dependent manner ($P<0.01$) (Table 6.8).

CAT activity in kidney was found to be significantly ($P<0.01$) decreased in the cisplatin alone treated group, and extract treatment enhanced the activity ($P<0.01$) (Table 6.8).

GPx activity in the kidney was decreased significantly ($P<0.01$) after the administration of cisplatin. The activity of this enzyme was restored to that of normal when the extract was administered prior to cisplatin injection ($P<0.01$) (Table 6.8).

The renal GSH concentration was significantly decreased ($P<0.01$) in cisplatin treated animals as compared to the normal animals. Administration of ethyl acetate extract was found to increase the renal GSH concentration (Fig.6.5).

The concentration of malondialdehyde was found to be significantly increased ($P<0.01$) in the cisplatin treated group as compared to the normal animals. Administration of the extract along with cisplatin decreased the MDA to the normal level ($P<0.01$) (Fig.6.6).

6.3.4.1 Antitumor efficacy of cisplatin in the presence of ethyl acetate extract

The cisplatin alone and extract along with with cisplatin treated groups of animals showed a significant reduction of solid tumor development compared to the control group. The reduction of tumor growth in the extract plus cisplatin treated group was more or less same to that of the cisplatin alone treated group indicating administration of ethyl acetate extract did not interfere the antitumor activity of cisplatin (Table 6.9).

6.3.4.2 Histopathological observations

Sections of kidney of cisplatin alone treated animals showed increased accumulation of plasma ultrafiltrate in the proximal convoluted tubule and renal cell necrosis. Treatment of *P. rimosus* extract before the cisplatin injection decreased these toxic manifestations to a moderate level (Fig. 6.7).

Table 6.1. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on serum GOT, GPT and ALP activities in rats with acute CCl_4 administration

Groups	Treatment (mg/kg)	SGPT (IU/l)	SGOT (IU/l)	ALP (IU/l)
Normal Control	Vehicle	120.0 ± 3.3	59.10 ± 5.4	123.8 ± 3.1
(CCl_4/Paraffin oil, 1:1)	--	449.8 ± 20.0*	276.3 ± 5.8*	324.3 ± 2.8*
EtOAc + CCl_4	10	306.0 ± 11.4[a]	240.8 ± 7.9[a]	280.8 ± 5.5[a]
,,	25	230.3 ± 7.1[a]	209.5 ± 3.2[a]	258.0 ± 4.0[a]
,,	50	177.5 ± 4.4[a]	147.1 ± 6.9[a]	184.3 ± 2.4[a]
Quercetin+CCl_4	25	229.1 ± 5.4[a]	173.3 ± 2.1[a]	265.5 ± 1.7[a]

Values are mean ± S.D, n=6 animals,

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control group.

Figure 6.1- Hepatoprotective activity of ethyl acetate extract (EtOAc) of *P. rimosus* against CCl_4 induced acute hepatotoxicity in rats. Liver sections stained with H&E a) Normal; b) CCl_4 / paraffin oil; c) EtOAc (10 mg/kg body wt) + CCl_4; d) EtOAc (25 mg/kg body wt) + CCl_4; e) EtOAc (50 mg/kg body wt + CCl_4 and f) quercetin (25 mg/kg body wt + CCl_4. Magnification x 20. (Arrowmark indicated cell necrosis).

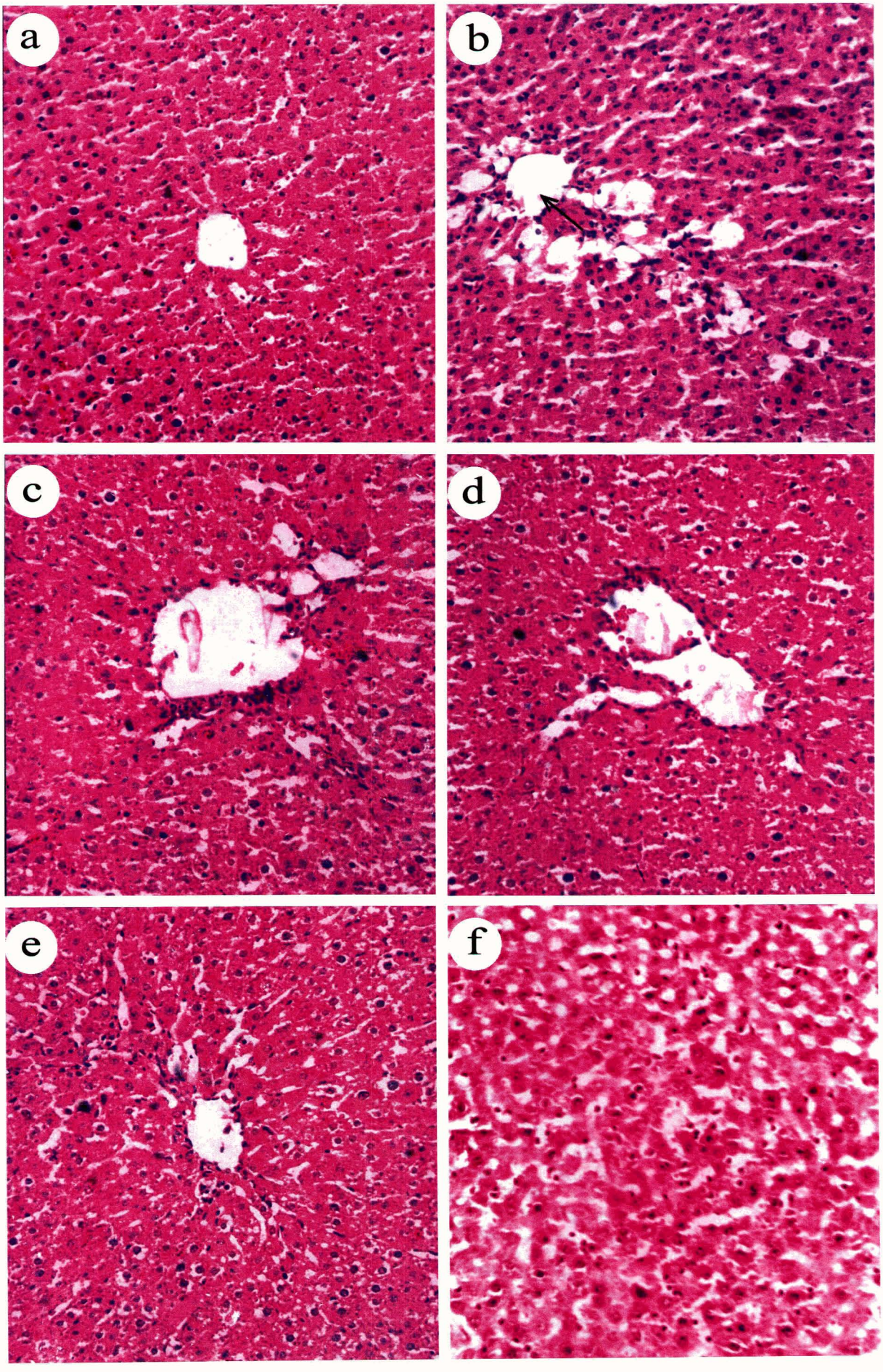
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Table 6.2. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on serum GOT, GPT and ALP activities in rats with chronic CCl_4 administration

Groups	Treatment (mg/kg)	SGPT (IU/l)	SGOT (IU/l)	ALP (IU/l)
Normal Control	Vehicle	116.5 ± 16.2	65.5 ± 5.4	179.9 ± 40.0
(CCl_4/Paraffin oil, 1:5)	--	959.8 ± 25.6*	231.1 ± 21.5*	437.3 ± 30.8*
EtOAc + CCl_4	25	516.7 ± 79.2[a]	103.5 ± 7.1[a]	259.8 ± 16.5[a]
,,	50	240.8 ± 16.9[a]	82.3 ± 4.7[a]	210.5 ± 7.8[a]

Values are mean ± S.D, n=6 animals

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control group.

Table 6.3. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on hepatic SOD, CAT and GPx activities in rats with chronic CCl_4 administration

Groups	Treatment (mg/kg)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Normal Control	Vehicle	20.2 ± 2.9	64.5 ± 4.5	22.5 ± 2.2
(CCl_4/Paraffin oil, 1:5)	--	14.5 ± 2.1*	46.3 ± 4.8*	16.9 ± 0.8*
EtOAc + CCl_4	25	17.2 ± 0.3[b]	53.9 ± 2.6[a]	18.8 ± 1.7[NS]
,,	50	19.1 ± 0.8[a]	60.9 ± 3.7[a]	20.5 ± 0.4[a]

Values are mean ± S.D, n=5 animals

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$ [b]$P<0.05$ significant (Dunnett's *t*-test) and NS non-significantly different from control group.

Table 6.4. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on blood and hepatic GSH level in rats with chronic CCl_4 administration

Groups	Treatments (mg/kg)	Blood GSH (μmol/ml)	Tissue GSH (nmol/mg protein)
Normal	Vehicle	3.87 ± 0.04	11.02 ± 0.14
Control (CCl_4/Paraffin oil, 1:5)	--	3.31 ± 0.39*	7.94 ± 0.65*
EtOAc + CCl_4	25	3.58 ± 0.33^{NS}	10.21 ± 0.81[a]
,,	50	3.87 ± 0.09[a]	11.38 ± 0.43[a]

Values are mean ± S.D, n=5

*$P<0.05$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) and NS non-significantly different from control group.

Table 6.5. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on blood and hepatic GST level in rats with chronic CCl_4 administration

Groups	Treatments (mg/kg)	Blood GST (mmol of CDNB conjugate formed /min/g Hb)	Tissue GST (μmol of CDNB conjugate formed /min/mg protein)
Normal	Vehicle	14.84 ± 1.10	748.4 ± 48.3
Control (CCl_4/Paraffin oil, 1:5)	--	14.70 ± 0.65^{NS}	775.6 ± 105.7^{NS}
EtOAc + CCl_4	25	18.32 ± 1.37[a]	1149.4 ± 44.0[a]
,,	50	20.10 ± 1.51[a]	1407.2 ± 89.1[a]

Values are mean ± S.D, n=5

^{NS} (lsd) non- significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control group.

Table 6.6. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on erythrocyte SOD, CAT and GPx activities in rats with chronic CCl_4 administration

Groups	Treatment (mg/kg)	SOD (U/g Hb)	CAT (k/g Hb)	GPx (U/g Hb)
Normal	Vehicle	1478.0 ± 74.9	94.5 ± 5.4	4974.0 ± 969.6
Control (CCl_4/Paraffin oil, 1:5)	1.5 ml	952.0 ± 194.4*	66.8 ± 5.7*	2469.6 ± 450.8*
EtOAc + CCl_4	25	1128.0 ± 76.5[b]	87.4 ± 1.8[a]	3133.6 ± 706.3[NS]
,,	50	1481.6 ± 63.2[a]	95.6 ± 3.6[a]	5102.4 ± 803.1[a]

Values are mean ± S.D, n=5 animals

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$ [b]$P<0.05$ significant (Dunnett's *t*-test) and NS non-significantly different from control group.

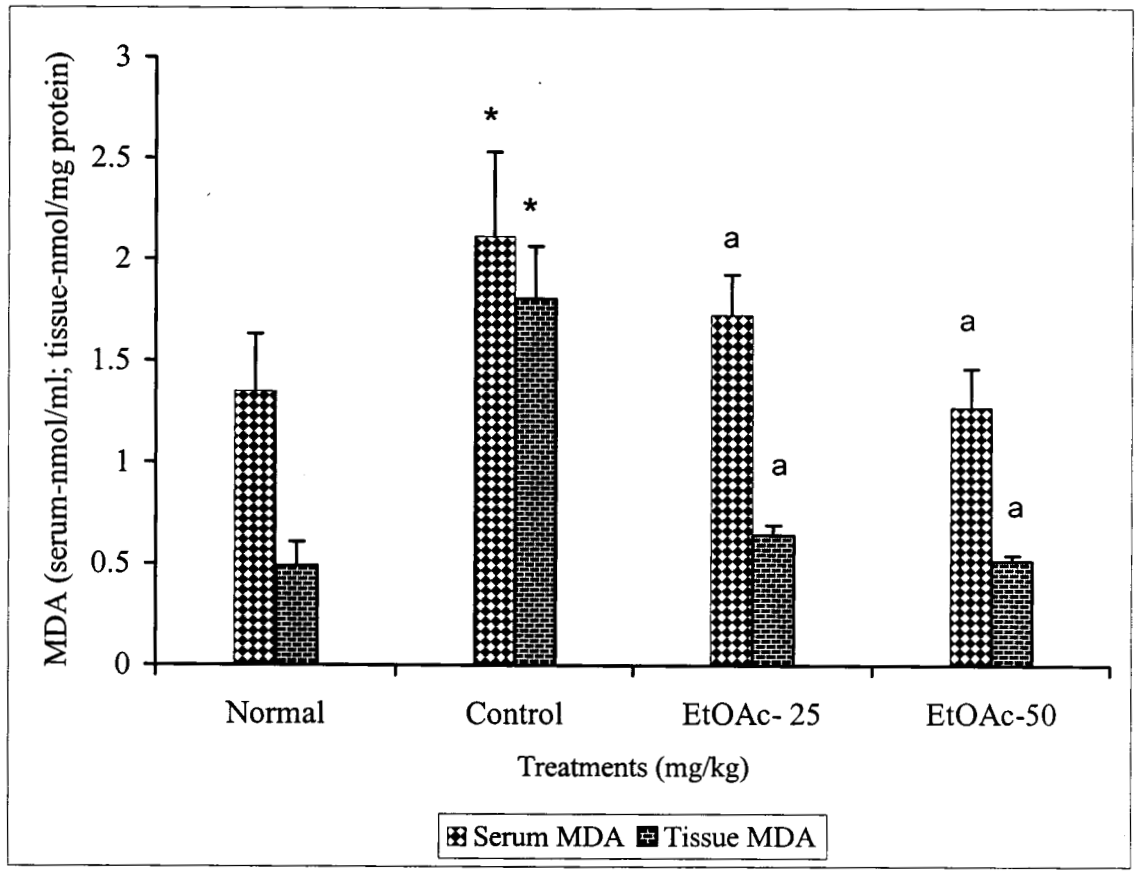

Figure 6.2. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on serum and MDA level in rats with chronic CCl_4 administration. Values are mean ± S.D, (n=6 in serum and n=5 in tissue). *P<0.01 (lsd) significantly different from normal. [a]P<0.01(Dunnett's t-test) significantly different from control group.

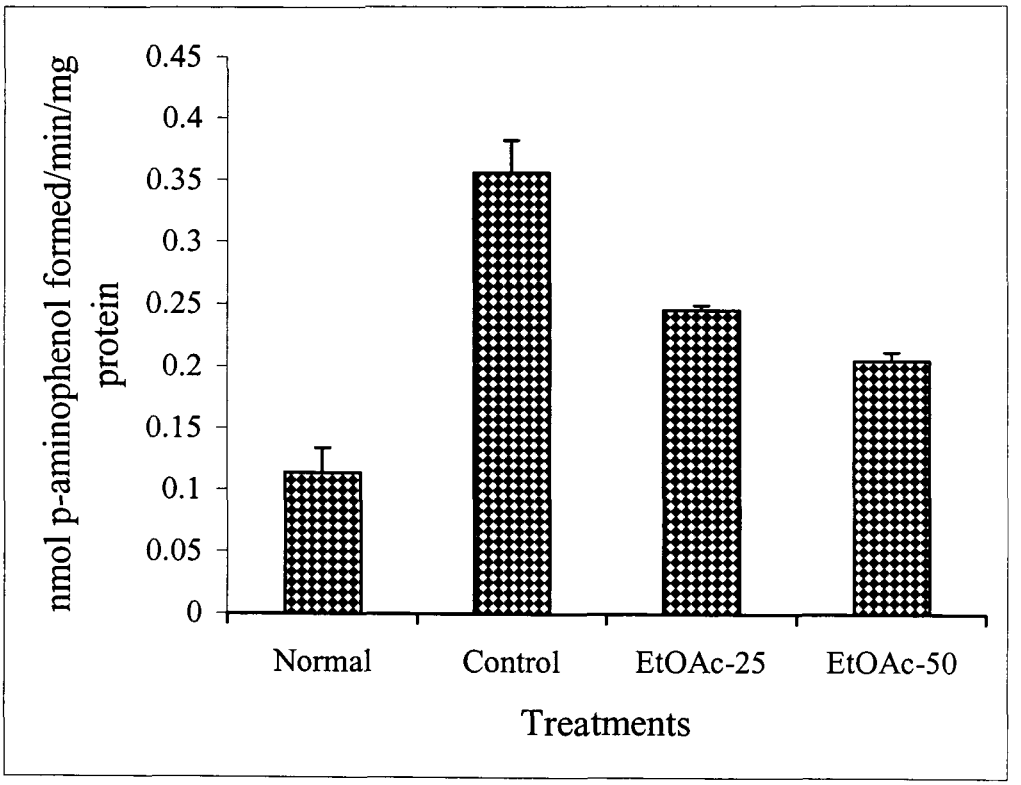

Figure 6.3. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on hepatic aniline hydroxylase activity in rats treated with chronic doses of CCl_4. Values are mean ± S.D, n=2

Table 6.7. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on renal urea and creatinine levels in mice treated with cisplatin.

Groups	Treatments (mg/kg)	Urea (mg/dl)	Creatinine (mg/dl)
Normal	Vehicle	67.4 ± 8.7	0.43 ± 0.06
Control (cisplatin)	16	317.2 ± 22.4*	3.93 ± 0.58*
EtOAc + cisplatin	25	102.6 ± 6.6[a]	0.99 ± 0.18[a]
,,	50	77.1 ± 4.5[a]	0.53 ± 0.15[a]

Values are mean ± S.D, (n=6 animals)

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control group.

Figure 6.4- Hepatoprotective activity of ethyl acetate extract (EtOAc) of *P. rimosus* against CCl_4 induced chronic hepatotoxicity in rats. Skin sections stained with H&E. a) Normal; b) CCl_4 / paraffin oil; c) EtOAc (25 mg/kg body wt) + CCl_4; d) EtOAc (50 mg/kg body wt) + CCl_4. Magnification x 20. (Arrowmark indicated ballooning degeneration).

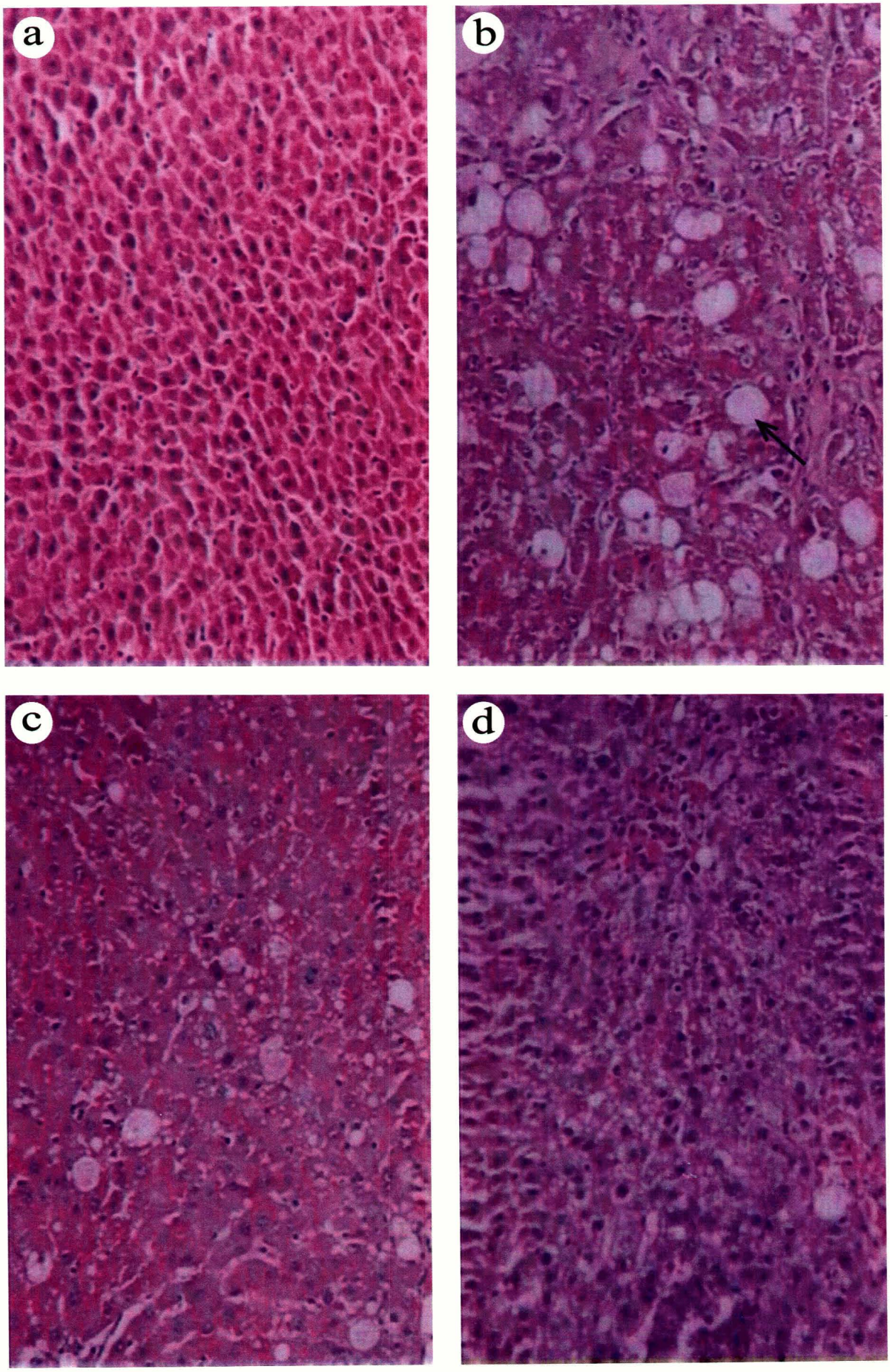
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Table 6.8. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on renal SOD, CAT and GPx activities in mice treated with cisplatin

Groups	Treatment (mg/kg)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Normal	Vehicle	11.8 ± 1.0	48.1 ± 5.9	26.3 ± 3.2
Control (cisplatin)	16	6.7 ± 1.2*	14.7 ± 2.3*	5.6 ± 1.8*
EtOAc + cisplatin	25	9.9 ± 1.2[a]	32.8 ± 3.5[a]	13.1 ± 2.3[a]
,,	50	13.4 ± 1.5[a]	46.3 ± 3.3[a]	22.4 ± 2.3[a]

Values are mean ± S.D, (n=6 animals)

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control group.

Table 6.9. Antitumor effect of cisplatin in the presence of ethyl acetate extract (EtOAc) of *P. rimosus*

Groups	Treatments (mg/kg)	Tumor weight
Contol	Vehicle	9.4 ± 2.3
Cisplatin	3	0.53 ± 0.05[a]
EtOAc + Cisplatin	50	0.13 ± 0.02[a]

Values are mean ± S.D, (n=6 animals)

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control group.

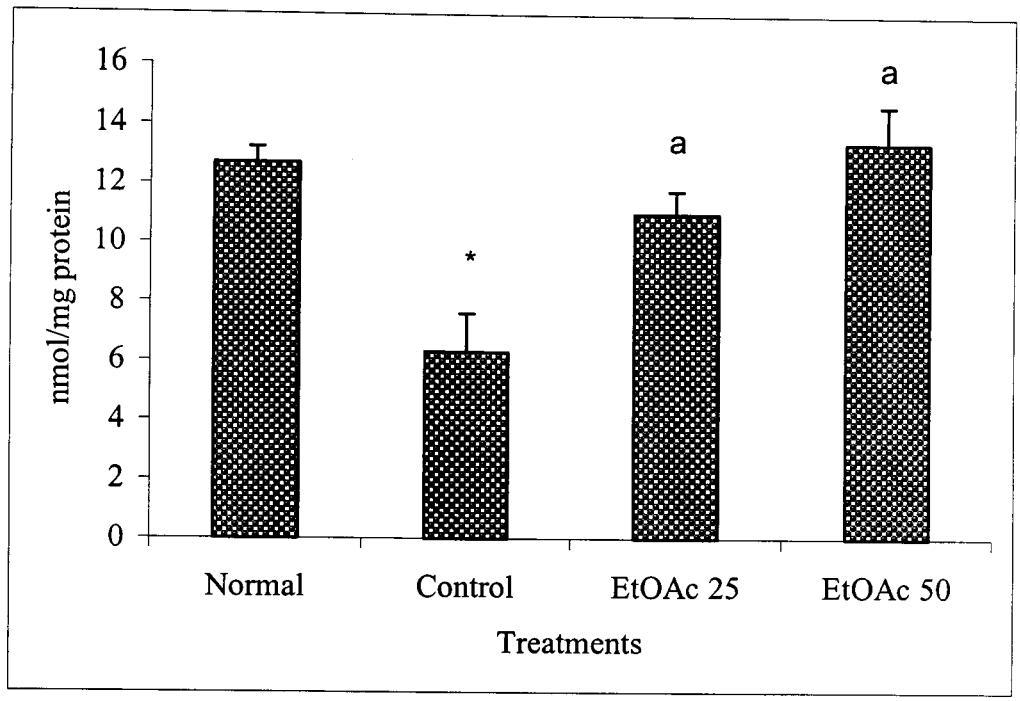


Figure 6.5. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on GSH level in mice treated with cisplatin. Values are mean ± S.D, n=6 animals.

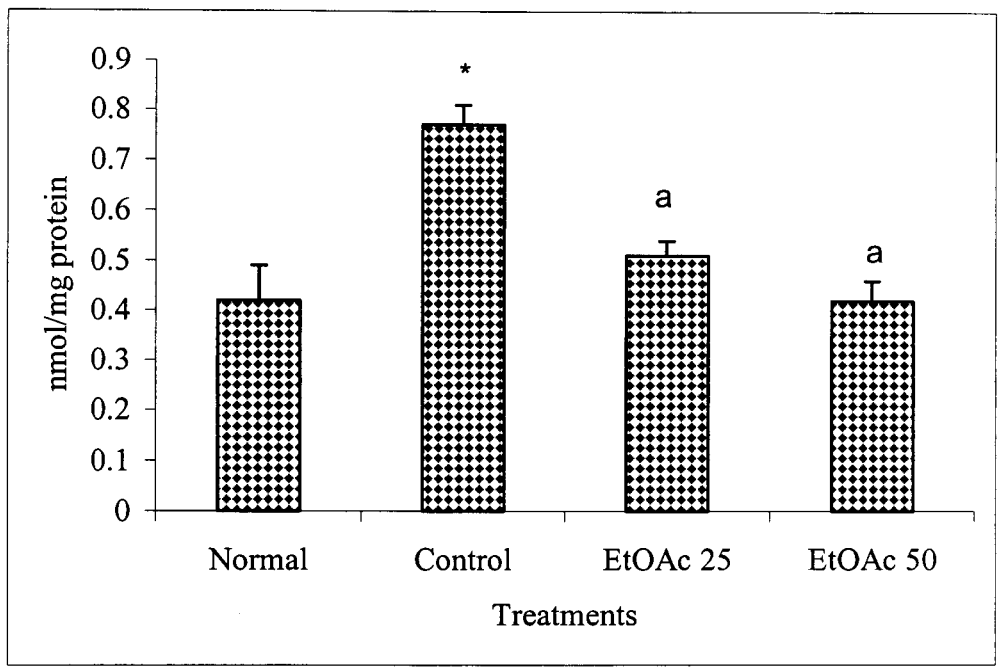
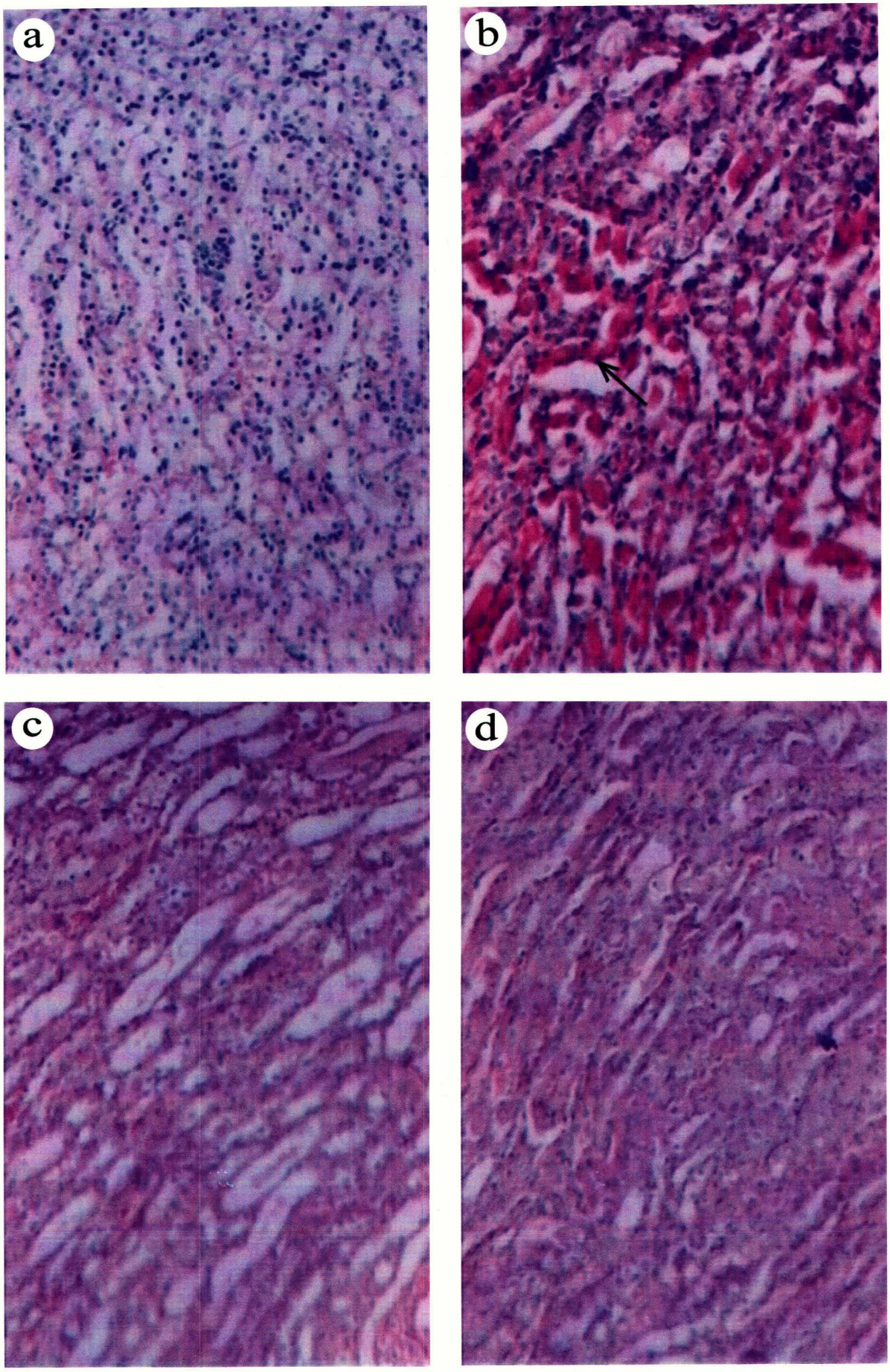


Figure 6.6. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on lipid peroxidation level (MDA) in mice treated with cisplatin. Values are mean ± S.D, n=6 animals. *$P<0.01$ (lsd) significantly different from normal. $^{a}P<0.01$(Dunnett's *t*-test) significantly different from control group.

Figure 6.7- Nephroprotective activity of ethyl acetate extract (EtOAc) of *P. rimosus* against cisplatin induced nephrotoxicity in rats. Kidney sections stained with H&E a) Normal; b) cisplatin; c) EtOAc (25 mg/kg body wt) + cisplatin and d) EtOAc (50 mg/kg body wt) + cisplatin. Magnification x 20. (Arrowmark indicated accumulation of plasma ultrafiltrate in the proximal convoluted tubule)


a
b
c
d

6.4 Discussion

Results of the present study reveal the significant hepatoprotective activity of the ethyl acetate extract of *P. rimosus* against acute and chronic CCl_4 hepatotoxicity. The activities of transaminases (SGPT and SGOT) were elevated significantly in the serum of CCl_4 alone injected animals. The elevation of SGPT in the serum indicates the hepatocyte necrosis. The altered ratio of SGPT to SGOT in animals injected with CCl_4 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 treatment, which indicates the hepatoprotective activity of the extract.

The antioxidant status of the hepatocytes was altered in the chronic CCl_4 treated animals. The treatment of *P. rimosus* extract prior to the CCl_4 injection 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). Studies indicated erythrocytes from selenium deficient

animals are more susceptible to haemolysis *in vitro* under conditions favoring lipid peroxidation than the normal erythrocytes (Halliwell and Gutaridge, 1985a).

The amount of ultimate toxic substances available for the interaction with its target represents, in part, a balance between activating and detoxifying reactions. Although this balance is under genetic control, it is easily modulated by a variety of factors including the exposure to drugs and other xenobiotics (Conney, 1982). The compounds elevate phase II enzyme activity without significant activation of phase I enzyme would be more desirable candidate to render protection from chemically induced liver toxicity (Prochaska et al., 1985 and Kensler, 1997). Direct measurement of phase II enzyme activities in blood cells have also been used to assess enzyme induction (Kensler, 1997). The total GST activity, a phase II enzyme, in the hepatocyte is enhanced significantly in the extract treated animals than the normal or control group of animals. The enhanced GST activity in the erythrocyte may also reflect its possible induction by the extract in the liver. Treatment with antioxidant compounds has been shown to elevate α rather than the μ or π or θ class subunits of liver GST (Kensler, 1997). Such increased activity of GST protects liver against damage by electrophilic metabolites. Nevertheless, enhanced level of the hepatic GSH, substrate of GST, could facilitate conjugation of glutathione to the active electrophilic radicals of CCl_4, and thus was able to reduce the hepatotoxicity. Elevation of aniline hydroxylase, a phase I enzyme, in the liver of CCl_4 treated animals and the inhibition of its activity by the extract supports its hepatoprotective effect. The enhanced activity of aniline hydroxylase in the CCl_4 intoxicated animals indicates the increased metabolism of CCl_4 and enhanced the toxicity of liver to active metabolites of CCl_4. By inhibiting the activation of phase I enzyme, ethyl acetate extract of *P. rimosus* render protection against CCl_4 mediated toxic metabolites.

The results of the nephroprotective investigation indicate that ethyl acetate extract of *P. rimosus* rendered significant protection against cisplatin-induced nephrotoxicity in a dose dependent manner. Previous experimental studies in animals have shown that a minimum dose of cisplatin (5 mg/kg body weight, i.p) was sufficient to induce nephrotoxicity in rats (Boogaard et al., 1991 and Ravi et al., 1995). The present study was carried out with higher dose of cisplatin (16 mg/kg body weight, i.p). This dose corresponds to the higher dose of cisplatin currently being used in clinical practice. The dose can also produce ototoxicity (Younes and Siegers, 1981). Administration of cisplatin shows a nine fold increase in serum creatinine and five fold

increase in serum urea levels compared to normal, which clearly indicates the renal failure. *P. rimosus* extract significantly prevents the elevation of cisplatin induced serum creatinine and urea levels.

Recent experimental findings have suggested that the free radicals and reactive oxygen species are involved in cisplatin induced oxidative stress because of depletion of GSH concentration and decreased antioxidant enzyme activity in the kidneys. The results of the present study show that renal SOD, CAT, GPx activities and reduced GSH level are significantly decreased in the cisplatin treated group of animals compared to the control. These observations also support the evidence that part of the mechanism of nephrotoxicity in cisplatin treated animals is related to depletion of antioxidant system. Treatment of *P. rimosus* (25 and 50 mg/kg body weight, p.o) along with cisplatin could significantly prevent the depletion of these renal antioxidant systems.

The concentration of MDA as a result of lipid peroxidation shows an increase in the cisplatin treated group. The decreased SOD activity can cause the initiation and propagation of lipid peroxidation in the cisplatin treated group. This decreased activity may be either due to loss of copper and zinc, which are essential for the activity of enzyme or reactive oxygen species-induced inactivation of enzyme (Sharma, 1985 and DeWoskin and Riviere, 1992). The activity of CAT and GPx also decreased in the cisplatin treated group, which in turn increased the H_2O_2 and lipid peroxides. *P. rimosus* extract treatment is found to prevent the lipid peroxidation by enhancing the renal antioxidant enzymes.

Reduced renal GSH, non-protein thiols in the cells, can markedly increase the toxicity of cisplatin. The depletion of GSH also seems to be a prime factor that permits lipid peroxidation in the cisplatin treated group. Concomitant treatment of extract rendered protection due to the increase in GSH level. Results of the antitumor study revealed that pretreatment of *P. rimosus* extract did not interfere with the antitumor efficacy of cisplatin.

Previous studies have demonstrated the significant *in vitro* radical scavenging activity of the ethyl acetate extract of *P. rimosus*. The presence of polyphenols and flavonoids in the extract might also be responsible for this property. Direct radical scavenging activity of the extract might also be involved in the hepatoprotective activity against acute or chronic CCl_4 exposure and nephroprotective activity against acute cisplatin administration.

Cytotoxicity and antitumor activities of P. rimosus

Ajith. T.A “Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrohungus, Phellinus rimosus (Berk) Pilat. ” Thesis. Amala Cancer Research Centre , University of Calicut, 2004

Chapter 7

Cytotoxicity and antitumor activities of *Phellinus rimosus*

7.1. 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, including 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.

Higher Basidiomycetes (mushrooms) have been found to possess significant pharmacological activities and physiological properties such as bioregulation (immunological enhancement), maintenance of homeostasis, regulation of biorhythm, cure of various diseases, prevention and improvement of conditions in cancer, cerebral stroke and heart diseases (Wasser and Weis, 1999a). A recent survey indicated that a large number of antitumor agents were produced by fungi (Hata, 1997). Some species of edible mushrooms have been found to markedly inhibit the growth of different kinds of tumors. There are approximately two hundred species of higher Basidiomycetes that have been reported to possess this activity. The searches for new antitumor and other medicinal substances from higher Basidiomycetes and the study of their medicinal value have become a subject of great interest. Extracts of the sporocarps of *P. rimosus* were evaluated for their *in vitro* cytotoxic and antitumor activities. The results are presented in this chapter.

7.2. Materials and Methods

7.2.1 Animals

Male Swiss albino mice weight 20 ± 2 g were used for the antitumor studies.

7.2.2 Cell lines

Ehrlich's ascites carcinoma (EAC) and Daltons lymphoma ascites (DLA) cell lines.

7.2.3 Preparation of extracts of P. rimosus

Ethyl acetate, methanol and aqueous extracts of *P. rimosus* were prepared as described in the section 3.2.1.

7.2.5 Assay for cytotoxicity

The *in vitro* short-term cytotoxicity of ethyl acetate, methanol and aqueous extracts of *P. rimosus* were assayed using DLA and EAC cell lines. Briefly $1x10^6$ viable cells of cell line suspended in 0.1 ml of phosphate buffered saline (PBS) (0.2 M,

pH 7.4), various concentrations of extracts (10 µg/ml-1 mg/ml) and phosphate buffer in a final volume of 1 ml were incubated at 37°C for 3 h. After the incubation, the viability of the cells was determined by trypan blue exclusion method (Gupta and Bhattacharya, 1983). Percent cytotoxicity was calculated after comparing with the untreated control using the formula, (number of dead cells in the extract treated tube-number of dead cells in the control tube/ total number of cells in the treated tube) x 100.

7.2.6 Antitumor activity

Antitumor activity of the extracts was determined using ascites tumor and solid tumor models.

7.2.6.1 Ascites tumor model

Animals were divided into 8 groups of 6 animals in each group. All the animals were injected intraperitoneally (i.p) with $1x10^6$ viable EAC cells in PBS (aspirated from 15 day old EAC ascites tumor in mice). After 24 hour of tumor inoculation ethyl acetate, methanol and aqueous extracts of *P. rimosus* was administered orally at doses of 25 and 50 mg/kg body weight and continued once daily for ten consecutive days. The group administered with vehicle alone was maintained as control. Cisplatin (2 mg/kg body weight, i.p) was used as the standard reference drug. The mortality rate were noted in each group and the percent increase in life span (ILS) was calculated using the formula % ILS = (1-T/C) x100 where T is mean survival time of treated group and C that of control group (Ahluwalia et al., 1984).

7.2.6.2 Solid tumor model

7.2.6.2.1 Effect of extracts when administered simultaneous with tumor inoculation

Animals were divided into 8 groups of 6 animals each. Viable DLA cells ($1x10^6$) in 0.1ml PBS were transplanted subcutaneously into the right hind limb of mice. Ethyl acetate, methanol and aqueous extracts were administered (25 and 50 mg/kg body weight) orally 24 h after tumor implantation and continued for 10 consecutive days. The control group was treated with vehicle and the standard reference group was treated with cisplatin (4 mg/kg body weight, i.p). The tumor development on animals in each group was determined by measuring the diameter of tumor growth in two perpendicular planes using a vernier calipers once every week for 5 weeks. The tumor volume was calculated using the formula $V= 4/3\ \pi\ r_1^2\ r_2$ where, r_1 is minor radius and r_2 is major radius. At the end of the 5th week, animals were sacrificed under anesthesia, tumor extirpated and weighed. The percent inhibition was

calculated by the formula: (1-B/A) 100 where, A is average tumor weight of the control group and B that of the treated group (Chihara et al., 1970).

7.2.6.2.2 Effect of extracts when administered after tumor development

Antitumor activity of the ethyl acetate, methanol and aqueous extracts was tested after tumor development in mice. Solid tumor development in mice was induced as described earlier. After 14 days, animals with tumor size around 0.394 ± .012 cm^3 were divided into 5 groups of 5 animals each. Extracts of *P. rimosus* (50 mg/kg body weight, p.o) was administered for 10 consecutive days. The group treated with vehicle was maintained as control. Cisplatin (4 mg/kg, i.p) was used as the standard reference drug. Tumor diameter was measured using vernier calipers once every week for a period of 3 weeks and volume was calculated. At the end of the 5^{th} week, animals were sacrificed, tumor extirpated and weighed. The percent inhibition was calculated as described earlier.

7.2.6.2.3 Effect of extracts when administered before tumor inoculation

Antitumor activity was also evaluated by administering the ethyl acetate, methanol and aqueous extracts (50 mg/kg body wt., p.o) for 10 consecutive days.1 h after the last dose of drug administration solid tumor was induced using DLA cell line in mice as described early. Animals were kept untreated after the tumor inoculation. The group treated with vehicle was maintained as control. Cisplatin (4 mg/kg, i.p) was used as the reference drug. Tumor growth was noted once every week for 4 weeks. Number of animals bearing tumor and percent of incidence were calculated.

7.3 Results

7.3.1 In vitro cytotoxicity

The ethyl acetate and methanol extracts of *P. rimosus* showed marked cytotoxic activity (Table 7.1). However, the aqueous extract did not possess cytotoxic activity up to a concentration of 1mg/ml. The concentration of methanol extract required for 50% death of the DLA and EAC cell lines (IC_{50}) was found to be 543 ± 24.7 μg/ml and 412 ± 20.4 μg/ml respectively. IC_{50} of ethyl acetate extract was 184 ± 3.4 μg/ml and 92 ± 10.4 μg/ml for DLA and EAC cell lines respectively.

7.3.2 Antitumor activity

In the ascites tumor bearing animals ethyl acetate extract administration at a dose of 50 mg/kg body weight increased 65 % ($P<0.01$) life span compared to the control group animals (Table 7.2). Methanol extract did not show activity at 25 mg/kg

but at 50 mg/kg treated group showed 33 % (P<0.01) increase in life span. However, the aqueous extract (25 and 50 mg/kg body wt) did not show any antitumor activity. The standard reference drug (cisplatin 2 mg/kg, i.p) exhibited 73 % (P<0.01) increase in life span.

All the extracts possessed significant (P<0.01) antitumor activity against solid tumor models (Table 7.3). Tumor volume of the control, extracts and the cisplatin treated groups at different weeks after the tumor induction were given in the figure 7.1 and 7.2. Among the 3 extracts, ethyl acetate could prevent 95.8 % of solid tumor growth at 50 mg/kg body weight when administered 24 h after tumor implantation. The weight (0.34 ± 0.07 g) and volume of the tumor (0.17 ± 0.08 cm^3) in ethyl acetate extract treated group (50 mg/kg body weight, p.o) at the end of 5^{th} week was significantly lower (P<0.01) than the control group. The aqueous and methanol extracts treated groups (50 mg/kg, p.o) also showed a significant reduction in tumor volume at the end of 5^{th} week. The ethyl acetate extract at 50 mg/kg inhibited the tumor proliferation as effectively as the standard reference drug cisplatin.

The ethyl acetate extract was also highly effective against developed solid tumor. The tumor volume in the extract treated group at different weeks intervals was decreased compared to the control group of animals (Fig. 7.3). The treatment of ethyl acetate, methanol and aqueous extracts (50 mg/kg body wt. p.o) for 10 consecutive days after tumor development showed 64 % (P<0.01), 51.1 % (P<0.01) and 56.2 % (P<0.01) tumor growth regression respectively as compared to control (Table 7.4).

Pretreatment of the aqueous and ethyl acetate extracts were also found to be effective in inhibiting the initiation of solid tumor growth induced by DLA (Table 7.5). Administration of ethyl acetate and aqueous extracts (50 mg/kg body wt., p.o) for 10 consecutive days prior to tumor inoculation inhibited the tumor incidence by 83.4 % and 66.7 % at the end of 4^{th} week. Methanol extract treated group exhibited only 33.4 % inhibition of tumor incidence. Infact only one animal in the ethyl acetate extract treated group and two animals in the aqueous extract treated group developed tumor the tumor volume of in the ethyl acetate extract treated group was higher than the aqueous extract treated group.

Table7.1. *In vitro* cytotoxic activity of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *Phellinus rimosus* IC_{50} (µg/ml).

Extracts	DLA Cell line	EAC Cell line
EtOAc	184 ± 3.40	92.0 ± 10.4
MeOH	543 ± 24.7	412 ± 20.4
AQ	Nil	Nil

Values are mean ± S.D, n=3.

Table 7.2. Antitumor activity of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* against EAC induced ascites tumor in mice.

Group	Treatments (mg/kg)	Survival time (Days)	% Increase in life span	Mortality at 25 day
Control	Vehicle	19.8 ± 2.0	--	6/6
Cisplatin	2	34.8 ± 5.1[a]	73	0/6
EtOAc	25	25.1 ± 1.1[a]	27	3/6
	50	34.5 ± 2.8[a]	65	0/6
MeOH	25	18.8 ± 1.6[NS]	---	6/6
	50	26.6 ± 3.9[a]	33	2/6
AQ	25	19.5 ± 1.0[NS]	---	0/6
	50	20.0 ± 0.8[NS]	---	0/6

Values are mean ±S.D, n=6

[a]$P<0.01$ (Dunnett's *t*-test) significantly and NS non-significantly different from control group.

Table 7.3. Antitumor activity of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* against DLA induced solid tumor model in mice when administered simultaneous with tumor inoculation.

Groups	Treatment (mg/kg)	Volume on 5th week (cm^3)	Wt. of tumor (g)	% inhibition
Control	Vehicle	4.27 ± 0.97	8.12 ± 1.40	--
Cisplatin	4	0.14 ± 0.06	0.15 ± 0.05[a]	98.1
EtOAc	25	0.66 ± 0.29	1.44 ± 0.11[a]	82.2
	50	0.17 ± 0.08	0.34 ± 0.07[a]	95.8
MeOH	25	0.82 ± 0.28	2.31 ± 0.25[a]	71.5
	50	1.10 ± 0.32	1.61± 0.57[a]	80.1
AQ	25	1.95 ± 0.79	3.74 ± 1.20[a]	53.9
	50	0.41 ± 0.12	1.10 ± 0.27[a]	86.4

Values are mean ± S.D, n=6.

[a] P< 0.01(Dunnett's *t*-test) significantly different from control.

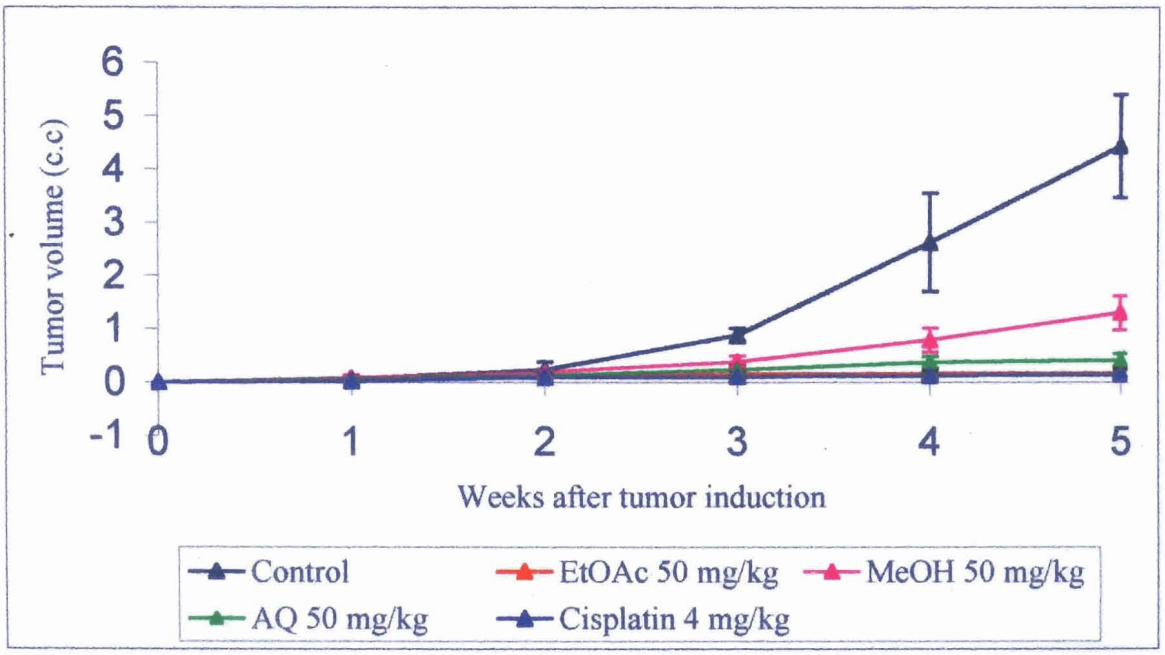


Figure 7.1. Antitumor activity (50 mg/kg body wt, p.o) of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* when treated simultaneously with tumor inoculation.

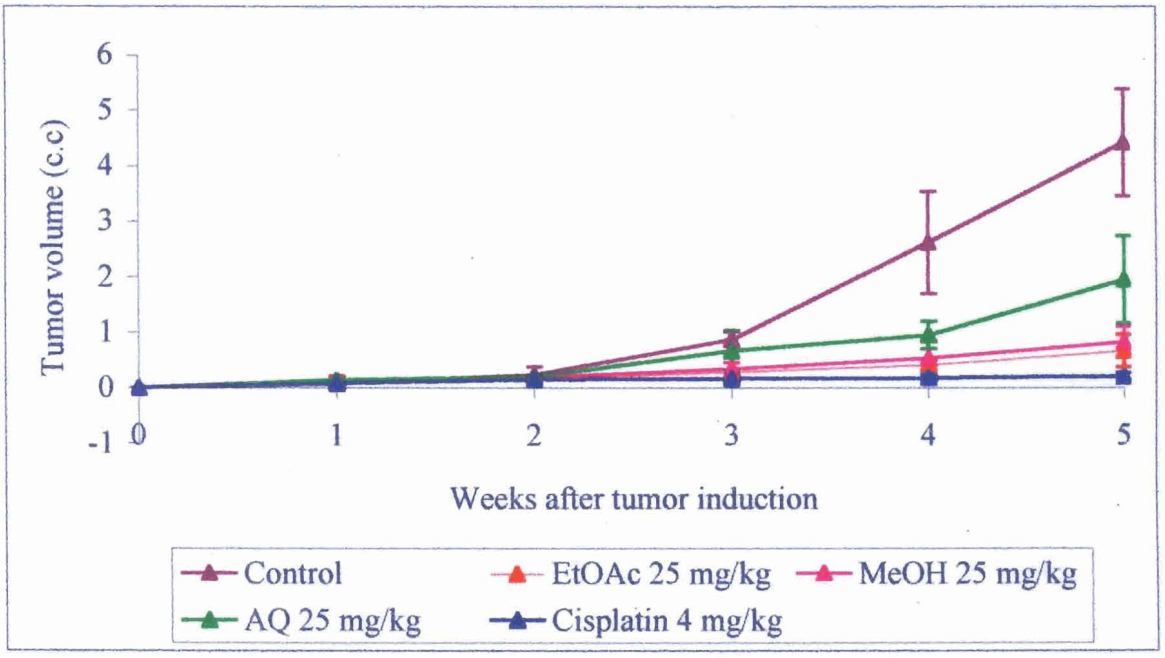


Figure 7.2. Antitumor activity (25 mg/kg body wt, p.o) of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* when treated simultaneously with tumor inoculation.

Table 7.4. Antitumor activity of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* (PR) on the developed tumor in mice.

Groups	Treatment (mg/kg)	Volume on 5th week (cm^3)	Wt. of tumor (g)	% inhibition
Control	Vehicle	3.22 ± 0.60	6.31 ± 0.26	--
Cisplatin	4	0.30 ± 0.04	1.53 ± 0.37[a]	75.7
EtOAc	50	1.07 ± 0.14	2.27 ± 0.07[a]	64.0
MeOH	50	2.06 ± 0.48	3.08 ± 0.12[a]	51.1
AQ	50	1.52 ± 0.29	2.76 ± 0.20[a]	56.2

Values are mean ± S.D, n=5.

[a] P< 0.01(Dunnett's *t*-test) significantly different from control.

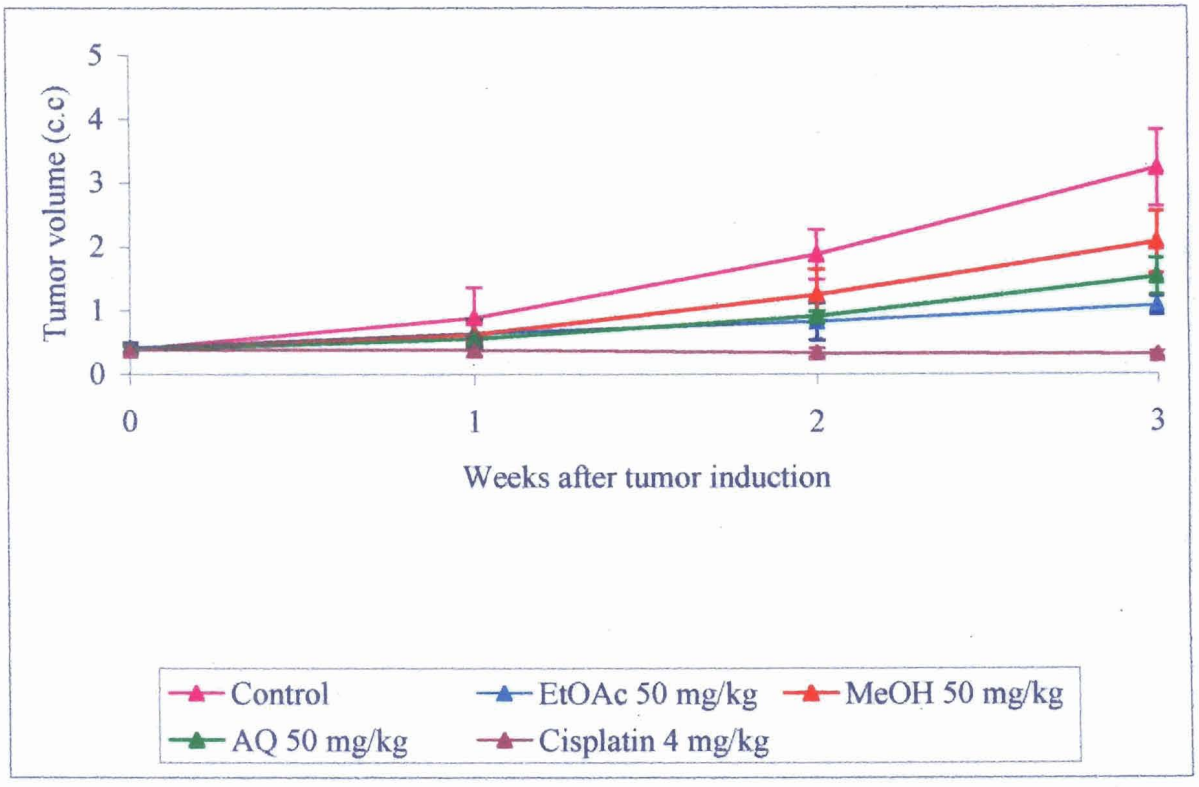


Figure 7.3. Antitumor activity of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts (50 mg/kg body wt, p.o) of *P. rimosus* when treated after the tumor development. Standard reference drug was cisplatin (4 mg/kg body wt., i.p)

Table 7.5. Antitumor activity of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* (PR) when treated before tumor induction in mice.

Groups	Treatment (mg/kg)	Volume on 4th week (cm^3)	No of animals with tumor on 4th week	Percent of incidence
Control	Vehicle	1.90 ± 0.94	6/6	100
Cisplatin	4	0.03 ± 0.04[a]	0/6	0
EtOAc	50	0.05 ± 0.10[a]	1/6	16.6
MeOH	50	0.11 ± 0.07[a]	4/6	66.6
AQ	50	0.05 ± 0.07[a]	2/6	33.3

Values are mean ± S.D. n=6

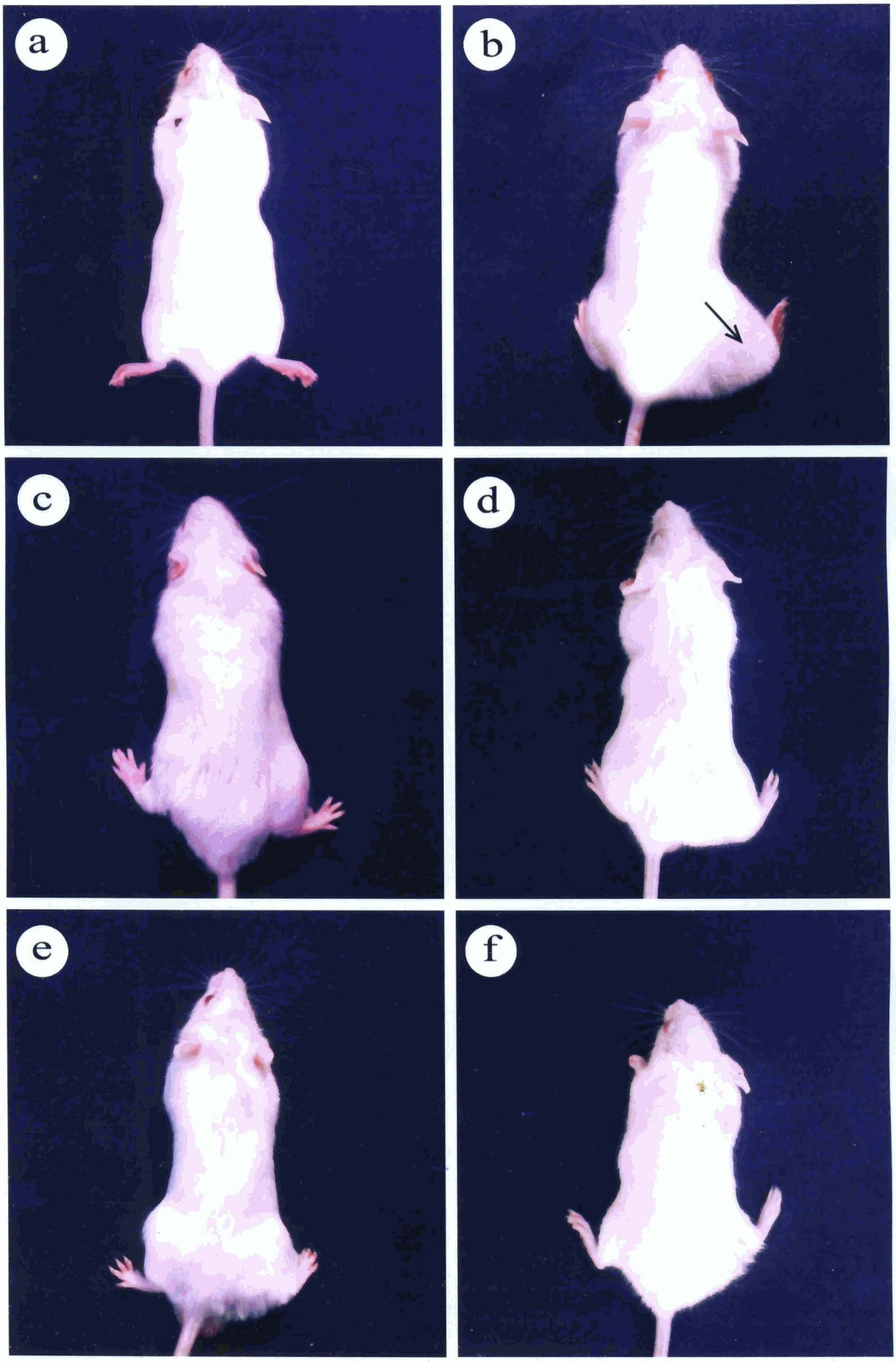
[a] P< 0.01(Dunnett's *t*-test) significantly different from control.

7.4 Discussion

The results of the present investigation demonstrate significant antitumor activity of ethyl acetate extract of *P. rimosus* against both ascites and solid tumors. Although, the methanol and aqueous extracts are significantly effective against solid tumor model, the former is weakly effective and latter ineffective against ascites tumor model. Results also reveal that ethyl acetate extract is a more effective antitumor agent than other extracts in all the models. However, its effect was found to be preventive rather than curative.

The antitumor activity of the ethyl acetate, methanol and aqueous extracts is in a dose dependent manner, with no signs of toxicity. In the ascites tumor model, the standard reference drug cisplatin at a dose of 4 mg/kg. body wt., i.p for ten consecutive days was found to be toxic and lethal to animals. This may be due to the combined toxic effect of intraperitoneal injection of cisplatin and cancer cell line in the animal.

Figure 7.4- Antitumor activity of ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* against DLA cell induced solid tumor in mice when treated simultaneously. a) Normal; b) control c) EtOAc (50 mg/kg body wt; d) MeOH (50 mg/kg body wt; e) AQ (50 mg/kg body wt) and f) cisplatin (4 mg/kg body wt. (Arrowmark indicated solid tumor)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e
f

Hence, lower dose of cisplatin (2 mg/kg body wt., i.p), which was significantly effective to inhibit ascites tumor, was selected.

Cytotoxicity is one of the chemotherapeutic targets of antitumor activity (Suffness and Pezzuto, 1991). Most of the clinically used antitumor agents possess significant cytotoxic activity in cell culture systems. The *in vitro* cytotoxic activity of ethyl acetate extract is higher than that of methanol extract. The higher cytotoxic activity of ethyl acetate extract against EAC and DLA cell lines explains its increased antitumor activity against ascites and solid tumors compared to other extracts.

Preliminary chemical examination of the ethyl acetate and methanol extracts show detectable amounts of polyphenols and flavonoids. A number of these compounds have been reported to possess antitumor activity (Carlo et al., 1994). Although the mechanism of tumor inhibiting activity of flavonoids is not clear, they are reported to decrease the high glycolytic activity of Ehrlich's ascites cells by inhibition of (Na^+, K^+)-ATPase in the plasma membrane (Pomilio et al., 1994).

The major constituent of the aqueous extract of *P. rimosus* is found to be polysaccharides. The polysaccharide-protein complex from a large number of mushrooms has been found to be effective against Ehrlich carcinoma or Sarcoma 180 cancerous tumors in mice (Jong and Donovick, 1989). The active substance exhibits neither toxicity nor side effects commonly seen with the use of conventional anticancer agents such as, decrease in the number of leucocytes, anemia, atrophy of the spleen, loss of body weight and loss of appetite.

A number of polysaccharides and protein bound polysaccharides isolated from mushrooms are clinically used for the treatment of cancer. Krestin (PS-K) isolated from *Coriolus versicolor,* lentinan from *Lentinus edodes* and Schizophyllan from *Schizophyllum commune* are sold in China and Japan as anticancer drugs and are extensively used in treatment (Fukushima, 1989). High molecular weight polysaccharide especially glucan are found to stimulate both nonspecific host resistance and specific immunological reactivity against tumors (Jong et al., 1991). The experimental results indicate that the antitumor activity of ethyl acetate and aqueous extracts of *P. rimosus* might be due to two different classes of substances. Immunomodulating polysaccharides might be responsible for the antitumor activity of aqueous extract. The activity of ethyl acetate extract could be due to substances other than polysaccharides, possibly polyphenols and flavonoids.

Antimutagenic activity of P. rimosus

Ajith. T.A “Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrohungus, Phellinus rimosus (Berk) Pilat. ” Thesis. Amala Cancer Research Centre , University of Calicut, 2004

Chapter 8

Antimutagenic activity of *Phellinus rimosus*

8.1 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.

Recently extract from *P. lintus, P. igniarius* are reported to possess antimutagenic activity (Shon and Nam, 2001). Among the activities studied for the extracts of *P. rimosus,* ethyl acetate and aqueous extract showed higher antioxidant, anti-inflammatory and antitumor activities were selected for the antimutagenic activity.

8.2 Materials and Methods

8.2.1 Preparation of extract of P. rimosus

Ethyl acetate and aqueous extract of *P. rimosus* was prepared as described in the section 3.2.1

8.2.2 Animals

Male Spraque Dawly rat (200 g) was used for the study.

8.2.3 Bacterial strains

Antimutagenic activity was determined using *Salmonella typhymurium* strains TA 98, TA 100, TA 102 and TA 1535.

8.2.4 Mutagens

NaN_3, MNNG, doxorubicin, B[*a*]P, 2-AF, NPDA and extract from deep fried beef meat were used as mutagens. NPDA, B[*a*]P, 2-AF and MNNG were dissolved in DMSO. NaN_3 and doxorubicin were dissolved in distilled water.

8.2.5 Preparation of extract from deep fried meat

100 g of fresh beef meat was fried in the absence of cooking oil under normal house cooking conditions in a non-sticky pan. The frying was repeated daily for 5 days. After the 5 th day, a 20 % homogenate of the repeatedly fried meat and normally fried meat was prepared in 50 % DMSO. The suspension was shaken vigorously and centrifuged for 5000 rpm for 10 min. The mutagenicity of the supernatant (0.1 ml) was tested against the *Salmonella typhimurium* strains. The *Salmonella* strain that showed maximum revertants was preferred for testing the antimutagenic activity of the ethyl acetate extract of *P. rimosus*.

8.2.6 Antimutagenic assay using direct acting mutagens

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), beef extract (0.1 ml/plate) and NPDA (20 μg/plate) were used as the direct acting mutagens.

Freshly grown overnight cultures of *Salmonella* tester strains (TA 98, TA 100, TA 102 and TA 1535) in nutrient broth (0.1 ml, approximately 10^9 bacterial cells/ml) were mixed with 0.1 ml of various concentrations of extract of *P. rimosus*, 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. Antimutagenic activity of ethyl acetate extract (0.5, 1 and 2 mg/plate) was tested against all the mutagens. Aqueous extract (1, 2.5 and 5 mg/plate) was tested against the mutagen, NPDA.

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 mg/plate ethyl acetate and 5 mg/plate aqueous extract) treated in the similar manner was employed.

8.2.7 Antimutagenic activity against mutagens needing activation

For this assay B[*a*]P (0.005 mg/plate) and 2-AF (0.025 mg/plate) were employed as mutagens. The antimutagenic activity against indirect acting mutagens was carried out by the Ames plate incorporation method (Maron and Ames, 1983). Activation was accomplished by treatment with the rat liver microsomal fraction before plating onto the minimal agar plate. The reaction mixture was prepared by adding 0.5 ml of S9 mix (section 3.2.33), 0.01 ml of mutagen, 0.1 ml of various concentration of the extract of *P. rimosus*, 0.1 ml of the freshly grown overnight *S. typhimurium* culture strains and 0.2 ml of 0.5 mM histidine/biotin solution to 2 ml of molten top agar at 45°C. The mixture was poured onto the minimal agar plate. The plates were incubated at 37°C for 48 h. Number of revertants and percentage of inhibition were calculated as described above. All the experiments were done in triplicate.

The ethyl acetate extract (0.5, 1 and 2 mg/plate) was tested using B[*a*]P and 2-AF against *Salmonella strain* TA 98. The aqueous extract (1, 2.5 and 5 mg/plate) was tested against B[*a*]P induced mutation of TA 98 strain.

8.2.8 Effect of P. rimosus extracts on in vitro aniline hydroxylase activity

Male Spraque Dawly rat (200 g) was treated with sodium phenobarbitone (80 mg/kg body wt, p.o) for 5 days (Waxman and Azaraff, 1992). After an overnight fasting, the animal was killed by decapitation and the cytosolic supernatant was prepared from the 10% liver homogenate in phosphate buffer (0.1M, pH 1.4) as described in the section 3.2.2. Aniline hydroxylase was determined as described in the section 3.2.11 in 1 ml of the cytosolic fraction in the presence and absence of various concentrations of the *P. rimosus* ethyl acetate (0.25, 0.5 and 1 mg) and aqueous extracts (1, 2.5 and 5 mg). The activity was compared to the reaction mixture devoid of the extract.

8.3 Results

8.3.1 Antimutagenic activity against direct acting mutagens

The ethyl acetate extract of *P. rimosus* showed significant antimutagenic activity against both direct acting mutagens and mutagens that require activation. The extract at 2 mg/plate exhibited 34 %, 50 % and 66.5 % inhibition of NaN_3 induced

revertants of *Salmonella* strains TA 100, TA 102 and TA 1535 respectively (Table 8.1). MNNG induced revertants were found to be reduced significantly in 2 mg ethyl acetate extract incorporated plate (Table 8.2). The inhibition was 60.2 % (TA100) and 92.4 % (TA102) compared to the control. The antimutagenic activity of the extract at 2 mg/plate against NPDA and doxorubicin induced revertants was also found to be significant (Table 8.3 and 8.4). Ethyl acetate extract at 2 mg/plate inhibited NPDA induced mutation of TA 98 (64.4 %) and TA 102 (63.3 %). Extract at 2 mg/plate also inhibited the doxorubicin induced mutations of TA 98 (89 %) and TA 100 (97.8 %) (Table 8.4). The extract at 2 mg/plate inhibited the repeated fried meat induced mutagenicity of TA 102 (93.7 %) (Fig. 8.1). The aqueous extract also showed antimutagenic activity against NPDA induced mutation of TA 100. The extract at 5 mg/plate inhibited (42.6 %) mutation of TA 100 induced by NPDA (Fig. 8.2).

8.3.2 Antimutagenic activity against mutagens needing activation.

The mutagenicity of metabolically activated B[*a*]P and 2-AF was also significantly inhibited by the ethyl acetate extract of *P. rimosus* . The ethyl acetate extract at 2 mg/plate inhibited the B[*a*]P (88.5 %) (Table 8.5) and 2-AF (88 %) induced mutation of TA 98 (Fig.8.3). Aqueous extract at 5 mg/plate inhibited 86.5 % the B[*a*]P induced mutation of TA 98 strains (Table 8.5). Ethyl acetate (2 mg/plate) and aqueous extract (5 mg/plate) treated plate in the presence and absence of S9 fraction showed clear background lawn and standard number of spontaneous revertants as that of respective strains.

8.3.3 In vitro aniline hydroxylase inhibiting activity P. rimosus extracts

Ethyl acetate and aqueous extracts of *P. rimosus* inhibited the *in vitro* aniline hydroxylase activity (Table 8.6). Ethyl acetate at 1 mg/ml and aqueous extract at 5 mg/ml inhibited the activity of aniline hydroxylase 50.6 % and 53.7 % respectively.

Table 8.1. Antimutagenic activity of ethyl acetate extract (EtOAc) of *P. rimosus* against sodium azide ($Na N_3$) induced mutations of TA 100, TA 102 and TA 1535.

Concentration (mg/plate)	Average number of revertants/plate			Percent inhibition		
	TA 100	TA 102	TA 1535	TA 100	TA 102	TA 1535
$Na N_3$ (0.002)	960 ± 53.6	320 ± 12	624 ± 17	--	--	--
$Na N_3$+ EtOAc (0.5)	833 ± 26.6	298 ± 7.2	549 ± 10	13	7.0	12
$Na N_3$+ EtOAc (1)	720 ± 17.7	242 ± 35	405 ± 14	25	24	35
$Na N_3$ + EtOAc (2)	636 ± 47.0	160 ± 7.2	209 ± 8.3	34	50	66.5

Values are mean ± S.D, n=3,

Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 100 (124 ± 4.1), TA 102 (170.5 ± 16) and TA 1535 (13 ± 2.0)

Table 8.2. Antimutagenic activity of ethyl acetate extract (EtOAc) of *P. rimosus* against *N*-methyl *N'*-nitro *N*-nitrosoguanidine (MNNG) induced mutations of TA 100 and TA 102.

Concentration (mg/plate)	Average number of revertants/plate		Percent inhibition	
	TA 100	TA 102	TA 100	TA 102
MNNG (0.001)	759 ± 31	293 ± 16	--	--
MNNG + EtOAc (0.5)	555 ± 74	194 ± 6.0	26.8	33.7
MNNG + EtOAc (1)	450 ± 18	135 ± 13	40.7	53.9
MNNG + EtOAc (2)	302 ± 21	22 ± 10	60.2	92.4

Values are mean ± S.D, n=3,

Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 100 (124 ± 14) and TA 102 (170.5 ± 16)

Table 8.3. Antimutagenic activity of ethyl acetate extract (EtOAc) of *P. rimosus* against 4-nitro-*o*-phenylenediamine (NPDA) induced mutations of TA 98 and TA 100.

Concentration (mg/plate)	Average number of revertants/plate		Percent inhibition	
	TA 98	TA 100	TA 98	TA 100
NPDA (0.020)	2483 ± 125	150 ± 5	--	--
NPDA + EtOAc (0.5)	2026 ± 64.3	127 ± 3	18.4	15.3
NPDA + EtOAc (1)	1790 ± 95.4	100 ± 6	28.0	33.3
NPDA + EtOAc (2)	883 ± 41.6	55 ± 4	64.4	63.3

Values are mean ± S.D, n=3,

Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 100 (124 ± 14) and TA 98 (29 ± 4)

Table 8.4. Antimutagenic activity of ethyl acetate extract (EtOAc) of *P. rimosus* against doxorubicin (DX) induced mutations of TA 98 and TA 102.

Concentration (mg/plate)	Average number of revertants/plate		Percent inhibition	
	TA 98	TA 102	TA 98	TA 100
DX (0.010)	245 ± 9.0	373 ± 60	--	--
DX + EtOAc (0.5)	81 ± 9.8	67 ± 9.1	67.0	82.0
DX + EtOAc (1)	55 ± 12	24 ± 2.0	77.5	93.5
DX + EtOAc (2)	28 ± 4.5	8 ± 3.5	89.0	97.8

Values are mean ± S.D, n=3,

Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 102 (240 ± 25) and TA 98 (35 ± 4)

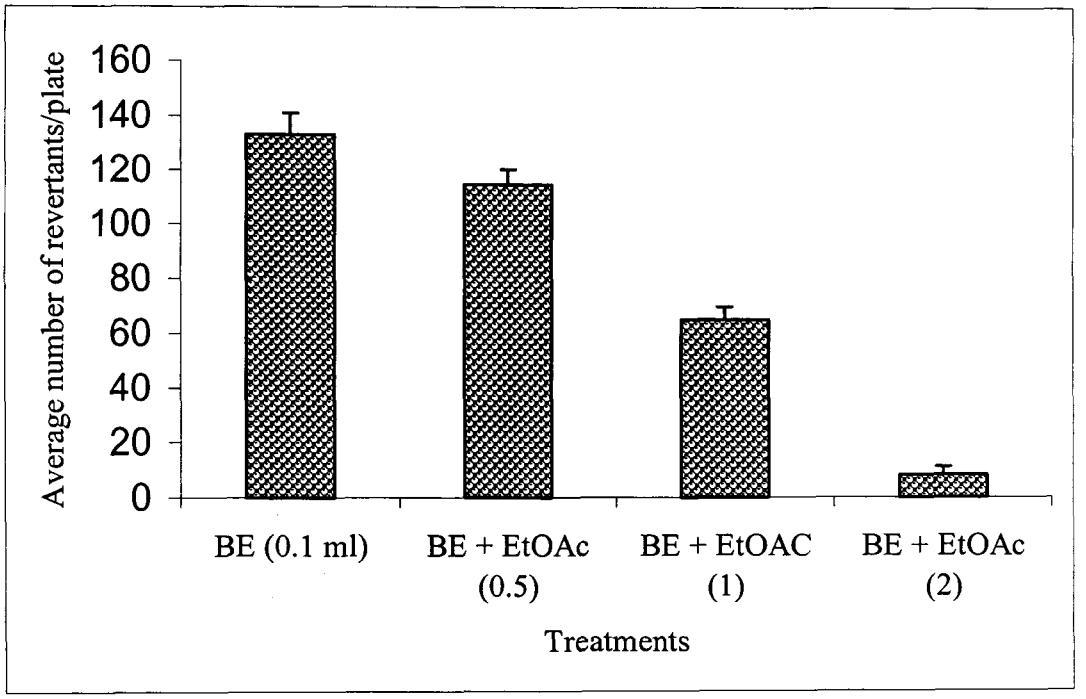

Figure 8.1. Antimutagenic activity of ethyl acetate (EtOAc) extracts of *P. rimosus* against repeated fried beef extract (BE) induced mutations of TA 102.

Values are mean ± S.D, n=3, Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 102 (235 ± 18).

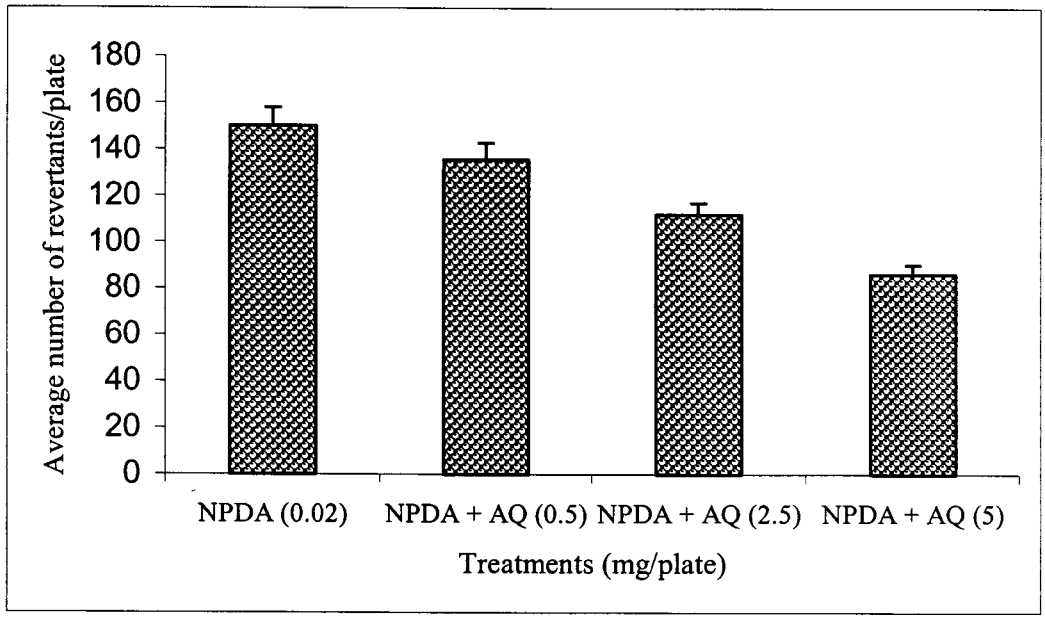

Figure 8. 2. Antimutagenic activity of aqueous (AQ) extracts of *P. rimosus* against 4-nitro-*o*-phenylenediamine (NPDA) induced mutations of TA 100.

Values are mean ± S.D, n=3, Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 100 (130 ± 13).

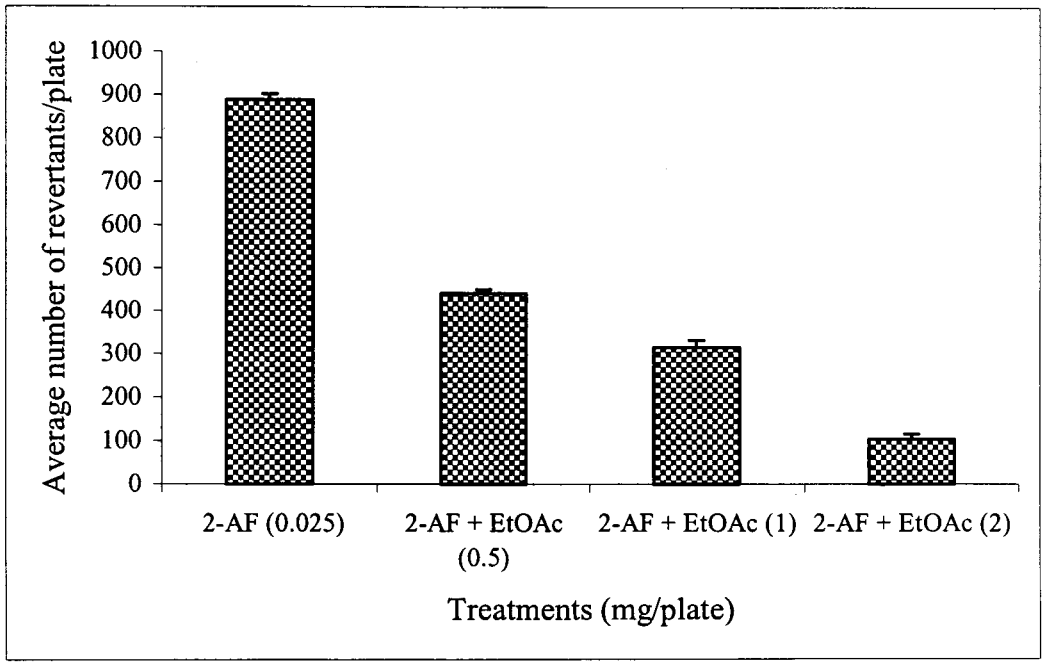

Figure 8.3. Antimutagenic activity of ethyl acetate (EtOAc) extracts of *P. rimosus* against 2-Acetamidofluorene (2-AF) induced mutations of TA 98.

Values are mean ± S.D, n=3, Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 98 (35 ± 8).

Table 8.5. Antimutagenic activity of ethyl acetate (EtOAc) and aqueous (AQ) extracts of *P. rimosus* against benzo[*a*]pyrene (B[*a*]P) induced mutations of TA 98.

Concentration (mg/plate)	Average number of revertants/plate	Percent inhibition
B[*a*]P (0.005)	71.0 ± 5.2	--
B[*a*]P + EtOAc (0.5)	54.0 ± 3.7	24.0
B[*a*]P + EtOAc (1)	23.3 ± 3.0	67.6
B[*a*]P + EtOAc (2)	8.00 ± 2.5	88.5
B[*a*]P + AQ (0.5)	49.0 ± 2.0	33.7
B[*a*]P + AQ (2.5)	38.0 ± 3.6	48.6
B[*a*]P + AQ (5)	10.0 ± 2.0	86.5

Values are mean ± S.D, n=3,

Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 98 (30 ± 2).

Table 8.6. *In vitro* aniline hydroxylase inhibiting activity of ethyl acetate (EtOAc) and aqueous (AQ) extracts of *P. rimosus*.

Groups	Treatments (mg/ml)	Aniline hydroxylase (nmol p-aniline formed/min/mg protein)	Percent inhibition
Control	Vehicle	1.58 ± 0.02	--
EtOAc	0.25	1.49 ± 0.01	5.60
	0.50	1.18 ± 0.03	25.3
	1.0	0.78 ± 0.03	50.6
AQ	1.0	1.43 ± 0.02	9.40
	2.5	1.12 ± 0.01	29.1
	5.0	0.73 ± 0.03	53.7

Values are mean ± S.D, n=2.

8.4 Discussion

Ethyl acetate and aqueous extract of *P. rimosus* showed significant inhibition of mutagenicity induced by both direct acting mutagens (NaN_3, MNNG, doxorubicin, NPDA and meat derived mutagen) and mutagen that require activation (B[*a*]P and 2-AF), in a dose dependent manner. The antimutagenic activity of the extract against direct acting 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 ethyl acetate extract showed maximum activity against the doxorubicin-induced mutation of TA 98 and TA 102. Doxorubicin and related quinoines are capable of undergoing an electron 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. Ethyl acetate extract may possibly also interfere in the intercalation of doxorubicin to DNA or scavenge the generated free radical.

Deep-fried beef and fish produces mutagenic compounds such as PAHs, heterocyclic aromatic hydrocarbons as well as *N*-nitroso compounds (Wakabayashi, 1990). Repeated frying also accelerates the rancidity reaction of fat in meat thus increase the mutagenic potential (Ames, 1983). The lipid peroxidation end products such as malondialdehyde and 4-hydroxynonenal were found to be mutagenic (Park and Floid, 1992). Among the *Salmonella* strains tested for the mutagenic effect of meat extract, TA 102 showed maximum number of revertants hence was employed for the antimutagenic activity studies of ethyl acetate extract of the *P. rimosus*. The antimutagenic activity of ethyl acetate extract might be mediated by neutralizing the meat derived oxidant muatgens. The extract obtained from fried meat prepared under normal condition not produced any mutagenicity to the *Salmonella* tester strains.

The experimental results also show that extract efficiently inhibited the B[*a*]P and 2-AF mediated mutagenesis. The microsomal fraction of rat liver containing mixed function oxidase (MFO) activate the B[*a*]P to an active mutagen benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) (Smith and Gupta, 1996).Two major adducts of BP-DNA has been noted ie. BPDE-deoxyguanosine (dG) and 9-OH-BP-dG-derived adducts (Smith and Gupta, 1996). MFO are generally inducible following exposure to various chemicals including drugs, steroids, pesticides, herbicides, food preservatives, polycyclic aromatic hydrocarbons and some of the naturally occurring constituents of

the diet. The MFO containing cytochrome P-450 along with other metabolically linked enzymes provide an important pathway whereby cells can metabolize and eliminate xenobiotics (Rastogi et al., 2002). The inhibition of B[*a*]P induced mutation by the extract might be mediated through both inhibition of the MFO system in the microsomal fraction of the liver and direct conjugation of BPDE. The effect can also attributed to the scavenging of the free radicals, which are generated during the activation of B[*a*]P by MFO (Lesko et al., 1975). Where as the inhibition of 2-AF induced mutagenicity may be mediated through the inhibition of the MFO or through the inactivation of activated 2-AF. The extract alone (2 mg ethyl acetate and 5 mg aqueous extract) treated plate did not produce any toxicity, which was evident from the background lawn. Moreover, the number of revertant colonies was similar to those of the spontaneous reversion. Hence the activity is not the consequence of the toxic effect of the extract on bacterial colony.

The ethyl acetate and aqueous extracts of *P. rimosus* inhibited the *in vitro* aniline hydroxylase activity in a dose dependent manner. The inhibition produced by ethyl acetate extract was found to be higher than the aqueous extract. The inhibition of phase I enzyme activity may also be responsible for the antimutagenic activity of these extracts.

Anticancer activity of P. rimosus

Ajith. T.A “Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrohungus, Phellinus rimosus (Berk) Pilat. ” Thesis. Amala Cancer Research Centre , University of Calicut, 2004

Chapter 9

Anticarcinogenic activity of *Phellinus rimosus*

Section I

9.1.1 Introduction

Chemoprevention is an area of cancer research that is considered to be of greatest potential for reducing mortality in particular, gastrointestinal cancers, skin, breast, lung and colon cancers (Lee et al., 1992 and Costa 1993). This intervention strategy appears helpful particularly in subjects at high risk for cancer development. The approach depends on the ability of certain chemical agents to block initiation and promotion events that occur during the process of neoplastic development. Compounds that prevent the initiational (mutational) events of neoplastic development can be expected to be chemopreventive agents.

The role of polycyclic aromatic hydrocarbons (PAH) are clearly implicated in the process of carcinogenesis especially 7,12-dimethylbenz[*a*]anthracene (DMBA) which is one of the most potent skin carcinogens known. Most of the metabolically activated PAHs are mutagenic to DNA (Miller, 1978). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a skin tumor promoter isolated from seed oil of *Croton tiglium* and has been extensively studied in DMBA induced mouse skin tumor model. Inflammation and free radicals have been associated with cancer in various tissues including skin, bladder, stomach and colon. The experimental evidences strongly suggested the role of free radical mediated tumor promotion in phorbol ester promoted papilloma on mouse skin (Lewis and Adams, 1987, Cerutti, 1985). The applications of croton oil have been shown to reduce antioxidant enzymes in both epidermal and inflammatory cells (Solanki et al., 1981). Inhibition of ROI generation can serve as an important system for the identification of agents that can inhibit oxidative DNA damage as well as tumor promotion.

Ethyl acetate extract of *P. rimosus* showed higher antioxidant, anti-inflammatory and antimutagenic activities, was selected for the antipromotional activity. The inhibition of tumor promoting activity was determined using the classic two-statge carcinogenesis model in mouse skin.

9.1.2 Materials and methods

9.1.2.1 Preparation of extract of P. rimosus

Ethyl acetate extract of *P. rimosus* was prepared as described in the section 3.2.1.

9.1.2.2 Animals

Female Balb/c mice (25 ± 2 g) were used for the study.

9.1.2.3 Determination of antipromotional activity using two-stage carcinogenesis

Female Balb/c mice were shaved on their back using surgical clippers 2 days before the experiment. Animals with complete hair growth arrest were grouped into 3 groups of eight animals each. The skin tumor was initiated with a single topical application of 390 nmol of 7,12-dimethyl benz[*a*]anthracene (DMBA) in 200 µl acetone (Mimura et al., 1994). One week after tumor initiation, the promotion was induced by topical application of 200 µl of freshly isolated croton oil (section 4.2.4) (10 % in acetone, v/v) twice weekly for 8 weeks to the same area (Verma and Boutwell, 1980 and Divan et al., 1985). The ethyl acetate extract of *P. rimosus* (1 mg and 5 mg in 200 µl acetone/mouse) was applied topically 40 minutes before each croton oil application. The group treated with croton oil alone served as positive control. Skin papilloma formation was recorded weekly in each experimental group. Average number of papilloma per mouse, percent of animals with papilloma and tumor latency period were recorded.

9.1.3 Results

9.1.3.1 Determination of antipromotional activity of ethyl acetate extract

Topical application of ethyl acetate extract inhibited skin papilloma initiated by DMBA and promoted by croton oil on mouse skin (Fig. 9.1.4). Group of animals applied with croton oil and DMBA showed 87.5 % tumor incidence at 15 weeks after DMBA treatment. Application of ethyl acetate extract of *P. rimosus* prior to croton oil reduced the percent of incidence. Topical application of extract at a dose of 1 mg showed 62.5 % incidence at 15 weeks and at a dose of 5 mg showed 37.5 % incidence at 15 weeks (Fig. 9.1.1). The percent animals with tumor in the control group of animals attained maximum at 7 week after tumor promotion by croton oil. The average number of tumor (1mm diameter) per animal in the control group was 5 nos at 7 weeks after the croton oil application, while the average number of tumor per animal in the 1 and 5 mg extract treated group of animals was 2 and 1.33 respectively (Fig. 9.1.2). The tumor latency period in the control, extract 1 and 5 mg treated groups was 39, 49 and 56 days respectively (Fig. 9.1.3).

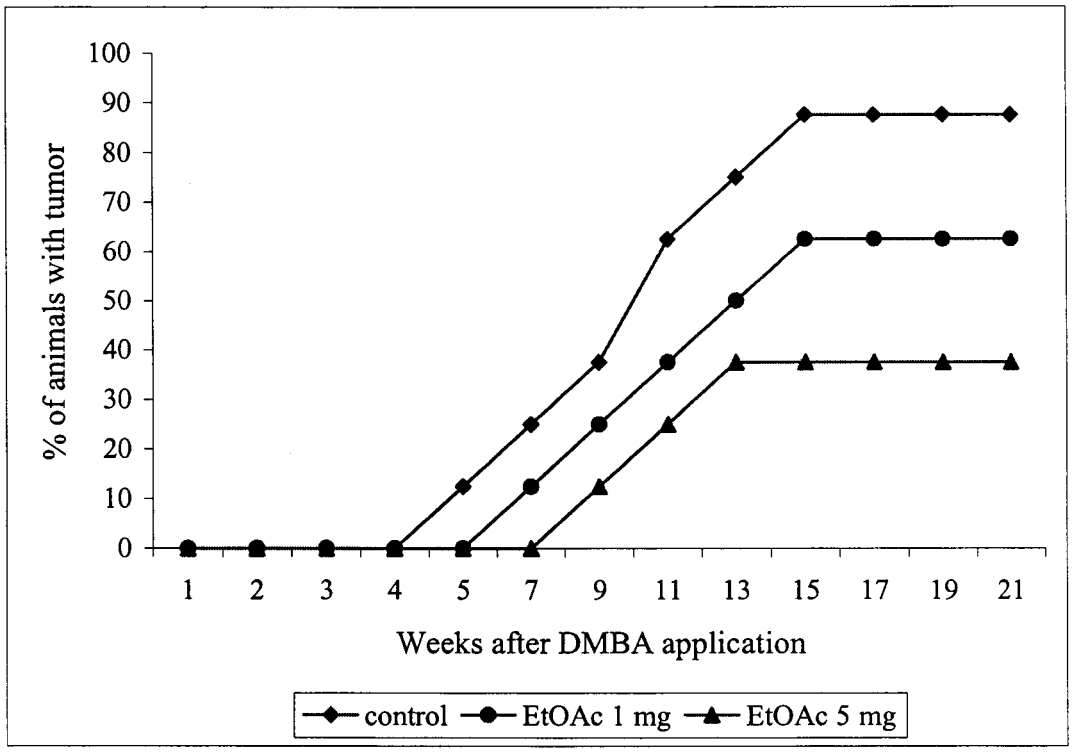

Figure 9.1.1. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on croton oil induced tumor promotion in mouse skin.

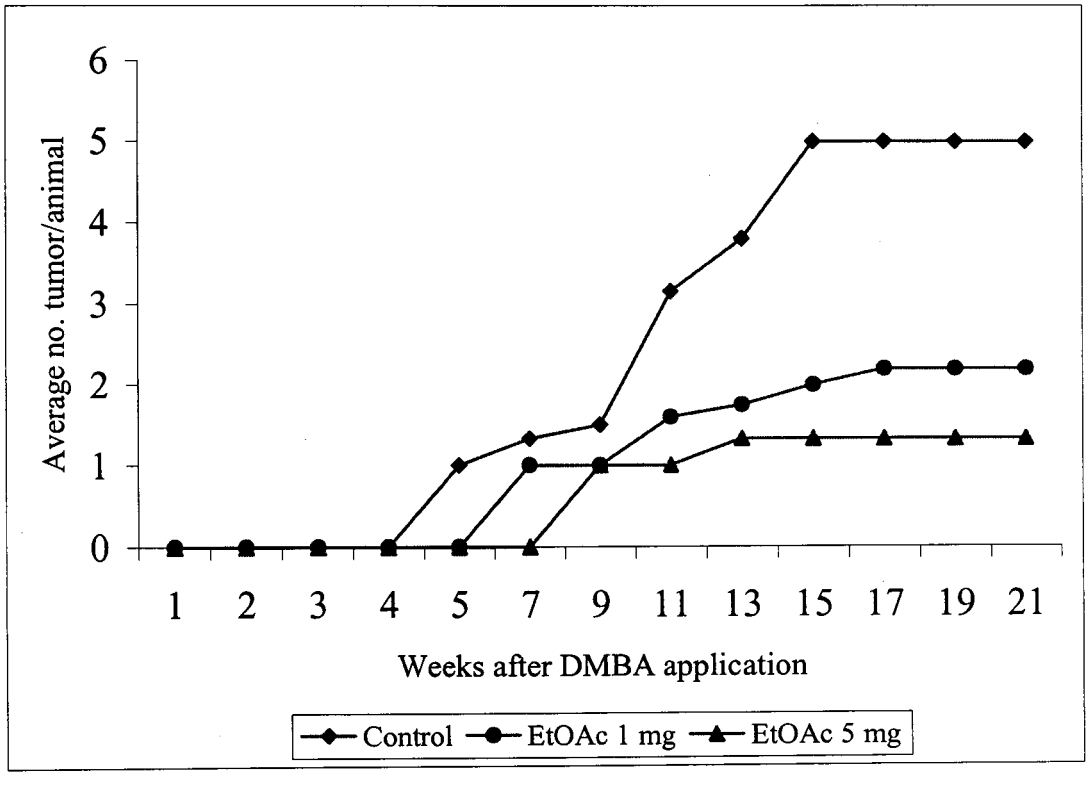

Figure 9.1.2. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on croton oil induced tumor promotion in mouse skin.

Figure 9.1.4- Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on DMBA induced and croton oil promoted skin papilloma on mice skin. a) and b) DMBA + croton oil; c) DMBA + croton oil + EtOAc (1 mg/skin) and d) DMBA + croton oil + EtOAc (5 mg/skin). (Arrowmark indicated papilloma)

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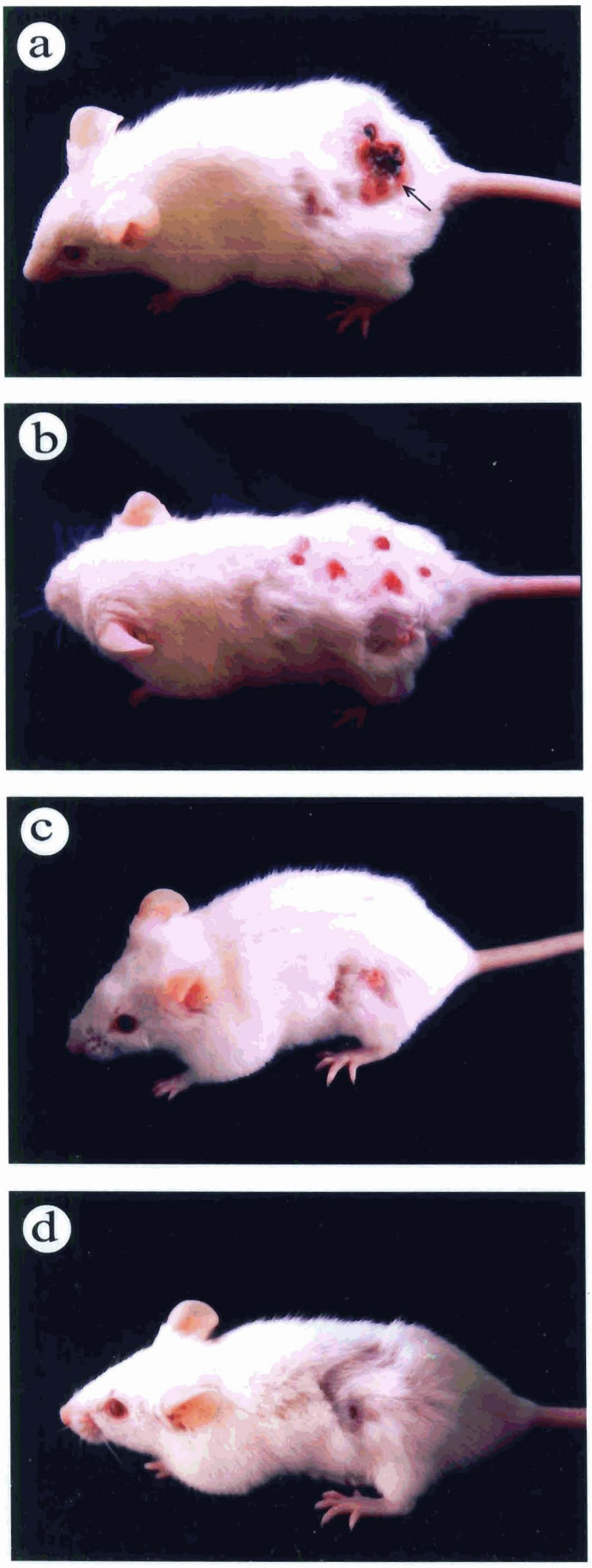


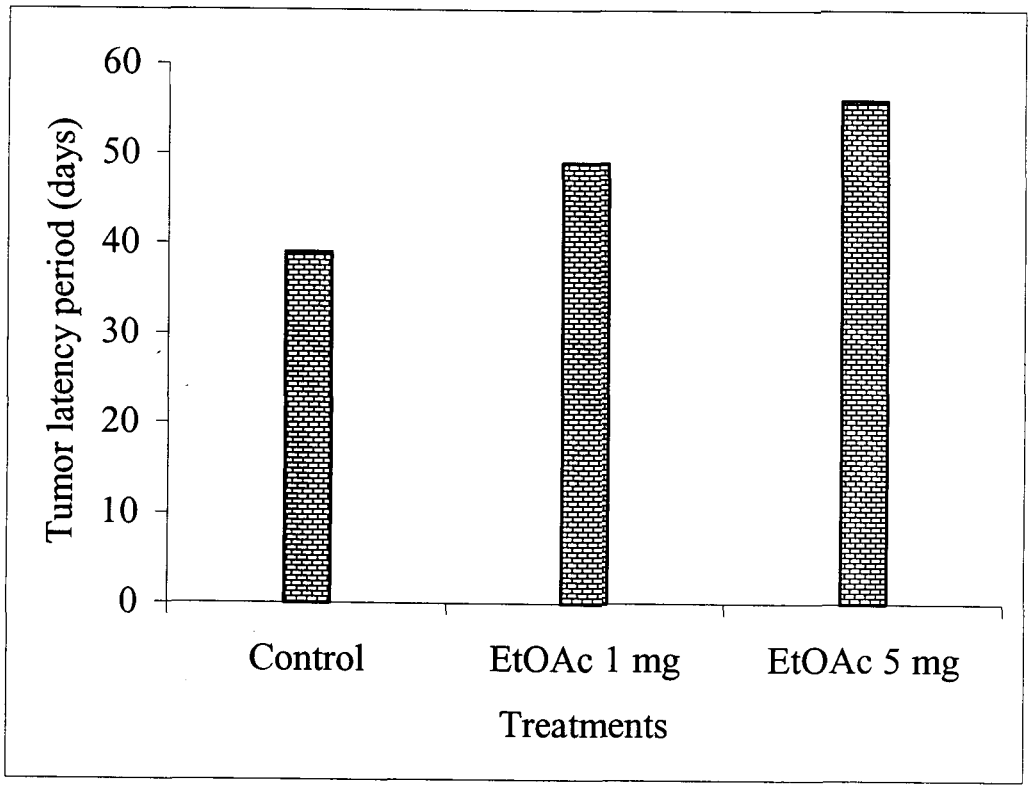


Figure 9.1.3. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on croton oil induced tumor promotion in mouse skin.

9.1.4 Discussion

Polycyclic aromatic hydrocarbon (PAH) must be metabolically activated to electrophilic intermediates, which can bind to DNA and exert their carcinogenic effects. Studies on the mutagenicity, tumorigenicity of the PAHs have indicated that bay region diol-epoxides are the ultimate carcinogenic species and the binding of these diol-epoxides to DNA can explain their carcinogeicity (Jerina and Daly, 1977; Jerina et al., 1979). Current evidence indicates that the metabolic activation of DMBA occurs primarily through the formation of a 3,4-diol-1,2-epoxide (Tang et al., 2000). ROS production by double or multiple TPA treatments is closely associated with the metabolic activation of proximate carcinogens and the increased levels of oxidized DNA bases. Persistent oxidative stress in cancer may also constantly activate transcription factors, such as NF-κB, through the intracellular signal transduction

system and induce expression of proto-oncogenes such as c-*fos*, c-*jun* and c-*myc* (Toyokuni et al., 1995). Oxidative stress induces DNA damage such as modified base products and strand breaks that may lead to further mutation and chromosomal aberration, in the single mutated clones.

Experimental results indicate that applications of ethyl acetate extract of *P. rimosus* before each application of croton oil directly scavenged the free radical or inhibited the generation of free radicals. This was evident from decreased skin lipid peroxidation induced by croton oil when pretreated with the extract (section 4.3.3). The extract also inhibited the croton oil mediated skin inflammation. Hence the antipromotional activity of extract is probably mediated through the radical scavenging activity of the extract (section 5.3.4). The tumor latency period in animals that were treated with 5 mg is extended significantly compared to the control as well as the 1 mg treated group. The average number of tumor/animal is also decreased in the extract treated groups compared to the control group. The results are indicating the efficacy of the extract in delaying the skin tumor incidence in animals.

Section II

9.2.1 Introduction

Hepatocellularcarcinoma (HCC) is one of the most common malignancies worldwide, with limited effective therapeutic options available. Chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and dietary aflatoxin B1 (AFB_1) contamination are considered as important etiological factors (Sheu et al., 1992). The absence of effective systemic chemotherapeutic agents and the mortality associated with intrahepatic, rather than metastatic growth of HCC have led many investigators to focus on developing better methods of local tumor control. Among the several group of chemical carcinogens implicated in human hepatocellularcarcinogenesis, nitrosocompounds form the largest group (Preussmann et al., 1984). The exposure to *N*-nitrosamines can be either from environment or from the diet. Majority of human cancers are currently thought to be caused by environmental factors (Doll and Peto, 1981) with food being one of the most important modifying agent (Waynder and Gobi, 1977). Cooking methods like roasting, grilling, baking and deep-frying in open furnaces, of foods, seem to increase the formation of *N*-nitrosamines. Similarly tumorigenic agents attributed to the initiation and promotion of tumor in the gas phase of cigarette smoke has also been identified as nitrosamines such as dimethylnitrosamine, diethylnitrosamine and nitropyrrolidine (Block, 1992). Heterocyclic amines in food and cigarette smoke are important exogenous source of free radical.

An alternative approach to control cancer is chemoprevention which refers to the administration of chemical agents to prevent the initiation (mutational) and promotional events that occur during the process of neoplastic development (Boone et al., 1990). Hence a chemopreventive agent exhibiting activities such as anti-inflammation, inhibition of carcinogen induced mutagenesis, inhibition of phase I enzyme activity and scavenging of free radical could play a decisive role in the inhibition of chemical carcinogenesis either at the initiation or promotion stage. Medicinal mushrooms useful against cancer are known in many countries. The anticancer activity of aqueous extract of *P. rimosus* was studied and the results are presented in this chapter.

9.2.2 Material and Methods

9.2.2.1 Preparation of extract of P. rimosus

The yield of aqueous extract was high and selected for the long-term carcinogenesis study using NDEA. The extract was prepared as described in the section 3.2.1.

9.2.2.2 Animals

Male Wistar rat (150 ± 30 g) were used for the study.

9.2.2.3 Effect of P. rimosus extract on NDEA induced hepatocellular carcinoma

Male Wistar rats were used for the experiment. HCC was induced according to the method of Jose et al., (1999) with some modifications. Animals were divided into 4 groups of 6 animals in each group. The group 1 treated with vehicle (distilled water) was maintained as normal. Group 2 treated with NDEA (4 mg/kg body wt, p.o.) for 5 days/week for 20 weeks were kept as untreated control. Group 3 and 4 were administered orally with 25 and 50 mg/kg body wt respectively with aqueous extract of *P. rimosus* 1 h prior to each NDEA administration. One week after the last dose of NDEA administration, animals were kept fasting overnight and then sacrificed. Coagulated and noncoagulated (heparin) blood were collected by heart puncture for serum and plasma respectively. Serum was used for the determination of glutamate oxaloacetate transaminase (GOT) (section 3.2.12), glutamate pyruvate transaminase (GPT) (section 3.2.13), alkaline phosphatase (ALP) (section 3.2.14), gamma glutamyl transpeptidase (GGT) (section 3.2.15), total protein (section 3.2.18), albumin (section 3.2.19), and lipid peroxidation (malondialdehyde) (section 3.2.21). Plasma was analyzed for fibrinogen (section 3.2.20).

Liver was removed and washed thoroughly in ice-cold saline and homogenate (10 %) was prepared in PBS (50 mM, pH 7). The homogenate was used for the estimation of reduced glutathione (GSH) (section 3.2.3), glutathione peroxidase (GPx) (section 3.2.6) glutathione *S*-transferase (GST) (section 3.2.7), and protein by the method as described in the section 3.2.10.

9.2.2.4 Histopathological examination

A portion of the liver was fixed in 10 % formalin and then embedded in paraffin. 5 μm microtome sections were prepared from each liver and stained with hematoxilin-eosin.

9.2.3 Results

9.2.3.1 Effect of extract on NDEA induced hepatocellular carcinoma

Aqueous extract of *P. rimosus* inhibited the NDEA induced hepatocellular carcinoma in a dose dependent manner (Fig. 9.2.2). Treatment of NDEA 5 days/week for 20 weeks induced hepatocellularcarcinoma in all the control group animals. The number of tumors and percent of incidence was reduced significantly in animals administered with the 50 mg/kg body wt extract. The activity of the SGOT (345 ± 10 IU/l), SGPT (798.1 ± 25.2 IU/l) and ALP (429.1 ± 20 IU/l) was elevated significantly ($P<0.01$) in the NDEA alone treated animals compared to the normal group of animals. Marked decline ($P<0.01$) was observed in the activities of these enzymes in group treated with the extract plus NDEA (Table 9.2.1).

The NDEA alone treated animal group also showed hyperfibrinogenemia (167.2 ± 14.5 mg/dl) compared to the extract plus NDEA treated animals 8.46 ± 0.92 g/dl (Table 9.2.2). The control animal group showed elevation of total protein (Table 9.2.2), hence the albumin/globulin ratio (Table 9.2.3) was altered ($P<0.01$) compared to the normal animal group. Treatment of the extract prevented the alteration of the A:G ratio. The activity of the serum GGT was found reduced significantly ($P<0.01$) in the extract plus NDEA treated animal group compared to the control group of animals (50 mg/kg body wt) (Table 9.2.4).

The index of lipid peroxidation, MDA was elevated ($P<0.01$) in the serum of the NDEA alone treated animal group (3.4 ± 0.2 nmol/ml) compared to the normal and *P. rimosus* treated groups (Table 9.2.4). The activities of GST (Fig. 9.2.1), GPx and GSH (Table 9.2.5) level in the liver homogenate of the extract (50 mg/kg) plus NDEA treated animals showed a significant decrease ($P<0.01$) compared to the NDEA treated group.

Histopathological analysis indicated that the NDEA alone treated liver cells were arranged mostly in solid and trabecular pattern, with cellular polymorphism, fatty infiltration, varying mitotic figures and focal necrotic changes. All these changes clearly indicated the hepatocellularcarcinoma. These pathological manifestations were decreased high to moderate level respectively in the 50 mg/kg and 25 mg/kg body wt extract treated group of animals (Fig.9.2.2).

Table 9.2.1. Effect of aqueous extract (AQ) of *P. rimosus* on serum GPT, GOT and ALP activities in rats with HCC induced by NDEA.

Groups	Treatments (mg/kg)	SGPT (IU/l)	SGOT (IU/l)	ALP (IU/l)
Normal	Vehicle	125.8 ± 10.6	71.1 ± 3.1	146.3 ± 11.5
Control (NDEA)	--	798.1 ± 25.2*	345.0 ± 10.0*	429.1 ± 20.0*
AQ + NDEA	25	442.8 ± 16.2[a]	210.3 ± 14.1[a]	247.6 ± 8.5[a]
,,	50	311.8 ± 14.7[a]	142.3 ± 9.1[a]	202.1 ± 14.7[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control.

Table 9.2.2. Effect of aqueous extract (AQ) of *P. rimosus* on serum fibrinogen and total protein levels in rats with HCC induced by NDEA

Groups	Treatments (mg/kg)	Fibrinogen (mg/dl)	Total protein (mg/dl)
Normal	Vehicle	96.6 ± 5.6	5.93 ± 0.08
Control (NDEA)	--	167.2 ± 14.5*	8.46 ± 0.92*
AQ + NDEA	25	123.0 ± 6.8[a]	7.19 ± 0.20[a]
,,	50	110.6 ±1.9[a]	6.17 ± 0.22[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control.

Table 9.2.3. Effect of aqueous extract (AQ) of *P. rimosus* on serum albumin, globulin and albumin/globulin (A/G) ratio in rats with HCC induced by NDEA

Groups	Treatments (mg/kg)	Albumin (mg/dl)	Globulin (mg/dl)	A/G (mg/dl)
Normal	Vehicle	3.16 ± 0.07	2.84 ± 0.24	1.11 ± 0.08
Control (NDEA)	--	2.78 ± 0.05*	5.67 ± 0.87*	0.49 ± 0.07*
AQ + NDEA	25	3.06 ± 0.05[a]	4.41 ± 0.76[a]	0.70 ± 0.10[a]
,,	50	3.21 ± 0.08[a]	2.95 ± 0.23[a]	1.09 ± 0.09[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control.

Table 9.2.4. Effect of aqueous extract (AQ) of *P. rimosus* on serum GGT and MDA activity in rats with HCC induced by NDEA

Groups	Treatments (mg/kg)	GGT (U/l) at 25°C	MDA (nmol/ml)
Normal	Vehicle	21.5 ± 6.09	1.46 ± 0.08
Control (NDEA)	--	70.5 ± 13.1*	3.40 ± 0.21*
AQ + NDEA	25	42.5 ± 3.67[a]	2.75 ± 0.18[a]
,,	50	31.1 ± 3.25[a]	1.90 ± 0.05[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$(Dunnett's *t*-test) significantly different from control.

Table 9.2.5. Effect of aqueous extract (AQ) of *P. rimosus* on hepatic GPx and GSH activity in rats with HCC induced by NDEA

Groups	Treatments (mg/kg)	GPx (U/l)	GSH (nmol/mg protein)
Normal	Vehicle	22.5 ± 1.8	8.40 ± 0.32
Control (NDEA)	--	36.8 ± 4.0*	11.10 ± 1.00*
AQ + NDEA	25	32.8 ± 2.6[b]	9.30 ± 0.47[a]
,,	50	24.7 ± 0.8[a]	8.55 ± 0.20[a]

Values are mean ± S.D, n=6

*$P<0.01$ (lsd) significantly different from normal.

[a]$P<0.01$ and [b] $P<0.05$ (Dunnett's *t*-test) significantly different from control.

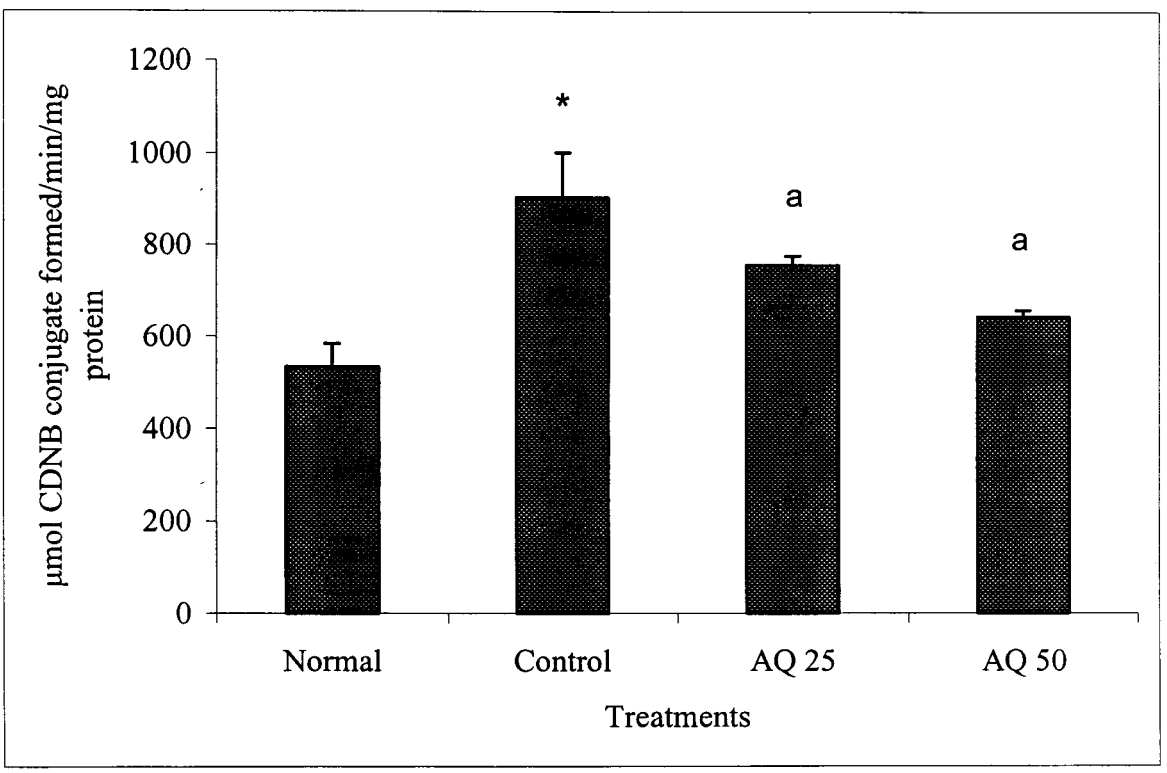
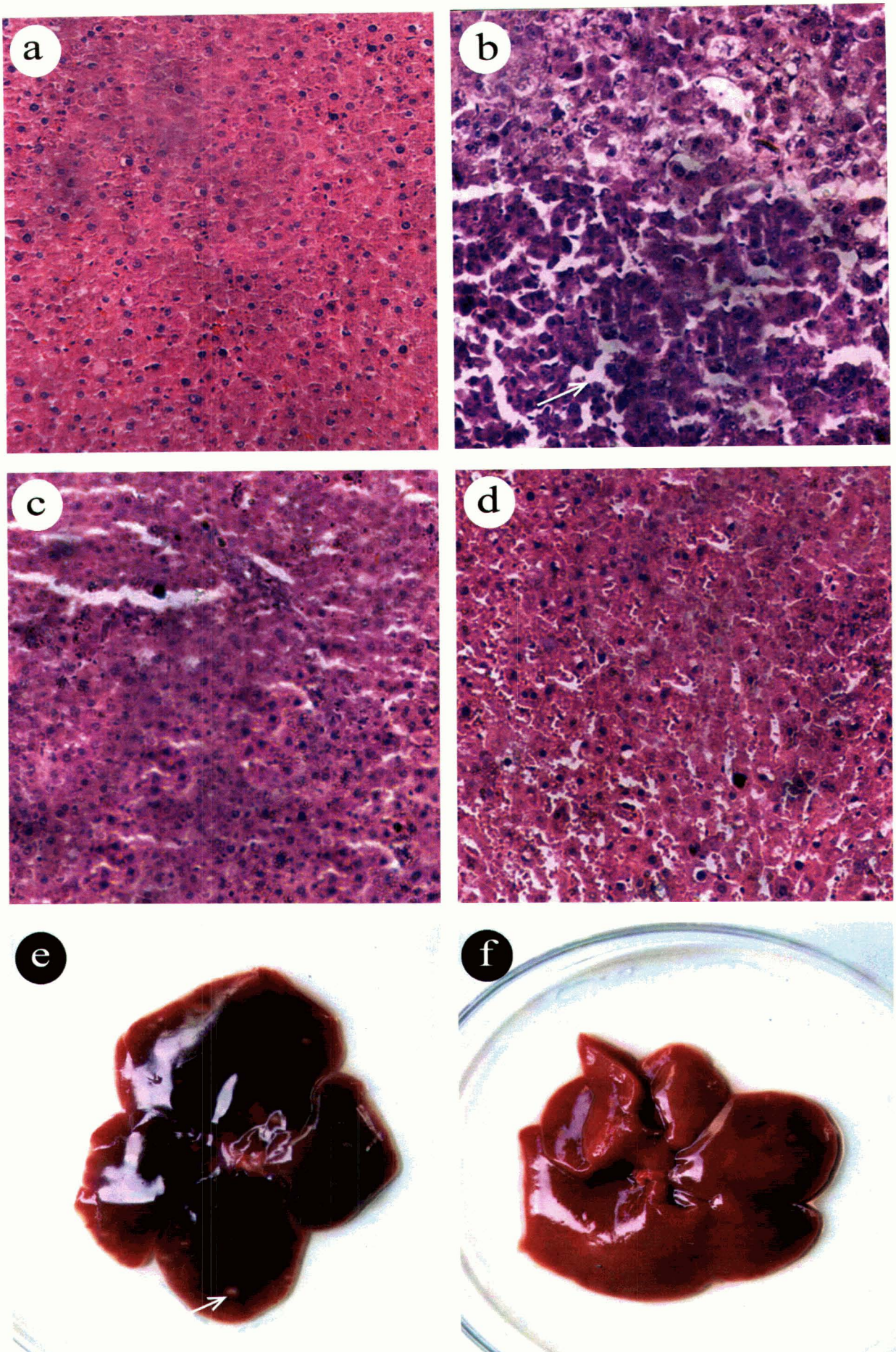


Figure 9.2.1. Effect of aqueous extract (AQ) of *P. rimosus* on hepatic GST activity in rats with HCC induced by NDEA. Values are mean ± S.D, n=6. *$P<0.01$ (lsd) significantly different from normal. [a]$P<0.01$(Dunnett's *t*-test) significantly different from control.

Figure 9.2.2- Histology of hepatocellularcarcinoma induced by NDEA in rat. (a) Normal, (b) NDEA ; (c) NDEA + aqueous extract (25 mg/kg body wt.) and (d) NDEA + aqueous extract (50 mg mg/kg body wt.). Magnification x 20. Stain H&E. (Arrowmark indicated necrotic changes).

Morphology of hepatocellularcarcinoma induced by NDEA in rat. e) NDEA applied liver and f) NDEA and aqueous extract (50 mg/kg body wt.) treated liver. (Arrowmark indicated liver tumor).


a
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9.2.4 Discussion

Results of the present investigations indicate that the aqueous extract of *P. rimosus* is an effective chemopreventive agent against the NDEA induced hepatocarcinogenesis. This conclusion is supported by various biological properties of the extract. Treatment of the extract prior to the NDEA administration significantly reduced the tumor incidence compared to the control group of animals. The serum GGT activity was significantly elevated in the NDEA alone treated group of animals indicating the induction of hepatocellularcarcinoma. However, treatment of the extract prior to NDEA showed a significant reduction of the tumor marker in a dose dependent manner. This is in agreement with elevated hepatic GST activity in the NDEA treated animal. Various hepatomas exhibited high levels of GST-P protein, as usually observed in pre-neoplastic and neoplastic lesions after chemical hepatocarcinogenesis (Satosh et al., 1991). The low level of the hepatic GST in the extract plus NDEA treated animal supports its ability to inhibit tumor progression. Further, the hepatocellularcarcinoma is associated with hyperfibrinogenemia (Amemiya et al., 1997). This was due to synthesis of this protein by the carcinoma cells. The plasma fibrinogen level decreased in the extract treated group. The elevated serum GOT, GPT, ALP and altered A:G ratios are indicative of hepatic damage in the NDEA treated animals compared to animals administered with extract prior to NDEA treatment. 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 GST, increased expressions of both γGT and GPx have been implicated in drug resistance (Tew, 1994). Decreased hepatic GPx, GST and serum γGT activity in the extract treated animal group compared to control support the efficacy of the treatment.

NDEA has been shown to metabolized by the microsomal mixed function oxidase (MFO) system to its active ethyl radical metabolites CH_3 CH_2^+. This 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 elevated MDA level in the serum of NDEA 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 prior to the NDEA administration 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. The liver of animals treated with the extract and NDEA shows a significant reduction of mitotic level and hyperplasia compared to the liver of NDEA alone treated 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 *P. rimosus* extract could also support the preventive role against NDEA 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 *P. rimosus* possesses antimutagenic and anti-inflammatory activities that may also contribute to the exhibited anticancer activity.

Phytochemical analyses of the extract show the presence of polysaccharides, protein bound polysccharides and polyphenols. A number of polysaccharides and protein bound polysaccharides isolated from mushrooms are clinically used for the treatment of cancer. Cancer preventive properties possessed by the aqueous extract of *P. rimosus* might be mediated through these active ingredients.

Toxicity studies of Phellinus rimosus

Ajith. T.A “Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrohungus, Phellinus rimosus (Berk) Pilat. ” Thesis. Amala Cancer Research Centre , University of Calicut, 2004

Chapter 10

Toxicity studies of *Phellinus rimosus*

10.1 Introduction

The importance of fungi in modern medicine was recognized long back with the discovery of pencillin. Some of the macrofungi have been used from time immemorial in folk medicine. Many pharmaceutical substances with potent and unique health-enhancing properties have been isolated from medicinal mushrooms. Most of the mushroom products are dried powders and extracts from naturally growing or commercially cultivated mushroom fruiting bodies or dried or extracted biomass of mycelium grown in a solid state or in a submerged culture. It is generally believed that many mushrooms can be safe because of their long history of usage. However, the scientific data on the safety of many of the mushroom preparations are lacking.

Clinical toxicology can be defined as the study of the clinically significant changes caused by xenobiotic and or therapeutic exposure, which are adverse in nature for the patient. Fundamentally, toxicology has two goals, identification of the tissues that are susceptible to the toxic effects of the xenobiotics and determination of the level of acute and chronic exposures that these tissues can be tolerate without clinical consequences (Parchment, 1998). Acute toxicity is usually defined as the adverse changes occurring immediately or at a short time following administration of a single exposure of substance (Walum, 1998).

A clear toxicity studies of extracts of *P. rimosus* is not reported. Present studies were carried out on the acute and sub-acute toxicity of ethyl acetate, methanol and aqueous extracts of *P. rimosus*. The findings are presented in this chapter.

10.2 Materials and Methods

10.2.1 Preparation of extracts

Extracts were prepared as described in the section 3.2.1. Ethyl acetate, methanol and aqueous extracts were employed for the studies.

10.2.2 Animals

Male Swiss albino mice weighing 25 ± 2 g were used for the study.

10.2.3 Acute toxicity study

Mice were divided into seven groups of five animals each. The drug was administered orally as a single dose as follows.

Group I	Vehicle
Group II	EtOAc 500 mg/kg body wt.
Group III	EtOAc 2500 mg/kg body wt.
Group IV	MeOH 500 mg/kg body wt.

Group V MeOH 2500 mg/kg body wt

Group VI AQ 500 mg/kg body wt.

Group VII AQ 2500 mg/kg body wt.

The animals were observed for mortality for 72 h.

10.2.4 Sub-Acute toxicity study

Mice were divided into seven groups of five animals each. The drug was administered orally once daily for 30 days.

Group I vehicle

Group II EtOAc 50 mg/kg body wt.

Group III EtOAc 250 mg/kg body wt.

Group IV MeOH 50 mg/kg body wt.

Group V MeOH 250 mg/kg body wt

Group VI AQ 50 mg/kg body wt.

Group VII AQ 250 mg/kg body wt.

The body wt changes were recorded weekly with simultaneous observation of toxic manifestation and mortality. One day after the last dose of extract administration animals were sacrificed. The blood was taken out by heart puncture. Haematological parameters such as haemoglobin (section 3.2.27), total erythrocytes count (section 3.2.28), total leukocytes count (section 3.2.29) and were determined. Serum was used for the determination of liver function test, transaminases (section 3.2.12 and 3.2.13) and alkaline phosphatase (section 3.2.14), and renal function tests such as urea (section 3.2.16) and creatinine (section 3.2.17).

10.3 Results

The animals administered with ethyl acetate, methanol and aqueous extracts of *P. rimosus* did not produce any external symptoms of toxicity or mortality up to the dose of 2500 mg/kg body wt orally. In sub-acute toxicity studies, treatment of extracts also did not produce any statistically significant change in the haematological or biochemical parameters when compared to the normal group of animals. However, treatment of ethyl acetate extract (250 mg/kg body wt.) for 30 days increased the total leukocyte count (11620 ± 940 cell/µl) compared to the normal group of animals (Table 10.1). The total leukocyte counts in other extract treated groups of animals were almost similar to those of the normal group animals. Similarly the total erythrocyte count in the aqueous extract treated (250 mg/kg) animals was slightly higher than the normal group animals.

Treatment of the extracts for 30 days did not produce any significant changes in the liver function or renal function tests compared to the normal group of animals (Table 10.2). The SGOT activities in the ethyl acetate, methanol and aqueous extracts (250 mg/kg body wt.) treated group of animals were 36.4 ± 0.9, 37.9 ± 0.8 and 36.3 ± 0.8 IU/l respectively. The SGPT activities in the ethyl acetate, methanol and aqueous extract (250 mg/kg body wt) treated group of animals were 101.4 ± 4.8 and 102.8 ± 4.1 and 97.5 ± 7.9 IU/l respectively. The ALP activities in the ethyl acetate, methanol and aqueous extract (250 mg/kg body wt) treated group of animals were 71.2 ± 12.5, 82.3 ± 6.3 and 68.9 ± 21.0 IU/l respectively.

The concentration of serum creatinine and urea are given in table 10. 3. The ethyl acetate, methanol and aqueous extracts (250 mg/kg body wt) treated group of animals showed serum urea level of 74.0 ± 7.9, 73.6 ± 8.6 and 71.0 ± 4.3 mg/dl respectively. Where as the creatinine concentration was 0.44 ± 0.03, 0.47 ± 0.05 and 0.45 ± 0.06 mg/dl respectively.

Table 10.1. Effect of ethyl acetate, methanol (MeOH) and aqueous (AQ) extracts of *P. rimosus* on total WBC, RBC counts and haemoglobin concentration.

Groups	Treatments (mg/kg)	WBC (cells/µl)	RBC 10^6 (cells/µl)	Hb (g/dl)
Normal	Vehicle	10880 ± 1490	72.5 ± 1.9	16.2 ± 1.0
EtOAc	50	10610 ± 326	69.2 ± 3.8	15.6 ± 0.5
	250	11620 ± 940	69.1 ± 5.1	16.2 ± 1.3
MeOH	50	10700 ± 787	70.0 ± 3.6	17.1 ± 0.6
	250	10310 ± 585	69.1± 3.4	15.6 ± 1.0
AQ	50	9890 ± 260	72.0 ± 1.2	16.9 ± 1.4
	250	10570 ± 901	78.6 ± 7.4	16.9 ± 1.6

Values are mean ± S.D, n=5.

Treatments are not significantly different.

Table 10.2. Effect of ethyl acetate, methanol (MeOH and aqueous extracts (AQ) of *P. rimosus* on serum GOT, GPT and ALP activities.

Groups	Treatments (mg/kg)	SGOT (IU/l)	SGPT (IU/l)	ALP (IU/l)
Normal	Vehicle	36.9 ± 2.3	101.0 ± 7.9	68.4 ± 14.6
EtOAc	50	36.9 ± 2.6	97.2 ± 8.0	70.0 ± 13.2
	250	36.4 ± 0.9	101.4 ± 4.8	71.2 ± 12.5
MeOH	50	36.3 ± 2.0	98.5 ± 5.5	67.4 ± 12.2
	250	37.9 ± 0.8	102.8 ± 4.1	82.3 ± 6.3
AQ	50	36.2 ± 1.5	96.6 ± 4.8	59.3 ± 4.0
	250	36.3 ± 0.8	97.5 ± 7.9	68.9 ± 21.0

Values are mean ± S.D, n=5

Treatments are not significantly different

Table 10. 3. Effect of ethyl acetate, methanol (MeOH) and aqueous extracts (AQ) of *P. rimosus* on serum urea and creatinine concentration.

Groups	Treatments (mg/kg)	Urea (mg/dl)	Creatinine (mg/kg)
Normal	Vehicle	72.3 ± 11.2	0.46 ± 0.03
EtOAc	50	67.5 ± 13.6	0.43 ± 0.05
	250	74.0 ± 7.9	0.44 ± 0.03
MeOH	50	62.6 ± 7.3	0.47 ± 0.01
	250	73.6 ± 8.6	0.47 ± 0.05
AQ	50	65.5 ± 8.9	0.45 ± 0.02
	250	71.0 ± 4.3	0.45 ± 0.06

Values are mean ± S.D, n=5.

Treatments are not significantly different

10.4 Discussion

Results of the study reveal that ethyl acetate, methanol and aqueous extracts of *P. rimosus* did not show any acute toxicity. The extracts administered up to 2500 mg/kg body wt. was not lethal to animals and LD_{50} could not be determined. The field of haematotoxicology includes the study of adverse effects of toxicants on mature blood cells and also the precursor cells in the haematopoitic tissues (Parchment, 1998). Ethyl acetate, methanol and aqueous extracts of *P. rimosus* did not produce any significant haematologic toxicity as evident from the normal counts of WBC and RBC. Haematologic toxicity following xenobiotic exposure can manifest clinically as leukocytosis. This can be due to abnormally high neutrophil or lymphocyte counts. Ethyl acetate extracts at 250 mg/kg body wt treated group of animals showed only a slight increase in the total leukocyte count of unknown reason. Serum transaminases

(GOT and GPT) and ALP activities are good indices of liver damage. The biochemical parameters to evaluate the liver function test indicate no significant increase in the activity of SGPT, ALP and SGOT in any of the extracts treated group of animals compared to the normal animals. A slight (statistically non-significant) increase in the ALP activity of methanol (250 mg/kg) extract treated group of animals compared to normal was observed. However, no considerable change in SGOT and SGPT activity occurred due to the treatment with the extracts. This indicated that the observed small changes in the liver function enzymes does not seem to be sufficient to support the toxicity of the extract.

The raised serum urea and creatinine concentrations have been observed with impaired renal function or acute renal failure. The extracts of *P. rimosus* did not induce any damage to kidney as evident from the normal level of urea and creatinine. Hence the current study indicated that ethyl acetate and aqueous extracts of *P. rimosus* are nontoxic to animal at the tested doses.

Summary and Conclusion

Ajith. T.A "Studies on the antioxidant and anticarcinogenic activities of a wood inhabiting macrohungus, Phellinus rimosus (Berk) Pilat. " Thesis. Amala Cancer Research Centre , University of Calicut, 2004

Chapter 11

Summary and Conclusion

Majority of human cancers are currently considered to be caused by environmental factors. Exogenous and endogenous sources of free radicals responsible for the genetic instability; have been correlated with augmented malignant potential. Hence antioxidants have significant importance in cancer chemoprevention. The *in vitro* free radical scavenging activities such as hydroxyl radical, superoxide anion and nitric oxide scavenging and lipid peroxidation inhibiting activity of ethyl acetate, methanol and aqueous extracts of *Phellinus rimosus* were studied. All the three extracts showed significant antioxidant activity. However, the superoxide anion scavenging (IC_{50}; 22 ± 1.0 µg/ml), hydroxyl radical scavenging (IC_{50}; 68 ± 4.1 µg/ml) and lipid peroxidation inhibiting (IC_{50}; 162 ± 7.0 µg/ml) activities of ethyl acetate extract was higher than the methanol and aqueous extracts. Nitric oxide scavenging and metal reducing activity of *P. rimosus* were found to be higher for the aqueous extract and methanol extract respectively.

Lipid peroxidation might be involved in tumor promotion and progression of carcinogenesis. Increased level of lipid peroxidation, including 4-hydroxynonenal and malondialdehyde are found in colon, liver, breast and kidney carcinogenesis. Investigations were carried out to evaluate the *in vivo* antiperoxidative activity of ethyl acetate extract. The extract significantly protected the ethanol induced lipid peroxidation in mouse liver, kidney and brain. Administration of the extract also protected croton oil induced lipid peroxidation on the mouse skin.

Chronic inflammation is regarded as an essential factor for the progression of the neoplastic process. Hence, the therapeutic intervention aimed at inhibiting inflammation, reducing angiogenesis and stimulating cell-mediated immune responses may have a major role in reducing the incidence of common cancers. The extracts of *P. rimosus* were, thus, evaluated for acute and chronic anti-inflammatory activity. The ethyl acetate, methanol and aqueous extracts of *P. rimosus* showed significant protection against acute inflammation induced by the carageenan and dextran and chronic inflammation induced by formalin in mouse paw edema model. The anti-inflammatory activity of the ethyl acetate extract was higher than that of methanol and aqueous extracts in all the inflammation models. The effective dose was found at 100 mg/kg. However, the activity of aqueous extract was better than the methanol extract.

Application of tumor promoting croton oil recruits inflammatory cells to the application site. Hence, anti-inflammatory activity of ethyl acetate extract against croton oil induced mouse skin edema was evaluated. The results showed that the extract

was highly effective against the inflammatory edema induced by the croton oil. Topical application of 20 mg extract showed significant anti-inflammatory activity. The effect was also found to be dose dependent. Histopathological observations supported the effective protection of the skin edema by the ethyl acetate extract.

Liver and kidney are the two important vital organs mostly affected by the drugs and xenobiotics. Oxidative stress in these organs might be one of the major etiological factors for the site specific carcinogenesis. Thus, compounds with higher antioxidant activity would be able to protect the vital organs from such chemically induced oxidative damage. The investigations were carried out to find out the protective effect of ethyl acetate extract of *P. rimosus* against CCl_4 induced acute and chronic toxicity in liver. Treatment with the ethyl acetate extract could restore the CCl_4 induced decline of the hepatic and erythrocyte antioxidant status. The extract also inhibited aniline hydroxylase (phase I enzyme) and activated GST (phase II) activity.

Majority of the anticancer drugs cause a large number of toxic manifestations to vital organs. Nephrotoxicity, is one of the dose limiting toxicities of the anticancer drugs such as cisplatin. Attempt was made to find out the protective effect of ethyl acetate extract on nephrotoxicity caused by cisplatin. Treatment of ethyl acetate extract could protect the cisplatin induced decline of the renal antioxidant status. The agents that can inhibit the nephrotoxicity produced by cisplatin without affecting its antitumor efficiency will be a welcome addition to the class of nephroprotective agents. Experimental results indicated that ethyl acetate extract of *P. rimosus* rendered complete protection against cisplatin induced nephrotoxicity without affecting the antitumor efficacy of the drug. Histopathological observation of the treated liver and kidney supported the efficacy of the hepatorenal protection by the extract.

Damage to DNA by oxygen-free radicals is frequently postulated to cause mutations that cause initiation and progression of cancers. *In vitro* antimutagenic activity of ethyl acetate and aqueous extracts of *P. rimosus* was evaluated using Ames antimutagenicity assay. The extracts were effective against the *Salmonella* tester strains carrying frame shift (TA 98) and base pair (TA 100, TA 102 and TA 1535) mutations. The animutagenic activity against the direct acting mutagens and mutagen needing activation were evaluated. The ethyl acetate extract showed highest antimutagenic activity against doxorubicin induced mutation of *S. typhimurium* tester strain TA100 (97.8 % at 2 mg/plate) and deep fried beef meat extract induced mutation of the strain TA 102 (93.7 % at 2 mg/plate). Laboratory studies of natural food stuffs and cooked

food have uncovered a variety of mutagens and possible carcinogens. Hence the antimutagenic activity of the ethyl acetate extract of *P. rimousus* against meat derived mutagens is of significant importance. The antimutaginc activity of the extracts against indirect acting mutagen also found to be significant. Ethyl acetate extract showed higher antimutagenic activity than aqueous extract. Clear background lone and spontaneous revertants of the extract alone treated plate indicated that the exhibited activity was not due to the antibacterial activity of the extracts.

Knowledge that metabolic activation is a critical determinant of tumor induction by chemicals has led to a search for non-toxic inhibitors of this enzyme mediated process. One promising approach to reduce the risk of developing chemically induced cancers might be modulating the activity of enzymes that are crucial for the metabolic activation and inactivation pathways. *In vitro* aniline hydroxylase, a P450 enzyme, inhibiting activity of ethyl acetate and aqueous extracts of *P. rimosus* were evaluated. Incubation of the extracts with the phenobarbitone induced liver microsomal fraction showed that the extracts inhibited the *in vitro* aniline hydroxylase activity in a dose dependent manner. The aniline hydroxylase activity was inhibited by ethyl acetate extract at 1mg/ml (50.6 %) and aqueous extract at 5 mg/ml (53.7 %).

In vitro cytotoxic activity of ethyl acetate, methanol and aqueous extracts of *P. rimosus* were determined using the DLA and EAC cancer cell lines. The results indicated that the activity of ethyl acetate extract was higher than that of methanol. Aqueous extract did not show cytotoxic activity up to 1 mg/ml concentration. Studies on the antitumor activity against DLA cell line induced ascites tumor in mice indicated that treatment of ethyl acetate (50 mg/kg body wt, p.o) could increase the life span 65 %. Antitumor activity against the EAC cell line induced solid tumor model showed that extracts were effective in preventing or delaying the tumor growth. The antitumor activity of the extracts (50 mg/kg body wt, p.o) was in the order of ethyl acetate > aqueous > methanol. Treatment of the extracts against the developed EAC induced solid tumor showed that activity of ethyl acetate extract was higher than the methanol and aqueous extracts. However, the antitumor activity was considerably lower than the standard reference drug, cisplatin, used in the experiment.

One of the hallmark activities of tumor promoters in animals is the ability of tumor promoters to recruit inflammatory cells to the application site and stimulate a respiratory burst in these cells. It is increasingly acknowledged that ROS and RNS play a key role in human cancer development. This hypothesis is supported by the increasing

report on the role of antioxidants in preventing or delaying the onset of some cancers. Free radicals are involved in both the initiation and promotion of multistage carcinogenesis. The croton oil promoted skin papilloma formation is mediated through the generation of free radicals from the inflammatory responses caused by the active component of croton oil i.e. 12-tetradecanoil-13-*O*-phorbolacetate. The compounds with the anti-inflammatory and antioxidant activities can effectively block the tumor promotion induced by croton oil. Hence the effect of ethyl acetate extract of *P. rimosus* on the induction of skin papilloma promoted by croton oil was evaluated. Topical application of ethyl acetate extract at a dose of 1mg and 5 mg effectively prevented the croton oil promoted skin papilloma formation in the mouse skin. Though there is no difference in the average number of tumor/animals between the two doses of extract tested, the 5 mg applied group of animals showed marked delay of tumor development (prolonged tumor latency period) and decreased the percent of incidence.

Strategies to inhibit $NO^{\bullet}$ generation during chronic inflammation or to scavenge reactive oxygen or nitrogen species may prove useful in decreasing the risk of cancer development in chronic inflammatory gastrointestinal diseases. Anticancer activity of the aqueous extract of *P. rimosus* was tested against the NDEA induced HCC in rats. Treatment of the extract (25 and 50 mg/kg, p.o) prior to the administration of the NDEA could effectively prevented the development of NDEA induced HCC. The effective dose was found to be 50 mg/kg.

The acute and sub-acute toxicity of the extracts of *P. rimosus* were studied in mice. The results indicated that all the three extract showed no acute or sub-acute toxicity. The liver function test, renal function test and hematological parameters after 30 days of drug administration showed that the tested doses (50 and 250 mg/kg) were non-toxic to the animals. However, a slight increase, though statistically insignificant, in the activity of ALP, SGPT and urea were noted in the 250 mg/kg ethyl acetate and methanol extract treated group of animals compared to the 50 mg/kg treated and normal group of animals. This suggested that the therapeutic dose should be lesser than 250 mg/kg especially for prolonged duration of treatment. The availability of drug is a critical factor for cancer treatment. Hence, the yield of the extract should be taken into consideration. The yield of the extracts were found to be in the decreasing order of aqueous > methanol > ethyl acetate.

Medicinal mushrooms produce several kinds of biologically active compounds such as polysaccharides, lectins, terpenoids, phenol etc. (Chang 1999; Mizuno, 1999

and Wasser and Weis, 1999 a,b). It was found that antitumor activity was connected with polysaccharides in most cases. The derivatives of polysaccharides and their partially hydrolyzed products were obtained from culture filtrate or by extracting the fruiting bodies or mycelia using different solvents (Babitskaya et al., 2000). Black or brown polyphenolic pigments in *Phellinus*, *Inonatus*, *Bjerkandera*, *Lentinus* genera play an important role in the antimuatgenic, anticarcinogenic, antioxidant, photoprotective, radioprotective and antibacterial activity (Gontcharova et al., 2001).

Preliminary phytochemical analysis of the extracts were done by thin layer chromatography on silica gel G using n-butanol: acetic acid: water (4:1:5 or 12:3:5), ethyl acetate: methanol: water (100:13.5:10), toluene: ethyl acetate: formic acid (60:30:10) or chloroform: methanol (90:10) as solvent systems (Harbone, 1973). The chromatogram was examined under u.v and also developed by the following reagents: $FeCl_3$: $K_3Fe(CN)_6$ (1:1), 1% alcoholic $FeCl_3$, vanillin-H_2SO_4, acetic anhydride and H_2SO_4 and 10% alcoholic KOH (quinones) (Wagner et al., 1984). Polysaccharide was detected by anthrone test (Yemm and Wills, 1954) and phenol-sulphuric acid reaction (Dubois et al., 1956). Yellow-muddy fluorescence spot and blue color with $FeCl_3$: $K_3Fe(CN)_6$ or 1% alcoholic $FeCl_3$ indicated the presence of polyphenol in the ethyl acetate and methanol extracts. Red color after alcoholic KOH treatment indicated the presence of quinones. Red colored or blue colored spots with vanillin-H_2SO_4 indicated the possibility of either polyphenols or flavonoids presents in the ethyl acetate and methanol extracts. A positive acetic anhydride-H_2SO_4 test indicated that terpenes might be present in the ethyl acetate extract. The aqueous extract responded to anthrone test and phenol-sulphuric acid reaction indicated the presence of polysaccharide. However, the ethyl acetate and methanol extracts did not respond to anthrone test and phenol-sulphuric acid reaction, which indicated the absence of polysaccharide components in this fraction.

Conclusion

The experimental findings indicate that ethyl acetate, methanol and aqueous extracts of *P. rimosus* possessed significant antioxidant, anti-inflammatory and antitumor activity. Ethyl acetate, and methanol extracts possessed *in vitro* cytotoxic activity. Ethyl acetate extract exhibited heptorenal protective activity. The activity can be correlated to the antioxidant activity of the extract. Ethyl acetate extract showed antipromotional activity. The activity can be traced partially due to the antiperoxidative and anti-inflammatory effect of the extract. Ethyl acetate and aqueous extracts

possessed marked antimutagenic activity. The activity might be due to the antioxidant and inhibition of phase I enzyme activity of the extracts. The aqueous extract showed profound effect on hepatocellularcarcinoma. The activity might be due to the antioxidant, anti-inflammatory and antimutagenic activity of the extract.

The results of the investigation indicate that extracts of *P. rimosus* especially, the ethyl acetate extract have significant medicinal properties. The findings, suggest the potential therapeutic use of this mushroom in cancer chemoprevention and treatments.

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List of Publications

1. Ajith, T. A. and Janardhanan, K. K (2001). Antioxidant and anti-inflammatory activities of methanol extract of *Phellinus rimosus*, *Ind. J. Exp. Biol.* 39, 1166-69.

2. Ajith, T.A., Jose, N and Janardhanan, K.K (2002). Amelioration of cisplatin induced nephrotoxicity in mice by ethyl acetate extract of a polypore fungus, *phellinus rimosus*, *J. Exp. Clin. Cancer Res.*,21, 487-91.

3. Ajith, T.A. and Janardhanan, K.K (2002). Antioxidant and antihepatotoxic activities of *Phellinus rimosus* (Berk) Pilat, *J. Ethnopharmacol.* 81, 387-391.

4. Ajith, T.A. and Janardhanan, K.K (2003). Cytotoxic and antitumor activities of a polypore macrofungus, *Phellinus rimosus*. *J. Ethnopharmacol.* 84, 157-162.