ANTIMUTAGENIC AND ANTICARCINOGENIC ACTIVITIES OF SELECTED MUSHROOMS FROM KERALA

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CERTIFICATE

I certify that the thesis entitled ***"Antimutagenic and Anticarcinogenic Activities of Selected Mushrooms from Kerala"*** *submitted to Calicut university, Calicut in partial fulfillment of the requirement for the award of Doctor of Philosophy in Microbiology (Faculty of Health Science) is an authentic account of the work carried out by Lakshmi. B under my supervision and guidance, and no part of this 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 here by declare that the thesis entitled " ***Antimutagenic and Anticarcinogenic Activities of Selected Mushrooms from Kerala****" submitted to Calicut university, Calicut is a bonafide record of research work done by me under the supervision of Dr. K.K. Janardhanan, Professor, Amala Cancer Research Centre, Amala Nagar, Thrissur for the award of Doctor of Philosophy and no part of this has been presented for any other degree, fellowship or titles of any university or society.*

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LAKSHMI. B

LIST OF ABBREVIATIONS

2-AF	:	2-Aminoflourene
AAPH	:	2.2'Azobis(2-amidopropane)dihydrochloride
ABTS	:	2,2-azobis-3-ethylbenzthiazoline–6- sulphonic acid diammonium salt.
AEAC	:	Ascorbic acid equivalent antioxidant capacity
ALP	:	Alkaline phosphatase
B [*a*] P	:	Benzo [*a*] pyrene
BHT	:	Butylated hydroxytoluene
CDNB	:	1-Chloro-2,4-dinitrobenzene
DMBA	:	7,12-dimethyl benz[*a*] anthracene
DMSO	:	Dimethyl sulphoxide
DPPH	:	1,1 –diphenyl 2- picryl hydrazyl
DTNB	:	5,5-Dithiobis-(2-nitrobenzoic acid)
EDTA	:	Ethylene diamine tetra acetic acid
GGT	:	γ-Glutamyl transpeptidase
GL	:	*Ganoderma lucidum*
GOT	:	Glutamate oxaloacetate transaminase
GPT	:	Glutamate pyruvate transaminase
GPX	:	Glutathione peroxidase
GSH	:	Reduced glutathione
GST	:	Glutathione- S-transferase
H_2O_2	:	Hydrogen peroxide
Hb	:	Haemoglobin
KCl	:	Potassium chloride
LP	:	Lipid peroxidation
MDA	:	Malondialdehyde
MeOH	:	Methanol

MFO	:	Mixed function oxidase
$MgCl_2$	:	Magnesium chloride
MNNG	:	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
NADPH	:	Reduced nicotinamide adeninedinucleotide phosphate(disodium)
NaN_3	:	Sodium azide
NBT	:	Nitroblue tetrazolium
NPD	:	4-Nitro-*o*-phenylene diamene
NDEA	:	*N*-Nitrosodiethyamine
O_2^-	:	Superoxide anion radical
OH^-	:	Hydroxyl radical
PAH	:	Polycyclic aromatic hydrocarbon
PBS	:	Phosphate buffered saline
PF	:	*Pleurotus florida*
ROI	:	Reactive oxygen intermediate
ROS	:	Reactive oxygen species
SDS	:	Sodium dodecyl sulphate
SGOT	:	Serum Glutamate oxaloacetate transaminase
SGPT	:	Serum Glutamate pyruvate transaminase
TBA	:	Thiobarbituric acid
TCA	:	Trichloroacetic acid
TPA	:	12-*O*-tetradecanoylphorbol-13-acetate
TPTZ	:	2,4,6-tripyridyl- s-triazine
WBC	:	White blood cell

CONTENTS

CHAPTER-1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 INTRODUCTION

Sudden heritable changes in the genetic material are called mutations. Mutations may occur spontaneously or may be induced by agents that interact with DNA and RNA. Some level of mutation is required for evolution. However, some mutations are detrimental. For many years, it has been recognized that most of the strongly mutagenic agents, such as ionizing radiations, ultraviolet light and chemicals that are carcinogenic, induce cancers. The observed correlation between mutagenicity and carcinogenicity is consistent with the theory that cancer is caused by somatic mutations.

Cancer is a disease of misguided cells, which have high potential of excessive proliferation without apparent relation to the physiological demand of the organ involved. It is a multifactorial, multistage and multimechanistic complex process. It is a genetic disease resulting from an accumulation of genetic abnormalities in various regulatory genes. Genomic instability is an eminent feature in the progression of a normal somatic cell to a transformed cancer cell . Cancer is a major threat to human in industrialized countries, with about one third to a one quarter of population dying of this disease (Fraumeni *et al.*, 1993). Genetic changes are often initiated by damage to DNA and a variety of environmental agents including X rays, UV light, chemicals etc are capable of altering DNA (Ames, 1989). Reactive oxygen species (ROS) are generated as by products of normal cellular metabolism. ROS and various aldehydes can damage DNA (Lendon *et al.*, 1988). The interactions between ROS and DNA may lead to mutation (Weitzman and Stossel, 1981) such as base substitution, deletion, rearrangements and insertion as well as sister chromatid exchange and chromosomal aberration. The genotoxic and carcinogenic properties of ROS are well documented (Ames, 1989., Ames and Gold, 1991., Breimer, 1990) and they appear to play an important role in tumor promotion .

Tumor initiation begins in cells through the mutation from exposure to carcinogens. These mutated cells may have an altered responsiveness to their microenvironment and a selective growth advantage compared with surrounding

normal cells. Tumor promotion results in further selective clonal expansion and proliferation of initiated cells thereby entering the probability of additional genetic damage through endogenous mutation or DNA damaging agents. During tumor progression malignant cells continue to exhibit progressive phenotypic changes and genomic instability including gene amplification chromosomal aberration and altered gene expression. It has become clear that cancer develop through the accumulation of various genetic alterations, i.e., a series of tumor suppressor genes are inactivated by mutation and chromosomal deletions.

Cancer treatment has evolved greatly in last decades when improved therapies has managed to increase in survival rates to >50% of diagnosed patients (De Vita, 1993). Besides, a much better knowledge of natural history of many types of cancer and the establishment of different novel strategies based on it, are still the most efficient ways for treatment than the classical methods such as surgery, radiotherapy and chemotherapy. It has been proposed that the clinical efficacy in cancer prevention and therapy depends on their ability to modulate cell growth differentiation and apoptosis in premalignant and malignant cells by regulating gene expression (De Luce, 1991.,1994., Lotan, 1995).

Most commonly used antineoplasic drugs produce toxicity in organs composed of self-renewing cell population such as bone marrow (Hoaglan, 1992) and gastrointestinal tract epithelium (Mitchel and Schein, 1992). Infertility can also follow chemotherapy (Mc Inness and Schilsky, 1996). Some antitumor agents are associated with more toxic effects such as cardiomyopathy (doxorubicin) (Von Hoff *et al.*, 1979), nephrotoxicity (cisplatin) (Blachely and Hill, 1981) and pulmonary fibrosis (bleomycin) (Comil, 1992). A major challenge for medical oncology is to develop therapeutic modalities that will prevent toxicity induced by antineoplastic treatments without impairing their antitumor effect. Different strategies have been developed previously to prevent toxicity of anticancer drugs. Some of them were based on thiol compounds. The concept of cancer intervention entails preventing, delaying or reversing any or all stages of cancer development by applying synthetic or natural compounds.

Traditionally cancer chemoprevention has been defined as a process facilitated by blocking induction of neoplastic process or preventing transformed cells from progressing to malignant phenotypes (Sporn *et al.*, 1976., Sporn and Hong, 1997). It may also involve reversal of progression. Chemopreventive agents may be consumed as part of diet or administered as pharmaceutical preparations. In principle, interventional agents can be introduced to enhance physiological process thereby protecting human against preneoplastic cell progression or neoplastic cell growth. Adequate therapeutic interventions may reduce the overall toxicity mediated by classical chemotherapeutic agents and thereby improve their efficacy.

Mushrooms have been reported to have significant medicinal properties. Attempts are being made to exploit mushrooms and their metabolites for the prevention and treatment of several diseases including cancer, diabetes etc. Various components of mushrooms are effective against tumor, thrombosis, hypertension, diabetes, and prevention of osteoporosis. Free radicals such as super oxide, nitric oxide, and their adducts peroxynitrite, hydrogen peroxide, hydroxyl radical, have been implicated in the causation of several diseases such as cancer (Halliwell and Gutteridge, 1985) and also in the pathophsyiology of various clinical disorder including cirrhosis of liver, arteriosclerosis, ischemia, reperfusion injury, myocardial infraction, rheumatoid arthritis, neurodegentative diseases, acute hypertension, hemorrhagic shock and diabetes mellitus (Henmani and Parihar, 1998). Hence, compounds that have the property to inhibit deleterious effects of reactive oxygen species are of significant importance in therapeutic intervention.

Oyster mushrooms (species of the genus *Pleurotus*) are excellently edible, nutritious and rank second among the commercially cultivated mushrooms in the world (Chang, 1999). In Chinese folklore, fruit bodies of *Ganoderma* have been regarded as a panacea for all types of diseases. The mushroom is highly valued in China and Japan for its medicinal properties.

The present investigations were undertaken to evaluate the antioxidant, antimutagenic, radioprotective and anticarcinogenic activities of two medicinal mushrooms, *Pleurotus florida* and *Ganoderma lucidum* occurring in South India. The findings derived from the investigations are reported in this dissertation.

1.2 REVIEW OF LITERATURE

1.2.1 Mutation

Mutation refers to a random event in which a change in the genetic make up or phenotype occurs. The process leading to mutation is called mutagenesis. Mutagenesis can be induced by chemical, physical or biological agents. Sometimes mutations arise spontaneously (Freifielder, 1988).

A mutation that involves only single base replacement is called as point mutation. Reverse mutation changes an altered nucleotide sequence back to its original arrangement (Watson *et al.*, 1987). The phenotype effect of point mutations can sometimes be reversed by a second mutation in a different gene, which is called suppressor mutation (De Robertis and De Robertis, 1980). An alteration that changes a codon specific for one amino acid to a codon specific for another amino acid is called as a missense mutation (Watson *et al.*, 1987). Non-sense mutation arises when a codon coding for amino acid is changed into a chain termination codon (UGA, UAA or UAG) (De Robertis and De Robertis, 1980). Many natural mutations destroy the function of a gene. These drastic changes are called null mutations (Watson *et al.*, 1987). Mutations that occur under all conditions are called as absolute defective mutation and those, which occur only at particular condition, are called as conditional mutation (Freifielder, 1988). An addition or deletion of one or a few base pairs that changes the transition reading frame within a protein coding sequence that completely disrupts its synthesis is often called as frame shift mutations (Watson *et al.*, 1987). When an amino acid substitution has no detectable effect on phenotype, it is called silent mutation and a base change without an amino acid alteration is a silent mutation (Freifielder, 1988).

1.2.2 Classification Of Mutagens

1.2.2.1 Physical Mutagens

Major source of physical mutation is various types of radiations. The radiation of the electromagnetic spectrum for which carcinogenicity has been established, includes ultraviolet rays and ionizing rays (Roentgen rays and gamma

rays). Other ionizing radiations with carcinogenic potency include the particulate radiation (alpha particles, beta particles, neutrons and protons).

Absorption of energy from electromagnetic radiation of broad wavelength spectrum results in a number of different type of DNA damage (Freifielder, 1965., Hariharan and Cerulte, 1972). For example, low linear energy transfer (LET) radiation such as gamma and X-rays cause strand breaks (McGrath and Williams, 1966) and ultraviolet radiation induces pyrimidine dimers (Wacker, 1963). These rays cause apurinic sites with consequent break down of DNA and the formation of free radicals, which can cause DNA break leading to somatic mutation. Formations of free radicals cause thirty different DNA adducts as well as DNA-protein cross links (Feig *et al.*, 1994).

Ultraviolet radiation catalyzes the formation of pyrimidine cyclobutane dimers (Beukers and Berends, 1960) and 6-4 photoproducts both of which are formed between adjacent thymine bases 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, which have marked susceptibility to melanoma and non-melanoma skin cancer. Skin cancer is the commonest type of cancer in the United States. The melanoma rates and mortality level have increased dramatically during the last 40 years.

1.2.2.2 Chemical Mutagens

Hundreds of chemicals are now known to have slight to very large mutagenic effects. The first chemical mutagen discovered was mustard gas (sulfur mustard). Chemical mutagens that transfer alkyl (CH^{-}_{3}, CH_3 CH_2^{-}, etc) groups to the bases in DNA are called alkylating agents. Chemical mutagens can be divided into two classes.

a. Chemicals, mutagenic to both replicating and non-replicating DNA, such as the alkylating agents and nitrous acid.

b. Chemicals, mutagenic only to replicating DNA, such as the acridine dyes and base analogs (Gardner, 1999).

1.2.2.3 Direct Acting Mutagens and Carcinogens

These are ultimate mutagens and carcinogens, and include for example alkylating and acylating agents. Alkylating agents are ubiquitous class of genotoxicants of environmental significance. Their most important representative being N-nitrosocompounds. Apart from being widespread in the environment, food (alcoholic beverages, tobacco and tobacco smoke), N-nitrosocompounds can also be formed in the human body from suitable precursors. They therefore constitute a class of genotoxic agents to which human are extensively exposed and they may contribute to etiology of human cancer (Bartsch and Montesano, 1984). They include the tobacco-specific nitrosoamines such as 4-(N-Nitrosomethyl amino 1-3-pyridyl)-1-butanone, N-Nitosomethyl amino propionitrile and N-Nitrosonornicotine. They can induce cancer in the lung. Some N-nitrosocompounds and alkylating agents are used as chemotherapeutic agents in the treatment of cancer. Examples are nitrosourea such as methyl-CCNU, bis chloroethyl nitrosourea (BCNU) and CCNU. However they can induce secondary cancer in treated patients.

Metals are known to be carcinogenic to humans. Important of this group include arsenic and arsenic compounds, chromium, nickel, cadmium and beryllium. They can induce the development of lung and prostrate cancers.

1.2.2.4 Precursor Carcinogens and Mutagens

Precursor carcinogens and mutagens include carcinogens and mutagens such as polycyclic aromatic and heterocyclic hydrocarbons, N-nitrosoamines, and N-nitrosoamides, nitroaryl and furan compounds, azodyes, alkyltriazines, dialkylhydrazines, and naturally occurring compounds such as aflatoxin, and pyrrolizidine compounds. If precursor carcinogens and mutagens are not converted into ultimate carcinogens and mutagens in the organism, they are non carcinogenic and non mutagenic to that organism (Miller and Miller, 1976).

1.2.2.5 Activation of Precursor Mutagens or Carcinogens

Precursor mutagens and carcinogens can be converted into ultimate reactants, depending on the presence of activating enzymes. The reactions are in most cases oxygenation reactions, catalyzed by membrane bound microsomal oxygenases. Some of the reactive intermediates catalyzed by the monooxygenase system are, for example, arylhydrocarbon epoxides, aryl halide epoxides, aliphatic halide epoxides and aliphatic epoxides. In a study of the ability of liver microsomes to metabolize benzo [*a*] pyrene (B [*a*] P) to reactive intermediates that bind to DNA. BP 7,8-dihydrol was the substrate that was metabolized most extensively to DNA-bound products. The two diastereoisomers possible for BP7, 8-diol-9, 10-epoxide are of interest because of their larger chemical reactivity. These two diol epoxide are highly mutagenic toward bacterial and mammalian cells (Conney, 1982)

1.2.2.6 Deactivation of Precursor Mutagens, Ultimate Mutagens or Carcinogens

Some enzyme-mediated reactions are directed towards deactivation of precursor carcinogens and mutagens, for example important reductive reactions, such as the reduction of the azogroup of aminoazo compounds, which yields two monocyclic amines, may be effective in detoxification steps. Primary and secondary amine may undergo conjugation reaction to yield N-glucuronide.

Mammalian tissue contain two enzymes systems, the glutathione-S-transferases and the epoxide hydrates, that are especially efficient in scavenging electrophillic compounds, thereby protecting cellular macromolecules from attack by carcinogens. The epoxide hydrates catalyse the hydrolysis of electrophillic epoxides, thereby producing diols. These reactions generally result in deactivation of reactive epoxides.

1.2.2.7 Biological Mutagens

1.2.2.7.1 Oncogenic Viruses

Another etiological factor of carcinogenesis is the integration of viral genes into the host DNA. The viral gene become part of the cellular DNA. The drive for multiplication by the virus genome overrules the regulatory checks and balances

of the cellular mechanism. Therefore, there is uncontrolled multiplication of the cells. This is called transformation by oncogenic virus. Viral oncogenes are known to cause mutation. Although a large number of DNA and RNA viruses are found to be onocogenic, only a few have been linked with human cancer such as human-T-cells leukemia virus (HTLV-1) causing T-cell leukemia or lymphoma that is endemic in certain parts of Japan.

1.2.3 Mutagens and Carcinogens In Food

A variety of carcinogenic and mutagenic substances occurs in the diet. The advent of rapid assays for mutagenicity has spurred the identification of many such compounds in food materials. Some of these substances occur naturally in the diet, where as others result from food additives, preparation and processing procedures, pesticide residues, environmental pollution and fungal contamination (Ames, 1983., Carr, 1985). Food mutagens, such as heterocyclic amines (HCAs), polycyclic aromatic hydrocarbons (PAHs) and N-nitroso compounds, play a key role in gastric carcinogenesis (Taj and Nagarajan 1996). Food preparation and preservation are major sources of dietary carcinogens, including hetrocyclic amines (HCA), formed during frying, broiling and grilling high protein foods and more prevalent in well-done meats; polycyclic aromatic hydrocarbons (PAH), formed during broiling and smoking food and N-nitrosocompounds (NOC), formed in smoked, salted, and pickled foods cured with nitrate or nitrite. (Zheng *et al.*, 1998., Skog *et al.,* 1998). A survey of mutagen formation during the cooking of a variety of protein rich foods that are minor sources of protein intake in the American diet is reported. Milk cheese, tofu and organ meats showed negligible mutagen formation except following high-temperature cooking for long periods. Naturally, occurring carcinogens include tannins found in the herbal teas (Korpassy, 1961), hydrazine's found in edible mushrooms (Toth, 1979) and safrole and related natural alkyl benzenes found in flavoring agents and spices (Miller *et al.*, 1979).

Fungal contamination of stored food can produce potent carcinogenic mycotoxins such as aflatoxins. Other notable sources of carcinogens in the human diet are the nitrosoamines, derived from the interaction of nitrite with secondary or tertiary amines. Rodents fed long-term diets containing mutagenic heterocyclic

amines consistent developed multiple tumors in the lung, liver, small and large intestine and colon. The mutagen caffeine may act through inhibition of DNA repair process. Individual exposure to dietary carcinogens and mutagens are variable in terms of dose, frequency and duration.

1.2.4 Carcinogenesis

Carcinogenesis can be roughly divided into stages of initiation, promotion and progression (Pitot, 1989). The initiation stage begins with exposure to carcinogens. Carcinogens are metabolized by host enzymes, generally resulting in addition of oxygen and /or conjugation with polar groups to facilitate excretion. During the process, reactive intermediates are formed. These intermediates can interact with cellular macromolecules such as DNA, RNA and protein to form covalently bound structures referred to as adducts. DNA adducts are important in initiation because they may cause mutations that activate oncogenes or inactivate tumor suppressor genes. This can result in uncontrolled growth. The clonal expansion of initiated cells is called promotion. During promotion, initiated cells expand to preneoplastic cells and visible tumors appear. In the progression stage, tumors that are essentially benign progress to metastasing and rapid growing neoplasms. The progression stage may involve multiple genetic damages caused by continuing carcinogen exposure. (Fig 1.1)

1.2.4.1 Relationship Between Mutagenesis And Carcinogenesis.

For many years, it has been recognized that most of the strongly mutagenic agents, such as ionizing radiation, ultraviolet light and chemicals can induce cancers in humans. The observed correlation between mutagenicity and carcinogenicity is consistent with the theory that cancer is caused by somatic mutations. This has received strong support from the discovery of cellular oncogenes and the demonstration that the oncogene responsible for human bladder carcinoma resulted from a single base pair change in its normal cellular counter part. Three major classes of genes that are linked to be molecular targets for development of neoplasia., proto-oncogenes (Temin, 1974), cellular oncogenes (Garrett, 1986), and tumor suppressor gene (Barrett, 1993).

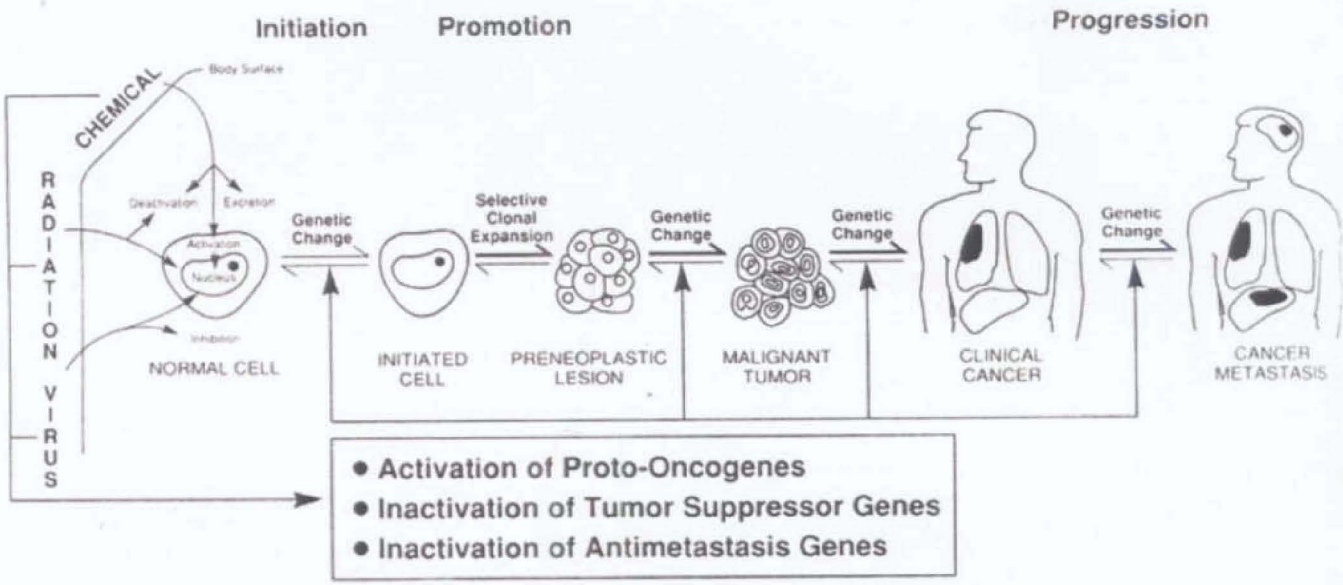


Fig.1.1 MULTISTAGE CARCINOGENESIS

Proto-oncogenes are those having a counter part within the genome of an oncogenic virus usually retrovirus. Today, more than 100 human proto-oncogene are known. They were located on specific chromosomes. Cellular oncogenes are genes that have been shown to be capable of inducing neoplastic transformation under the specific circumstances, but they have no viral counterpart. They occur normally, within the cell, but activated during mutagenesis, either that of a simple transition, transversion, deletion or in other instances. The mutational alteration of tumor suppressor genes leads to complete loss of function or to a change in function as such its activity suppressing the neoplastic phenotype is drastically altered. In human, mutation in *p53* tumor suppressor gene, appear to be related with neoplasm development. All cancers exhibit a loss of the normal control of cell division, with the resultant formation of tumors. Cell division is undoubtedly, at least, in part under genetic control. Mutation in a gene is involved in the control of cell division, like mutation in any other gene, can cause a loss of function and thus a loss of the normal control of cell division. A number of different human tumors have been shown to have specific base pair substitutions in *C-ras* gene, which confers an ability on DNA from these tumors to transform mouse cell line neoplastically. Thus it is reported that exposure of humans to chemicals that can cause such mutation would increase the probability of developing cancer.

Several studies on the mutagenicity and carcinogenicity of cigarette smoke and cigarette smoke condensate have led to the realization that smoking is one of the most important sources of human exposure to mutagens and/or carcinogens (Bridges *et al.,* 1979).

1.2.4.2 Oncogenic Activation By Translocation

In Burkett's lymphoma, translocation of chromosome 8 to14 with consequent activation of *C-myc* gene. In chronic myeloid leukemia, deletion of short chromosome 22, called Philadelphia chromosome is seen in 80% cases. In the rest, there is translocation of 9 to 22 leading to activation of *C-abl* present in chromosome 9. In non-Hodgkin's lymphoma, translocation of chromosome 14 to 18 is very common, involving the *bcl-2* oncogene. The *bcl-2* product suppresses programmed cell death leading to tumor formation.

1.2.4.3 Oncogene Activation By Point Mutations

The C-H *ras* oncogene isolated from a human bladder carcinoma cell line was the first oncogene shown to be activated in a human tumor cell line. This gene was activated by a single point mutation that resulted in a change from a glycine to a valine codon in the 12^{th} aminoacid position (Reddy *et al.*, 1983., Tabin *et al.*,1982 and Taparowsky *et al.*, 1983). The *ras* oncogene has been identified in many animal and human tumor samples. *Ras* oncogenes in tumors have been shown to be activated by mutations in the 12^{th}, 13^{th} and 59^{th} to 61^{st} codons. In addition, studies have shown that mutation created *in vitro* by site directed mutagenesis in codons 63, 116 and 119 could also activate *ras* as transforming gene (Levinson, 1986).

Although it is not completely clear, how these point mutation or codon changes result in the *ras* protein activation. The normal gene product binds guanidine nucleotides and possesses GTP ase activity (Shih and Weeks, 1984) where as, mutant *ras* oncogene products have generally reduced GTP ase activity suggesting that this dysfunction may be a factor. Studies show that *ras* activation can also plays a role in tumor progression (Vousden and Marshall, 1984). With the exception of the new gene in rodents, the *ras* family represents the only known oncogenes that can be activated by a single point mutation. This may explain the predominance of *ras* gene mutation found in animal and human cancers.

1.2.4.4 Inactivation of Oncosupressor Gene By Mutation

A part of short arm of chromosome 17 was shown to be deleted in various human cancer. This region contains an oncosuppressor gene, called *p53*. Patients with germline mutations at the *p53* locus are at very high risk for cancer development as seen in Li-Fraumeni hereditary cancer syndrome characterized by early onset of breast carcinoma, childhood sarcoma and other tumors. Somatic mutations at *p53* locus, usually point mutations substituting one amino acid for another and inactivating suppressor activity, are the most common genetic change in human cancers and occur about 50% of them including carcinoma of breast, colon, stomach, bladder, and testis, melanoma and soft –part sarcoma. Sequential deletion

or mutational losses of function of both allelic genes at *Rb* locus on chromosome 13q14 are required for the development of retinoblastoma .

Mutagenesis by ROS/RNS could contribute to the initiation of cancer in addition to being important in the promotion and progression phases. It is increasingly proposed that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a key role in human cancer development (Feig *et al.,* 1994).

1.2.5 Free Radicals

A free radical is any species, which is capable of independent existence and contains 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. Free radicals such as superoxide ($O_2^{\cdot-}$), nitric oxide (NO) and their adducts peroxy nitrite ($ONOO^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) as well as alkyl peroxy radicals are involved in several disease conditions. Reactive oxygen species (O_2-, H_20_2 and $OH^{\cdot}$) have been implicated in the pathophysiology of several disorders, including ischemia, reperfusion injury, myocardial infraction, rheumatoid arthritis, neuro-degenerative disorders, atherosclerosis, acute hypertension, hemorrhagic shock and diabetes mellitus. Some tumor cells produce reactive oxygen species (ROS), although the source of these products and their contribution to the transformed phenotype is not known.

1.2.5.1 Generation Of Reactive Oxygen Species (ROS)

Several sources of ROS in the cells are proposed, from leakage of electrons on to oxygen from mitochondrial electron transport chains, microsomal cytochrome P-450 and their electron donating enzyme systems (Beal, 1997., Fridovich, 1989). Reactive oxygen species are produced in living cells as by products of normal metabolism of xenobiotics (Stohs and Bagehi, 1995., Winston and Digiulio, 1991), during exposure to high temperature (Parihar and Dubey, 1996), or radiation (Sen, 1995). ROS, e.g. O_2, HOCl and H_2O_2 are produced from activated phagocytes (Barbior and Woodman, 1990., Prakash *et al.,* 1998) The univalent

reduction of O_2 forming O_2^- also occurs from other normal biochemical oxidation-reduction reactions, both enzymatic (e.g. xanthine oxidase, aldehyde oxidase and peroxidases) and non-enzymatic reactions (e.g. autooxidation of catecholamines). H_2O_2 is additionally generated *in vivo* by several oxidase enzymes, viz monoamine oxidase (MAO), tyrosine hydroxylase and L-amino acid oxidase (Coyle and Puttfarcken, 1993).

1.2.5.2 Role of ROS/RNS In Mutation

ROS/RNS cause structural alteration in DNA such as base pair mutations, re-arrangements, deletions, insertions and sequence amplification (Weisman and Halliwell, 1996). ROS can produce gross chromosomal alterations in addition to point mutation and 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 and progression, allowing expression of the mutated phenotype.

1.2.5.3 DNA Damage by ROS/RNS

ROS/RNS can cause DNA base changes, strand breaks, damage to tumor suppressor genes and enhanced expression of proto-oncogenes and has been shown to induce malignant transformation of cells (Weitzman and Gordon, 1990). Damage to DNA by ROS, as measured in a single stranded DNA, *E. coli* based, forward mutation assay, was found to induce a wide spectrum of mutations, which depended not only on the ROS used but also on the DNA replication apparatus that encountered the lesion (Feig *et al.*, 1994). The most frequent mutations found in this system were C to T transitions. However, mutations arising from C to T transitions are not diagnostic for mutagenesis by ROS (Weisman and Halliwell, 1996).

1.2.5.4 Pathways Leading From Initial Base Damage To Subsequent Mutation.

The endogenous reactions that are likely to contribute to ongoing DNA damage are oxidation, methylation, depurination and deamination. Nitric oxide or more likely reactive products derived from its such as NO_2, $ONOO^-$, N_2O_3 and HNO_2 are mutagenic agents with potential to produce mutation, nitrosation and deamination

reactions on DNA bases. The H_2O_2 induced mutations included deletions and base substitutions (Weisman and Halliwell, 1996). Methylation of cytosine in DNA is important for regulation of gene expression, and normal methylation patterns can be altered during carcinogenesis (Weitzman *et al.*, 1994). Damage to DNA by ROS/RNS appears to occur naturally, in that low steady levels of base damage products have been detected in nuclear DNA from human cells and tissues. The pattern of damage to the purine and pyrimidine bases suggests that at least some of the damage occurs by $OH^{\cdot}$ attack, suggesting that $OH^{\cdot}$ is formed in the nucleus *in vivo*.

1.2.5.5 Damage to Proteins, Lipids and Deoxyribose

ROS/RNS induced mutations could not only cause DNA damage, but also the protein. Protein damage is a major consequence of excess ROS generation *in vivo* and damage to DNA polymerase could alter their fidelity. It has been suggested that an alteration in the conformation of DNA polymerase could explain the frequency of close proximity double mutations that occur secondarily to a wide range of genetic stresses (Feig *et al.*, 1994). Oxidative protein damage could also affect the activity of DNA repair enzymes. Another possible mutagenic effect of ROS involves their attack on lipid to initiate lipid peroxidation. Peroxidation can decompose to a range of mutagenic carbonyl products. For example, 4-hydroxynonenal is genotoxic to lymphocytes and hepatocytes, and disrupts gap-junction communication in cultured endothelial cells.

Ionizing radiation is a well known mutagenic and carcinogenic agent and much of the cell damage caused by ionizing radiation involve the formation of $OH^{\cdot}$ radicals by radiolysis of water. The deoxyribose is also fragmented by $OH^{\cdot}$ radical, yielding a multiplicity of products. Irradiated solutions of 2-deoxy-D-ribiose produce carbonyls and dicarbonyls, which are mutagenic to *Salmonella typhimurium*. Radicals can also attack nuclear proteins.

1.2.5.6 Mitochondrial DNA Damage

ROS/RNS can also damage mitochondrial DNA. Mitochondrial damage has been suggested to be important in several human diseases and in the aging

process. Oxidative damage could contribute to the deletion and other mutations in mitochondria DNA that accumulate with age at a higher rate than in nuclear DNA. ROS generated from mitochondrial electron transport chain are responsible for such DNA damage. The apparent increased damage in mitochondrial DNA compared with nuclear DNA could be due to the proximity of mitochondrial DNA to ROS. The lack of histone proteins to protect the DNA against attack or inefficient repair caused accumulation of the base damage to higher level (Weismann and Halliwell, 1996).

1.2.6 Antioxidants

Antioxidant defenses in the organism against reactive oxygen species (pro-oxidants and free radicals) produced during normal cell aerobic respiration may be of endogenous (enzymatic and nonenzymatic) or dietary origin (vitamins carotenoids and flavanoids, etc) (Harman, 1995). When natural defenses are overwhelmed by an excessive generation of prooxidants, a situation of oxidative stress evolves and cellular and extracellular macromolecules can suffer oxidative damage, causing tissue injury (Halliwell and Gutteridge, 1989., Halliwell and Aruoma, 1991., Halliwell and Chirico 1993) affecting immune function (Meydani *et al.*, 1995). Increased intakes of dietary antioxidant may help to maintain an adequate antioxidant, defined as the balance between antioxidants and oxidants in living organisms (Halliwell *et al.*, 1995).

The antioxidant system comprises different types of functional components classified as first line, second line and third line defenses.

1.2.6.1 First Line of Defense

The first line defense comprises preventive antioxidants that act by quenching of O_2-, decomposition of H_2O_2 and sequestration of metal ions. The antioxidants belonging to this category are enzymes like superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase etc.

1.2.6.1.1 Antioxidant Defense Enzymes

In 1969, McCord and Fridovich reported that the erythrocyte protein is able to remove the superoxide radical catalytically, i.e. it functions as a superoxide dismutase enzyme. The CuZn SOD enzymes isolated from eukaryotes have molecular mass of about 32,000 and contain two protein subunits, each of which bears an active site containing one copper and one zinc ion.

All CuZn SOD catalyse the same reaction: they greatly accelerate the dismutation of $O_2^{\cdot-}$+

$$O_2^{\cdot-}+2H^+ \rightarrow H_2O_2+O_2$$

The copper ions in CuZnSODs appear to function in the dismutation reaction by undergoing alternate oxidation and reduction, i.e.

$$EnzymeCu^{2+}+O_2^{\cdot-} \rightarrow Enzyme\text{-}Cu^{+}+O_2$$

$$Enzyme\text{-}Cu+O_2^{\cdot-}+2H^+ \rightarrow Enzyme\text{-}Cu_2^{+}+H_2O_2$$

The Zn^{2+} does not function in the catalytic cycle but helps to stabilize the enzyme. This conclusion is drawn from experiments in which the metals are removed from the active sites and replaced either singly or together.

Dismutation of $O_2^{\cdot-}$ generates H_2O_2, a species also generated by several oxidase enzymes *in vivo*, including xanthine, urate and D-aminoacid oxidases. Hydrogen peroxide is usually removed in aerobes by two types of enzyme. The catalases directly catalyze decomposition of H_2O_2 to ground state O_2

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

Peroxidase enzymes remove H_2O_2 by using it to oxidize another substrate.

$$SH2+H_2O_2 \rightarrow S+2H_2O$$

Glutathione peroxidase (GPX) removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione, GSH.

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

Glutathione peroxidases are not generally present in higher plants or bacteria, although they have been reported in few algae and fungi. GSH is a low molecular mass thiol – containing tripeptide. It is present in animals and many aerobic bacteria, at intracellular concentration that are often in millimolar range, but rarely present in anaerobic bacteria. Glutathione is also involved in the metabolism of herbicide, pesticides and xenobiotics generally in both animal and plant tissues. Many xenobiotics supplied to living organisms are metabolized by conjugation with GSH, catalyzed by glutathione-S-transferase (GST) enzymes.

$$RX+GSH \rightarrow RSG+HX$$

Liver is especially rich in these enzymes and the resulting glutathione conjugates are often excreted into bile using ATP-dependent glutathione S conjugate 'efflux pumps'.

1.2.6.1.2 Antioxidant Defense: Sequestration of Metal Ions

Iron and copper are essential in the human body for the synthesis of a huge range of enzymes and other proteins involved in respiration, O_2 transport, $NO^{\cdot}$ formation and other redox reactions. Yet these metals are potentially dangerous, their ability to undergo one-electron transfer enables them to powerful catalyst's of auto oxidation reactions (e.g. oxidation of adrenalin, dopamine and ascorbate), conversion of H_2O_2 to $OH^{\cdot}$ and decomposition of lipid peroxides to reactive peroxyl and alkoxyl radicals. It is only 'free' metal ions that are catalytic: haem and certain haem proteins can decompose lipid peroxides and interact with H_2O_2 to cause damage. Many dietary components influence iron uptake, e.g. phytates present in cereals, and nuts and legumes chelate iron. Transferrin also accepts iron released by the destruction of aged red blood cells. A protein similar to transferin known as lactoferrin is found in saliva, vaginal mucosa, seminal fluid, tears, bile, nasal secretion, milk and other secretary fluids and is released by activated neutrophills at site of inflammation. Metallothionein molecule can bind 5-7 ions of such metals as zinc (Zn^{2+}), silver (Ag^{+}) copper (Cu^{+}), cadmium (Cd^{2+}) and mercury (Hg^{2+}). Binding of metal to metallothioneins is achieved by association of cysteine-SH groups with the metal ion.

1.2.6.2 Second Line of Defense

The antioxidants belonging to second line defense serves as a scavenger of different free radicals. These include glutathione (GSH), vitamin C, uric acid, albumin, bilirubin, vitamin E, carotenoids, flavonid and ubiquinol. Glutathione is the most abundant non-protein thiol, synthesized in the liver and acts as a substrate for glutathione peroxidase enzyme.

1.2.6.3 Third Line of Defense

Third line antioxidants are a complex group of enzymes for repair of damaged DNA, damaged protein, oxidized lipids and peroxides and to stop chain propagation of peroxyl lipid radical. These enzymes repair the damage to biomolecules and reconstitute the damaged cell membrane e.g., lipase, proteases, DNA repair enzymes, transferase, methionine sulphoxide reductase etc.

1.2.6.4 Antioxidant Protection by Low Molecular Mass Agents

Low molecular mass compounds are thought to be important in antioxidant defense. These compounds are formed *in vivo* and/or compounds obtained from the diet. *In vitro*, bilirubin is a powerful scavenger of peroxy radicals and singlet oxygen. Several keto acids including pyruvate and α-keto glutarate react non-enzymatically with H_2O_2 and acts as 'H_2O_2 scavengers'. The female sex hormones oestradiol, oestrone and oestriol can inhibit lipid peroxidation *in vivo*, at micro molar concentration, because they possess phenolic –OH, groups and so can act as chain-breaking antioxidants in a way similar to that of vitamin E. Melatonin produced by pineal gland at the base of the brain shows antioxidant activity *in vitro*.

Epidemiological studies support the protective effect of dietary antioxidants, increased intake of fruits and vegetable have been related to a reduction of the risk of cardiovascular disease and certain types of cancer (Gillman *et al.*, 1995., Kohlmeer *et al.*, 1995., Hininger *et al.*, 1997.,Ness and Powles, 1997). Vitamin E is lipid soluble and is the most effective chain breaking antioxidant. Vitamin C or ascorbic acid is the important aqueous phase antioxidant. Dietary antioxidants other than vitamin (i.e. polyphenolic compounds such as flavanoids)

have been shown to be a major dietary factor responsible for such protective effects (Hertog 1996, Hertog *et al.*, 1994,1995,1997).

1.2.7 Radioprotection

A large number of chemical substances have been reported to possess radioprotective activity (Table 1.1). They can be classified into three groups

1) Radio protectors
2) Adaptogens
3) Absorbents.

The first group of protectors is generally sulfhydryl compounds and other antioxidants (Livesey and Reed, 1987). These include several myelo-, entero- and cerebro-protectors. Adaptogens act as stimulators of radio resistance. These are natural protectors, which offer chemical protection under low levels of ionizing radiation. These are generally extracted from the cells of plants and animals and have least toxicity. They can influence the regulatory system of exposed organisms, mobilize the endogenous background of radioresistance, immunity and intensify the overall nonspecific resistance of an organism. Absorbents protect organisms from internal radiation and chemicals. These include drugs, which prevent the incorporation of radioiodine by the thyroid gland and the absorption of ^{137}Cs, ^{90}Sr, ^{239}Pu, etc. (Nair *et al.,* 2001). The radio protectors can elicit their action by various mechanisms, such as suppressing the formation of reactive species, detoxification of radiation induced reactive species, target stabilization and enhancing the repair and recovering processes. The removal of radiation-induced reactive aqueous free-radical species with short life times in the nano-second range can significantly mitigate radiation damage. Several free radical scavengers are known to interact with aqueous free radicals and to prevent the radiation-induced lethality of cells. The radio protectors may also react with hydroxyl radicals or radicals of bio molecules by donating hydrogen atom to repair the radical species (Nair *et al.*, 2001)

Table 1.1 List of Various Categories of Radio Protectors and Their Mechanism of Action. (Nair *et al.*, 2001)

RADIO PROTECTORS	MECHANISM OF ACTION
A) Sulfhydryl compounds: - Cysteine, Cysteamine, Glutathione	Free radicals scavenging, donations of H atom.
B) Antioxidants Vitamin A, E & C, TMG, Melatonin etc.	Free- radical scavenging
C) ACE inhibitors Captopril, Elanopril, Penicillamine	Protease inhibition (through renin-angiotensin system), antioxidation, collagen synthesis inhibition.
D) Cryoprotective agents Mesna, Dexrazoxane, Amifostin (WR 2127)	Reduced toxicity of chemotherapeutic drugs, decrease of urothelial toxicity and nephrotoxicity.
E) Metalloelements: Manganese chloride, Cadmium salts, Bismuth etc.	Metallothionine induction
F) Immunomodulators:- Gamma-interferon, Polysaccharides AM5, AM218, Heat killed lactobacillus cells.	Immune stimulation, Increased production of cytokines.
G) Lipopolysaccharides & prostaglandins	Prostaglandin synthesis, elevated levels of cAMP, DNA repair.
H) Plants extract and isolated compounds:- Orientin, Vicimin	Free-radicals scavenging, antioxidation
I) DNA binding ligands: - Hoechst 33342	Electron transfer, free radical scavenging
J) Other Compounds Melatonin, Carnosin	Free radical scavenging, Antioxidant.

Several plant extracts, herbal preparation and phytochemical have been reported to have radio protective action in *in vitro* and *in vivo* studies. However, no clinical trails for their efficacy in clinical use have been reported so far. The radio protecting abilities have been attributed to their antioxidant and free radical scavenging properties.

1.2.8 Chemoprevention

Epidemiological studies indicate that approximately 80% of human cancer is caused by exposure to chemical carcinogens in tobacco smoke, in the diet or in the work place (Harris,*et al.*, 1992). Given these observations, at least three approaches to the prevention of cancer can be envisioned. First, reduce human exposure to environmental carcinogens through careful monitoring of the work place and through education approaches to encourage changes in life style. Second, identify individuals at high risk for cancer development through predisposing genetic or biochemical factors, followed by appropriate clinical follow up. Third, provide chemoprevention by dietary or synthetic means. For several reasons, chemoprevention has received growing considerations as means of cancer control. Primary cancer prevention requires removal of exposure to causal agents. Although this is an important approach to cancer prevention, it is not always effective, evidenced by marginal success of tobacco cessation programs. Moreover, numerous populations at high risk of certain types of cancer may already have received considerable exposure to etiological agents, and many human cancers cannot be ascribed to specific agents.

Cancer chemoprevention can be defined as the prevention, inhibition, or reversal of carcinogenesis by administration of one or more chemical entities, either as individual drugs or naturally occurring constituents of the diet. Knowledge on chemoprevention and its application in clinical studies have been growing rapidly over the past decade .

An ideal chemopreventive agent should have the following qualities: (i) little or no untoward or toxic effects; (ii) high efficacy; (iii) capability of oral administration; (iv) a known mechanism of action; (v) low cost.

The target population for cancer chemoprevention consist of high risk groups, such as, individuals with high exposure to carcinogens (e.g., tobacco smokers and populations that consume food stuffs contaminated with fungal toxins and nitrosamines); those who are known to be genetically predisposed to the development of cancer (e.g., patients with familial colonic polyposis); individual

with premalignant lesions (e.g., oral leukeoplakia, Barrets oesophagus etc); individuals with occupational exposure to known carcinogens ; survivers of primary cancer with high degree of recurrence or a marked tendency towards the formation of second primary tumors.

1.2.8.1 Classes of Chemopreventive Agents

The classification scheme developed by Wattenberg (Wattenberg, 1985) is based essentially on the period during which agents appear to exhibit activity in animal models of carcinogenesis. On this basis, chemopreventive agents are classified as inhibitors of carcinogen formation, blocking agents and suppressing agents. Blocking agents are inhibitors of tumor initiation, while suppressing agents are inhibitors of tumor promotion/progression. Examples of the major classes of chemopreventive agents are given below.

1.2.8.1.1 Inhibitors of Carcinogen Formation

Chemopreventive agents that inhibit the formation of carcinogens act predominantly to prevent the formation of nitrosamine from secondary amines and nitrate in an acidic environment. When present in appreciable amounts, ascorbic acid decreases nitrosamine production from secondary amines and nitrate in stomach (Mirvish, 1981) thus leading to diminished lung tumor response in mice (Hartman and Shankael, 1990). Other compounds that inhibit nitrosamine formation include phenols such as ferulic, gallic acid and caffeic acid (Kuenzig *et al.*, 1984) as well as several sulfhydryl compounds (Shenoy and Choughuley, 1992). Compounds of this class may have utility when incorporated into the diet of populations with suspected high rates of endogenous formation of nitrosamines.

1.2.8.1.2 Blocking Agents

There are several means of chemical intervention at the initiation stage of carcinogenesis. It is well known that environmental procarcinogen must be metabolically activated to electorphillic forms that damage DNA, while to some extent avoiding pathways of metabolic detoxification. The electrophillic species react with DNA, forming adducts that result in base mispairing and mutation. On this

basis, most blocking agents can be assigned to one, or more of five major categories. Inhibitors of cytochrome P450 enzymes; inducers of cytochrome P450 enzymes; inducers of phase II enzymes and scavengers of electrophiles and free radicals.

1.2.8.1.3 Suppressing Agents

Suppressing agents prevent the evolution of the neoplastic process in cells which otherwise would become malignant. Suppressing agents can be classified as compounds that inhibit polyamine metabolism, induce terminal cell differentiation, modulate signal transduction; modulate normal/growth factor activity, promote intercellular communication; restore immune response, induce apoptosis, correct DNA methylation imbalances, and inhibit arachidonic acid metabolism.

1.2.9 Mushrooms

Of the approximately 70,000 species of fungi, about 10,000 belong to the highly evolved class of basidomycetes, recognized by their fleshy fruiting bodies called mushrooms. Edible mushrooms have long been used as garnishes or in folk medicine, but they actually represent a high quality proteinaceous food and produce industrially useful enzymes and biologically active principles.

Mushrooms are considered more nutritious than many other vegetables. They contain the essential amino acid, as well as the most commonly occurring nonessential amino acids, vitamins and minerals. Their chemistry and food value have been extensively reviewed. (Chang, 1999) thousands of years. The medicinal part of mushroom is usually the carpophores. Metabolites of some of the mushrooms are currently being investigated for the inhibition of HIV virus and the management of AIDS.

Table 1.2 Reported Medicinal Benefits Of Mushrooms

Analgesic	Hypocholesterolemic
Antibiotic, Antiparasitic,	Hypolipidemic
Anticarcinogenic	Immunomodulatory
Antidiabetic	Metabolic regulatory
Antitumor	Radioprotective
Antirheumatic	Respiratory stimulatory
Chronic disease remedy	Mitogenic
Cardiotonic	Sedative
Coronary heart disease remedy	Tonic
Digestion stimulating	
Hypotensive	
Hepatoprotective	

1.2.9 .1.2 Mushrooms and Antitumor Activity

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 (Table1.2) and their metabolites that attracted the attention of the public is their antitumor property. A number of mushrooms have been reported to possess antitumour properties. Most of the investigations on the antitumor effects of mushrooms were carried out in Japan. It started in the 1960's when they tested numerous basidiomycetes for their anticarcinogenic potential. Intra-peritoneal injection of aqueous extracts of seven edible mushroom species when tested were found to inhibit the tumors arising from sarcoma 180 ascites cells implanted in Swiss albino mice by 72 to 92% versus controls (Ikekewa *et al.*, 1969, Breene, 1990). It was found that antitumor active fractions were polysaccharides in almost all mushrooms. Metabolites isolated from some mushrooms namely *Coriolus versicolor, Lentinus*

edodes and *Schizophyllum commune* are sold in China and Japan as anticancer drugs and are extensively used for the treatment of cancer in these countries. Lucas and his collaborators first demonstrated the antitumor activity of the higher basidiomycetes and their effectiveness 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 from many of the mushrooms (Wasser and Weis, 1999).

Methanolic extract from the fruiting bodies of *Pleurotus florida* (Jose and Janardhanan, 2000), *Pleurotus pulmonaris* (Fr.) Quel. (Jose *et al.*, 2002) and *Ganoderma lucidum* (Curt.: Fr.).P.Krast.Reshi (Jones and Janardhanan, 2000) occurring in South India were demonstrated to 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 isolated from mushrooms were also found to be effective against various tumor models in animals.

Polysaccharides from mushrooms do not attack cancer cells directly, but produce their antitumour effects by activating different immune response in the host. (Table 1.3) Immunomodulators work mainly by increasing macrophage activity. Macrophages are white blood cells that "eat up" and destroy pathogens, such as bacteria, yeast cells, virus-infected cells and so on. They reside in great numbers in the mucous membranes of the body-especially through out the digestive, urinary and respiratory tracts. Macrophages and other phagocytes can be regarded as the body's protective shield.

TABLE 1.3 ANTITUMOR POLYSACCHARIDES FROM MUSHROOMS.

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

Currently it is known that many mushroom polysaccharides isolated from *Tremella fuciformis, Schizophyllum commune, Dendropolyporus umbellatus, Grifola frondosa, Hericium erinaceus, Inonotus obliqaus, Ganoderma lucidum, G.applanatum, Lentinus edodes and Flammulina velutipes* etc, have been shown the ability to stimulate marcophage activity and strengthen immune system.

Of all mushroom immunomodulators investigated, the most effective is lentinan, from *Lentinus edodes*. Lentinan appears to act as a host defense potentiator (HDP), which is able to restore or augment the responsiveness of host cells to lymphocytokines (interleuckins), hormones and other biologically active substances by stimulating maturation, differentiation or proliferation of cells involved in host defense mechanism (Chihara *et al.*,1987). HDP's are functionally different from biological response modifiers. Thus, lentinan is able to increase host resistance against various kinds of cancer and infectious disease, including AIDS.

Lentinan stimulates various kinds of natural killer cells (NK cells) T cell, B cell and macrophage-dependable immune system responses. The antitumour effect of lentinan is abolished by the neonatal thymectomy and decreased by the administration of antilymphocyte serum, supporting the concept that lentinan requires immunocompetent T-cell compartments. The effect of lentinan was also inhibited by antimacrophage agents, such as carrageenan. (Maeda and Chihara, 1973, Hamuro and Chihara, 1985, Chihara *et al.,* 1987, Maeda *et al.*, 1988).

Using the blood of healthy donors and cancer patients, some authors (Arinaga, 1992., Tani *et al.*, 1993) have shown that lentinan is able to stimulate peripheral blood lymphocytes *in vitro* to increase interleukin mediated LAK cell and NK cell activity levels achievable *in vivo* by administration of clinical doses of lentinan. Lentinan has been shown to inhibit suppressor T cell activity *in vivo* and to increase the ratio of activated T cells and cytotoxic T-cells in the spleen when administered to gastric cancer patients undergoing chemotherapy (Miyakoshi, 1984., Aoki, 1984)

Lentinan's immune- activating ability may be linked with its modulation of hormonal factors that are known to play a role in tumor growth. Aoki (1984)

showed that the antitumor activity of lentinan is strongly reduced by administration of thyroxin or hydrocortisone. Lentinan can also restore a tumor-specific antigen-directed delayed type hypersensitivity reaction.

Lentinan is not formally included among the nonspecific immuno stimulants but it augments the induction of antigen- specific cytoxic T lymphocyte, marcophges, and other non-specific immune response.

1.2.9.1.3 Medicinal Value Of Pleurotus And Ganoderma Species

1.2.9.1.3.1 Pleurotus

Pleurotus species are wide spread throughout the hardwood forest of the world. Unlike the local use of many medicinal plants and fungi, the beneficial effect of *Pleurotus* was discovered independently on different continents and locations. According to Pharmacopeia Sinica, the medicinal part of the oyster mushroom is its fruiting body .The fruiting bodies of the oyster mushrooms have become a basis for dietic preparations in Asia, Europe and United States but their use as health ingredients is still rather limited, although it has an important commercial potential.

Pleurotus is well known for its medicinal properties in Central America and Mexico. *Pleurotus* has been used in ceremonial as well as in traditional medicine. The medicinal properties of *P.smithii* and *P.ostreatus* reported from these countries are very similar to those described in Asia. Oyster mushrooms are supposed to be efficient as a blood pressure lowering agent, diuretic, cholesterol reducer and adjuvant (Guzman, 1994).

According to Eastern folklore, *Pleurotus* can also prevent high blood pressure and atherosclerosis, impart long life and vigor and assist people in recovering from fatigue (Breene, 1990). The mushroom can be used to treat headache, fever and cold, asthma, nervous disorders and stomach pain.

The polysaccharides isolated from *Pleurotus* species consisted mainly of D-glucose and mannose polymers. Their derivatives and partially hydrolised products were prepared from culture filtrates or by extracting the fruit bodies,

Fig 1.2 Pleurotus florida

Fig 1.3 Ganoderma lucidum

sclerotia and mycelia of the fungi with hot water or alkaline aqueous solutions. (Jong and Donovick, 1989).

Pleuran isolated from *Pleurotus* species is an immunomodulatory compound, acts as an antiviral substance, when administered prophylactically. Recently, antiviral substances comprised of a mixture of low molecular weight polysaccharides and zeatine like substances having cytokinin activity, similar to zeatine were obtained from water extracts of *Pleurotus* mycelium. These antiviral agents obtained from this mushroom had no side effects or toxicity. A preparation of the mycelium of *Pleurotus* was used for the treatment of kidney inflammation without reduction in immunity.

Antibiotic substances obtained from *Pleurotus* spp are divided in to low molecular weight polysaccharides, which in most cases also exert anticarcinogenic and antiviral activity by the stimulation of the immune response of the organism, and secondary metabolites of mainly terpenoid origin and different protein derivatives. Other antibiotic substances produced by *Pleurotus* spp, are the volatile C8 compounds that give oyster mushrooms their specific aroma and taste.

It is a generally accepted that lowering of serum cholesterol levels reduces the risk of atherosclerosis. Natural substances with hypocholesterolemic activity are useful in prevention or treatment of hypercholesterolemia and especially relevant in countries with persistent progression of coronary artery disease. The addition of 5% dried oyster mushroom to a high cholesterol diet effectively reduces cholesterol accumulations in the serum and liver of rats, it redistributes cholesterol in favour of HDL (high density lipo-protien), reduces production of VLDL (very low density lipoprotein) and LDL (low density lipoprotein), reduces cholesterol absorption and reduces HMG CoA reductase (3 hydroxy-3 methyl glutaryl-coenzyme A reductase). The methanolic extract of *P. florida* mushroom activity showed significant antioxidant, and antitumor activities. (Jose and Janardhanan, 2000).

1.2.9.1.3.2 Ganoderma

Ganoderma lucidum, commonly known as Reishi, is one of the most popular medicinal mushrooms in China, Japan and the United States. In Chinese, it is called as Ling Zhi.

Ganoderma grows on logs or tree stumps. It has a shiny, hard, asymmetrical cap that ranges in color from yellow to black. The fruiting bodies of *G. lucidum* contain a variety of chemical substances. The mushroom is a rich source of triterpenes, and is currently reported to contain 119 different triterpenes (Kim and Kim, 1999). *G lucidum* was reported to contain some intensely bitter compounds such as lucidenic acid A, B, C, D, E, lucidone A, and ganoderic acid B and C, which were known to inhibit histamine release from mast cells, an angiotensin converting enzyme that is responsible for hypertension and growth of liver cancer cells (Eo *et al.*, 1999).

The polysaccharides of *G.lucidum* are the other major source of its biological activity and therapeutic use. This mushroom has attracted great attention owing to its antitumor and hypoglycemic activities (Ooi and Liu, 1999). Many fungal polysaccharides have been reported to be active against humans. The area of immune-stimulating polysaccharides is rather an attractive field of tumor therapy. (Jones and Janardhanan, 2000)

Ganoderma is also used in treating conditions of the nervous system. It is used to calm the nerves, cure insomnia, reduce stress, eliminate nervousness and increase determination and focus. *Ganoderma* is also frequently used to treat allergies, hay fever, bronchial asthma, and to reduce skin inflammation. Recent studies have also shown that compounds found in *Ganoderma* do lower blood sugar and interfere with the clotting of blood platelets. This reduction in clotting may account for *Ganoderma*'s effectiveness against stroke and atherosclerosis. Investigations show that *G.lucidum* occurring in South India possessed significant antioxidant antitumor, anti-inflammatory and antinociceptive properties (Jones and Janardhanan, 2000., Sheena *et al.*, 2003).

CHAPTER-2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals

Swiss albino mice and Sprague Dawley and Witsar rats were purchased from Small Animal Breeding Center, Kerala Agricultural University, Mannuthy, Thrissur. The animals were kept for a week under environmentally controlled conditions with free access to standard food (Lipton, India) and water.

2.1.2 Bacterial strains

Salmonella typhimurium strains TA98, TA100, TA102 were originally obtained from Prof.B.N.Ames, University of California, Berkely, USA. The strains were subcultered in nutrient broth for 12h and stored at –70°c as frozen permanent. 40µl of frozen permanent was used to inoculate for the fresh overnight culture of each strain in nutrient broth (5ml). The inoculated nutrient broth was incubated overnight at 37°c and used for the antimutagenic assay.

2.1.3 Chemicals

Ascorbic acid, 2,2-azobis-3-ethylbenzthiazoline–6-sulphonic acid diammonium salt (ABTS), butylated hydroxytoluene (BHT), 1,1 –diphenyl 2- picryl hydrazyl (DPPH), ethylene diamine tetra acetic acid (EDTA), ferric chloride, hydrogen peroxide, methanol (HPLC grade), myoglobin, potassium ferricyanide, sodium acetate, Trolox (6-hydroxy- 2,5,7,8 – tetra methyl chroman –2-carboxylic acid), 2,4,6-tripyridyl- s-triazine (TPTZ), 2-thiobarbituric acid, triphenyl phospene (TPP), trichloro acetic acid and xylenol orange were purchased from Sigma Chemical Co. USA. Hydrogen peroxide (H_2O_2) was purchased from Merck, India Ltd, Mumbai and 2.2'Azobis(2-amidopropane) dihydrochloride (AAPH) was from Aldrich Chemical Co, USA.

Sodium azide (NaN_3), reduced glutathione (GSH), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT), 1-chloro-2,4-dinitrobenzene (CDNB), riboflavin, glucose-6-phoshate, L-histidine and D-biotin were purchased from Sisco Research Laboratories, Mumbai. 2-Aminoflourene (2-

AF), Benzo [*a*] pyrene (B [*a*] P), 4-Nitro-*o*-phenylene diamine (NPD) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were purchased from Sigma Chemicals, St.Louis, USA. Amberlite XAD-4 was purchased from Lancaster Synthesis, England.

All other chemicals and reagents used in the experiments were of analytical reagent grade.

2. 2 METHODS

2. 2.1. Preparation of the extract

Sporocarps of *G.lucidum* growing on gulmohar trees (*Delonix regia*. Raf) were collected from the out skirts of Thrissur, Kerala. Type specimen was deposited in the Madras University Botany Laboratory Herbarium (MUBL 3172). Fruiting bodies of *P.florida* was obtained from Kerala Agricultural University, Mannuthy, Thrissur. The sporocarps were cut into small pieces, dried at 40-50°C for 48 h and powdered. Two hundred gram samples of the powdered materials were extracted with petroleum ether. The defatted materials were air dried then extracted with 70% methanol for 8-10 h using Soxhlet apparatus (Suffness and Douros, 1979). The extracts were pooled and solvents completely evaporated at 40°C using a rotary vacuum evaporator. The residues thus obtained were designated as methanol extract. The extracts were dissolved in distilled water and used for the experiment.

2.2.2. Preparation of tissue homogenate

Animals were sacrificed after completion of treatments. Livers were excised and rinsed in ice-cold saline to remove the blood. They were then gently blotted between the folds of a filter paper and weighed. 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. 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.

2.2.3. Determination of tissue reduced glutathione (GSH)

Reduced glutathione (GSH) 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 and 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 n mole/mg protein.

2.2.4. Determination of tissue Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) 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, reduces the NBT to a formazan blue. The SOD in the sample inhibits the formazan production.

Procedure

0.1ml 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 determined against distilled water as blank at 560 nm. All the tubes were uniformly illuminated for 15 min and absorbance of the blue color formed were measured again. Percent of inhibition was calculated after comparing absorbance of sample with that 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.

2.2.5. Determination of tissue catalase (CAT) activity

Tissue catalase (CAT) activity was determined according to the method of Beer and Seizer (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, every 1 min interval for 3 min immediately after adding 0.1 ml of 10 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 μmolar extinction coefficient of 40 cm^{-1}.

nmoles of H_2O_2 decomposed/ min/ mg protein

or

$$(U/mg\ protein) = \frac{\Delta A/min \times 1000 \times 3}{40 \times mg\ protein\ in\ sample}$$

2.2.6 Determination of tissue glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) 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. A sample without the tissue homogenate processed in the same manner was kept as the blank.

$$U/mg\ protein = \frac{O.D\ Blank - O.D\ Test}{0.001 \times mg\ protein\ in\ test\ vol} \times \frac{1}{10}$$

2.2.7. Determination of tissue glutathione -S-transferase (GST) activity

Glutathione-S-transferase (GST) 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 tissue homogenate. 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.

$$\text{µmol of CDNB-GSH conjugate-formed/ min/ mg protein} = \frac{\Delta A/\text{min} \times 1000 \times 3}{9.6 \times \text{mg protein in sample}}$$

2.2.8. 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

A 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, mixed 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.

2.2.9 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

0.01ml of the homogenate was made up to 1 ml of distilled water, 5 ml of alkaline $CuSO_4$ (0.5 % $CuSO_4$ in 1 % sodium potassium tartrate and 2% Na_2CO_3 in 0.1 N NaOH mixed in the ratio 1:50) kept for 10 min at room temperature. 0.5 ml of 1 N Folin phenol reagent 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).

2.2.10 Determination of serum glutamate oxaloacetate transaminase (GOT) activity.

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

Principle

Glutamate oxaloacetate transaminase (GOT) 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 .0.1ml 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) and incubated at 37°C for 60 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 to the reaction mixture and read the absorbance after 10 min at 505 nm. A control tube containing buffered substrate with out 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.

2.2.11 Determination of serum glutamate pyruvate transaminase (GPT) activity

GPT 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.0.1ml 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. 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, 2mM) calibration curve.

2.2.12 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 pH10 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. 0.05ml of serum was incubated with 0.5ml of the buffered substrate (1ml of 0.254g of disodium phenyl phosphate dihydrate/dl water mixed with 1ml of the carbonate buffer pH10) and 1.54ml of distilled water at 37°c for 15min. After incubation, 2ml of chromogen (1ml of 0.6g 4 aminoantipyrene/dl water and 1ml of potassium ferricyanide 2.4g/dl water) reagent was added and O.D measured at 510 nm. Phenol (10mg%) was used as the

standard for the calibration curve. The activity (KA/dl) is converted to IU/l by multiplying with 7.1

$$\text{Serum ALP (IU/l)} = \frac{\text{O.DT - O.DCx10x7.1}}{\text{O.DS - O.D b}}$$

2.2.13. 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-nitrobenzoate. The rate of the reaction is measured per minute for 3 min at 405 nm against distilled water as blank.

Procedure

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

Serum GGT (U/l) = (ΔA/min) x1158

2.2.14 Determination of serum total protein

Serum protein was determined by the method of Reinhold (1953)

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 against reagent blank 0.01ml of the standard solution, which was treated in the same way.

Procedure

The reagents used were from Span diagnostic kit . 1ml of working biurett reagent was mixed with 0.01ml of serum and absorbed at 530nm. The reagent blank, 0.01ml of standard solution was treated in same way.

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

$O.D_T$ - Optical density of test solution

O.Ds - Optical density of standard solution

2.2.15 Determination of serum albumin

Serum albumin was determined using bromocresol green reagent (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 640nm.

Procedure

Reagents used were from Ranbaxy diagnostic kit. 0.01ml of serum was mixed with 1 ml of BCG reagent (Succinate buffer 75mM 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 standard.

$$\text{Albumin (g/dl)} = \frac{\text{O.DT x3.8}}{\text{O.Ds}}$$

2.2.16 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 precipitation 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 0.5ml 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 rpm for 10 min, the precipitate was washed and estimated the TBARS by procedure given under tissue lipid peroxidation determination (section 2.2.8). The result was expressed as nmol MDA /ml of serum.

2.2.17 Statistical Analysis

To compare the effect of various treatments employed in the study the experimental data were analysed statistically using the ANOVA technique. Where ever only one set of treatment was to compared single factor ANOVA was used , for two set of treatments two factor ANOVA and three set of treatments three factor ANOVA was used. The mathematical model employed for one factor ANOVA was $X_{ij}=\mu+\alpha_i+\varepsilon_{ij}$

Where, X_{ij} is the observation using the i^{th} treatment in the j^{th} replication, μ the overall effect , α_i the i^{th} treatment effect and ε_{ij} random error.

The model used for two factor ANOVA was

$X_{ij}=\mu+\alpha_i+\beta_j+\varepsilon_{ij}$

And the model for three factor ANOVA is $X_{ij}=\mu+\alpha_i+\beta_j+\gamma_k+\varepsilon_{ijk}$

Where ever the treatment effect were found to be significant, least significant difference (LSD) were calculated and the significant effects were separated.

CHAPTER -3

FREE RADICAL SCAVENGING ACTIVITY OF *PLEUROTUS FLORIDA* AND *GANODERMA LUCIDUM*

3.1 INTRODUCTION

Potentially harmful reactive oxygen species (ROS) are produced as a consequence of normal aerobic metabolism. The free radicals are capable of inducing damage to all cellular molecules that can lead to diseased states (Weisman and Halliwell, 1996). The ROS are usually inactivated *in vivo* by a team of antioxidants. The study of lipid peroxidation (LP) is attracting much attention in recent years due to its role in disease processes. Membrane lipids are particularly susceptible to LP. Since membranes form the basis of many cellular organelles like mitochondria, plasma membranes, endoplasmic reticulum, lysosomes, peroxisomes etc, the damage caused by LP is highly detrimental to the functioning of the cell and its survival. It has been implicated in the pathogenesis of a number of diseases and clinical conditions. These include atherosclerosis, cancer, adult respiratory distress syndrome, Alzheimer's disease, Parkinson's disease, ischemia-reperfusion injury of various organs, chemical and radiation-induced injury, diabetes etc. Experimental and clinical evidence suggest that aldehyde products of LP can also act as bioactive molecules in physiological and pathological conditions (Devasagayam *et al.*, 2003). It is now generally accepted that LP and its products play an important role in liver, kidney and brain toxicity (Poli *et al.*, 1987., Cojocel *et al.*, 1989., Usyal *et al.*, 1989)

Exogenous chemicals and radiation produce peroxidation of lipids leading to structural and functional damage to cellular membranes (Rayleigh., 1987). Antioxidants are deployed by physiological process to prevent generation of ROS or to scavenge those formed. Thus, the oxidatively induced tissue damage is minimized. However, deficiency of antioxidant defenses may lead to oxidative stress, which might be associated with a variety of disorders including coronary heart disease, neural disorders, diabetes, arthritis and cancer (Spiteller, 2001). When natural defenses are overwhelmed by excessive generation of prooxidants, a situation of oxidative stress evolves and cellular macromolecules might suffer oxidative damage (Sies, 1996). A number of methods have been developed to measure the efficiency of antioxidants. These methods focus on different mechanisms of the antioxidant defense such as scavenging or inhibiting free radicals or chelating of metal ions that otherwise may lead to free radical formation.

A number of natural antioxidants have already been reported from various plant materials such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs (Ramarathnam *et al.*, 1995., Packer and Ong, 1997., Cullen *et al.*, 1997., Kamat, *et al.*, 2000., Devasagayam *et al.*, 2001., Nigris *et al.*, 2003., Negi and Jayaprakasha, 2003). Chinese herbs have been used for diet therapy for several millennia. Some of them are reported to exhibit significant antioxidant activity (Su, 1992., Kim *et al* 1994., Weng and Chen, 1996., Yun., 1999., Jose *et al* ., 2002).

Mushrooms are traditionally used in Chinese medicine and are commonly utilized for pharmaceutical purposes and health foods. Increasing experimental evidence indicates that mushrooms contain a large number of biologically active components that offer health benefits and protection against degenerative diseases. Since the role of free radicals has been implicated in a large number of diseases, the antioxidant activity of the mushrooms is of significant importance in exploiting their therapeutic potential. The proof of their antioxidant activity can also explain their mechanism of action. Hence, it was considered desirable to evaluate the mechanism of antioxidant activity of *P. florida* and *G. lucidum* by various methods using standard equivalent references, acting at different levels. The findings are presented in this chapter.

3.2 MATERIALS AND METHODS

3.2.1 PREPARATION OF EXTRACTS

P. florida and *G. lucidum* extracts were prepared as described in section 2.2.

For *in vitro* antioxidant activity studies, except for DPPH assay, the extracts of *P. florida* and *G. lucidum* were dissolved in distilled water.

3.2.2 ASSAY FOR FREE RADICAL SCAVENGING ACITIVITIES

Antioxidant activity of the extracts was assayed by FRAP, DPPH and ABTS methods. For the assays the extracts at 0.1%, 0.5%, 1% concentrations were employed.

3.2.2 .1 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing ability was measured at low pH (Benzie and Strain, 1996., Pulido *et al.*, 2000). The stock solution of 10 mM 2,4,6- tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It was prepared freshly and warmed to 37^0C. Then, 900 µl of FRAP reagent was mixed with 90 µl of distilled water and 30 µl test sample/distilled water/standard solutions were added and the reaction mixture was then incubated at 37^0C for 30 min and absorbance was recorded at 595 nm. An intense blue colour complex was formed when ferric tripyridyl triazine (Fe^{3+}TPTZ) complex was reduced to the ferrous (Fe^{2+}) form. The calibration curve was plotted with absorbance at 595 nm versus concentration of $FeSO_4$ in the range of 0-1 mM. The concentrations of $FeSO_4$ were in turn plotted against concentrations of standard antioxidants (L-ascorbic acid).

3.2.2.2 DPPH radical scavenging assay

In this method a commercially available and stable free radical ($DPPH^+$, 2,2-diphenyl-1-picrylhydrazil), which was soluble in methanol, was used (Aquino *et al.*, 2001). DPPH in its radical form has an absorption peak at 515nm, which disappeared on reduction by an antioxidant compound. An aliquot (37.5 µl) of the extract was added to 1.5 ml of freshly prepared DPPH solution (0.25 g/l in methanol). Absorbance was measured at 515 nm 20 min after the reaction was started. The DPPH concentration in the reaction medium was calculated. The calibration curve was plotted with % DPPH scavenged versus concentration of the standard antioxidant (L-ascorbic acid).

3.2.2.3 ABTS radical scavenging assay by spectrophotometry

In this assay, the radical scavenging activity of the extracts was determined by using ferryl myoglobin/ABTS protocol. The stock solutions of 500 µM ABTS diammonium salt, 400 µM myoglobin, 740 µM potassium ferricyanide and 450 µM H_2O_2 were prepared in phosphate buffered saline (PBS) (pH7.4).

Metmyoglobin (MbIII) was prepared by mixing equal amounts of myoglobin and potassium ferricyanide solutions. The reaction mixture (total volume 2 ml) contained the following substances (final concentrations in the reaction mixture): ABTS (150 μM), MbIII (2.5 μM), 16.8 μl of the sample and 978 μl PBS. The reaction was initiated by adding 75 μM H_2O_2 (330 μl) and the lag time in seconds, before absorbance of $ABTS^{\cdot +}$ at 734 nm began to increase was recorded. The calibration curve was plotted with lag time in seconds versus concentration of the standard antioxidants (L-ascorbic acid).

For all the above assays, Ascorbic acid Equivalent Antioxidant Capacity (AEAC) was obtained by dividing concentration of ascorbic acid % by concentration of extract % .

3.2.2.4 ABTS radical scavenging by Pulse radiolysis

Antioxidant activity of the extracts was also assayed by ABTS radical scavenging by pulse radiolysis technique using a linear accelerator. For pulse radiolysis measurements, the absorbed dose was kept to a minimum to avoid decomposition of the test compound. Typical maximum doses with 50 pulses were 15 Gy. The standard pattern of decay with ascorbic acid having four different concentrations of 2.5, 5, 7.5 and 10 μg per ml showed typical concentration dependent curves (Scotl *et al.*, 1993). The ascorbic acid equivalent was computed by extrapolating the results with the standard graph.

3.2.2.5 Oxygen Radical Absorbance Capacity (ORAC) assay

In ORAC assay oxygen, radical absorbance capacity was measured on detection of chemical damage to β-phycoerythrin through the decrease in the fluorescence emission. The fluorescence was recorded every 5 min till the last reading is less than 5% of the zero minute reading. ORAC values were calculated in terms of μmoles/g of fresh weight.

3.2.3 ASSAY FOR INHIBITION OF OXIDATIVE DAMAGE INDUCED BY AAPH (2.2'AZOBIS(2-AMIDOPROPANE) DIHYDROCHLORIDE)

The effect of extracts on the antioxidant damage caused by AAPH was determined using rat liver/brain mitochondria.

3.2.3.1 Isolation of mitochondrial fraction

Three months old female Wistar rats (weighing about 250 g) were used for the preparation of mitochondria. In brief, rat liver and brain tissues were excised, homogenized in 0.25 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at 3000-x g for 10 min to remove cell debris and the nuclear fraction. The resultant supernatant was centrifuged at 10,000 x g for 10 min to sediment mitochondria. This pellet was washed thrice with 50 mM phosphate buffer, pH 7.4, to remove sucrose. The protein was estimated and pellets were suspended in the same buffer (Kamat and Devasagayam, 1996)

The mitochondria (2.0 mg protein/ml) were exposed to AAPH (10 mM) with or without extract for 30 minutes. The mitochondria after exposure to AAPH were evaluated for lipid peroxidation.

3.2.3.2 ASSAY FOR LIPID PEROXIDATION:

3.2.3.2.1 Lipid Hydroperoxide (LOOH)

Aliquots (90 µl) of brain/liver mitochondria, after exposure to AAPH sample were transferred to micro centrifuge tubes together with 10 µl of triphenyl phospene (TPP) in methanol/10 µl of methanol in blank and test samples respectively. The samples were then vortexed and subsequently incubated for 30 min at room temperature. 900 µl of Fox II reagent (Xylenol orange (100µM), Butylated hydroxy toluene (4.4µM), sulphuric acid (25mM), ammonium ferrous sulphate (250µM)) was then added and samples were incubated for a further 30 min in dark. The samples were centrifuged at 12,000 x g for 10 min and absorbance of supernatant was measured at 560 nm. The level of the peroxide in sample was then determined from the difference between the mean absorbance of samples with and

without TPP treatment and the final volume was extrapolated to H_2O_2 concentrations from the standard graph. (Nourooz-zadeh *et al.*, 1996) The effect of mushroom extracts on hydroperoxide induction (rat brain mitochondria) by AAPH at varying time interval was also determined.

3.2.3.2.2 Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS assay was performed by standard method using malonaldehyde equivalents derived from tetramethoxypropane. Malonaldehyde and other aldehydes have been identified as products of lipid peroxidation that react with thiobarbituric acid (TBA) to give a pink colored species at 532 nm. The method involved heating of the samples (extracts and mitochondria) after exposure to AAPH with TBA reagent for 20 min in a boiling water bath. TBA reagent contains 50ml TCA (20%), TBA (500mg) 25ml, 2.5M HCl, EDTA 224mg and final volume made up to 100ml. After cooling, the solution was centrifuged at 2,000-x g for 10 min and precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm against a blank that contained all the reagents minus the sample.

The malonaldehyde equivalents of the sample were calculated using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$(Hunter, 1963).

3.3 STATISTICAL ANALYSIS

To compare the effect of various treatments employed in the study the experimental data were analysed statistically using the two way ANOVA technique.

3.4 RESULTS

3.4.1 Antioxidant Power Of The Extracts – FRAP ASSAY

The ferric reducing antioxidant power of the extracts was found to increase in a concentration dependent manner. *P.florida* and *G. lucidum* at a concentration of 1% showed AEAC values of 0.021, and 0.020 respectively. There was no significant difference in activity between extracts at 5% level of significance but between concentrations, effects were highly significant ($P<0.001$). Between 1% and 0.5%, there was no significant difference in activity. (Fig-3.1) Non-enzymatic

antioxidants react with pro-oxidants and inactivate them. In this redox reaction, antioxidant acts as a reductant. In this context, the antioxidant power can be referred to as reducing ability. In FRAP assay, an easily reducible oxidant Fe III is used in excess. Thus on reduction of Fe III- TPTZ complex by antioxidant, Fe II –TPTZ is formed which can be measured spectrophotometrically at 595 nm. The results indicate that all the extracts have hydrogen-donating capacity. The first line of defense is the preventive antioxidants, which suppress the formation of free radicals.

3.4.2 DPPH assay

P. florida and *G. lucidum* at concentration of 1% showed AEAC values of 0.016, and 0.015 respectively. There was no significant difference in activity between extracts but between concentrations, the difference was significant ($P<0.001$). 0.1% showed significantly lower value than 1%. Between 0.5% and 1%, the difference was significant. This assay showed the second line of defense to scavenge free radicals by suppressing chain initiation and /or by breaking the chain propagation reactions. In DPPH assay, the antioxidant scavenged the stable purple colored primary radical DPPH and the depolarization was determined spectrophotometrically at 515nm (Aquino *et al.*, 2001) (Fig-3.2).

3.4.3 Ferrylmyoglobin/ABTS assay

There was a concentration dependent increase in radical scavenging activities (Fig 3.3). There was no significant difference in activity between extracts but between concentrations, the difference was significant ($P<0.001$). 1% concentration showed significantly higher value compared to blank, 0.1% and 0.5%. This method showed the scavenging of secondary radicals. In this assay, *G.lucidum* and *P.florida* extracts showed 0.13 and 0.11 AEAC values respectively.

3.4.4 Pulse radiolysis assay of ABTS

Pulse radiolysis study of ABTS indicated the scavenging of primary radicals. In this assay, extract of *P. florida* showed highest activity (4.8 μg ascorbic acid equivalent per ml) (Fig 3.4b) followed by *G. lucidum* (4.2 μg ascorbic acid

equivalent per ml)(Fig 3.4c). In this system, also the mushrooms showed significant antioxidant activities.

3.4.5 Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC values for the mushroom extracts determined by spectrofluorimetric assay were, *P.florida* (45.65 ± 5.85) and *G.lucidum* (38.94 ± 9.0) as μmoles of ascorbic acid equivalent/ g fresh weight. Hence, *P. florida* was the most potent against scavenging peroxyl radicals on ORAC assay. The ORAC values are used as 'standard' measures for comparing antioxidant activity of food materials

3.4.6 Inhibition of lipid peroxidation induced by AAPH

The data on the effect of mushroom extracts on LOOH induced by AAPH in rat liver mitochondria is presented in Fig 3.5. There was no significant difference between extracts at 5% level but there was significant difference between 0 minutes and 30 minutes ($P<0.001$). The data on the effect of mushroom extracts on TBARS formation induced by AAPH in rat liver mitochondria are given in Fig 3.7. There was significant difference between extracts and between 0 minutes and 30 minutes ($P<0.001$). Lipid hydroperoxide formation induced by AAPH at varying time intervals in brain mitochondria. There was no significant difference between extracts at 5% level but there was significant difference between 0 minutes and 60 minutes and also between CO and extract treated group ($P<0.001$). After 60 min lipid hydroperoxide, formation was inhibited in all treated groups. Fig 3.6.

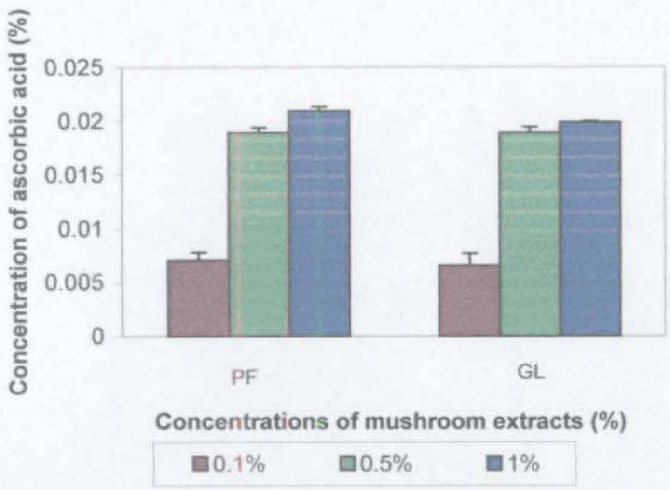


Fig 3.1 Ferric reducing antioxidant power of methanolic extract of *P.florida* (PF) and *G.lucidum* (GL).

Values are mean ± S.D, n=4. There was no significant difference between extracts at 5% level of significance but between concentrations (0.1%&1%) , effects were highly significant (P<0.001).

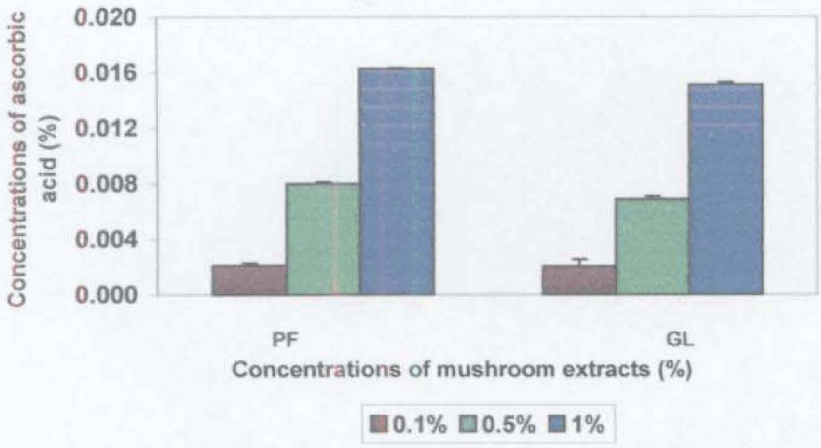


Fig 3.2 DPPH radical scavenging activity of methanolic extracts of *P.florida* (PF) *and G.lucidum*(GL) .

Values are mean ±S.D n=4. 0.1% showed significantly lower value than 1% .

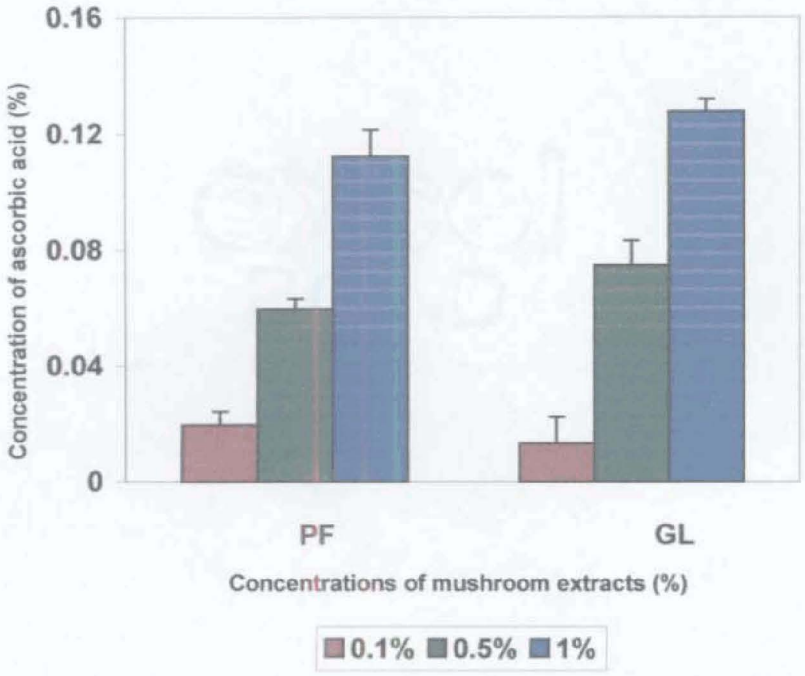
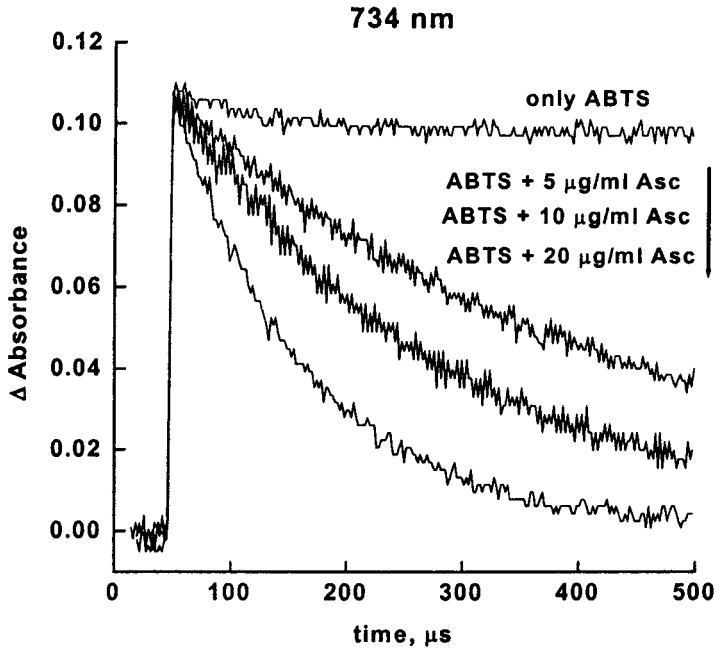
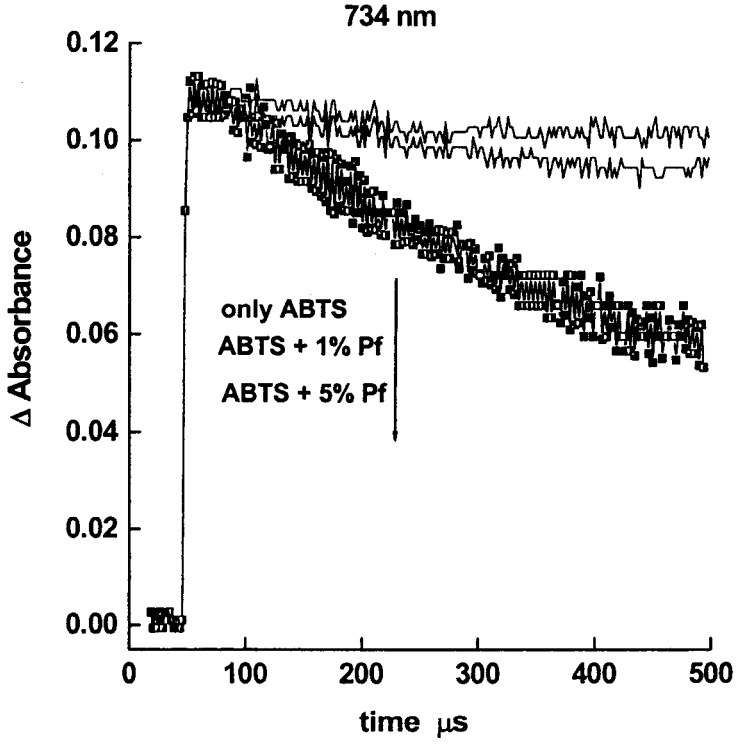


Fig 3.3 Inhibitory effect of methanolic extract of *P.florida* (PF) and *G.lucidum* (GL) on formation of Ferryl myglobin ABTS radical .

Values are mean ±S.D, n=4 There was no significant difference between extracts but between concentration the difference was significant ($P<0.001$). 1% concentration showed significantly higher value compared to 0.1% and 0.5%.


734 nm
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Δ Absorbance
only ABTS
ABTS + 5 μg/ml Asc
ABTS + 10 μg/ml Asc
ABTS + 20 μg/ml Asc
0
100
200
300
400
500
time, μs


734 nm
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Δ Absorbance
only ABTS
ABTS + 1% Pf
ABTS + 5% Pf
0
100
200
300
400
500
time μs

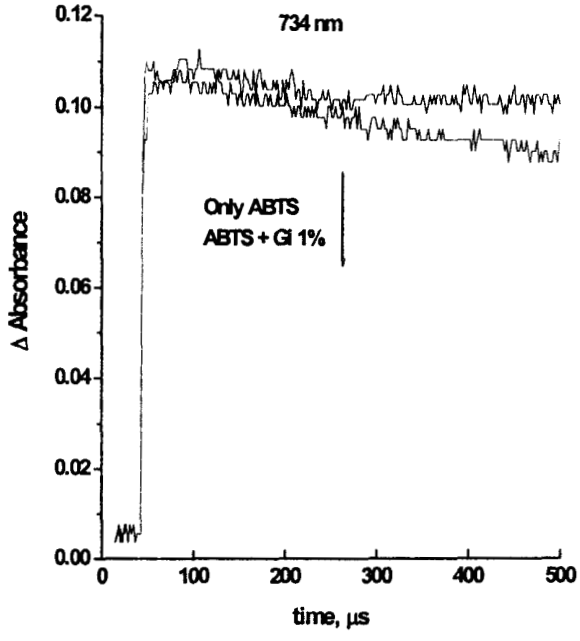


Fig-3.4 The antioxidant capacities of mushroom extracts as determined by pulse radiolysis by measuring the decay of ABTS.

Fig 3.4a Represents the decay of in the absence and the presence of standard antioxidant , ascorbic acid 5 μg /ml, b 10 μg /ml,c 20 μg /ml

Fig 3.4 b shows ABTS radical scavenging activity of *P.florida*

Fig 3. 4c shows ABTS radical scavenging activity of *G.lucidum*

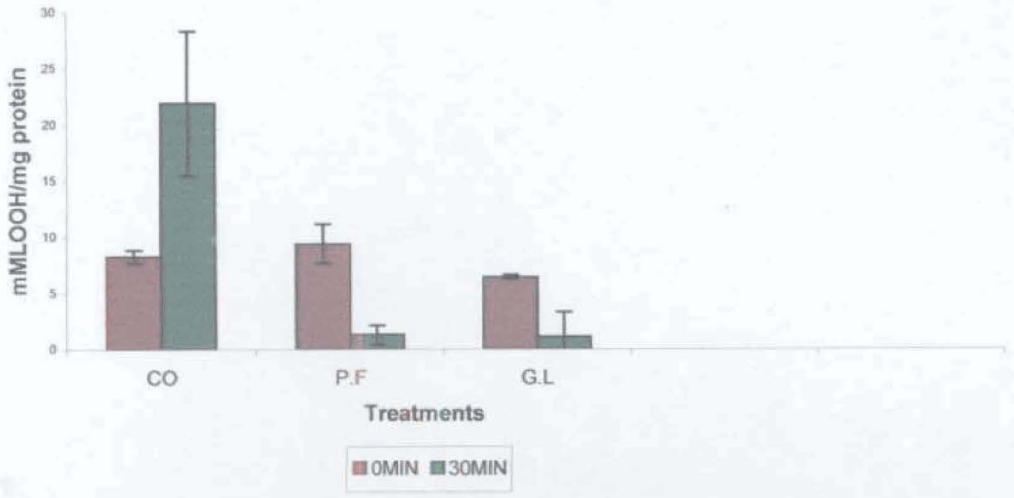


Fig-3.5 Inhibitory effect of methanolic extract of *P.florida*(PF) and *G.lucidum* (GL) on LOOH formation induced by AAPH.

Values are mean ±SD n=4 CO-control . There was no significant difference between extracts at 5% level but there was significant difference between 0 minutes and 30 minutes ($P<0.001$).

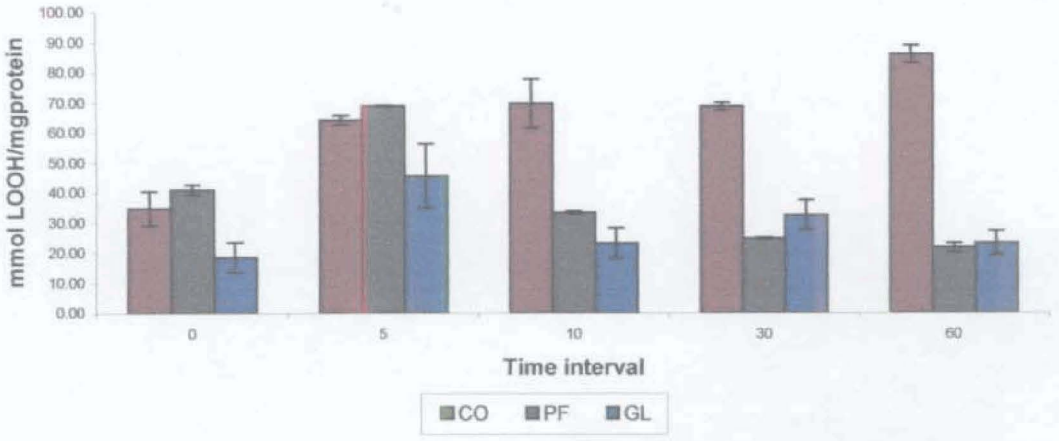


Fig 3.6 Inhibitory effect of methanolic extract of *P.florida* (PF) and *G.lucidum* (GL) on LOOH formation at varying time interval in brain mitochondria Control (CO).

Values are mean ±SD n=4. There was no significant difference between extracts at 5% level but there was significant difference between 0 minutes and 60 minutes and also between CO and extract treated group ($P<0.001$).

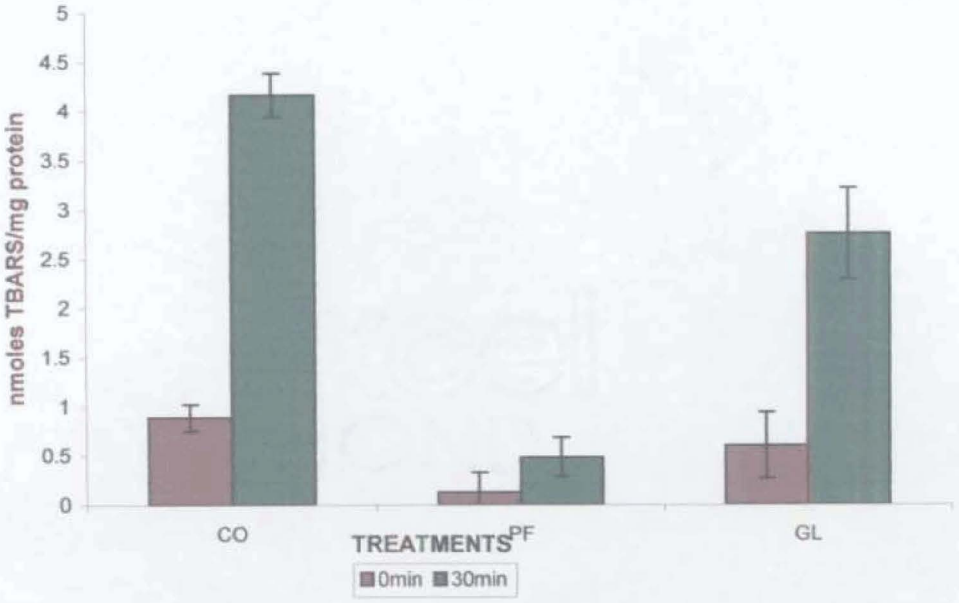


Fig-3.7 Inhibitory effect of methanolic extract of *P.florida*(PF) and *G.lucidum* (GL) on TBARS formation induced by AAPH. Control (CO) ±SD n=3

There was significant difference between extract and between 0 minutes and 30 minutes ($P<0.001$).

3.5 DISCUSSION

The results of the investigation reveal that extracts of *P. florida,* and *G. lucidum* possess profound property to inhibit free radical formation and scavenging activity. The extracts act at two different levels as primary antioxidants. Antioxidants show activities at different levels of protection (Cadenas and Packer, 1996). Although organisms are bestowed with antioxidant and repair systems they have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage totally (Hemnani and Parihar, 1998). Hence, antioxidants in diet are of importance as possible protective agents to help human body to reduce oxidative damage. Recently a large number of natural antioxidants have been isolated from different plant materials (Packer and Ong, 1997; Jovanovic and Simic, 2000; John *et al.*, 2002).

Mushrooms are functional foods and are traditionally used in folk medicine of several systems of medicine. Medicinal mushrooms possessing antioxidant properties in human diet would be potentially useful to help human body to reduce oxidative damage. In Chinese medicine fruiting bodies of *G. lucidum* is considered as a panacea because of its proven ability to treat a number of diseases. The broad-spectrum medicinal property of *G. lucidum* might be due its significant antioxidant activity. Antioxidant activities of the edible mushrooms have significant importance because this activity greatly contributes to their nutraceutical property, and enhancing their nutritive value.

Non-enzymatic antioxidants react with pro-oxidants and inactivate them. In this redox reaction, antioxidant acts as a reductant. In this context, the antioxidant power can be referred to as reducing ability. In FRAP assay, an easily reducible oxidant Fe III is used in excess. Thus on reduction of Fe III- TPTZ complex by antioxidant, Fe II –TPTZ is formed which can be measured spectrophotometrically at 595 nm (Benzie and Strain, 1996). The results indicate that all the extracts have hydrogen-donating capacity. The first line of defense is the preventive antioxidants, which suppress the formation of free radicals. In ferryl myoglobin- ABTS assay, on addition of antioxidant, formation of ABTS radical by reaction of ferryl myoglobin and ABTS is delayed and inhibition of formation of the radical is measured as the lag time in seconds (Alzoreky and Nakahara, 2001). All the extracts had the ability to prevent the radical formation and the ability was more in the case of *P. florida.* The second line of defense scavenges free radicals to suppress chain initiation and /or break the chain propagation reactions. In the DPPH assay the ability of antioxidant to scavenge stable purple colored primary radical DPPH is tested by its depolarization spectrophotometrically at 515 nm (Aquino *et al.*, 2001). In the pulse radiolysis study, ABTS radicals are generated as 'primary radicals' due to electron bombardment in a linear accelerator. In this system too, the mushroom extracts show significant antioxidant activities. *P. florida* shows the highest scavenging of ABTS radicals in terms of ascorbic acid equivalent (4.8 μg/ml). In ORAC assay inhibition of peroxyl radical-induced β-phycoerythrin oxidation and the protection by the mushroom extracts is measured. The ORAC values are used as 'standard' measures for comparing antioxidant activity of food materials. The results show that the values of

mushroom extract ranging from 30 to 45 are higher than the values observed for some fruits and vegetables including those for garlic (19.4), spinach (12.6) and onion (4.5) μmoles of trolox equivalent/g fresh weight. Being a free radical chain reaction, lipid peroxidation causes membrane damage as well as oxidative modification of critical targets. Therefore, agents, which can interact with these secondary radicals formed during peroxidation and scavenge them, would be effective in inhibiting lipid peroxidation and in turn protect against AAPH induced damage. Since the removal of excess of reactive oxygen species, suppression of their generation or protection against peroxidation by repair of membrane damage may be an efficient way of preventing cancer and other diseases. The effects of mushroom extracts on lipid peroxidation show significant inhibition of LOOH and TBARS formation. Protection of membranes at both primary and secondary levels explains the possible mechanism by which mushrooms inhibit the lipid peroxidation by AAPH. The present finding strongly suggests that the use of these mushroom extracts to prevent lipid peroxidation leading to membrane damage consequent to the exposure certain chemicals which can generate potent ROS in the form of $OH^{\cdot}$ or $ROO^{\cdot}$. This also explains the possible mechanisms behind observed health benefits of these mushrooms.

In conclusion, the studies reveal that extracts of the mushrooms, *P. florida,* and *G. lucidum* show high free radical scavenging activities and potent reducing power when assayed by five standard assays.

CHAPTER -4

ANTIMUTAGENIC ACTIVITY OF *GANODERMA LUCIDUM* AND *PLEUROTUS FLORIDA*.

4.1 INTRODUCTION

Alteration in genetic material results in mutation. In 1901, Hugo de Vries advanced the hypothesis of mutation, as abrupt spontaneous origin of new character. Any agent, which increase DNA damage or cell proliferation, can cause increased rate of mutation also. Mutation may alter the regulatory control; such as mutation with a somatic cell may results in uncontrolled cell division leading to cancer. A mutagen is considered an agent capable of destroying the integrity of hereditary mechanism of the cell or organism. Any substance causing increased mutation can also increase the probability of cancer. Vast majority of cancers are initiated by genetic changes. A carcinogen is a chemical capable of increasing the incidence of cancer in any species. The carcinogenesis appears to be linked with mutagenesis. Majority of known cancer causing agents are mutagens. The rate of tumor evolution and progression is accelerated by mutagenic agents. Human body is continuously and unavoidably exposed to a plethora of structurally diverse chemicals (polyaromatic hydrocarbons, aromatic amines and heterocyclic amine) which have established carcinogenic activity in animal model and / or mutagenic activity in short term tests. A characteristic of the above major classes of chemical carcinogens namely polyaromatic hydrocarbons, heterocyclic amines and aromatic amines is that in order to express their genotoxicity and carcinogenicity, they must be metabolized to reactive intermediates that have the capacity to interact covalently with DNA. Damage to DNA is likely to be a major cause of cancer and other diseases. Pollution of the environment by man made chemical toxicants is difficult to control for obvious reasons. Hopefully the genotoxic effects of toxicants can be minimized by modulation of the physiological detoxification. Many naturally occurring compounds with antioxidant activity are known to protect cellular components from oxidative damage and prevent diseases. A number of such compounds can activate the phase II detoxification enzymes, which can remove the toxic elements from our system. Exposure to such phytochemical is therefore beneficial to human health. A considerable emphasis is being placed on the use of dietary constituents to prevent mutagenesis and carcinogenesis due to their relative non-toxic properties.

There has been growing interest in the identification of naturally occurring dietary factors as potential anticarcinogens. The identification of such dietary components and definition of their antitumor effects could lead to strategies for reducing the risk of human cancer. Mushrooms have been used in folk medicine throughout the world since ancient times. Medicinal mushrooms useful against cancer are known in China, Russia, Japan and Korea as well as in USA and Canada (Wasser and Weis, 1999). *Ganoderma lucidum* has been used for thousands of years in traditional oriental medicine. This medicinal mushroom has been reported to be effective in the treatment of hypertension allergy, inflammation, hyperglyceamia etc. *Pleurotus* species are excellently edible oyster mushrooms. About seven edible species of *Pleurotus* tested highly inhibited growth of tumors arising from sarcoma 180 ascities cells implanted in Swiss albino mice (Ikekawa *et al.,* 1969). Agents that are capable to inhibit mutagenicity might be able to interfere with the process of carcinogenesis and tumor promotion. Chemoprevention aimed at inhibiting or delaying the onset of carcinogenesis is a rapidly growing area of cancer research. Several line of evidence indicates the relation between mutagenesis and carcinogenesis. Hence, the antimutageinic property of the methanolic extracts of *G.lucidum* and *P.florida* was examined. The findings are reported in this chapter.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of mutagens

All the chemical mutagens, 2-Aminoflourene (2-AF), Benzo [*a*] pyrene (B [*a*] P), 4-Nitro-*O*-phenylene diamene (NPD) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were dissolved in DMSO and sodium azide (NaN_3) was dissolved in water.

4.2.2 Preparation of extract

P. florida and *G. lucidum* extracts were prepared as described in section 2.2.

4.2.3 Animals

Four groups of six male Witsar rats (180-200g) in each group were used for the study of *in vivo* antimutagenic activity .

4.2.4 Determination of antimutagenic activity

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

4.2.4.1 Reagents

0.5mM histidine/biotin solution- 12.36 mg biotin was dissolved in 100 ml of hot distilled water. 9.6 mg histidine was added to the solution after cooling. The solution autoclaved at 121^0C for 20 min.

Spizizen's salt solution (10x)- 0.2g $MgCl_2$ $7H_2O$, 1g trisodium citrate, 14 g anhydrous K_2H PO_4, 6 g KH_2PO_4 and 2g $(NH_4)_2SO_4$ were dissolved in 70ml of distilled water and made up to 100ml. Autoclaved the solution at 121^0C for 20 min. 40 g glucose was dissolved in 100 ml distilled water and autoclaved at 121^oc for 20 min.

Top agar- 600 mg agar and 500 mg NaCl were dissolved in 100ml distilled water and 2ml of this was poured into test tubes and autoclaved at 121^0 C for 20min. Before pouring the top agar onto minimal agar, 0.2 ml of the sterilized histidine/biotin solution was added to each tube.

Minimal agar plates 1.5 g agar was dissolved in 85 ml of distilled water. The solution was autoclaved at 121^0 C for 20 min. After sterilization, 10ml of sterilized Spizizens salt (10x) and 5ml of 40% glucose were added. 20ml of this solution was poured into sterile petriplates under sterile condition.

4.2.4.2 Confirming genotype of Salmonella typhimurium strains

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

A)Histidine requirement

Histidine requirement of the tester strains TA 98, TA 100, and TA 102 were confirmed by streaking across the histidine/biotin plate and across the biotin

control plate. The plates were incubated overnight at 37^0c and examined for growth on the histidine/biotin plates.

B) Rfa mutation

Strains having rfa mutation was tested using crystal violet sensitivity test. 0.1ml of each tester strains TA 98, TA 100 and TA102 was mixed with 2ml 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, (8mm diameter) containing 10µl of a 1mg/ml crystal violet were transferred to the strain seeded plates. After 12h incubation, the zone of inhibition was measured.

C) UvrB mutation

The uvrB mutation was tested by UV sensitivity test. With a sterile cotton swabs, the tester strains TA98 TA 100 and TA 102 were streaked across standard nutrient plate, in parllel strips. A piece of cardboard was placed over the uncovered plate so that half of each bacterial streak was covered. The plates were irradiated with a 15W germicidal lamp at a distance of 33cm. TA 98, TA 100 and TA 102 were irradiated for 8 sec. The irradiated plates were incubated at 37^0c for 12-24h. Strains with uvrB deletion will grow only on the unirradiated side of the plate.

D) R-factor

R-factor was tested by ampicillin resistance on ampicillin plate. R-factor of the tester strains TA 98, TA 100 and TA102 was confirmed by streaking across the surface of ampicillin plate. The plates were incubated overnight at 37°c and examined for growth.

4.2.4.3 Preparation of rat liver microsomal fraction (S9)

Male Sprague Dawley rat (200g) was treated with sodium phenobarbitone (0.1%) in drinking water for 4 days (Maron and Ames, 1983). After an overnight fasting, animal was killed by decapitation; liver removed and washed several times in chilled 0.15 M KCl. Homogenate was prepared aseptically in 0.15M

KCl (3ml/g wet liver). The homogenate was centrifuged in a cooling centrifuge at 8,600 rpm for 10 min at 40^0c. The supernatant was used as the S9 fraction.

Preparation of S9 mix

5ml of the S9 mix was prepared by adding sterile reagents in the following order, 1.675 ml sterile distilled water, 2.5 ml 0.2M sodium phosphate buffer (pH 7.4), 0.2ml 0.1 M NADP, 0.025 ml 1M glucose-6-phosphate, 0.1 ml $MgCl_2$-KCl solution (1.64M KCl +0.4M $MgCl_2$) and 0.5 ml of rat liver S9.

4.2.4.4 Determination of in vitro antimutagenicty

4.2.4.4.1 Direct acting mutagens: Antimutagenicity of *G.lucidum* and *P.florida* extracts against direct acting mutagens was determined according to the methods of Maron and Ames (Maron and Ames , 1983). For this 2 ml of top agar, containing 0.5 mM histidine -biotin was mixed with mutagens (NaN_3, MNNG and NPD), at a concentration given in the Tables 4.2-4.3, 4.6-4.7. Different concentration of the extract of *G.lucidum* / *P.florida* (3mg, 2mg, 1mg) dissolved in distilled water and 0.1 ml freshly grown *S. typhimurium* (TA 100/ TA102/ TA 98) (10^9 cells/ml approximately) were poured onto minimal agar plates and incubated at 37^0 C for 48 h. After the incubation, the revertants colonies were counted using a colony counter.

4.2.4.4.2 Mutagens requiring activation: Antimutagenic assay against mutagens that require metabolic activation (2 AF and B [*a*] P) was carried out as follows. Liver microsomal fraction (S9) was prepared from Sprague Dawley rat (200 g). The rat was treated with 0.1% phenobarbitone for 4 days (Ames *et al.* 1973). After overnight fasting the animal was killed by decapitation, the liver was removed and homogenate was prepared aseptically. Activation mixture was prepared by mixing 50 µl of S9 fraction, and various concentration of *G.lucidum* /*P.florida* extracts (3 mg, 2mg, 1mg) mixed with the mutagens at a given concentration (Tables 4.4 - 4.5,4.8-4.9) and 0.1 ml freshly grown *S. typhimurium* (TA 100 and TA 98) (10^9 cells/ml approximately) poured onto minimal agar plates and incubated for 48 h at 37°C. After incubation, number of revertants were counted using a colony counter.

Toxicity of *G.lucidum* and *P.florida* extracts, if any, against bacterial strains was determined by incubating different concentration of *G.lucidum* / *P.florida* extracts with cultures of different tester strains of *Salmonella* for 48 h and checking the number of revertants and background lawn. Percent inhibition of mutagenicity in all assays was determined by the following formula

$$\% \text{ Inhibition of mutagenicity} = \frac{(R_1 - SR) - (R_2 - SR)}{(R_1 - SR)} \times 100$$

Where R_1 is the number of revertants without mushroom extracts, R_2 is the number of revertants with *G.lucidum* / *P.florida* extracts and SR is the spontaneous revertants.

4.2.4.5 Determination of *in vivo* antimutagenic activity

Male Wistar rats were divided into four groups of six animals. Group I animals were given distilled water orally which served as normal. Group II animals were given B [*a*] P (10 mg/rat) by i.p injection as a single dose, served as control. Group III animals were fed with *G.lucidum* extract (500mg/kg b wt) for 30 days. Group IV animals were fed with *P.florida* extract (500mg/kg b wt) for 30 days and on 31st day B [*a*] P (10 mg/rat i.p) were administered as a single dose to Group III and IV. The urine was collected from all the animals for 24h in metabolic cages. The urine, thus collected was filtered using Whatman No.1 filter paper, and 20 ml of urine was passed through XAD-4 amberlite column (40 mm x 10 mm) to concentrate the mutagen (Yamasaki and Ames, 1997). The weakly anionic components adsorbed were eluted with 10 ml acetone. The eluents were evaporated to dryness at 60^{0}C and stored at –20^{0}C and reconstituted in 1.5 ml DMSO just before the antimutagenicity assay (Polosa *et al.*, 1991). *S. typhimurium* strains TA 98 and TA 100 were used for the assays. Fresh *Salmonella* culture (10^{9}cells/ml) and 0.1 ml of urine concentrate were mixed with 2 ml top agar containing 0.5mM histidine and biotin on minimal glucose agar plate. The revertants were counted after incubation for 48 h at 37^{0}C. The percent inhibition of revertants was calculated compared to control.

4.2.5 STATISTICAL ANALYSIS

To compare the effect of various treatments employed in the study the experimental data were analysed statistically using the Two- way ANOVA technique.

4.3 RESULTS

4.3.1 Confirming genotype of *Salmonella typhimurium* strains

A)Histidine requirement

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

B) Rfa mutation

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

C) UvrB mutation

TA 102 strain with excision repair enzymes showed growth on both sides of the plate.

D) R-factor

R-factor of the tester strains TA 98, TA 100 and TA102 was confirmed by the presence of growth on ampicillin containing media.

4.3.2 *In vitro* antimutagenicity of the *G.lucidum* extract

Methanolic extract of *G.lucidum* showed significant inhibition of the mutagenicity induced by direct acting mutagens. The extract at a concentration of 3 mg/plate, inhibited NaN_3 induced mutagenicity by 51.4 % (TA100) and 43.78 % (TA102)(Table 4.2). Between concentrations, there was significant difference in activity ($P<0.001$). Control showed significantly higher number of revertants compared to 1 mg, 2 mg and 3 mg extracts. MNNG induced mutagenicity was

inhibited by 65.5 % (TA100) and 33.2 % (TA 102) (Table 4. 2). TA 100 showed significantly higher number of revertants than TA102. Between concentrations, effects were significant (P<0.001). Control and 1mg gave significantly higher number of revertants than 3 mg. NPD induced mutagenicity was inhibited by 40.4% (TA 98) and 64.2% (TA 100) (Table 4.3). TA 98 gave significantly higher value than TA 100. Between concentrations, effects were significant (P<0.001). Control gave significantly higher number of revertants than 1, 2 and 3 mg.

Methanolic extract of the mushroom was also found to inhibit mutagenicity elicited by mutagens requiring activation .The extract at concentration of 3 mg/plate inhibited 2-AF induced mutation by 71.8 % (TA98), and 51.1 % (TA100) (Table 4. 4). The concentration effects were significant (P<0.001). Control and 1mg gave significantly higher number of revertants than 3mg. The extract at the same concentration inhibited benzo [*a*] pyrene induced mutation by 57.1 % (TA 98) and 70.36 %(TA 100) (Table 4.5). Between concentrations, the effect was significant (P<0.01). Control showed significantly higher number of revertants than 2 and 3mg. The mushroom extract did not show any toxic effects on any of the *Salmonella* tester strain at the tested concentrations of 2 or 3 mg /plate (Table 4.1)

4.3.3 *In vitro* antimutagenicity of the extract *P.florida*

Methanolic extract of *P. florida* showed significant inhibition of the mutagenicity induced by direct acting mutagens. The extract at a concentration of 3 mg/plate, inhibited NaN_3 induced mutagenicity by 57.5 % (TA100) and 81.2 % (TA 102) (Table 4.6). MNNG induced mutagenicity was inhibited by 51.9 % (TA102) and 81.6 % (TA 100) (Table 4.6). There was significant difference between concentrations. Among concentration, control and 1 mg showed significantly higher number of revertants than 3 mg. NPD induced mutagenicity was inhibited by 49.2 % (TA 98) and 79 % (TA 100) (Table 4.7). There was significant difference in activity between concentration (P<0.001). Among concentrations control and 1mg showed significantly higher revertants than 2 and 3 mg. Methanolic extract of the mushroom was also found to inhibit mutagenicity elicited by mutagens requiring activation .The concentration of the extract at 3 mg/plate inhibited 2-AF induced mutation by 84.1% (TA98), 79.8% (TA100) (Table 4.8). TA 98 gave significantly higher number of

revertants than 1, 2 and 3 mg. The extract at the 3mg/plate inhibited B [*a*] P induced mutation by 74.3 % (TA 98), 79.6 % (TA 100) (Table 4.9). Control and 1mg gave significantly higher number of revertants than 3mg. The mushroom extract did not show any toxic effects on any of the *Salmonella* tester strains at the tested concentrations of 2 or 3 mg /plate (Table 4.1).

4.3.4 *In vivo* antimutagenic activity of *G.lucidum* and *P. florida* extracts

Antimutagenicity test of the urine of animals treated with B [*a*] P showed that methanolic extracts of *G.lucidum* inhibited mutagenicity induced by B [*a*] P. The extract at 500 mg/kg of body weight inhibited B [*a*] P induced mutation by 58.8 % (TA 98) and 39.1 % (TA 100) (Table –4.10). Antimutagenicity test of the urine of test animals treated with B [*a*] P showed that methanolic extracts of *P. florida* inhibited mutagenicity induced by B [*a*] P. The extract at 500 mg/kg of body weight inhibited B [*a*] P induced mutation by 63.7 % (TA 98) and 52.8 % (TA 100) (Table 4.10).

TABLE 4.1 Effect of methanolic extracts of *G.lucidum*(GL) and *P.florida* (PF) on *Salmonella typhimurium* spontaneous revertants in the presence (+S9) or absence (-S9) of liver microsomal fraction.

Extract	S_9	Average no of revertants per plate		
		TA98	TA100	TA102
GL (3 mg)	-	42.0 ±2.	116.0 ± 8.6	225.3 ±9.2
GL (3 mg)	+	46 ±2	117 ± 15.4	241.0 ±14.5
PF (3 mg)	-	41.0 ±1.4	100.0 ± 2.7	217.0 ± 5.6
PF (3 mg)	+	45.3 ± 2.5	104.0 ± 3.4	240.0 ±1.6
SR	-	38.7 ± 3.6	92.0 ± 4.0	235.0 ± 25.8
SR	+	43.7 ± 3.3	110.0 ± 2.7	240.0 ± 37.0

SR-Spontaneous Revertants

TABLE 4.2. Antimutagenic activity of *G. lucidum* extract (GL) against sodium azide (NaN_3) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)

Concentration (mg/plate)	Average number of revertants/plate		% Inhibition	
	TA 100	TA 102	TA100	TA102
NaN3 (0.0025)	1354.7±75.1	431±22.6	----	----
NaN_3+ GL (3)	658.7 ±36.7	242.3±42.5	51.4	43.78
NaN_3+ GL (2)	823.7 ± 21.1	321.7±12.5	39.1	25.3
NaN_3+ GL (1)	913.3 ±17.2	352.6±14.1	32.5	18.19
MNNG (0.001)	1105.6 ±47.6	631±19.6	----	----
MNNG + GL (3)	381.3 ± 16.5	421±15.04	65.5	33.2
MNNG + GL (2)	671.7 ±20.9	526 ± 22.1	39.2	16.6
MNNG + GL (1)	761 ± 32.0	576 ± 17.4	31.16	8.72
SR	96.3 ± 7.5	232.6 ± 32.2	----	----

Values are mean ± S.D, (n = 3). Average no. of revertants after deducting the SR. .(3),(2),(1) denotes concentration of extracts/plate .

NaN_3- Control gave significantly higher number of revertants than 1mg, 2 mg and 3 mg ($P<0.001$). MNNG-. Control and 1mg gave significantly higher number of revertants than 3mg ($P<0.001$).

TABLE 4.3 Antimutagenic activity of *G. lucidum* extract (GL) against 4-Nitro-*O*-phenylene diamene (NPD).

Concentration (mg/plate)	Average number of revertants/plate		% Inhibition	
	TA 98	TA 100	TA98	TA100
NPD (0.020)	1125±90.1	755.0±24.8	-	-
NPD + GL (3)	670.6 ± 14.7	270±14.7	40.4	64.2
NPD + GL (2)	781 ±27.4	422.0 ± 23.1	30.6	44.1
NPD + GL (1)	823 ± 33	615.3 ± 27.3	26.8	18.5
SR	38.6±3.5	88 ± 5.3	---	---

Values are mean ± S.D, (n = 3) (3),(2),(1) denotes concentration of extracts/plate. SR-spontaneous revertants. Control gave significantly higher number of revertants than 1,2 and 3 mg. ($P<0.001$).

TABLE 4.4. Antimutagenic activity of methanol extract of *G. lucidum* (GL) against 2-aminofluorene (2-AF).

Concentration (mg/plate)	Average number of revertants/plate		% Inhibition	
	TA 98	TA 100	TA98	TA100
2-AF (0.020)	912± 95.5	466 ± 57.6	---	---
2-AF + GL (3)	256.7 ± 53.9	227.7 ± 24.4	71.8	51.1
2-AF + GL (2)	374 ±67.8	320 ± 20	58.9	31.3
2-AF + GL (1)	550 ± 38.2	414± 32.2	39.6	11.2
SR	39.5±7.02	120.7±19.3	---	---

Values are mean ± S.D, (n = 3). Average no. of revertants after deducting the SR. (3),(2),(1) denotes concentration of extracts/plate. SR-spontaneous revertants. Control and 1mg gave significantly higher number of revertants than 3mg. ($P<0.001$).

TABLE 4.5 *In vitro* Antimutagenic activity of *G.lucidum* extract (GL) against Benzo[*a*]pyrene (B[*a*]P)

Concenteration (mg/ per plate)	Average number of revertants per plate		% Inhibition	
	TA98	TA100	TA98	TA100
B[*a*]P (0.010)	75.3 ±4.5	488 ±30.2	---	---
B[*a*]P + GL (3)	32.3 ± 6.6	144.6± 44.9	57.1	70.36
B[*a*]P + GL (2)	50 ±5.2	251 ±33.7	33.7	48.56
B[*a*]P + GL (1)	63 ± 6.7	326.3 ±13.6	16.3	33.13
SR	36.6±8.9	95±10	---	---

Values are mean ± S.D, (n=3). Average no. of revertants after deducting the SR. (3),(2),(1) denotes concentration of extracts/plate. SR-spontaneous revertants. Control showed significantly higher value of revertants compared to 2 and 3 mg ($P<0.001$).

TABLE 4.6 Antimutagenic activity of *P.florida* (PF) extract against sodium azide (NaN_3) and *N*-methyl *N'*-nitro-*N*-nitrosoguanidine (MNNG).

Concentration (mg/plate)	Average number of revertants/plate		% Inhibition	
	TA 100	TA 102	TA100	TA 102
NaN_3 (.0025)	1263.4 ± 55.6	140 ± 18.2	---	---
NaN_3+ PF (3)	536.7 ± 18 .2	26.3 ± 11.9	57.5	81.2
NaN_3+ PF (2)	629.0 ± 9.5	82.3 ±10.3	50.2	41.4
NaN_3+ PF (1)	781.7 ± 31.6	113 ±16.9	38.1	19.2
MNNG (.001)	940.4 ± 54.0	427 ± 10.3	-	-
MNNG + PF (3)	124.4 ±10.0	205.4 ± 44.8	81.6	51.9
MNNG + PF (2)	354.7 ±11.6	278 ± 18.8	62.2	34.8
MNNG + PF (1)	577.4 ± 29.7	363 ± 29.8	38.6	14.9
SR	98.30 ± 7.6	235 ± 25.8	-	-

Values are mean ± SD (n=3). (3), (2), (1) denotes concentration of extracts/plate. Average no. of revertants after deducting the SR SR-spontaneous revertants Control gave significnantly higher number of revertants compared to 2mg and 3mg ($P<0.001$)..

TABLE. 4.7 Antimutagenic activity of *P. florida* extract (PF) against 4-nitro-O-phenylenediamene (NPD) induced mutation of TA98 and TA100.

Concentration (mg/plate)	Average number of revertants per plate		(%) Inhibition	
	TA 98	TA 100	TA 98	TA100
NPD (.02)	1062.0± 54.0	843.7 ± 58.8	--	--
NPD+ PF (3)	539.0 ±11.8	176.7 ±15.1	49.2	79.0
NPD+ PF (2)	580.6 ±10.6	239.7 ± 21.8	45.3	71.6
NPD+ PF (1)	824.0 ± 23.0	379.0±16.1	22.4	55.0
SR	38.0 ± 3.3	92.3 ± 4.0	-	-

Values are mean±SD (n=3). Average no. of revertants after deducting the SR (3),(2),(1) denotes concentration of extracts/plate. SR-spontaneous revertants Average no. of revertants after deducting the SR. Control and 1mg gave significantly higher value of revertants than 2 and 3mg. ($P<0.001$).

TABLE 4.8 Antimutagenic activity of *Pleurotus florida* extract (PF) against 2-aminofluorene (2-AF).

Concentration (mg/Plate)	Average number of revertants per plate		Inhibition %	
	TA98	TA100	TA98	TA100
2-AF (0.02)	881.3 ± 78.3	332.3 ± 27.7	--	--
2-AF + PF (3)	139.5 ± 8.8	67.0 ± 14.4	84.1	79.8
2-AF+ PF(2)	242.3 ± 15.3	134.0 ± 18.2	72.5	59.6
2-AF+ PF(1)	321.9 ± 32.3	216.3 ± 7.8	63.5	34.9
SR	34.7 ± 37.6	112 .0± 11.5	-	-

Values are mean ± SD (n=3). Average no. of revertants after deducting the SR (3),(2),(1) denotes concentration of extracts/plate. SR-spontaneous revertants. Control gave significantly higher value of revertants compared to 1, 2 and 3 mg.

TABLE 4.9 Antimutagenic activity of *Pleurotus florida* extract (PF) against benzo[*a*]pyrene (B[*a*]P) induced mutation of TA98 and TA100.

Concentration (mg/plate)	Average number of revertants per plate		Inhibition (%)	
	TA98	TA100	TA98	TA100
B[*a*]P (0.025)	32.7 ± 2.4	392.6 ± 10.7	-	-
B[*a*]P+ PF(3)	8 .40 ± 2.5	80.0 ± 74.0	74.3	79.6
B[*a*]P+ PF(2)	14.4 ± 5.1	165.6 ± 11.0	56.0	57.8
B[*a*]P+PF(1)	26.4 ± 2.5	367 .0± 38.8	19.3	6.5
SR	32.6 ± 3.9	117 ± 12.8	---	---

Values are mean ±SD (n=3), Average no. of revertants after deducting the SR (3),(2),(1) denotes concentration of extracts/plate. SR-spontaneous revertants. Control and 1mg gave significantly higher number of revertants compared to 3mg. ($P<0.001$).

TABLE 4.10 *In vivo* Antimutagenic activity of *Ganoderma lucidum* (GL) and *Pleurotus florida* extract (PF) against Benzo[*a*]pyrene (B[*a*]P).

Mutagen/ Extract per animal	Average number of revertants per plate		Inhibition (%)	
	TA98	TA100	TA98	TA100
B[a]P (10mg/rat)	128.4±4.5	373.5±20	-	-
B [*a*] P+ GL (500mg/Kg)	52.8±3.5	227.6±26.5	58.8	39.1
B[a]P+PF(500mg/Kg)	46.6±3.5	176.3±14	63.7	52.8
Normal	27±2.6	101±11	-	-
SR	40.5±5.6	130±18	-	-

Values are mean ± SD (n=3), Average number of revertants after deducting the SR. SR-spontaneous revertants. There was significant difference in activity between extract treated group and B[*a*] P alone treated group($P<0.01$).

4.4 DISCUSSION

Methanolic extracts of *P.florida* and *G.lucidum* showed significant inhibition of mutagenicity induced by both direct acting mutagens (NaN_3, MNNG, NPD) and mutagens that require metabolic activation (B[a]P and 2-AF) in a dose dependent manner. The mushroom extracts did not show any toxicity to the tester strains in the doses tested. The conclusion was based on the number of revertants and background lawn. Hence, the activity is not the consequence of the toxic effect of the extract on bacterial colony. The antimutagenic activity of the extract against direct acting mutagens probably may be due to the inactivation of the mutagens. MNNG induces a wide spectrum of mutations by alkylating purines and pyrimidines. The major adduct reported was O^6 –methylguanine.

Significant correlations have been observed between the carcinogenicity of a series of polycyclic aromatic hydrocarbons (PAH) and their covalent binding to mouse epidermal DNA (Brookes and Lawley *et al* .1964; Hoel *et al.*1983; Miller *et al.* 1978). Based on extensive evidence accumulated in the last two decades, it is believed that PAH must be metabolically activated to electrophillic intermediates, which can bind to DNA and exert its carcinogenic effects (Pelkonen and Nebert, 1982). B [*a*] P is metabolized by mixed function oxidase (MFO) of rat liver to active intermediate benzo [*a*] pyrene 7,8 diol, 9,10 epoxide [BPDB] (Smith and Gupta, 1996). These can attack cellular macromolecules like DNA, RNA, proteins, membranes etc and cause dysfunction and damage. Reactive oxygen species increase the lipid peroxidation, which in turn alter the integrity of membrane bound enzymes. The free radical scavenging efficiency of the extract thus might be playing, an important role in the antimutagenic activity. The activity might be mediated through the inhibition of the MFO or through the inactivation of activated intermediates . The experimental results indicate that the extracts of both the mushrooms, *G.lucidum* and *P.florida* were highly effective in preventing *in vitro* and *in vivo* mutagenicity induced by benz [*a*] pyrene. This indicates the possible protective effect of the extract against the attack of cellular macro molecules by polyaromatic hydrocarbons such as benzo[*a*]pyrene.

Most cancers are thought to originate from a single cell that has experienced an initial mutation. The rate of tumor evolution and progress is accelerated both by mutagenic agents, tumor initiators and by tumor promoters that effect gene expression, stimulate cell proliferation and alter ecological balance of mutant and non mutant cells. A large number of cancer causing agents are mutagens. Tumor progression can be correlated with mutations that activate specific oncogenes and inactivate tumor suppressor genes. However, the present investigations are unable to explain the mechanism by which the mushroom extracts inhibit the mutagenicity induced by both direct and indirect acting mutagens. Nevertheless, the extracts are significantly effective in preventing mutagenic activity induced by both direct and indirect acting mutagens, indicating their potentials in chemoprevention.

CHAPTER-5

PROTECTIVE EFFECT OF *PLEUROTUS FLORIDA* AND *GANODERMA LUCIDUM* AGAINST CARCINOGEN INDUCED HEPATIC DAMAGES.

5.1 INTRODUCTION

Several lines of evidence indicate the crucial role of environmental predisposition, especially toxic chemicals, in the neoplastic transformation. The human population is persistently exposed to low levels of carcinogen such as polyaromatic hydrocarbons. Tumor initiation begins in cells through mutation from exposure to carcinogens. Oxidative reactions are involved in the promotion of tumor-initiated cells, which finally are transformed to highly undifferentiated cancer cells. Removal or suppression of oxygen free radicals enzymatically or non-enzymatically has been shown to reduce the incidence of cancer in animals as well as in clinical trials (Smith and Gupta, 1996).

The environmental procarcinogens are metabolized by mammalian cells to electrophillic derivatives, which bind to cellular macromolecules, initiating the process of carcinogenesis. Many chemical carcinogens are metabolized by enzymes to non carcinogenic as well as to proximate and ultimate carcinogenic metabolites, the effects of inducers of these enzymes on the carcinogenicity of a chemical will depend upon the effects of the inducers on the ratio of metabolism of the carcinogen to inactive and active metabolites by Phase I as well as Phase II enzymes. Many organs are capable of metabolizing chemicals to toxic intermediates. The liver protects the body from potentially injurious substances as well as toxic by products of metabolism. The most important detoxification process is that of the microsomal drug metabolizing system of the liver.

In recent years, there has been an increased emphasis on dietary modulators of carcinogenesis. Use of medicinal plants or their active principles for the prevention and treatment of chronic diseases in modern medicine suffers from lack of scientific evidence and as such only few medicinal plants attracted the interest of scientists. The mushrooms are mainly represented by macrofungi and they represent one of the untapped sources of powerful pharmaceutical products. Some mushrooms have been reported to possess significant hepatoprotective activity (Ajith and Janardhanan, 2002). Investigations were carried out to evaluate the protective effect of methanolic extracts of *P. florida* and *G. lucidum* against

carcinogen induced hepatic damages. Polyaromatic hydrocarbons, benzo [*a*] pyrene was used as carcinogen in this study.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of the extract

P.florida and *G.lucidum* extracts were prepared as mentioned in Chapter-2 Materials and Method section 2. 2.1

5.2.2 Animals and treatments

Animals: Male Witsar rats (180-200g) (Section 2.1.1)

Male Witsar rats were divided into four groups of six animals. Group I animals without any treatment served as normal. Group II animals were given distilled water orally daily for 30 days. Group III and IV animals were fed with *P.florida* and *G.lucidum* extract (500 mg/kg b wt, per day) for 30 days. On the 31st day B [*a*] P (10 mg/rat i.p) was administered to group II, III and IV animals as a single dose.

5.2.3 Determination of the effect of mushroom extracts on serum alkaline phosphatase and transaminases consequent to B [a] P challenge.

The animals were sacrificed by cervical dislocation 24 hrs after B [*a*] P administration and blood was collected directly from the heart and serum was separated. Serum was used for the determinations of the activities of glutamate pyruvate transaminase (GPT) (section 2.2.11), glutamate oxaloacetate transaminase (GOT) (section 2.2.10) and alkaline phosphatase (ALP) (section 2.2.12).

5.2.4 Determination of the effect of mushroom extracts on the antioxidant status of liver consequent to B [*a*] P challenge.

Liver homogenate was prepared as described in section 2.2.2 and was used for the estimation of reduced glutathione (GSH) (section 2.2.3) , glutathione s-transferase (GST) (section 2.2.7) and glutathione peroxidase (GPx) (section 2.2.6). Protein (section 2.2.9), superoxide dismutase (SOD) (section 2.2.4) and catalase

(CAT) section 2.2.5 activities. The level of lipid peroxidation was also determined (section 2.2.8).

5.2.5 Histopathological examination of liver

A portion of liver was fixed in 10% formalin and embeded in paraffin; 6 μm microtome sections were prepared from each liver and stained with heamatoxylin and eosin. The sections were examined under light microscope.

5.2.6 Statistical analysis

To compare the effect of various treatments employed in the study the experimental data were analysed statistically using the ANOVA technique.

5.3 RESULTS

Effect of *P. florida* and *G. lucidum* extracts on hepatic enzymes consequent to B [a] P challenge.

The activity of SGOT, and SGPT was drastically elevated in B [*a*] P treated group of animals. The level of these enzymes was effectively brought down in the case of animals treated with extracts of *G.lucidum* (GL) and *P.florida* (PF) (Table5.1). B [*a*] P treated group of animals showed higher SGPT than other groups ($P<0.01$). *G.lucidum* (GL) and *P.florida* (PF) extracts also showed significant effect on serum GOT consequent to B [*a*] P challenge ($P<0.01$). B [*a*] P treated group of animals showed significantly higher value of SGOT than mushroom extract treated group of animals. *G.lucidum* (GL) and *P.florida* (PF) extracts also showed significant effect on serum ALP consequent to B[*a*]P challenge. There was significant difference between B [*a*] P treated group and normal ($P<0.05$).

5.3.1 Effect of *P. florida* and G. *lucidum* extract on the hepatic antioxidant status consequent to B [a] P challenge:

The activity of GST, GPX, and the level of GSH were decreased consequent to B [*a*] P treatment. Both *P. florida* and G. *lucidum* extract elevated the activity of these antioxidant enzymes (Fig 5.1,5.2,5.3). B [*a*] P alone treated group

of animals showed significantly lower antioxidant activity than mushroom extract treated group of animals prior to B [*a*] P challenge. Between mushroom extract treated group of animals prior to B [*a*] P challenge and normal there was no significant difference. *G.lucidum* (GL) and *P.florida* (PF) extract showed higher GPx activity consequent to B [*a*] P challenge. *G.lucidum* (GL) and *P.florida* (PF) extract showed significant effect on GSH consequent to B [*a*] P challenge. B[a] p treated group showed significantly lower activity of GSH than P.F treated group ($P<0.01$). *G.lucidum* (GL) and *P.florida* (PF) extracts showed significant effect on SOD consequent to B [*a*] P challenge There was significant difference in SOD activity between B[a] p treated group and extract treated groups ($P<0.05$). The level of SOD and catalase was significantly reduced in the B [*a*] P treated group of animals but the level was markedly enhanced in animals that were treated with the extract (Fig 5.4, 5.5). There was no significant difference in activity between *P.florida* and *G.lucidum* extracts

The lipid peroxidation was elevated in the tissue of B [*a*] P treated animals than animals that were treated with *P.florida* and *G.lucidum* extracts (Fig5.6). B[*a*]P treated group showed significantly higher value than extract treated group. Between extracts there was no significant difference.

5.3.2 Histopathological observation

Sections of liver of animals challenged with B [a] P showed fatty infiltration and mild to moderate necrosis (Fig.5.7). Pretreatment of animals with the mushroom extracts were able to ameliorate these histopathological manifestations caused by B [*a*] P to a great extent.

Table -5.1 Effect of *G.lucidum* (GL) and *P.florida* (PF) extracts on serum ALP, SGOT and SGPT consequent to B[*a*]P challenge.

Groups	Treatments (mg/kg)	SGPT (IU/l)	SGOT (IU/l)	ALP (IU/l)
Normal		61± 10.8	125.6 ± 13	98 ± 22.3
Control (B[*a*]P)	10mg/rat	231.25± 34.9	197.75± 9.8	242 ± 18.8
PF+ (B[*a*] P)	500	174± 18.5	149.5± 11.3	124.8 ± 21.5
GL+ (B[*a*]P)	500	182.5± 18.9	160.96± 17.9	173.5 ± 15.2

Values are mean ±S.D n=6.

B [*a*] P treated group of animals showed higher SGPT than other groups (P<0.01). *G.lucidum* (GL) and *P.florida* (PF) extracts also showed significant effect on serum GOT consequent to B [*a*] P challenge(P<0.01). B [*a*] P treated group of animals showed significantly higher value of SGOT than mushroom extract treated group of animals.

G.lucidum (GL) and *P.florida* (PF) extracts also showed significant effect on serum ALP consequent to B[*a*]P challenge. There was significant difference in activity between B [*a*] P treated group of animals and normal (P<0.05).

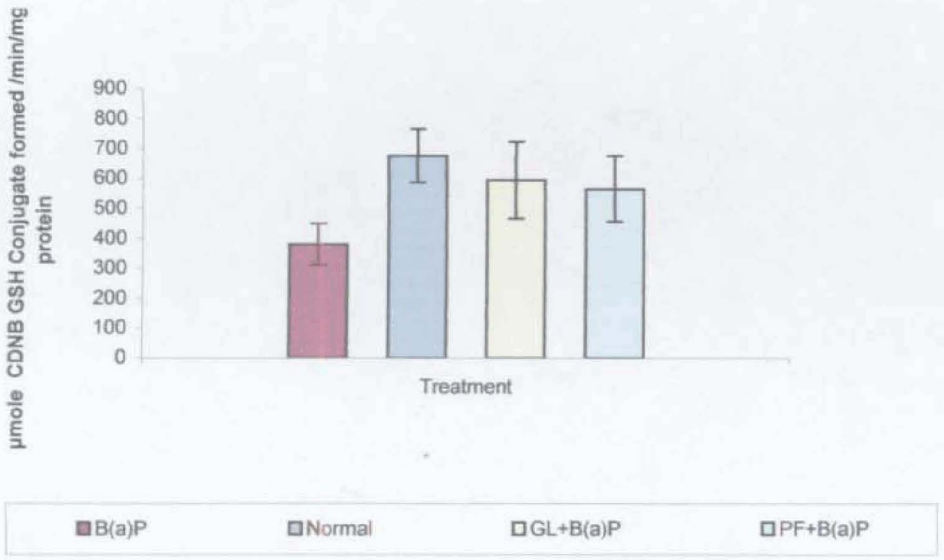


Fig –5.1 Effect of *G.lucidum* (GL) and *P.florida* (PF) extracts on tissue GST consequent to B[*a*]P challenge.

Values are mean ±S.D n=6.There was significant difference between B[a]P treated group and extract treated group (P<0.01). B [*a*] P treated group showed significantly lower GST activity than extract+B [*a*] P treated group.

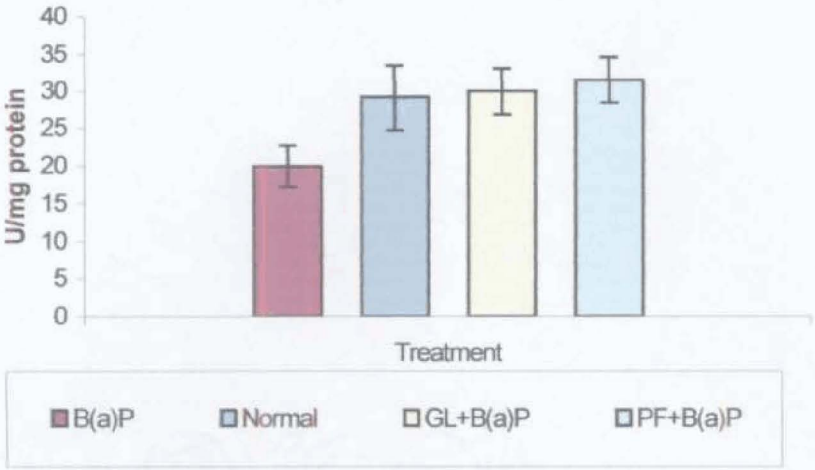


Fig-5.2 Effect of *G.lucidum* (GL) and *P.florida* (PF) extracts on GPX consequent to B[*a*]P challenge .

Values are mean ±S.D n=6.Extracts of the mushrooms (PF and GL) ($P<0.05$) and normal showed significantly higher activty than treatment with B[*a*]P alone. ($P<0.05$) There was no significant difference between *P.florida* extract + B [a] P and *G.lucidum* extract + B [*a*] P.

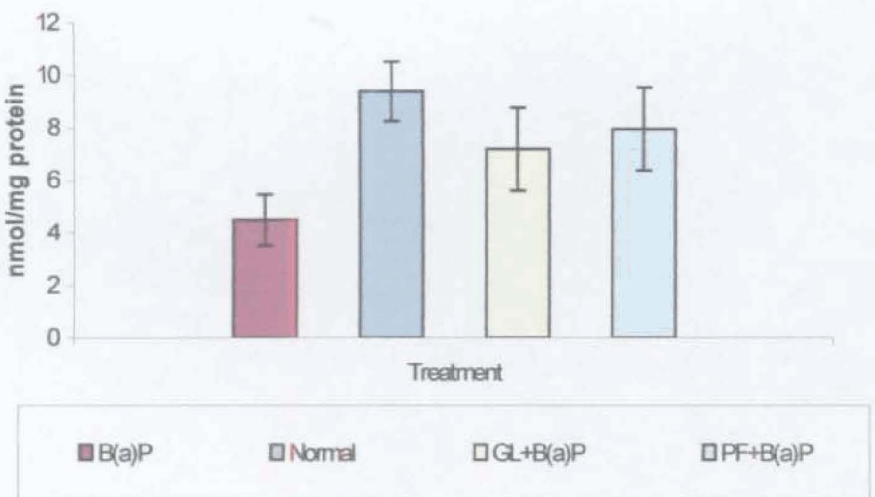
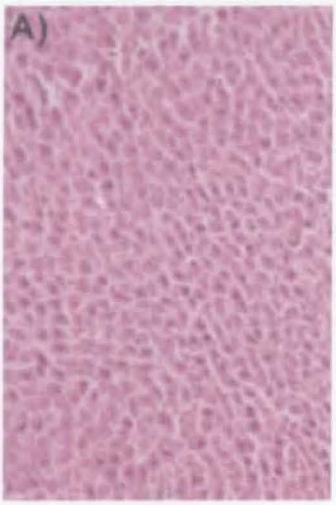


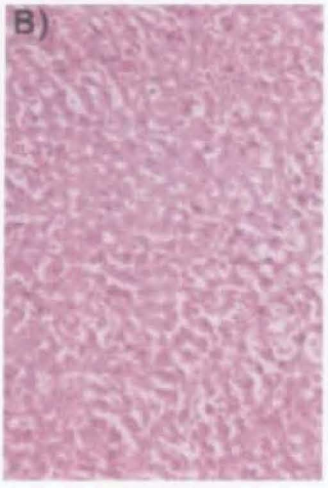
Fig-5.3 Effect of *G.lucidum* (GL) and *P.florida* (PF) extracts on tissue GSH consequent to B [*a*] P challenge.

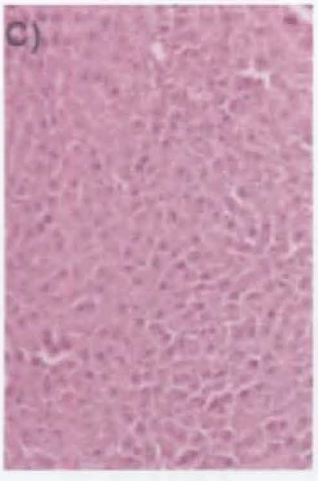
Values are mean ±S.D n=6. B[*a*] p treated group showed significantly lower activity of GSH than treatments with mushroom extracts (P.F and G.L) along with B[*a*] p ($P<0.01$).

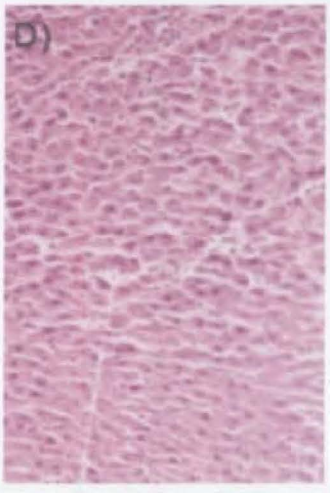
Fig 5.7 Effect of *P.florida* and *G.lucidum* extracts on B[*a*]P challenge. Liver sections stained with H&E (A) Normal (B) Control (B[*a*]P) (C) *P.florida* (500mg/kg) + B[*a*]P (D) *G.lucidum*(500mg/kg) + B[*a*]P

24.B









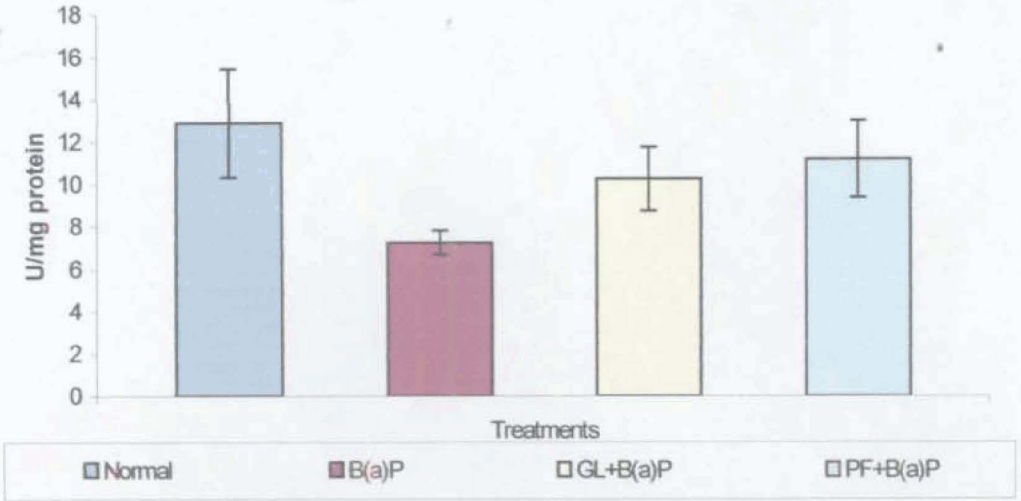


Fig –5.4 Effect of *G.lucidum* (GL) and *P.florida* (PF) extracts on tissue SOD consequent to B [*a*] P challenge.

Values are mean ±S.D n=6. There was significant difference in activity between B[*a*]P treated group and extract treated groups (P<0.05). There was no significant difference between *P.florida* extract + B [*a*] P and *G.lucidum* extract + B [*a*] P.

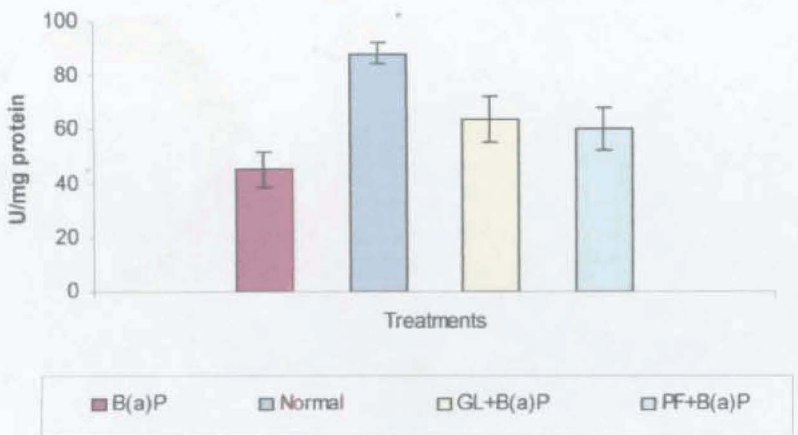


Fig-5.5 Effect of *P.florida* (PF) and *G.lucdum* (GL) extracts on tissue catalase consequent to B [*a*] P challenge.

B[*a*]P treated group showed significantly lower activity than extract treated group (P<0.001). There was no significant difference between *P.florida* extract + B [a] P and *G.lucidum* extract + B [a] P.

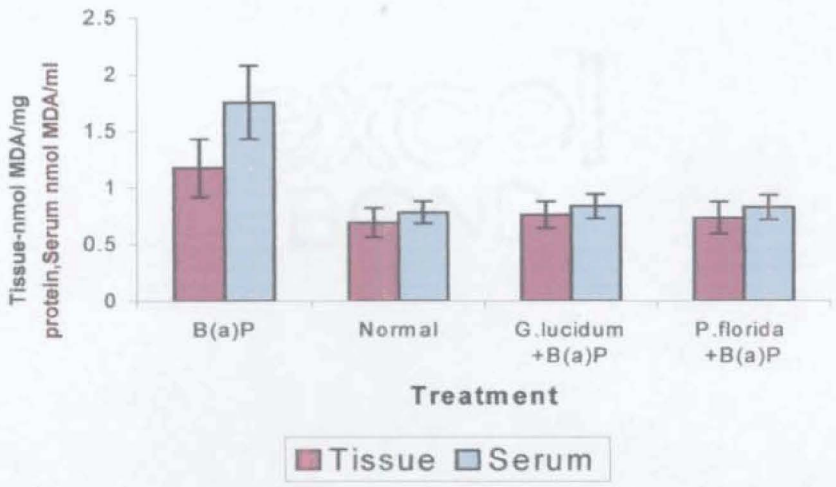


Fig-5.6 Effect of *P.florida* (PF) and *G.lucidum* (GL) extracts on tissue and serum lipid peroxidation consequent to B [*a*] P challenge.

Values are mean ±S.D n=6. B[*a*]P treated group showed significantly higher value than extract treated group (P<0.001). There was no significant difference of activity between *P.florida* extract + B [a] P and *G.lucidum* extract + B [a] P.

5.4 DISCUSSION

Environmental factors such as diet, exposure to chemicals, physical agents etc, have been increasingly implicated in the development of human cancer. The identification of naturally occurring inhibitors of carcinogenesis could lead to important new strategies of cancer prevention, especially by dietary intervention. Living systems have effective endogenous defense systems involving enzymatic and non-enzymatic processes. Reactive oxygen species are important as direct and indirect initiators as well as promoters of mutagenesis and carcinogenesis (Ames *et al.* 1986). Antioxidants in food have drawn notable attention as agents to scavenge and eliminate active oxygen species and act as potential agents to prevent mutagenesis and carcinogenesis. Dietary antioxidants are reported to reduce tumor incidence in animals (Namiki *et al* .1986; Osawa *et al*.1990; Cutler and Proyor. 1984).

Significant correlations have been observed between the carcinogenicity of a series of polycyclic aromatic hydrocarbons (PAH) and their covalent binding to

mouse epidermal DNA (Brookes and Lawley, 1964; Hoel *et al.*, 1983., Miller, 1978). Based on extensive evidence accumulated in the last two decades, it is believed that PAH must be metabolically activated to electrophillic intermediates, which can bind to DNA and so exert carcinogenic effects (Pelkonen and Nebert, 1982). B [*a*] P is metabolized by mixed function oxidase (MFO) of rat liver to active derivative benzo [*a*] pyrene 7,8 diol, 9,10 epoxide [BPDB] (Smith and Gupta, 1996). This can attack cellular macromolecules like DNA, RNA, proteins, membranes etc and cause dysfunction and damage. Reactive oxygen species increase the lipid peroxidation, which in turn alter the integrity of membrane bound enzymes. The free radical scavenging efficiency of mushroom extracts thus might be playing an important role in the anticarcinogenic activity. The treatment of animals with B [*a*] P shows a significant increase in lipid peroxidation. Antioxidants lower the carcinogenicity of B [*a*] P by acting on antioxidant response elements and thereby increasing synthesis of enzymes involved in detoxification. The blocking of carcinogenicity induced by B [*a*] P might be mediated by the changes in the GSH content, which can detoxify the intermediate epoxide of B [*a*] P (Burke *et al* .1981; Pezzuto *et al.* 1976; Pietropaole and Weinstein, 1975). The significant increase of superoxide dismutase in *P. florida* and *G. lucidum* treated group of animals appears to facilitate removal of superoxide anions and H_2O_2 formed in the process of oxidative stress induced by B [*a*]P .

The results of the investigation indicate that methanolic extract of *G.lucidum* and *P.florida* possess significant protective effect against carcinogen induced hepatic damages. The activity might be due to the capacity of the extracts to restore the depleted antioxidant defense consequent to the challenge by the carcinogen, B [*a*] P.

CHAPTER-6

RADIOPROTECTIVE EFFECT OF *GANODERMA LUCIDUM* AND *PLEUROTUS FLORIDA*

6.1 INTRODUCTION

Ionizing radiation damages cellular molecules directly by transferring energy or indirectly by generation of oxygen derived free radicals, excited states and other reactive species collectively known as ROS (Von Sonntag, 1987). The effects of low-LET radiation are mainly mediated by generation of ROS. Excess reactive oxygen species (ROS) generation can result from exposure to various physical and chemical agents, including ionizing radiation, air pollutants, chemical toxicants, photosensitization etc. besides various pathophysiological states. Most of the radiation induced damage to biomolecules in aqueous media; such as those, prevailing in living systems is caused by the formation of free radicals resulting from radiolysis of water and macromolecules. ROS such as superoxide anion radical ($O_2^{\cdot}$) hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) are considered to be important in the etiology of several pathological conditions such as cardiovascular diseases, neurological disorders, arthritis, diabetes, inflammation, cancer etc. (Hennmani and Parihar, 1998)

Ionizing radiation inflicts deleterious effects on living cells and damage vital cellular targets such as DNA and membrane. Protecting the onslaught of ionizing radiation have significant importance. Radiation protection is also important in radiation therapy. Radiation therapy evolved as a treatment of cancer because it permitted tumor eradication with preservation of normal tissue functions. Mammalian cells are most sensitive to radiation-induced damage in the late G_2 and M phase of the cell cycle. Cellular damage produced by the radiation therapy is an indirect result of ionizing chemicals in the cell to very reactive compounds. Cytotoxicity is primarily caused by oxygen-derived free radicals such as hydrogen peroxide (H_2O_2), superoxide ($O^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$) (Parker,1990). In modern clinical practice, radiotherapy is frequently combined with surgery and chemotherapy to provide the most effective tumor control with least damage to normal tissue. One of the major draw backs of radiotherapy is that it produced severe side effects generated due to the damage of normal tissues during irradiation (Maunch *et al.*, 1995) .The sub lethal whole body gamma irradiation also inflicts drastic changes in the haematopoietic system as it is very sensitive to the ionizing

effects on irradiation (Anderson, 1990). Radiation can induce the formation of free radicals and this radiation induced free radicals produce peroxidation of lipids, leading to structural and functional damage to cellular membranes (Rayleigh, 1987). The natural antioxidant system of the body, consisting of reduced glutathione (GSH) and the related enzymes as well as superoxide dismutase (SOD) are believed to be the major cellular constituents involved in the defense against lipid peroxidation. Reduced glutathione (GSH), which amounts to about 90% of the nonprotein thiols in the cell is involved in a number of reductive reactions in the cell and acts as a substrate or co-factor for the antioxidant enzymes, GSH peroxidase, GSH transferase and reductases which are involved in the termination of peroxidation. GSH has been shown to protect cells against oxidative stress by reacting with peroxides and hydroperoxides. Increase in GSH was found to be correlated with thiols induced radioprotection and chemoprotection in mouse. Superoxide dismutase (SOD) reacts with superoxide radicals and converts them to H_2O_2, which is catalyzed by catalase or GSH peroxidase (Uma Devi and Ganasoundari 1999).

Antioxidant enzymes and other scavengers of reactive oxygen intermediates are involved in numerous defense systems in cells. Antioxidants may exert their effects on biological systems by different mechanisms including electron donation (as reducing agent) metal ion chelation (there by eliminating potential free radicals) sparing by antioxidants (co-antioxidants) or by gene expression regulation (Yoshikawa *et al.*, 2000)

Development of effective radioprotective agents with least side effects is a compelling urgency because radiotherapy is increasingly found useful for cancer treatment. The only drug approved for clinical use in cancer therapy patients is amifostine, a synthetic compound that also induces minor side effects like nausea, vomiting and hypotension. Moreover, amifostine is very expensive. Therefore, there is need to find non-toxic and less expensive drugs for clinical radiation protection. Although a large number of biological properties and clinical use of *Ganoderma* and *Pleurotus* species have been reported, no attempt has been made to exploit the radioprotective effects of these medicinal mushrooms. Prevention of radiation

induced cellular damage by the methanolic extracts of *G. lucidum* and *P.florida* was investigated and the findings are reported in this chapter.

6.2 MATERIALS AND METHODS

6.2.1 ANIMALS

Swiss albino female mice weighing 20-25 g were purchased from Veterinary College Mannuthy, Thrissur. Animals were fed with standard mouse feed and free access to water.

Irradiation – Animals were exposed to Cobalt-60 gamma rays (9 Gy (Goel *et al*,2002)) at Radiation Therapy Unit, Amala Cancer Hospital, Thrissur.

Three months old female Wistar rats (weighing about 250 g) were used for isolation of mitochondria.

6.2.2 METHODS

6.2.2.1 Preparation of the extract

P. florida and *G. lucidum* extracts were prepared as described in section 2.2.

6.2.2.2 *Determination of haemoglobin (Hb) in blood*

Principle

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

$$\text{Haemoglobin (g/dl)} = \frac{\text{O.D}_{\text{T}} \times 60 \times 0.251}{\text{O.Ds}}$$

$O.D_T$ – Optical density of test solution

O.Ds – Optical density of standard solution

6.2.2.3 Determination of total white blood cell (WBC) count

Total WBC count was determined using haemocytometer as described by Chaudari (2000b).

Principle

The whole blood was diluted using a diluent which haemolysed 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

0.2ml blood was added to 0.38 ml of diluting fluid and charged the Neubauer Counting Chamber .The total number of white blood cells in the four large corner squares of chamber was counted.

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

6.2.*3 In vivo* Radioprotection Assay

6.2.3.1 Experiment I – Survival studies

Six groups of ten animals in each group were used for the study

Group I (Normal)

Group II (Control): Distilled water was given to the animals for five consecutive days orally and whole body irradiated with 9 Gy gamma rays.

Group III Methanolic extract of *G.lucidum* (1000 mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group IV Methanolic extract of *G.lucidum* (500 mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group V Methanolic extract of *P.florida* (1000 mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group VI Methanolic extract of *P.florida* (500 mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Animals were observed for survival daily up to 30 post irradiation days. Data were presented as % survival after post irradiation days.

6.2.3.2 Experiment II –Hematological studies

Six groups of six animals in each group were used for the study

Group I (Normal): Distilled water was given to the animals for five consecutive days orally.

Group II (Control): Distilled water was given to the animals for five consecutive days orally and whole body irradiated with 9 Gy gamma rays.

Group III Methanolic extract of *G.lucidum* (*1000* mg/kg) was given orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group IV Methanolic extract of *G.lucidum* (*500* mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group V Methanolic extract of *P.florida* (1000 mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group VI Methanolic extract of *P.florida* (500 mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Blood was collected on 1^{st},3^{rd},5^{th}, 7^{th},9^{th} day after irradiation by tail bleeding and the total leukocyte count and heamoglobin content were estimated as described in section 6.2.2.3 and 6.2.2.2 respectively

6.2.3.3 Experiment III-Bonemarrow cellularity

Six groups of fifteen animals in each group were used for the study .

Group I (Normal):

Group II (Control): Distilled water was given to the animals for five consecutive days orally and whole body irradiated with 9 Gy gamma rays.

Group III Methanolic extract of *G.lucidum* (*1000* mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group IV Methanolic extract of *G.lucidum* (*500* mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group V Methanolic extract of *P.florida* (1000 mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group VI Methanolic extract of *P.florida* (500 mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Three animals from each group was sacrificed on 1^{st},3^{rd},5^{th}, 7^{th},9^{th} day after irradiation respectively and bone marrow was collected from both the femur and the cell count was estimated.

6.2.3.4 *Experiment IV-Radiation induced liver damage*

Ninety Swiss albino mice were used in the experiment. Animals were divided into six groups of 15 animals per group. Each group was again divided into five groups of three animals per group.

Group I (Normal)

Group II (Control): Distilled water was given to the animals for five consecutive days orally and whole body irradiated with 9 Gy gamma rays.

Group III Methanolic extract of *G.lucidum* (*1000* mg/kg) was given orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group IV Methanolic extract of *G.lucidum* (*500* mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group V Methanolic extract of *P.florida* (1000 mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Group VI Methanolic extracts of *P.florida* (500 mg/kg) was given to the animals orally for five consecutive days and then whole body irradiated with 9 Gy gamma rays.

Three animals from each of the six groups were sacrificed on 1^{st},3^{rd},5^{th} 7^{th},9^{th} day after the irradiation and the liver was excised. Liver homogenate was prepared as described in section 2.2.2 and used for the estimation of reduced glutathione (GSH) (section 2.2.3) , glutathione s-transferase (GST) (section 2.2.7) and glutathione peroxidase (GPX) (section 2.2.6). Protein (section 2.2.9), superoxide dismutase (SOD) (section 2.2.4) and catalase (CAT) section 2.2.5 activities. The level of lipid peroxidation was also determined (section 2.2.8).

6.2.4 *In situ* Radioprotection Assay

6.2.4.1 *Isolation of mitochondrial fraction*

Mitochondria were isolated from liver of 3 months old female Witsar rat as described in section 3.2.3.1.

6.2.4.2 *Exposure of mitochondria to γ-radiation*

The mitochondria were suspended in the buffer (50 mM phosphate buffer, pH 7.4), and exposed to γ-radiation from ^{60}Co source (Atomic Energy of Canada Ltd) at a dose rate of 15 Gy/min. The effect of extract on the oxidative damage caused by radiation was studied at a dose of 450 Gy. Mitochondria (2.0 mg protein/ml) were suspended in the buffer and exposed to radiation with or without the

extracts. The lipid peroxidation caused by radiation was studied after exposure of mitochondria to γ-radiation .

6.2.4.3 *Lipid Hydroperoxide (LOOH)*

Aliquots (90 µl) of liver mitochondria, after exposure to radiation were transferred to microcentrifuge tubes together with 10 µl of triphenyl phospene (TPP) in methanol/10 µl of methanol in blank and test samples respectively. The samples were then vortexed and subsequently incubated for 30 min at room temperature. 900 µl of Fox II reagent (xylenol orange (100µM), butylated hydroxy toluene (4.4µM), sulphuric acid (25mM), ammonium ferrous sulphate (250µM)) was then added and samples were incubated for a further 30 min in dark. The samples were centrifuged at 12000 X g for 10 min and the absorbance of supernatant was read at 560 nm. The level of the peroxide in sample was determined from the difference between the mean absorbance of samples with and without TPP treatment and the final reading was extrapolated to H_2O_2 concentrations in the standard graph (Nourooz-zadeh *et al.*, 1996).

6.2.4.4 Thiobarbituric acid reactive substances (TBARS)

Malonaldehyde and other aldehydes have been identified as products of lipid peroxidation that react with thiobarbituric acid (TBA) to give a pink colored species with absorption maxima at 532 nm. The method involved heating of the samples after exposure to radiation and AAPH with TBA reagent for 20 min in a boiling water bath. TBA reagent contains 50ml TCA (20%), TBA (500mg) 25ml, 2.5M HCl, EDTA 224mg and final volume made up to 100ml. After cooling, the solution was centrifuged at 2,000 x g for 10 min and precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm against a blank that contained all the reagents minus the sample. The malonaldehyde equivalents of the sample were calculated using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$. For measuring endogenous TBARS, fresh samples were boiled without radiation exposure, and values were subtracted (Hunter, 1963).

6.2.5 Statistical Analysis

To determine the effect of various treatments employed in the study the experimental data were analyzed statistically using the three-way ANOVA technique.

6.3 RESULTS

6.3.1 Experiment-I: Survival Studies

Survival of animals up to 30 days of post irradiation is shown in Fig-6.1. 30 days after irradiation survival of 50% animals was achieved by the administration of 1000mg/kg of *G.lucidum* or *P.florida* extract.

6.3.2 Experiment-II: Bone Marrow Cellularity

Irradiation of animals with 9Gy resulted in decrease of bone marrow cells from 18 million cells to 2.5 million cells in the irradiated group. Treatment of the animals with *G.lucidum* or *P.florida* extract, the bone marrow count was maintained above 5 million cells (Fig-6.2) nine days after irradiation. There was significant difference in counts between extract treated irradiated group and irradiated group ($P<0.001$).

Normal animals showed significantly higher value of counts than other groups. There was no significant difference between the *G.lucidum* extract treated irradiated and *P.florida* extract treated irradiated groups.

6.3.3 Experiment-III: Heamatological Parameters

Irradiation of animals with 9Gy resulted in decrease of total leukocyte count to 2000 cells per mm cube,on the 9^{th} day where as in the irradiated group of animals treated with *G.lucidum* and *P.florida* extracts , the total leukocyte count remained above 5000 cells mm^3 after irradiation. (Fig-6.3). Between days after irradiation difference in counts was significant ($p<0.001$), first day showed significantly higher value than 3^{rd} , 5^{th}, 7^{th} and 9^{th} days in radiation alone treated group. Between 5^{th} , 7^{th} and 9^{th} day there was no significant difference in radiation

alone treated group. There was significant difference in counts between extract treated irradiated group and irradiated group ($P<0.001$). Between irradiated group of animals treated with *G.lucidum* and *P.florida* extracts there was no significant difference in counts.

Heamoglobin content was also reduced by irradiation. There was significant reduction in heamoglobin content in animals which received radiation alone treated group on 7^{th} and 9^{th} day. However, treatment of animals with *G.lucidum* and *P.florida* extracts prior to irradiation, hemoglobin content was maintained near normal on the 9^{th} day after irradiation (Fig-6.4).

6.3.4 Experiment-IV: Radioprotective Effect Of *G.lucidum* And *P.florida* On Liver Damage

Radiation caused several deleterious changes at cellular level. Significant radiation induced metabolic changes in the liver of irradiated animal were observed. Radiation caused severe oxidative stress and damage to the antioxidant defense. The *G. lucidum* or *P.florida* mushroom extracts were found to delimit these changes to a great extent. The effect of the extract was evident from the restoration of the activities of GSH, GPX, GST, SOD, CAT and inhibition of lipid peroxidation

6.3.4.1Reduced Glutathione (GSH)

G.lucidum or *P.florida* extract treatment before irradiation prevented the radiation induced depletion of GSH in liver (Fig 6.5). There was significant difference in GSH level between group of animals received irradiation and treatment with extract prior to irradiation. ($P<0.001$). There was no significant difference in GSH activity between the treatment of *G.lucidum* or *P.florida* extract prior to irradiation.

6.3.4.2 Glutathione peroxidase (GPX)

G.lucidum or *P.florida* extract treatment prior to irradiation prevented the radiation induced depletion of liver GPX (Fig 6.6). There was significant difference in GPX activity between irradiation alone and extract treatement prior to

radiation on 7^{th} and 9^{th} day ($P<0.001$). There was no significant difference in GPX activity between the treatment of *G.lucidum* or *P.florida* extract prior to irradiation.

Normal group gave significantly higher value of GPX than others. There was no significant difference in GPX activity between the treatment with *G.lucidum* or *P.florida* extract prior to irradiation.

6.3.4.3 Glutathione- s- transferase

G.lucidum or *P.florida* extract treatment before radiation prevented the radiation induced decrease in GST activity (Fig 6.7).

There was significant difference between irradiation alone and extract treatment prior to radiation treated group . There was no significant difference in GST activity between the treatment of *G.lucidum* or *P.florida* extract prior to irradiation.

6.3.4.4 Superoxide dismutase

G.lucidum or *P.florida* extract pretreatment checked the initial fall of SOD activity after irradiation and maintained the activity at normal level thereafter (Fig 6.8). There was significant difference in activity in extract treated group as than animals that received radiation alone ($P<0.001$). There was no significant difference in activity between treatment with *G.lucidum* or *P.florida* extract prior to irradiation.

6.3.4.5 Catalase

There was significant difference in activity of catalase between days ($P<0.001$). 9^{th} day showed significantly higher catalase activity in extract treated group than animals that received irradiation alone. There was significant difference in activity between control and extract treated groups ($P<0.001$). There was no significant difference in catalase activity between the groups treated with *G.lucidum* or *P.florida* extract prior to irradiation.(Fig 6.9)

6.3.4.6 Lipid peroxidation

Irradiation resulted in a highly significant increase of lipid peroxidation. (Fig 6.10). There was significant difference in activity between days ($P<0.05$). 7^{th}

day and 9th day showed significantly higher activity than 1st and 3rd day. Among extract treated group, control and normal variation was highly significant ($P<0.001$). Control showed significantly higher activity than other groups. There was no significant difference in activity between the two mushrooms extracts treated groups.

6.3.5 In situ Radioprotection Assay

Exposure to radiation, as a function of dose, ranging from a dose of 0 to 750 Gy, resulted in enhanced lipid peroxidation as evident from the formation of TBARS. The increase in TBARS formation was significant with the increasing doses examined. Exposure to 300-450 Gy showed steep increases while higher doses were effective only in marginally enhancing peroxidation. Hence, a dose of 450 Gy was selected for the experiments as this dose caused optimum damage in terms of lipid peroxidation in rat liver and brain mitochondria.

Mitochondria are crucial targets for radiation and free radical mediated damage. Since mitochondria are devoid of cystolic antioxidants, as a whole cell, they are fairly resisitant to γ radiation , hence a dose of 450 Gy is needed to achieve optimum concentration of free radicals to induce significant damage measurable by simple spectrophotometric means. This dose is much higher than the dose used in radiotherapy (1-6Gy) or for radioprotection pertinent to mammals (LD50 in the range of 5-7Gy).

The data on the radiation induced lipid peroxidation of rat liver mitochondria and its protection by mushrooms extracts are given in Fig 6.11 and 6.12 *P. florida* and *G.lucidum* extract showed significant ability to inhibit radiation induced lipid peroxidation in rat liver mitochondria. There was significant difference in activity between control and the extract treated group ($P<0.001$) . The formation of LOOH, an intermediate of peroxidation, showed that LOOH formation induced by γ-radiation in rat liver mitochondria was inhibited by extract. *P. florida* and *G.lucidum* at a concentration of 1% reduced TBARS formation significantly when it was present at the time of irradiation .

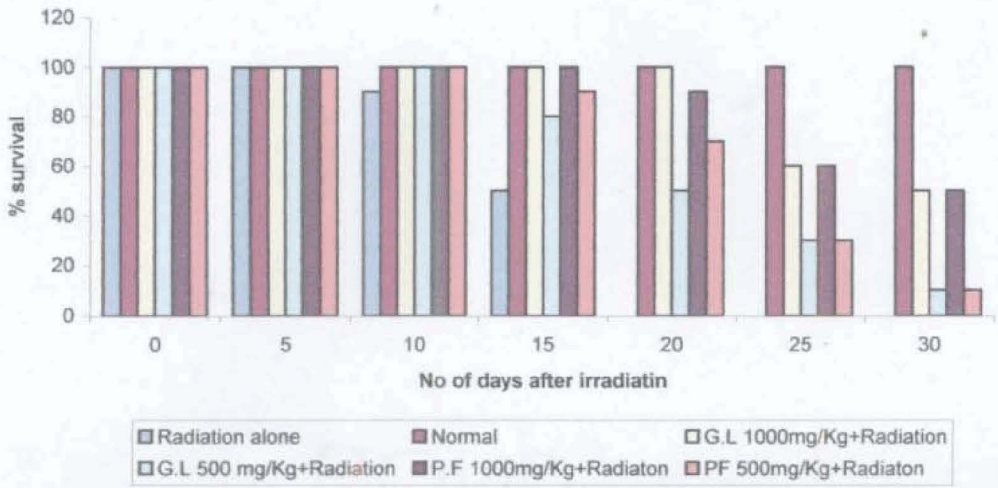


Fig 6.1 Effect of mushroom extract on radiation induced survival rate

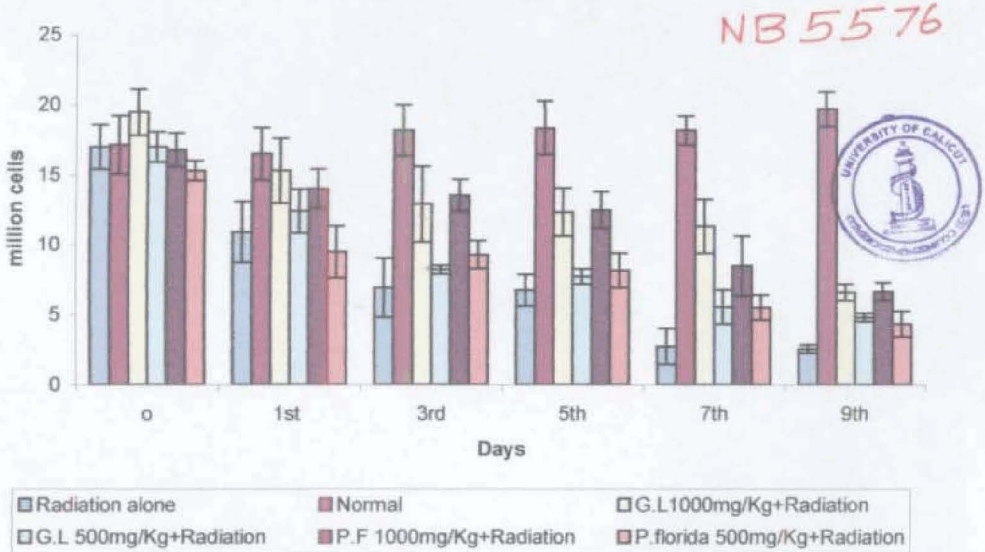


Fig- 6. 2 Effect of mushroom extracts on radiation induced changes in bonemarrow cellularity

Values are mean ± SD (n=3). There was significant difference in counts between extract treated irradiated group and irradiated group ($P<0.001$). Normal animals showed significantly higher value of counts than other groups. There was no significant difference between the *G.lucidum* extract treated irradiated and *P.florida* extract treated irradiated groups.

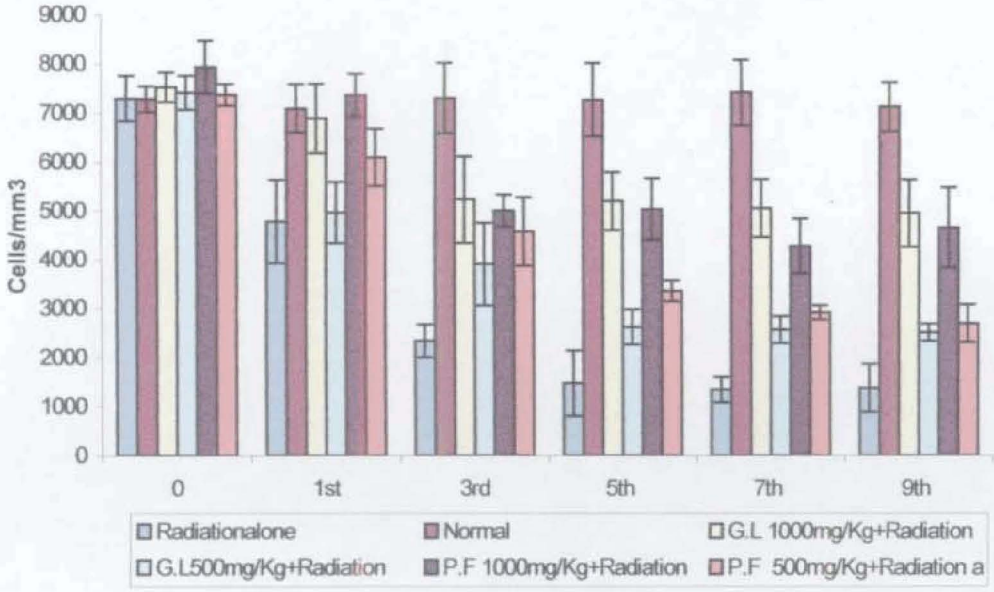


Fig-6.3 Effect of mushroom extracts on radiation induced changes in total leukocyte count

Values are mean ± SD (n=6). Between days after irradiation difference in counts was significant ($p<0.001$), first day showed significantly higher value than 3rd, 5th, 7th and 9th days in radiation alone treated group. Between 5th, 7th and 9th day there was no significant difference. There was significant difference in counts between extract treated irradiated group and irradiated group ($P<0.001$). Between irradiated group of animals treated with *G.lucidum* and *P.florida* extracts there was no significant difference in counts.

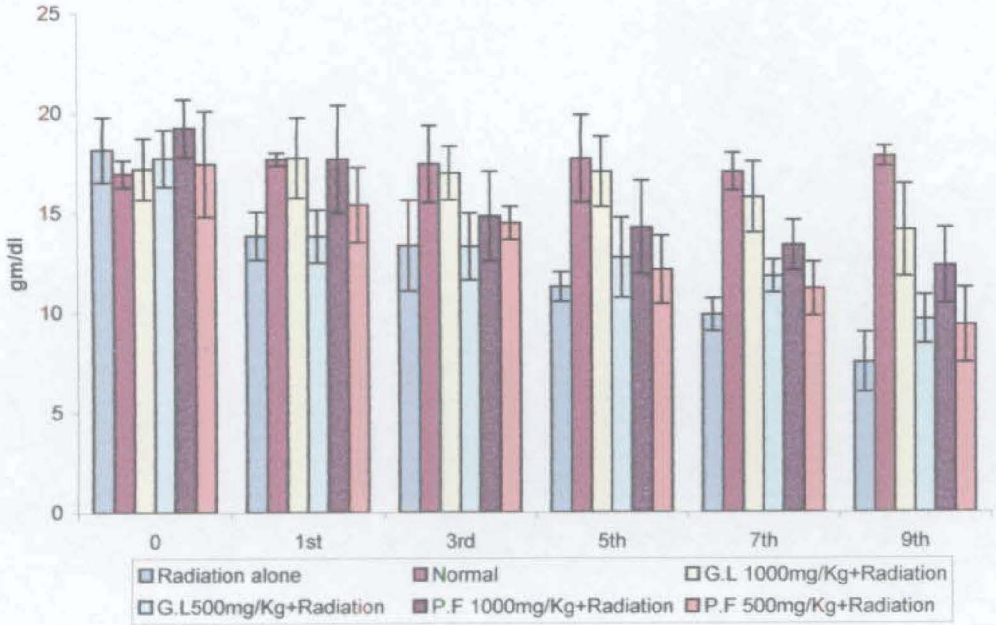


Fig-6.4 Effect of mushroom extracts on radiation induced changes in heamoglobin count.

Values are mean ± SD (n=6). Treatment of animals with *G.lucidum* and *P.florida* extracts prior to irradiation the heamoglobin content was maintained near normal on the 9th day after irradiation. There was significant reduction in heamoglobin content in radiation alone treated group on 7th and 9th day when compared to normal ($P<0.001$). .

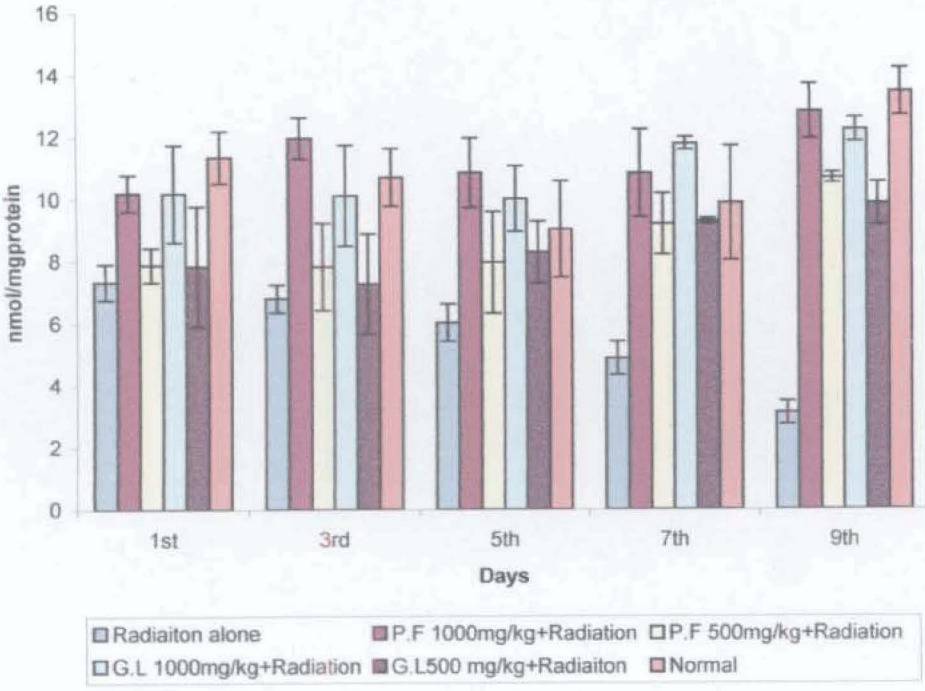


Fig-6.5 Effect of mushroom extracts on radiation induced changes of GSH levels.

Values are mean ± SD (n=3). There was significant difference in GSH level between group of animals received irradiation and treatment with extract prior to irradiation. ($P<0.001$). There was no significant difference in GSH activity between the treatment of *G.lucidum* and *P.florida* extract prior to irradiation.

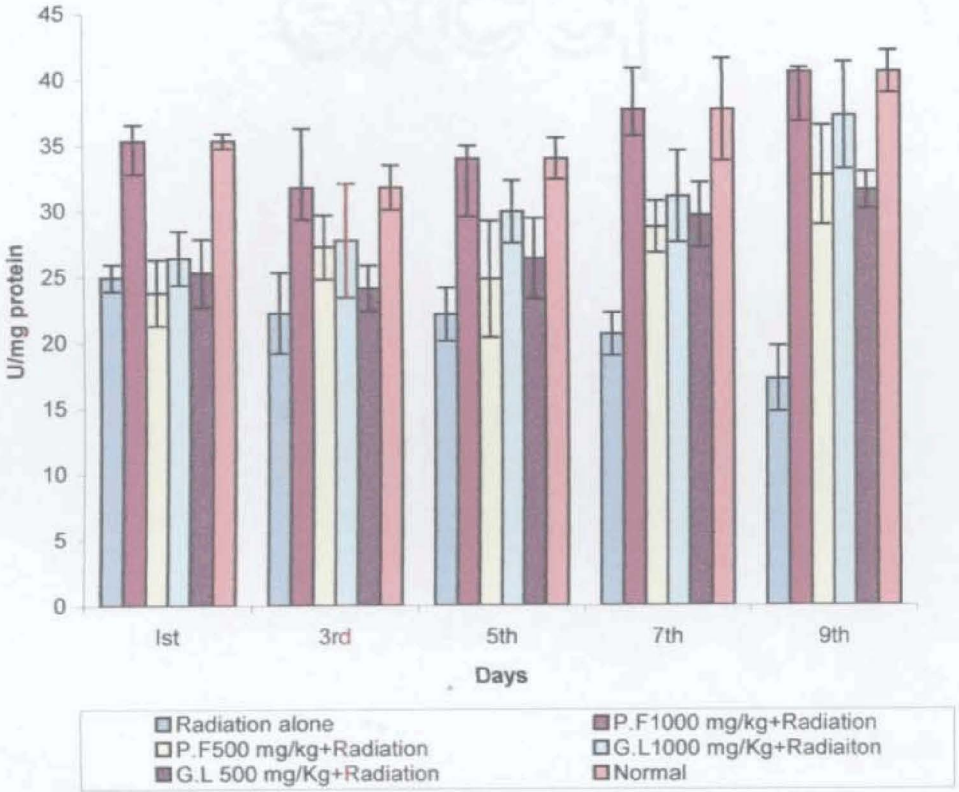


Fig-6.6 Effect of mushrooms on radiation induced changes in GPX activity.

Values are mean ± SD (n=3). There was significant difference in GPX activity between irradiation alone and extract treatement prior to radiation on 5th, 7^{th} and 9^{th} day ($P<0.001$). There was no significant difference in GPX activity between the treatment of *G.lucidum* and *P.florida* extract prior to irradiation.

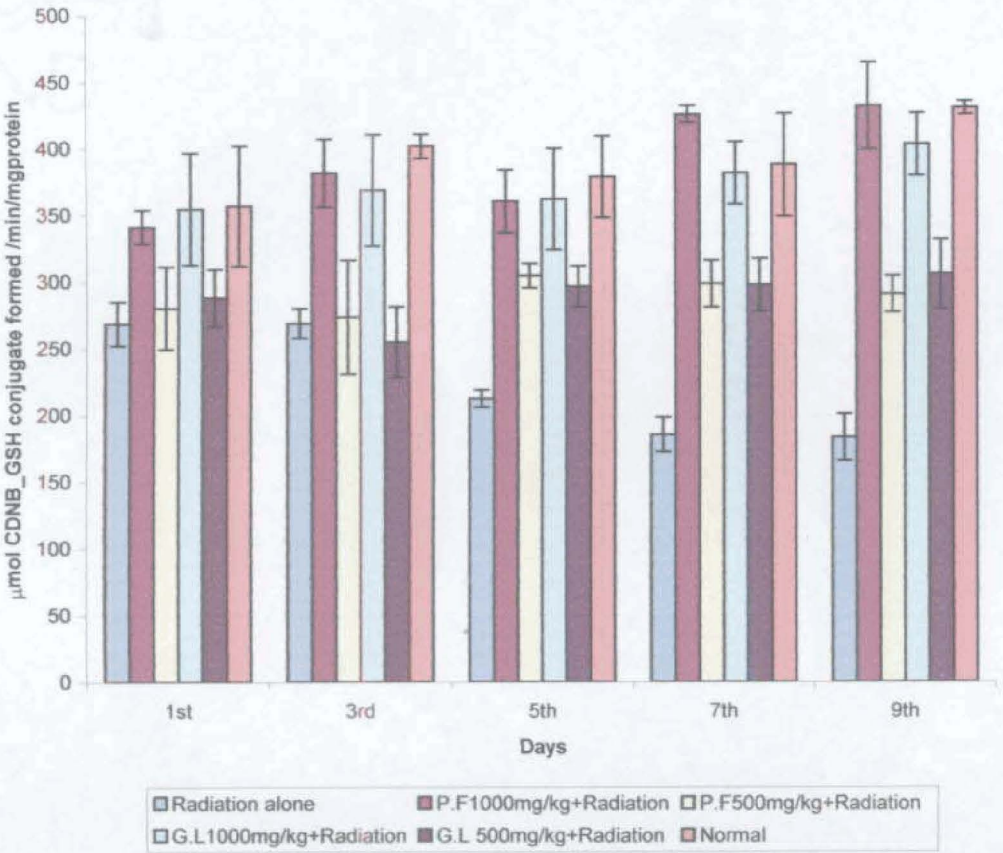


Fig-6.7 Effect of mushroom extracts on radiation induced changes in GST activity.

Values are mean ± SD (n=3). There was significant difference between irradiation alone and extract treatment prior to radiation treated group . There was no significant difference in GST activity between the treatment of *G.lucidum* and *P.florida* extract prior to irradiation.

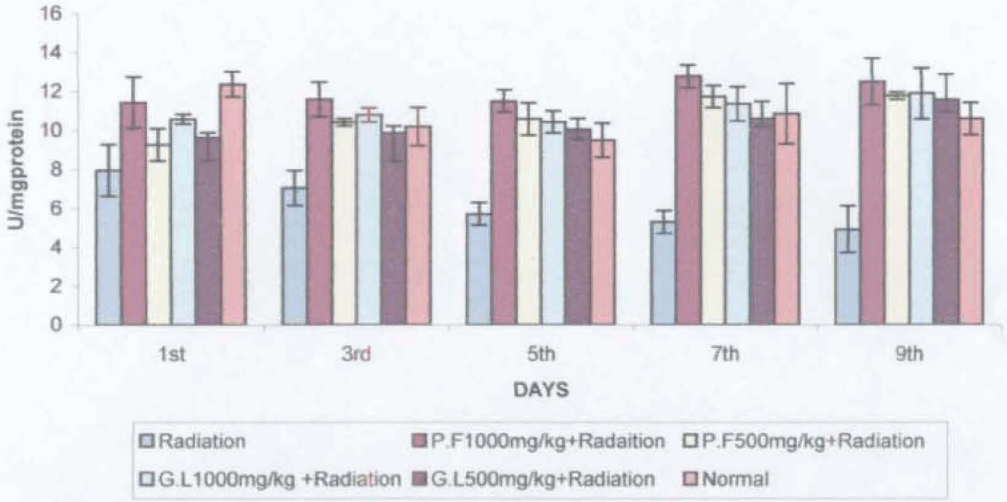


Fig-6.8 Effect of mushroom extracts on radiation induced changes in SOD activity

Values are mean ± SD (n=3). There was significant difference in activity between irradiation alone and mushroom extract treatment prior to radiation ($P<0.001$).

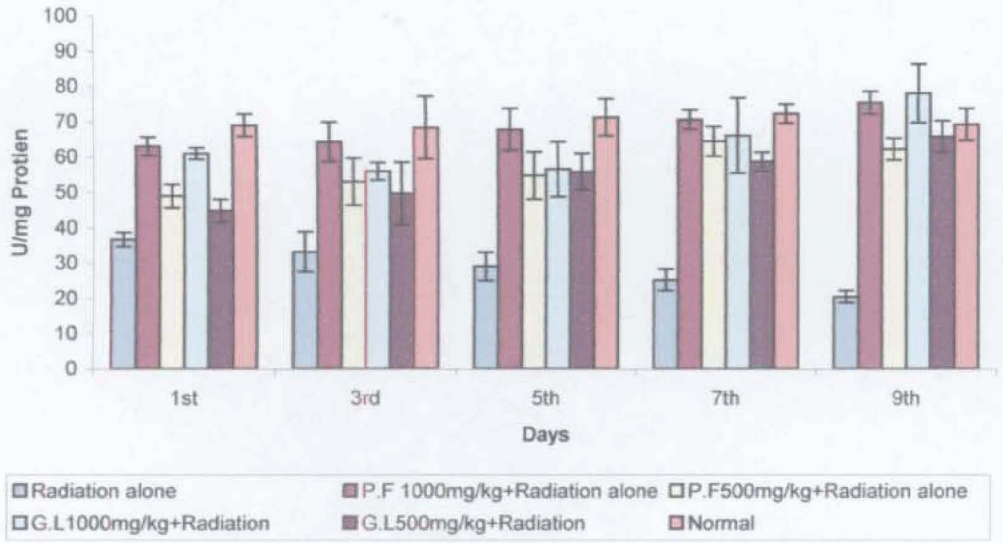


Fig-6.9 Effect of mushroom extracts on radiation induced changes in catalase activity

Values are mean ± SD (n=3). There was significant difference in activity between control and mushroom extracts treated groups ($P<0.001$).

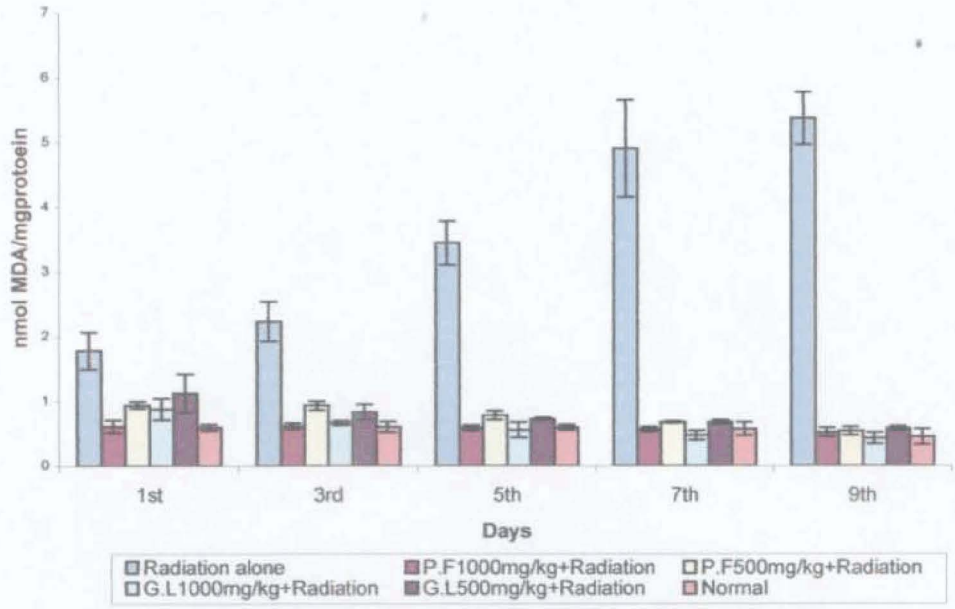


Fig 6.10 Effect of mushroom extracts on radiation induced changes in lipid peroxidation.

Values are mean ± SD (n=3). The lipid peroxidation in radiation alone group was significantly higher than other groups (P<0.001).

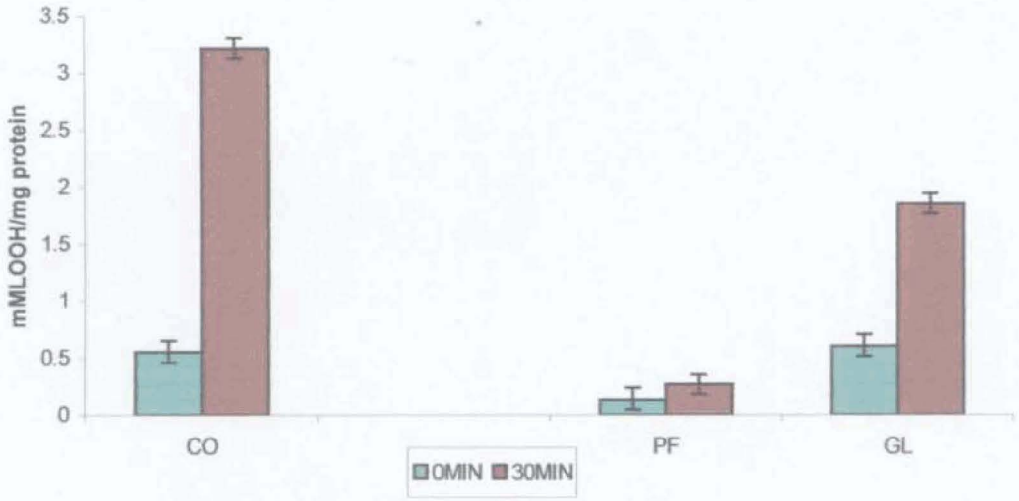


Fig 6. 11 *In situ* effect of mushroom extracts on radiation induced lipid hydro peroxide (LOOH).

Values are mean ± SD (n=4). (CO-control, PF-*Pleurotus florida*, GL-*Ganoderma lucidum*). Control showed significantly higher value of hyderoperoxide than other groups.(P<0.001)

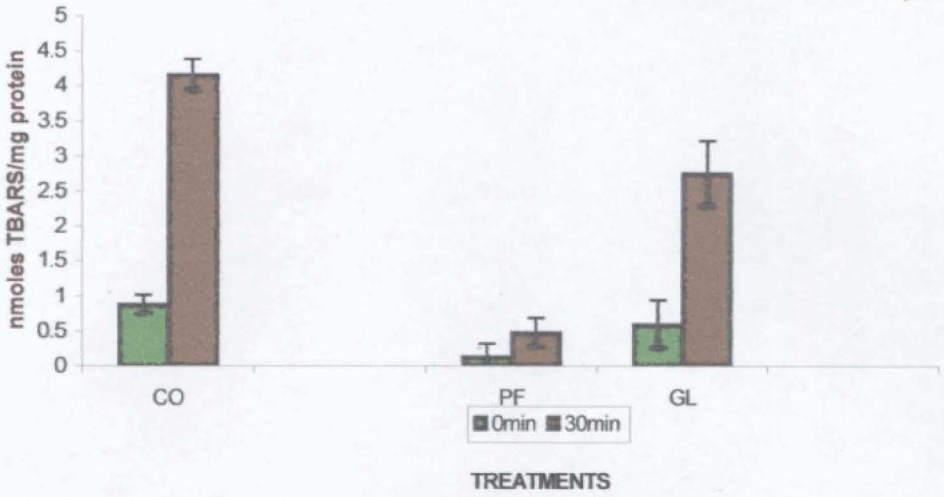


Fig 6.12 *In situ* effect of mushroom extracts on radiation induced lipid peroxidation (TBARS)

Values are mean ± SD (n=4). C0- Control, PF-*P.florida*,GL-*G.lucidum* There was significant difference in lipid peroxidation between 0 min and 30 min. ($P<0.001$). Control gave significantly higher value than other groups.

6.4 DISCUSSION

Protecting living systems from the dangers of ionizing radiation is of paramount importance in radiaition biology. However radiation protection has also significant relevance in radiotherapy of cancer. The result of the investigations reveals that administration of *G.lucidum* and *P.florida* extracts prior to irradiation rendered 50% survival in mice against 9 Gy whole body irradiation. Untreated irradiated mice suffered 100% mortality within 20 days. The maximum protective effect was achieved at a concentration of 1000 mg/kg of the extract .

After whole body irradiation the majority of bone marrow cells are damaged or dead and resulting in their inability for division or maturation. At the end of 9^{th} day the bone marrow cellularity and total leukocytes count of animals received radiation alone group were very low as compared to animals administered with *G.lucidum* and *P.florida* (1000mg/kg) extracts prior to irradiation. The radiation induced losses in bone marrow cellularity and total WBC count have been well

compensated by treatment with *G.lucidum* and *P.florida* extract . The results thus indicate that mushroom extracts render significant protection against radiation.

Radiation protection by *G.lucidum* and *P.florida* extracts at cellular and subcellular level reflects both wholesome effect of scavenging of radiation induced free radicals and the repair of damaged targets and molecules. However, repair of damage and replenishment of cells to substitute apoptotic and necrotic cell are important manifestations of an ideal radioprotector (Goel *et al*, 2002). This may contribute towards recovery of a number of tissues like bone marrow, intestine etc. This could be possible due to proliferative stimulation rendered to the stem cells of the tissue system factors etc. A large number of mushroom compounds, both cellular and secondary metabolites have been shown to affect the immune system and could be used to treat a variety of diseases (Chihara, *et al* 1987., Jong and Birmingham 1992., Sakagmi and Takeda, 1993)

A large number of chemicals have been investigated during the last 50 years for radiation protection. Among them, some are natural amino acids like cysteine, derivatives, vitamins, protein like glutathione and synthetic molecules. However, the inherent toxicity of these compounds in useful concentrations has warranted further exploration. In last decade, natural herbal products have received significant attention owing to their utility in traditional system of medicine. Among the different isolated compound the flavones, flavanoids, flavins, glucosides, polyphenols, catachins, epigallocatachins etc. have been reported to render radioprotection because of their free radical scavenging potential. Scavenging of free radicals by an agent plays a pivotal role in radioprotection activity. This probably explains the radioprotective effect of *G.lucidum* and *P.florida* extract.

Antioxidants in diet are of significant importance as possible protective agents to help human body to reduce oxidative damage. Recently a large number of natural antioxidants have been isolated from different plant materials (Packer and Ong, 1997; Jovanovic and Simic, 2000; John *et al.*, 2002). Mushrooms are functional foods and are traditionally used in folk medicine of several systems of medicine. Medicinal mushrooms possessing antioxidant properties in human diet would be potentially useful to help human body to reduce oxidative damage.

Ionizing radiation is toxic to organisms since it induces deleterious structural changes in essential macromolecules (Navaro *et al*, 1997). The interaction of ionizing radiation with biological system results in generations of free radicals, H and OH radicals, H_2 and H_2O_2. Radiations induced free radicals in turn impair the antioxidant defense mechanism leading to increased membrane lipid peroxidation, which results in the damage of membrane, bound enzymes (Halliwell and Gutteridge, 1989). The increased lipid peroxidation is due to the low concentration of GSH. Antioxidant enzymes are among the endogenous system that are available for the removal or detoxification of these free radicals and their products formed by ionizing radiation. The GSH/GST detoxification system is an important part of cellular defense against a large array of endogenously or exogenously formed injurious agents. GSH offers protection against oxygen-derived free radicals and cellular lethality following exposure to ionizing radiation. GST enzymes also possess peroxidase activity and can directly attack the peroxides that may be generated via oxidative reduction recycling, resulting in decreased cytotoxicity.

The present study demonstrates that a significant reduction in GSH and the activities of all antioxidant enzymes in radiation treated group of animals . This could be due to the enhanced utilization of antioxidant defense system in an attempt to detoxify the radicals generated by radiation. In the intact and healthy cells, the enzymes are restored immediately after each interaction. GSH is restored by synthesis (Meister and Anderson, 1983). But in the irradiated animals, the normal synthesis/repair will be disrupted due to damage to DNA and membranes. As a result, restoration will be delayed until the cells are recovered. This could explain the slow recovery in the levels of GSH and antioxidant enzymes after radiation treatment.

One of the most deleterious effects of radiaition is the drastic increase of lipid peroxidation. The result of present study indicates that methanolic extract of *G.lucidum* and *P.florida* inhibits lipid peroxidation .

Radiation causes increase in the levels of superoxide radicals. The increased SOD activity with the treatment of *G.lucidum* and *P.florida* extracts was therefore due to the elimination of the superoxide radicals. Because of this

dismuttation reaction, highly reactive H_2O_2 are formed. Catalase and GPX are enzymes responsible for degradation of H_2O_2,. *G. lucidum* treatment increases the activity of catalase in liver.

GPX is an important scavenger enzyme and has an incorporated selenium molecule to which it owes its activity . The treatments with *G.lucidum* and *P.florida* extracts also increase the level of GPX .

The level of GSH was decreased in radiation-exposed group as compared to normal group of animals. This may be due to the electrophillic burden generated because of irradiation. The data clearly indicate that treatment of *G.lucidum* and *P.florida* extracts increase the level of liver GSH. The increased activity of GPX in the group of animals that were treated with the extract prior to irradiation might be due to the increased level of its substrate GSH.

The results of the investigations indicate that methanolic extracts of *G.lucidum* and *P.florida* possess significant property to protect radiation induced cellular damage. This might be due to their profound antioxidant activity. The findings thus suggest the potential therapeutic use of the extracts of these mushrooms in radiation protection.

CHAPTER-7

ANTI CARCINOGENIC ACTIVITY OF *GANODERMA LUCIDUM* AND *PLEUROTUS FLORIDA*

7.1 INTRODUCTION

The evolution of an invasive cancer cell form a normal cell, in other words the process of transformation of a normal cell to an invasive cell is called carcinogenesis. Carcinogenesis can be divided into stages of intitation, promotion and progression. The initiation stage is more clearly understood than subsequent stages. It begins with exposure to carcinogens. Carcinogenesis may result from the action of any one or a combination of chemical, physical, biological and/or genetic insult to cells. Chemical carcinogenesis and co- carcinogens are considered responsible for many of the cancers of humans (Higginson, 1976). Because of the nature of occupation, certain people more than others are exposed to higher concentration of carcinogens prevailing in the work environment. Many chemical compounds are carcinogenic only after the metabolic activation. Exposure to carcinogens such as PAH, causes an increase in the expression of the enzyme responsible for this activation. These enzymes consist of members of CYP1A and 1B sub families. They generate genotoxic epoxide metabolites of the parent hydrocarbon that can bind to DNA forming adducts (Gilbert, 1994). These adduct if not repaired, can cause specific mutation leading to cellular transformation. Therefore, the activation and expression of carcinogen activating enzymes are key components in chemically induced carcinogenesis and the inhibition of their activity, either by direct enzyme inhibition or through modulation of their expression is thought to be an important mechanism in the prevention of carcinogenesis. (Gilbert and Marinduque, 1990). Prevention of carcinogenesis is one of the major strategies for cancer control. The inhibitory effect of several preventive agents in experimental carcinogenesis has been reported. However, some of these agents have harmful effects and are also usually expensive

The presence of nitroso compounds in diet together with the possibility of their endogenous formation in human body from precursor has been a matter of concern, as these compounds are known carcinogenic agents. (Lai and Acroos, 1980). Nitrosoamines have been found in foods such as meat and dietary products and alcoholic beverages and industrial waste (Tricker *et a l.*, 1991). The average intake of volatile nitrosamines from food is approximately 1μg/day (Scanlan, 1983).

Since the liver is the major site in the body that metabolizes ingested material, it is more susceptible to carcinogenic insult. Nitrosamines such as N-nitrosodiethylamine (NDEA) has been considered to a potent hepatocarcinogen in rats. NDEA produces a reproducible tumor after repeated administration. The mechanism of action is due to metabolism of NDEA to alkylating reactants and reactive oxygen species and further interaction with the DNA molecule (Czygan *et al.*, 1973., Leoppky and Li, 1991). Moreover, due to the high tolerance of liver hepatocarcinoma is seldom detected at the early stage and once detected treatment has a poor prognosis in most cases. Hepatocarcinoma (HCC) is a major disabling disease that affects a large population in the world, and there are only a few drugs that can reduce the onset and course of the disease. Hence a chemopreventive agent exhibiting activities such as antiinflammation, 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.

Breast cancer is a major cause of cancer death in women worldwide. Life style and environmental risk factors account for a great proportion. Genotoxic compounds implicated in human breast carcinogenesis include endogenous compounds such as estrogens and dietary or environmental xenobiotics heterocyclic amine, aromatic amine, polycyclic hydrocarbons. Almost 600,000 new cases of breast cancer are identified each year worldwide. In North America breast cancer accounts for over one quarter of all cancer related deaths (Parker *et al.*, 1977). Despite abundant information about its etiopathogenesis and early detection, effective therapeutic modalities for patients with advanced stages of the disease are still needed. Adjuvant therapy after ablative surgery is effective only when the tumor is detected early.

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 and breast carcinogens known. Most of the metabolically activated PAHs are mutagenic to DNA (Miller, 1978). 12-*O*- tetradecanoylphorbol-12-acetate (TPA) is a 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 tumor, bladder stomach and colon. The experimental evidence strongly suggests the role of free radical mediated tumor promotion in phorbol ester promoted papilloma on the skin (Cerutti, 1985). The applications of croton oil have been shown to reduce antioxidant enzymes in both epidermal and inflammatory cells. (Solanki, *et al.*, 1984). Inhibition of ROI (Reactive Oxygen Intermediates) generation can serve as an important system for the identification of agents that can inhibit oxidative DNA damage as well as tumor promotion.

Accumulating evidence derived from laboratory studies and study cohorts drawn form the general population have led to search for "chemoprotection" and agents to attenuate the risk of breast cancer. Based on observation that most human cancers are associated with a long period of latency (Benner and Hong, 1993) . Several nonnutritive phytochemicals found in natural products associated with pharmacological attributes reveal that they inhibit /delay/ and or reverse cancer evoked by either environmental insults and/or life style (Waladkhani and Clemens, 1998). Several of these chemopreventive agents act at the initiation, promotion or progression stages conceptually associated with the ontogeny of multistage carcinogenesis. Anticarcinogenic potential of methanolic extracts of *G.lucidum* and *P.florida* were investigated. N-nitrosodiethylamine induced hepatocarcinogenesis, DMBA inititated and croton oil promoted mouse skin pappiloma and DMBA induced rat mammary tumor were employed as experimental models. The results of the investigations are reported in this chapter.

7. 2 MATERIALS AND METHODS

7.2.1 Preparation of extracts

Extracts of *P.florida* and *G.lucidum* were prepared as described in Chapter 2 section 2.2.1

7.2.2 Determination of anticarcinogenic activity using NDEA induced Hepatocellular carcinoma. (HCC)

Male Witsar rats (150± 20g) were used for the experiment HCC was induced according to method of Jose *et al* (1999) with slight modifications. Animals were divided into four groups of six animals in each group. The group 1 treated with distilled water was maintained as normal. Group 2 administered with NDEA 94mg/kg body wt .p.o for 5 days/week for 20 weeks was kept as control. Group 3 and 4 were administered orally with 500 mg /kg body wt methanolic extract of *G.lucidum* or *P.florida* respectively 1hr prior to each NDEA administration. 12 weeks after the last dose of NDEA administration, animals were kept fasting overnight and then sacrificed. Blood was collected from the heart for serum. Serum was used for the determination of gammaglutamyl transpeptidase (GGT) (section 2.2.13), glutamate pyruvate transaminase (GPT), (section 2.2.11) glutamate oxaloacetate transaminase (GOT)) (section 2.2.10), total protein (section 2.2.14), albumin (section 2.2.15), alkaline phospatase (section 2.2.13) and lipid peroxidation (section 2.2.8).

Liver homogenate was prepared as described in section 2.2.2 and was used for the estimation of reduced glutathione (GSH) (section 2.2.3) , glutathione s-transferase (GST) (section 2.2.7) and glutathione peroxidase (GPX) (section 2.2.6). Protein was determined by the method of Lowry *et al* (1957).

7.2.3 Histopathological examination

A portion of the liver was fixed in 10% formalin and then embedded in paraffin. 6μm microtone sections were prepared from each liver and stained with hematoxylin-eosin.

7.2.4 Determination of anticarcinogenic activity using mammary tumor model

Female Sprague Dawley rats 40-50 days old (140 g) were grouped in to 6 groups of 6 animals in each group.

Group I: Untreated animals: Normal

Group II 10mg 7,12 dimethyl benz (*a*) anthracene (DMBA) in olive oil/animal by gavage once a week (3 weeks) .

Group III Methanolic extract of *P. florida* (500mg/kg) was administered orally twice weekly for two weeks. Thereafter 10 mg DMBA was administered as in group II.

Group IV Methanolic extract of *P. florida* (1000 mg/kg) was administered twice weekly to two weeks orally. Thereafter 10 mg DMBA was administered as in Group II.

Group V Methanolic extract of *G. lucidum* (500 mg/kg) was administered orally twice weekly to two weeks. Thereafter 10 mg DMBA was administered as in Group II.

Group VI Methanolic extract of *G. lucidum* (1000 mg/kg) was administered twice weekly to two weeks orally. Thereafter 10 mg DMBA was administered as in Group II.

Average number of tumor per rat, percent of animals with tumor and tumor latency period were recorded for a period of 160 days.

7.2.4. Histopathological examination

A portion of the mammary gland was fixed in 10% formalin and embedded in paraffin 6μm microtone sections were prepared from each and stained with hematoxylin-eosin.

7.2.5. Determination of antipromotional activity using two stage carcinogenesis

Fourty female Balb/c mice (20-25g) shaved on their back using surgical clippers 2 days before the experiment. Animals with complete hair growth arrest were grouped into five 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 200μl acetone (Mimura *et al*, 1994). One week after tumor initiation, the promotion was induced by topical application of 200μl of freshly isolated croton oil (10% in acetone, v/v) twice weekly for 8 weeks on to the same area (Verma and

Boutwell, 1980., Divan *et al* 1985). The methanolic extract of *P.florida* and *G.lucidum* (2mg or 10 mg in 200µl acetone/mouse) was applied topically 40 minutes before each croton oil application. The group treated with croton oil alone served as positive control. Skin pappiloma formation was recorded weekly in each experimental group. Average number of pappilloma per mouse, percent of animals with pappilloma and tumor latency period were recoded.

7.2.6 Statistical analysis

To compare the effect of various treatments employed in the study the experimental data were analysed statistically using the one way ANOVA technique.

7.3 RESULTS

7.3.1 Effect of *P.florida* (PF) and *G.lucidum* (GL) extracts rats with HCC induced by NDEA

All the animals in the control group developed liver tumors by the end of 32 week. The number of tumors and percent of incidence was reduced significantly in animals administered with the *P.florida* and *G.lucidum* extract treated group. The activities of SGOT (440 ± 35.7 IU/L) , SGPT (1100 ± 334.66 IU/L) and ALP (394.89 ± 48.17IU/L) were elevated significantly in the NDEA alone treated ($P<0.001$) animals compared to the normal group of animals. Marked decline was observed in the activities of these enzymes in the group of animals administered with mushroom extracts prior to NDEA treatement (Table 7.1). There was significant difference in activities of SGOT, SGPT and ALP between control and extract treated groups ($P<0.001$). The control group of animal the albumin/globulin ratio A: G (Table 7. 2) was altered 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 when animals were pretreated with mushroom extracts. The activity of GGT was lowered significantly in NDEA alone treated group of animals (Table 7.3). There was significant difference in GGT activity between control and extract treated group of animals ($P<0.001$). NDEA alone treated animals showed significantly higher GGT value than extract treated group. Between extract treated group and normal group of animals there was no significant difference in GGT activity.

The index of lipid peroxidation (MDA level) was elevated in the serum of the NDEA alone treated group of animals (Table 7.3). There was significant difference in lipid peroxidation between treatments ($P<0.001$). NDEA alone treated group of animals showed significantly higher MDA levels than normal, and mushroom extract treated group of animals. The activities of GST, GPX and GSH (Table 7.4) level in the liver homogenate of the extract treated animals showed significant decrease compared to the NDEA alone treated group of animals. There was significant difference in GST activity between control and extract treated group of animals ($P<0.001$). NDEA alone treated animals showed significantly higher activity of GST than animals treated with mushroom extract. Between extract treated group and normal group of animals there was no significant difference. NDEA alone treated group showed significantly higher GPX activity than normal and *P.florida* treated group of animals. Between normal, *P.florida* and *G.lucidum* treated group of animals there was no significant difference in GPX activity. NDEA alone treated group of animals showed significantly higher GSH level than extract treated group. Between normal and *G.lucidum* treated group of animals, there was no significant difference in GSH level.

Histopathological examination indicated that in NDEA alone treated group of animals liver cells were arranged mostly in solid and trabecular pattern, with cellular polymorphism, fatty infiltration, varying mitotic figures and focal necrosis. All these changes clearly indicated the hepatocellular carcinoma. These pathological manifestations were decreased to moderate level when animals were treated with mushroom extracts (Fig7.2)

TABLE- 7.1. Effect of *P.florida* (PF) and *G.lucidum* (GL) extracts 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		148.3±20.4	72.3±7.3	142.4±31.9
Control(NDEA)	94	1100±334.6	440± 35.7	394.89±48.1
PF+NDEA	500	403±64.0	235± 30.2	188.1±41.57
GL+NDEA	500	385±104.3	256.7± 29.4	190.24±48.4

Values are mean ±S.D, n=6.

SGPT: There was significant difference between treatments (P<0.001). Control showed significantly higher value than normal and mushroom extract treated group.

SGOT. Control showed significantly higher values than normal, and mushroom extract treated group.

ALP: There was significant difference between control and extract treated groups (P<0.001).

TABLE-7.2 Effect of *P.florida* and (PF) and *G.lucidum* (GL) extracts on serum albumin, globulin and albumin globulin ratio (A/G) in rats with HCC induced by NDEA.

Groups	Treatments (mg/kg)	Total Protein	Albumin (mg/dl)	Globulin (mg/dl)	A/G (mg/dl)
Normal		5.85 ± 0.2	3.53 ± 0.06	2.32 ± 0.9	1.75 ± 0.7
Control (NDEA)	94	6.6 ± 1	2.4 ± 0.6	4.2 ± 1.5	0.5 ± 0.09
PF+NDEA	500	6.97 ± 0.5	3.301 ± 0.3	3.67 ± 0.9	1.07 ± 0.25
GL+NDEA	500	6.8 ± 0.9	3.92 ± 0.43	2.9 ± 1.08	1.37 ± 0.4

Values are mean ±S.D, n=6

Normal gave significantly higher A/G ratio than control (P<0.001). Between control and GL treated group there was 5% level of significance. Between normal and PF treated group there was no significant difference.

TABLE-7.3 Effect of *P.florida* and (PF) and *G.lucidum* (GL) extracts on serum GGT activity and MDA level in rats with HCC induced by NDEA.

Groups	Treatments (mg/kg)	GGT (U/l)	MDA (nmol/ml)
Normal	Vehicle	16.7±6.08	1.07±0.24
Control (NDEA)	94	40.8±11.5	3.36±0.29
PF+NDEA	500	16.6±5.02	1.21±0.284
GL+NDEA	500	18.49±6.8	1.28±0.22

Values are mean ±S.D n=6 .GGT. Control showed significantly higher activity of GGT than mushroom extract treated group ($P<0.001$). Between mushroom extract treated groups and normal there was no significant difference in GGT value.

MDA: ($P<0.001$). Control showed significantly higher level of MDA than normal, and mushroom extract treated group of animals ($P<0.001$).

TABLE- 7.4. Effect of *P.florida* (PF) and *G.lucidum* (GL) extracts on hepatic GPX, GST activity and GSH level in rats with HCC induced by NDEA.

Groups	Treatments (mg/kg)	GPX (U/l)	GSH (nmol/mg protein)	GST (µmol of CDNB-GSH conjugate formed /min/ mg protein)
Normal	Vehicle	28.2 ± 3.6	9.35 ± 0.9	396.2 ± 50.1
Control (NDEA)	94	35.7 ± 2.6.	11.9 ± 1.4	730.7 ± 67.9
PF +NDEA	500	29.5± 4.42	8.78 ± 1.13	476.03 ± 93
GL +NDEA	500	32.8 ± 3.08	9.4 ± 1.29	538 ±110

Values are mean ±S.D n=6 .

GST: Control showed significantly higher activity of GST than mushroom extract treated group of animals ($p<0.001$). There was no significant difference between normal and *P. florida* extract treatment along with NDEA.

GPX: Control showed significantly higher activity of GPX than normal and *P. florida* extract treated group ($P<0.05$).. There was no significant difference between normal and *P.florida* extract treatment along with NDEA.

GSH. Control showed significantly higher level of GSH than *P. florida* extract + NDEA treated group ($P<0.05$). There was no significant difference between normal and mushroom extract treatment along with NDEA.

7.3.2 Effect of mushroom extracts on DMBA induced mammary tumor model

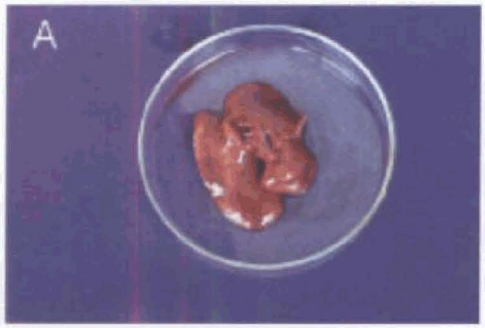
First mammary tumor was observed in the group of animals after a period of 73 days after the first administration of DMBA. In the group of animals treated with *P. florida* or *G. lucidum* extract prior to DMBA administration the first tumor appeared only after 105 and 98 days respectively (Fig-7.3). All the surviving animals in the DMBA group had mammary tumors. An average of 4 mammary tumors were observed per animal in the DMBA alone treated group while the animals treated with *P. florida or G. lucidum* (1000 mg/kg) groups showed an average of one mammary tumor per animal at 15^{th} and 14^{th} weeks respectively where as animals treated with low dose (500mg/kg) of *P.florida* or *G.lucidum* showed and average of two tumor per animal at 13^{th} week (Fig-7.4, Fig 7.9). DMBA alone treatment induced 100 % tumor incidence where as in animals treated with *P. florida* or *G. lucidum* extracts (1000mg/kg) the percentage of tumor incidence was 33.33% and animals treated with lower dose i.e. 500mg/kg the tumor incidence was 50%. This showed a dose depended decrease in the tumor incidence (Fig- 7.5). Microscopic examination showed that tumor had the histological pattern of undifferentiated carcinoma with marked nuclear pleomorphism and high mitotic index. The treatment with mushroom extract prevented these histopathological changes to a great extent.(Fig 7.10)

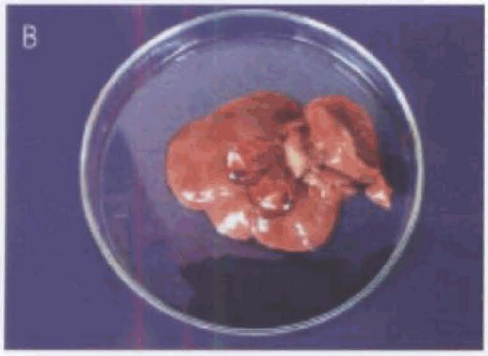
7. 3. 3 Antipromotional activity of *G. lucidum* and *P. florida* extracts

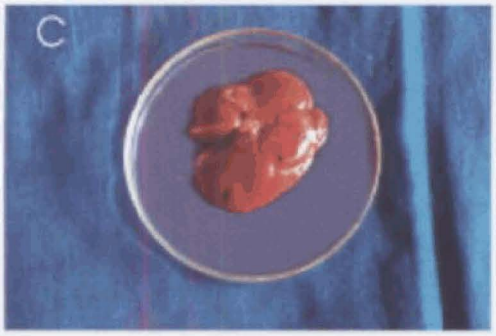
Topical application of methanolic extracts of *G. lucidum* and *P. florida* inhibited mouse skin pappiloma initiated by DMBA and promoted by croton oil . Group of animals applied with croton oil and DMBA showed 87.5% tumor incidence, 13 weeks after DMBA treatment. Application of methanolic extract of *G. lucidum* or *P. florida* prior to croton oil application reduced the percent of tumor incidence. Topical application of methanolic extract of *G. lucidum* and *P.florida* at a concentration of 10 mg showed 37.5% and 25% tumor incidence respectively at 13

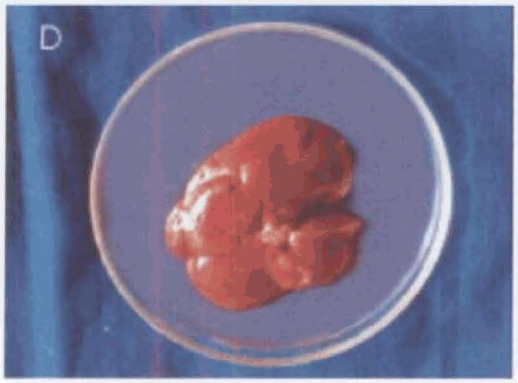
Fig 7.2 Anticarcinogenic activity of *P.florida* and *G.lucidum* against HCC induced by NDEA. Liver section statined with H&E. (A) Normal (B) Control (NDEA) (C) *G.lucidum* 500mg/kg + NDEA (D) *P.florida* 500mg/kg + NDEA

123. B

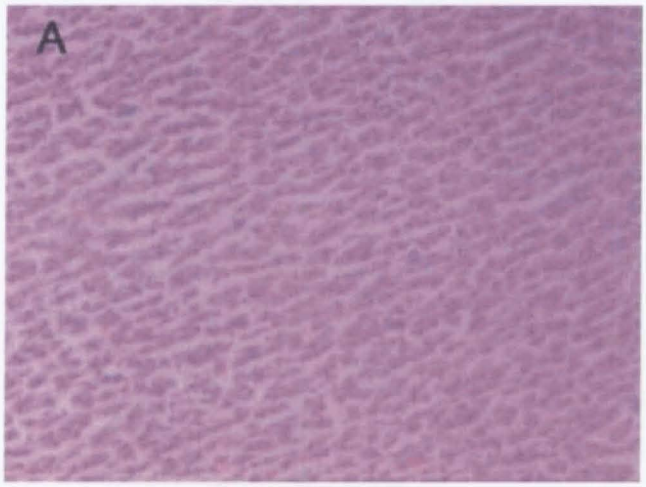


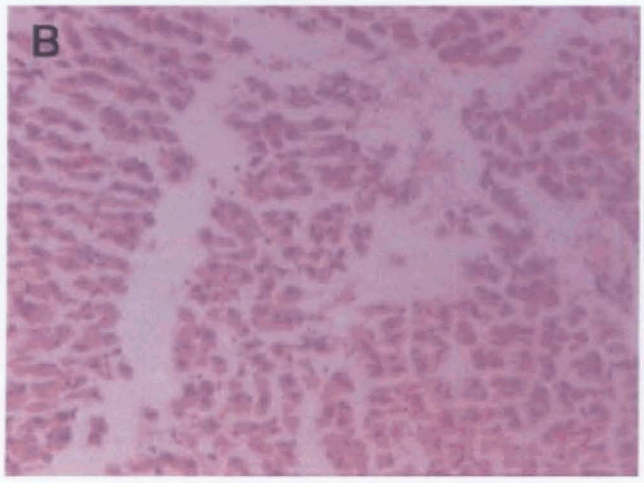


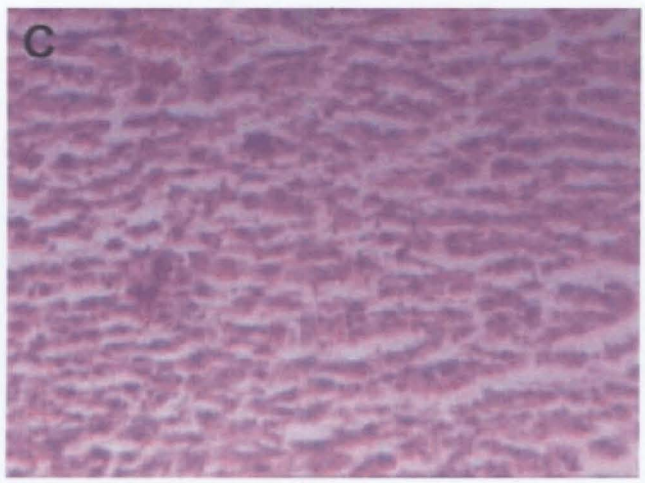


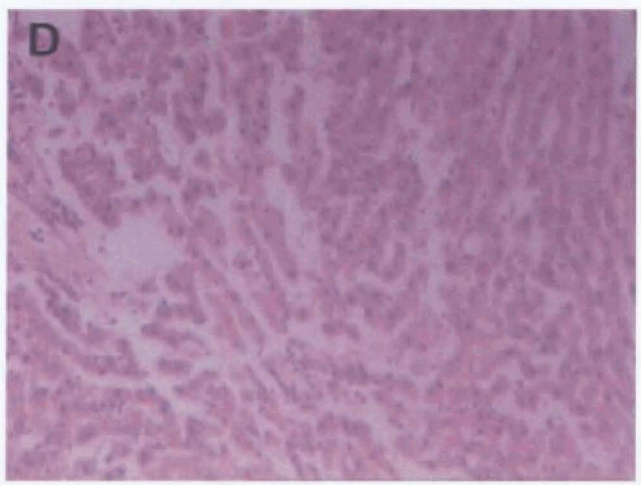


123. D









weeks (Fig-7.8). The average number of tumor per animal in the control group was 7 at 16 weeks after the croton oil application. The average number of tumor per animal in the 2 mg and 10 mg *G.lucidum* extract treated group of animals was 2 and 1.66 respectively (Fig-7.7,7.11). The average number of tumor per animal in the 2 mg and 10 mg *P. florida* extract treatment (2mg and 10 mg) reduced the tumor development in animals (1.5 and 1.25 respectively) (Fig-7.7). The tumor latency period in the positive control, *G. lucidum* (10 mg) and *P. florida* (10 mg) extract treated group of animals was 35,56 and 63 days respectively (Fig 7.6)

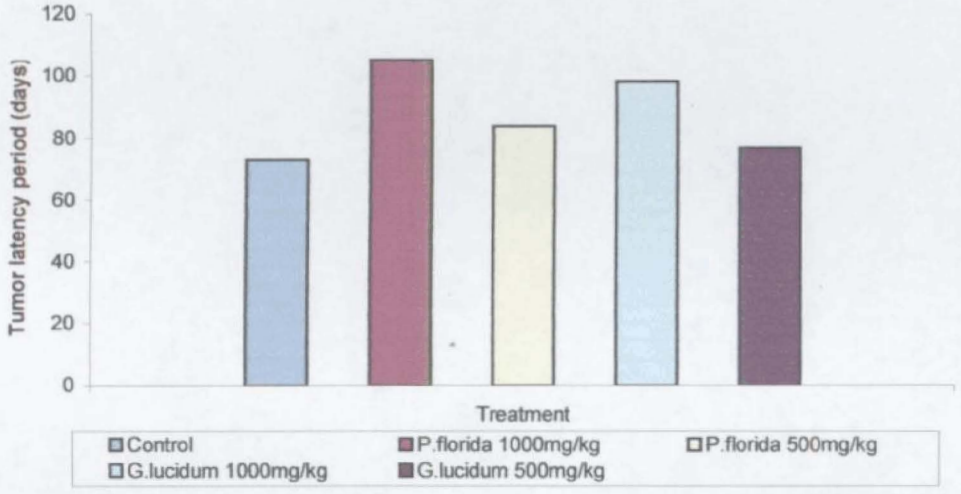


Fig-7.3 Tumor latency period: Effect of *P.florida* and *G.lucidum* extracts on DMBA induced mammary tumor

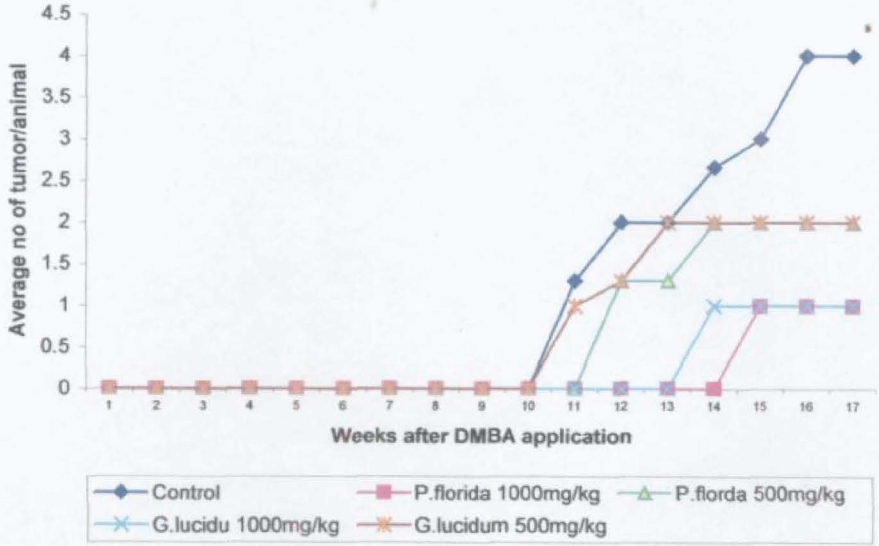


Fig-7.4 Cumulative no of tumors per animal: Effect of *P.florida* and *G.lucidum* extracts on DMBA induced mammary tumor

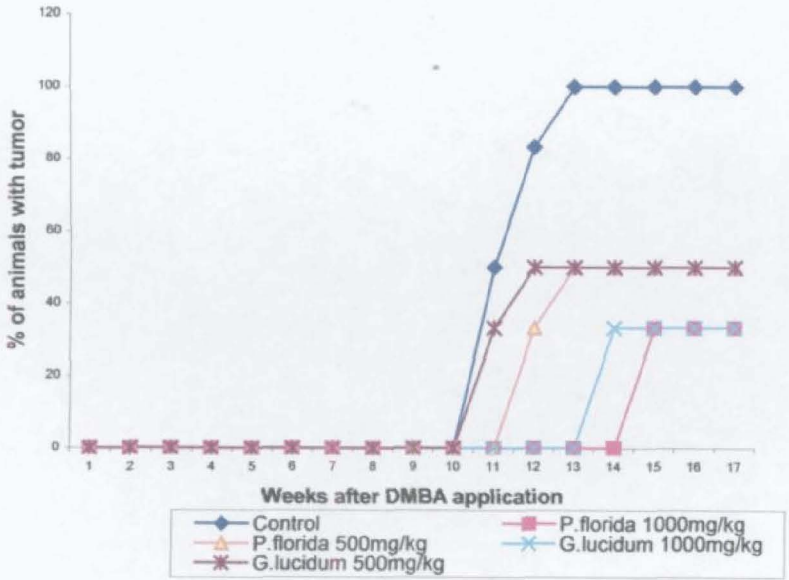


Fig –7.5 Tumor incidence: Effect of *P.florida* and *G.lucidum* extracts on DMBA induced mammary tumor

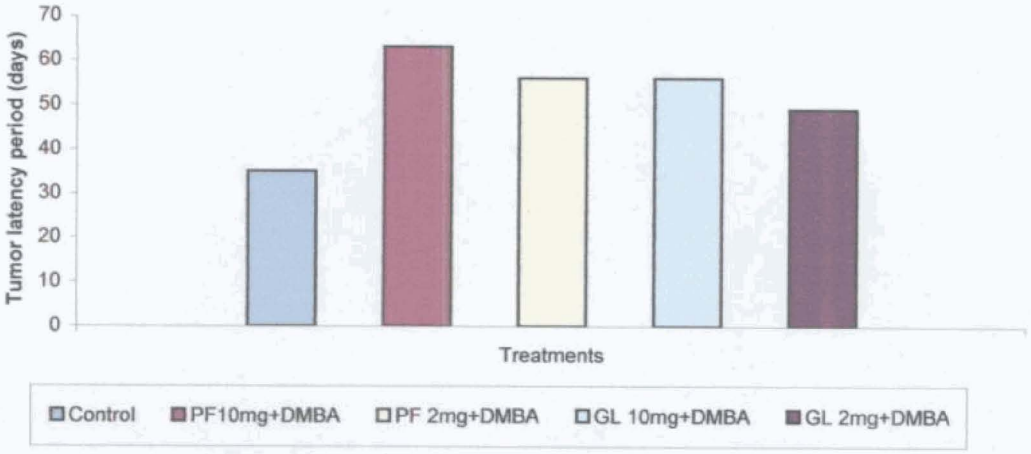


Fig-7.6 Effect of *P.florida* (PF) and *G.lucidum* (GL) extracts on tumor latency period: mouse skin tumor induced by DMBA and promoted by croton oil .

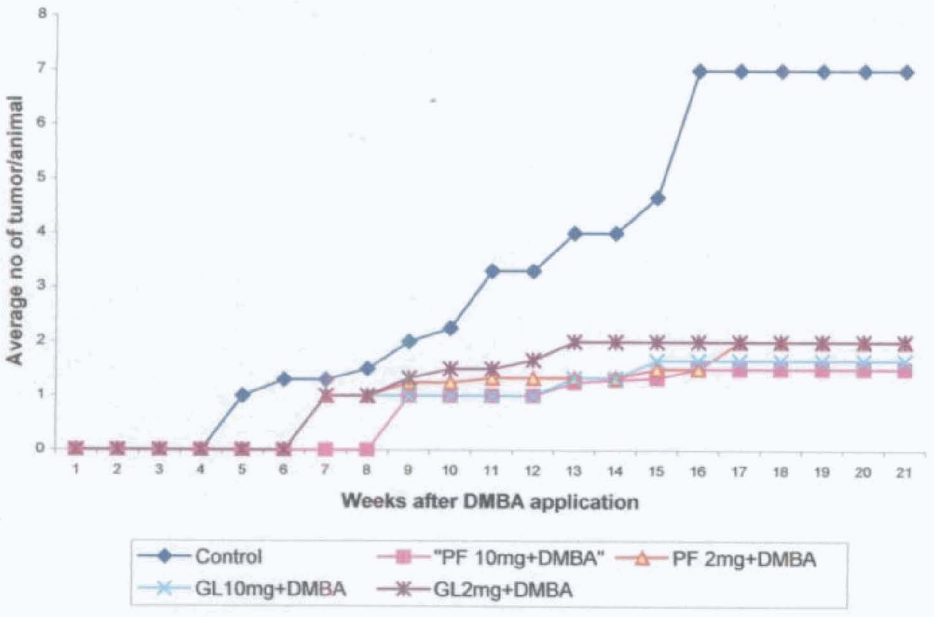
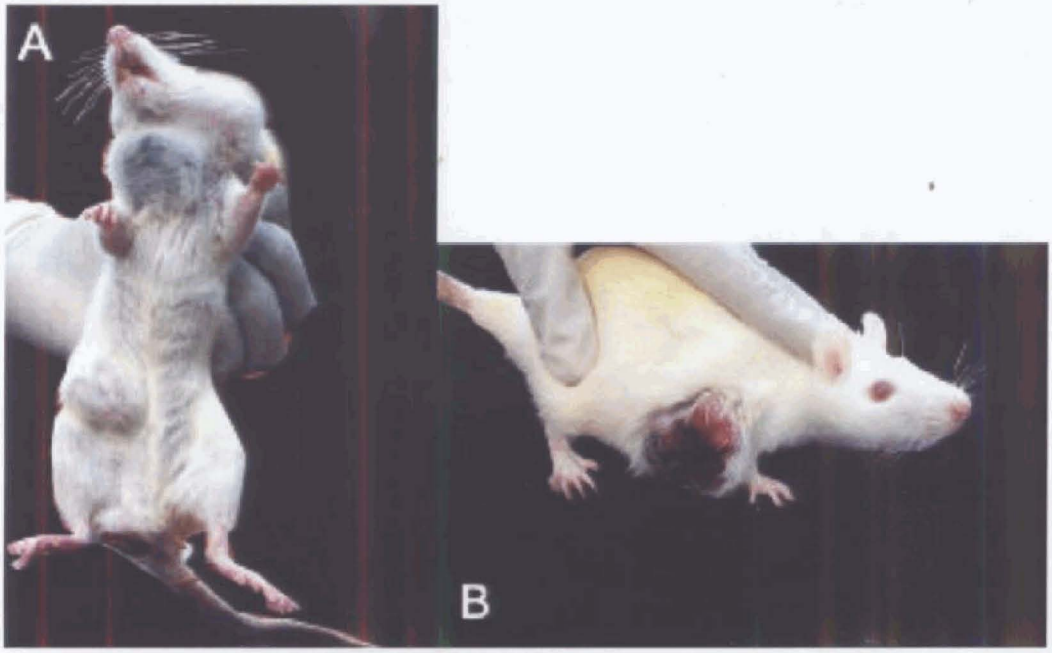
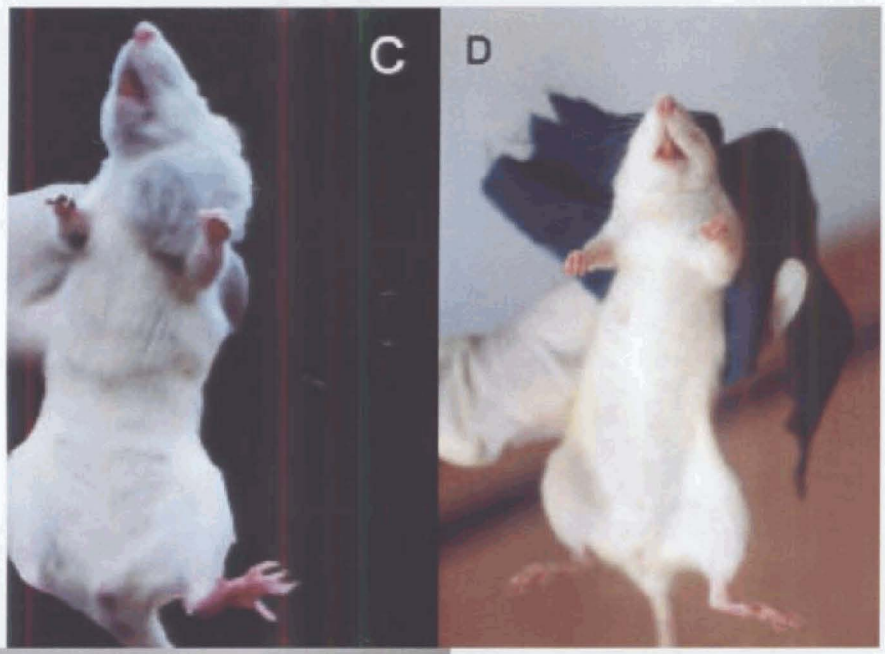


Fig-7.7 Effect of methanolic extract of *P.florida* and *G.lucidum* extracts on average number of tumor/animal induced by DMBA and promoted by croton oil

Fig 7.9 Effect of *P.florida* and *G.lucidum* extracts against DMBA induced mammary tumor A) Control (DMBA) B) *G.lucidum* (500mg/kg) + DMBA C) *P.florida*(500mg/kg) + DMBA D) *P.florida* (1000mg/kg) + DMBA E) *G.*lucidum (1000mg/kg) + DMBA F) Normal


A
B


C
D

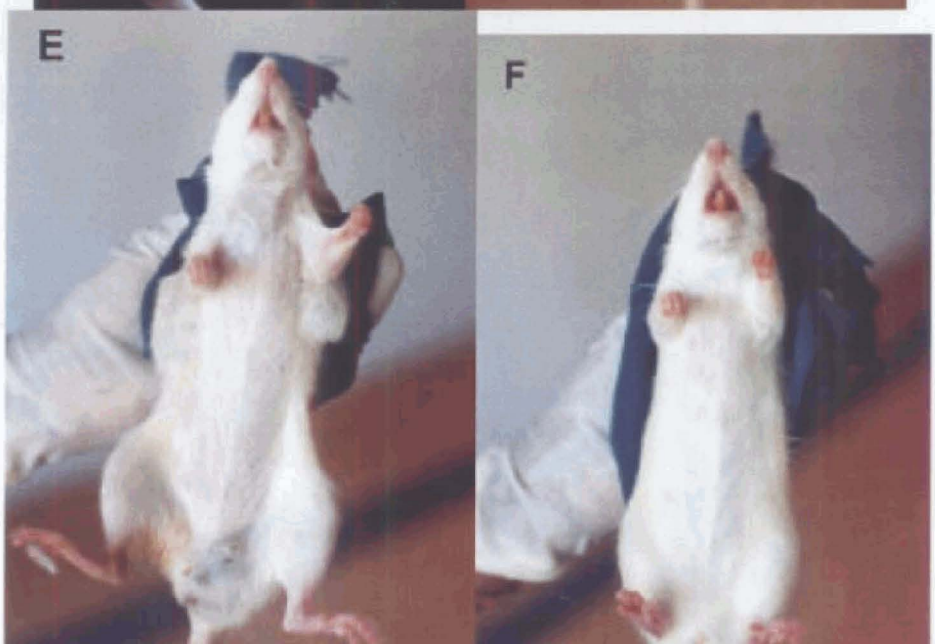
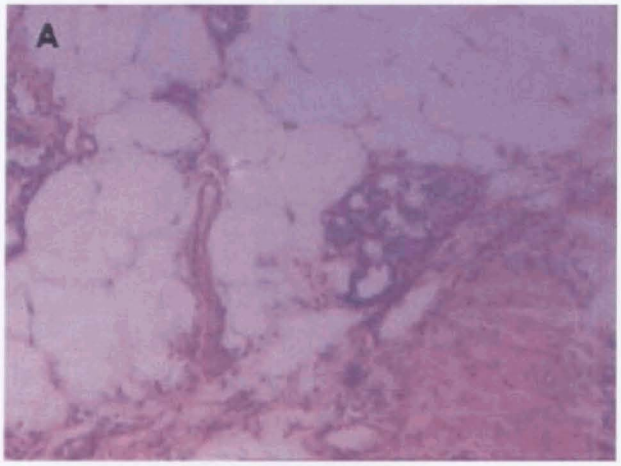
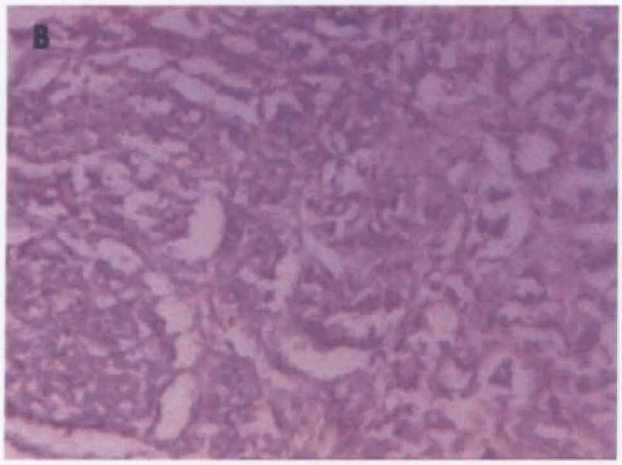

E
F

Fig 7-10 Anticarcinogenic activity of *P.florida* and *G.lucidum* against DMBA induced mammary tumor. Tumor section stained with H&E A) Normal B) Control (DMBA) C) *P.florida* (1000 mg/kg) + DMBA D) *G.lucidum* (1000 mg/kg) + DMBA


A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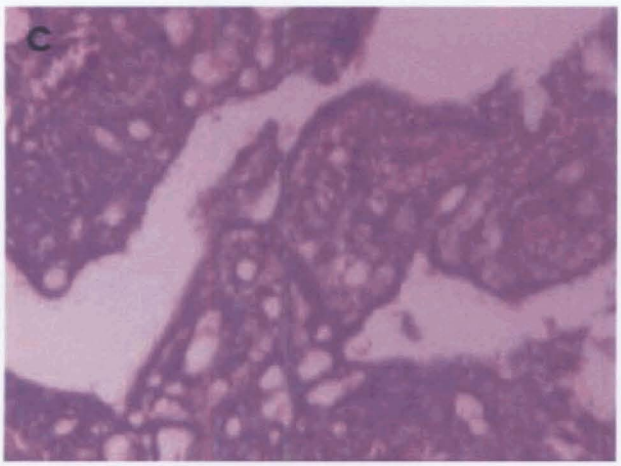

C

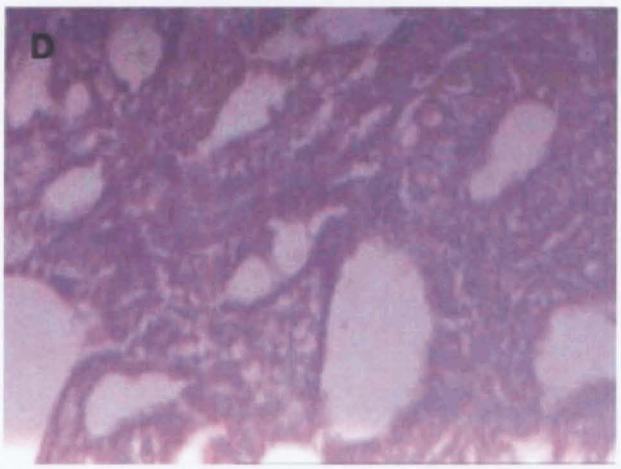
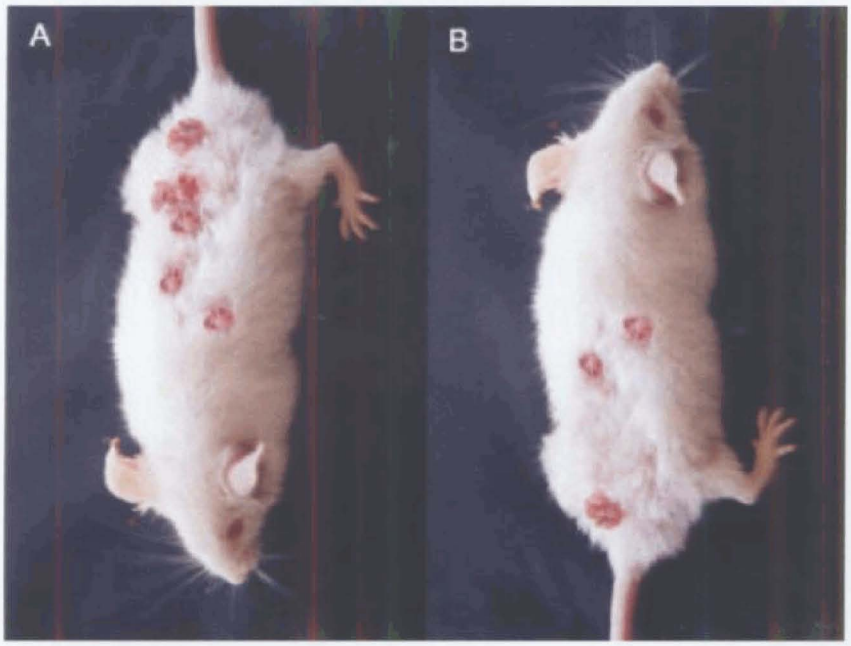

D

Fig 7-11 Effect of *P.florida* (PF) and *G.lucidum*(GL) extracts on DMBA induced and croton oil promoted skin pappiloma on mice skin a) DMBA +Croton oil B) DMBA +Croton oil +PF 2mg C) DMBA +Croton oil+ GL 2mg D) DMBA +Croton oil +10 mg PF E)DMBA +Croton oil +10 mg GL


A
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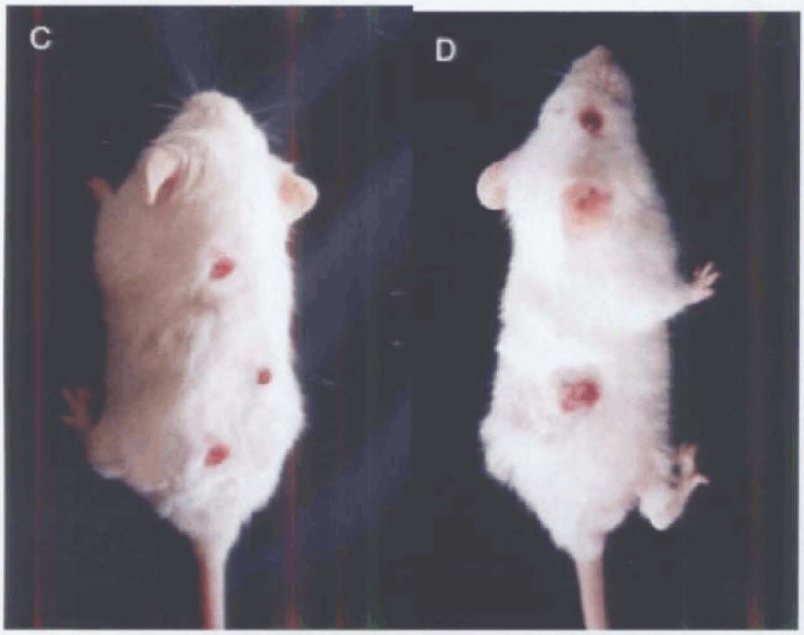
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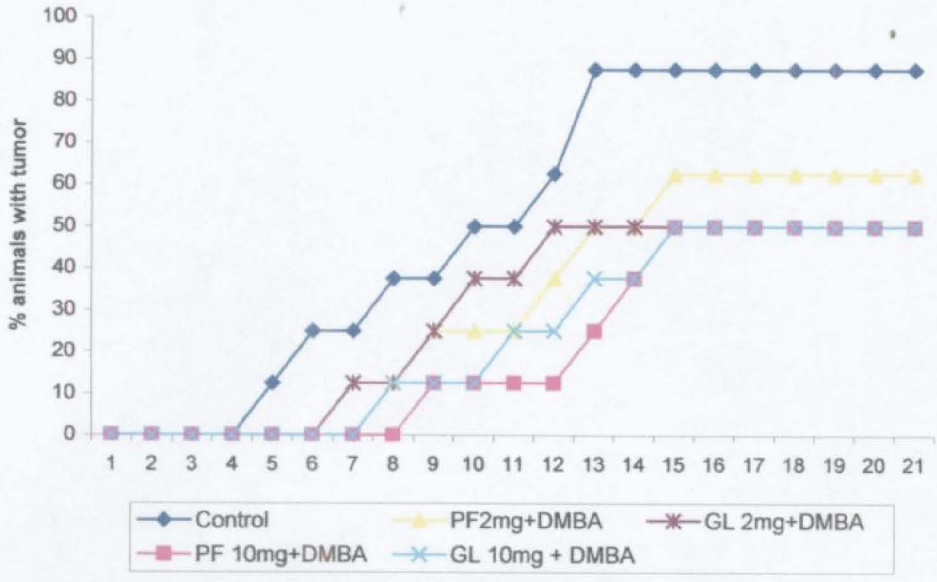

Fig-7.8 Effect of *P.florida* (PF) and *G.lucidum* (GL) extracts on mouse skin tumor induced by DMBA and promoted by croton oil

DISCUSSION

The experimental results indicate that methanolic extracts of *P.florida* and *G.lucidum* are able to delimit the incidence of hepatocarcinoma caused by NDEA effectively. Both *P.florida* and *G.lucidum* show almost similar hepatoprotective efficacy. Treatment of the extracts 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 hepatocellular carcinoma. However, treatment with 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. 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 the extracts prior to NDEA treatments. 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 expression of both γGT and GPx has 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 be metabolized by the microsomal mixed function oxidase (MFO) system to its active ethyl radical metabolites CH_3 $CH_2^{+\cdot}$. 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 etal 1995). The *in vitro* radical scavenging activity of the extract partially explains its mechanism in the prevention of hepatocarcinogenesis.

The present study demonstrates the chemopreventive activity of *P.florida* and *G.lucidum* extracts. In recent years, cancer chemoprevention by biologically active dietary or nondietary supplements has generated immense interest in view of their putative role in attenuating the risk of developing cancer. The administration of mushroom extracts for the prevention of tumor formation in both DMBA initiated mammary tumor and skin pappiloma promoted by croton oil open up new approach in chemoprevention. Important mechanism of action of many chemopreventive agents is through their ability to modulate metabolic activation of a procarcinogen or by increasing detoxification of reactive metabolites. In polycyclic aromatic hydrocarbon induced tumorigenicity, oxidative phase I biotransformation results in highly reactive diolepoxides that form covalent adduct with DNA. Reports indicate that the level of polycyclic aromatic hydrocarbon-DNA adducts is related to the level of CYP1A1 expression (Mollerup, 2001). Furthermore, mushroom extracts also induces phase II enzymes as evident in our earlier studies. Thus, one may interpret the observed chemopreventive activity of mushroom extracts might be at the level of inhibition of procarcinogen activation, leading to reduced bioactivated DMBA metabolites as well as increased expression of phase II detoxification enzymes. The experimental results indicate that methanolic extracts of *P.florida* and *G.lucidum* possess significant protective and antipromotional effect against hepatocellular

carcinoma induced by NDEA and DMBA induced mammary tumors and skin papilloma. The findings suggest the therapeutic potential of *G.lucidum* and *P.florida* mushroom extract in cancer prevention.

SUMMARY AND CONCLUSION

Vast majority of cancers are initiated by genetic changes caused by mutations. Cancer is not a product of just a single mutation, but of multiple different mutations. With each additional mutation, the tumor becomes more metastatic and aggressive. Cancer starts as a single genetically altered cell which provides it with a slight growth advantage. Because of this, the cell begins to replicate faster than its surrounding cells and forms a mass (hyperplasia). If the cell incurs more mutations during this time, then it will become more aggressive and replicate faster, becoming dysplastic. After even more mutations, the cell will lose more growth regulatory checkpoints and become an *in situ* cancer, which is still localized to the initial area. Eventually the cell will become the most aggressive type of cancer, invasive, and spread to the surrounding areas.

Natural products have been of exceptional value in drug discovery programme to discover anticancer drugs. Among the plant products, microbial sources, especially fungi are currently of major interest in the long search for anticancer compounds. Mushrooms have been valued throughout the world as both food and medicine for thousands of years. In terms of nutraceuticals, the mushroom category has been built upon a handful of mushrooms most commonly found in dietary supplements, such as maitake (*Grifola frondosa*), reishi (*Ganoderma lucidum*), shiitake (*Lentinula edodes*), Cordyceps (*Cordyceps sinensis*), turkey tail (*Trametes versicolor*) and lion's mane (*Hericium erinaceus*) (Wasser *et al* ,2000). Some up-and-coming mushrooms that have received a lot of attention recently in the research community are agaricus (*Agaricus brasiliensis*), oyster mushrooms (*Pleurotus ostreatus*). Mushrooms are known to posses significant medicinal properties. They have been used in folk medicine throughout the world since ancient times. There has been growing interest in the identification of naturally occurring dietary factors as potential anticarcinogens. The identification of such dietary components and definition of their antitumor effects could lead to strategies for reducing the risk of human cancer. *Ganoderma lucidum* has been used for thousands of years in traditional oriental medicine. The polysaccharides of *G.lucidum* are the other major source of its biological activity and therapeutic use. This mushroom has attracted great attention owing to its antitumor and hypoglycemic activities (Ooi and Liu, 1999). Many fungal polysaccharides have been reported to be active against

humans. According to Eastern folklore, *Pleurotus* (Oyster mushroom) can also prevent high blood pressure and arteriosclerosis, impart long life and vigor and assist people in recovering from fatigue (Breene, 1990). The mushroom can be used to treat headache, fever and cold, asthma, nervous disorders and stomach pain. However no significant information is available on the medicinal properties especially the antimutagenic and anticarcinogenic activity of oyster mushrooms form India. Current investigations were under taken to evaluate the antioxidant, anti-mutagenic , anticarcinogeic and radioprotective properties of *G.lucidum* and *P.florida* mushrooms.

In biological systems, the normal processes of oxidation (plus a minor contribution from ionizing radiation) produce highly reactive free radicals. These can readily react with and damage other molecules, but the presence of extremely easily oxidisable compounds in the system can "mop up" free radicals before they damage other essential molecules. Antioxidants are a group of substances which when present at low concentrations in relation to oxidizable substrates, inhibit or delay oxidative process, while often being oxidized themselves. Antioxidant enzymes and other scavengers of reactive oxygen intermediates are involved in numerous defense systems in cells. Antioxidant may exert their effect on biological systems by different mechanisms including electron donation (as reducing agent) metal ion chelation or by gene expression. Reactive oxygen species (ROS) produced by sunlight, UV light, ionizing radiation, chemical reaction and metabolic process have a wide variety of pathological effects such as DNA damage, carcinogenesis and cellular degeneration related to ageing. Non-enzymatic antioxidants react with pro-oxidants and inactivate them. The free radical scavenging activity of the mushroom extracts of *P.florida* and *G.lucidum* was studied. The ferric reducing antioxidant power (FRAP) of the extracts was found to increase in a concentration dependent manner. There was no significant difference in activity between *P.florida* and *G.lucidum* extracts at 5% level of significance . However , the activity of the extract with reference to control was significant ($P<0.001$). Non enzymatic antioxidant react with prooxidant and inactivate them. In this redox reaction , the antioxidant power can be referred to as reducing ability The FRAP assay , thus indicated the non enzymatic antioxidant activity of the extracts of both the mushrooms. This showed

the hydrogen- donating capacity of the extracts; the first line of defense which suppress the formation of free radicals. The ferryl myoglobin- ABTS assay, also showed the inhibition of formation of the radical The extracts of both the mushroom had the ability to prevent the radical formation . The abililty was more in case of *P.florida* than *G.lucidum* There was no significant difference in activity between extracts but between the extract treated and control, the difference in activity was significant ($P<0.001$). The second line of defense scavenged free radicals to suppress chain initiation and /or break chain propogation reaction. In this respect DPPH assay showed significant activity equivalent to ascorbic acid (AEAC). *P.florida* extract (0.016) showed higher activity than *G.lucidum* extract (0.015) at concentration of 1%. Pulse radiolysis assay also confirmed this conclusion. ORAC values (trolox equivalent) of the mushroom were also higher than some trait and vegetables. Being a free radical chain reaction, lipid peroxidation causes membrane damage as well as oxidative stress in critical targets. Thereafter , agents that can react with these secondary radicals formed during peroxidation and scavange them would be effective in inhibition lipid peroxidaiton. The effect of both the mushroom extracts on LOOH and TBARS formation induced by AAPH in rat liver mitochondria was estimated. *P.florida* extract showed 94.1 % and *G.lucidum* extract 94.9% inhibition on LOOH formation. TBARS formation also inhibited by the extracts. The result thus indicated that extracts of *P.florida* and *G.*lucidum possessed profound antioxidant activity to inhibit free radical formation and scavenging activity . This hypothesis is supported by five antioxidant assays. The findings strongly suggest the ability of mushroom extract to prevent lipid peroxidation leading to membrane damage.

A mutagen is considered as an agent capable of destroying the integrity of hereditary mechanism of a cell or organism. Any substance that cause increased mutation can also increase the probability of cancer. The rate of tumor evolution and progression is accelerated by mutagenic agents. Hence, peroxidation of mutation is of paramount importance for the prevention of cancer.

A considerable emphasis is being placed on the use of dietary constituents to prevent mutagenisis and carcinogenesis due to their relative non-toxic

effects (Weisberger, 1999; Stranic, 1994; Fergnson, 1994). The antimutagenic activity of the extracts of *P.florida* and *G.lucidum* was evaluated by Ames assay. The antimutagenic activity of the extracts of both the mushrooms were tested using *Salmonella* tester strain, TA100, TA102 and TA98 employing Sodium azide (NaN_3) *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), Nitorso-O-Phenylene diamine (NPD), 2-aminoflourene (2-AF) and benzo [*a*] pyrene. The mushroom extracts did not show any toxic effects on any of the *Salmonella* tester strains at the tested concentrations of 2 or 3 mg /plate. Methanolic extracts of *P..florida* and *G.lucidum* was also found to inhibit mutagenicity elicited by both direct acting mutagens and also with those requiring metabolic activation. A dose dependent increase in antimutagenic activity was observed in both the extracts. Antimutagenicity test of the urine of animals treated with B [*a*] P showed that methanolic extracts of *G.lucidum* and *P.florida* inhibited mutagenicity induced by B [*a*] P. *P.florida* extract showed higher *in vivo* antimutagenic activity than *G.lucidum*. The results thus indicated that methanolic extracts of *P.florida* and *G.lucidum* possessed significant antimutagenic activity. The effect of extracts on hepatic antioxidant status consequent to B [*a*] P treatment was studied. The activity of SGOT, and SGPT was elevated in B [*a*] P treated group of animals. The level of these enzymes was effectively brought down when experimental animals were treated with extracts of *G.lucidum* (GL) and *P.florida* (PF). The activity of GST, GPx, and the level of GSH were decreased consequent to B [*a*] P treatment. Both *P. florida* and *G. lucidum* extract elevated the activity of these antioxidant enzymes. The results of the investigations indicate that methanolic extracts of *P.florida* and *G.lucidum* significant protective effect against carcinogen induced hepatic damages. The activity might be due to the capacity of the extract to restore depleted antioxidant defense.

Ionizing radiations deleterious effects on living cells and damages vital cellular targets such as DNA and cellular membranes. Protecting the onslaught of ionizing radiation has significant importance not only in radiation therapy but also in accidental nuclear explosion. Mammalian cells are most sensitive to radiation-induced damages at certain cell cycle phase. Ionizing radiation damages molecules directly by transferring energy or indirectly by generation of oxygen derived free radicals. Cellular damage produced by the radiation therapy is an indirect result of

ionizing chemicals in the cell to very reactive compounds. Cytotoxicity is primarily caused by oxygen-derived free radicals such as hydrogen peroxide (H_2O_2), superoxide ($O^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$) (Parker,1990). Mitochondria are crucial targets for radiation and free radical mediated damage. Since mitochondria are devoid of cystolic antioxidants, as a whole cell, they are resistant to γ radiation, hence a dose of 450 Gy is needed to achieve optimum concentration of free radicals of inducing significant damage measurable by simple spectrophotometric means. This dose is much higher than the dose used in radiotherapy (1-6Gy) or for radioprotection pertinent to mammals (LD50 in the range of 5-7Gy). The protective effect of mushroom extracts (*P. florida* and *G.lucidum*) on radiation-induced damage was evaluated.

P. florida and *G.lucidum* extracts showed significant ability to inhibit radiation induced lipid peroxidation in rat liver mitochondria. The formation of LOOH, an intermediate of peroxidation, showed that LOOH formation induced by γ-radiation in rat liver mitochondria was markedly inhibited by extracts *P. florida* and *G.lucidum* at a concentration of 1% reduced TBARS formation significantly when it was present at the time of irradiation.

The interaction of ionizing radiation with biological system results in generations of free radicals, $^{\cdot}H$ and $^{\cdot}OH$ radicals, H_2 and H_2O_2. Radiations induced free radicals in turn impair the antioxidant defense mechanism leading to increased membrane lipid peroxidation, which results in the damage of membrane, bound enzymes (Halliwell and Gutteridge, 1989). The result of the investigations reveal that administration of *G.lucidum* and *P.florida* extracts prior to irradiation rendered 50 % survival in mice against 9Gy whole body irradiation. Untreated irradiated mice suffered 100 % mortality within 20 days. The maximum protective effect was achieved by the administration of 1000 mg/kg body weight of the extract to animals.

After whole body irradiation the majority of bone marrow cells were damaged or dead and there after not available for division or maturation. At the end of 9^{th} day the bone marrow cellularity and total leukocytes count in untreated radiation received alone group of animals were very low as compared to *G.lucidum*

and *P.florida* (1000mg/kg) treated group. The radiation induced losses in bone marrow cellularity and total WBC count has been well compensated by *G.lucidum* and *P.florida* extract treatment

The experimental results indicated that a significant reduction in GSH and the activities of all antioxidant enzymes in radiation treated group of animals. *G.lucidum* or *P.florida* extracts checked the fall of catalase activity caused by irradiation. The results of present study showed that methanolic extract of *G.lucidum* and *P.florida* inhibited lipid peroxidation in a dose dependent manner. Radiation caused increase in the levels of superoxide radicals. The increased SOD activity after *G.lucidum* and *P.florida* extract treatment was therefore due to the elimination of the superoxide radicals. Because of this dismutation reaction, highly reactive H_2O_2 are formed. Catalase and GP_X are enzymes responsible for degradation of H_2O_2. *G. lucidum* and *P.florida* treatment increased the activity of catalase in liver. The treatment of *G.lucidum* and *P.florida* also increased the activity of GP_X significantly. The results of the investigations indicate that methanolic extract of *G.lucidum* and *P.florida* posses significant property to protect radiation induced damages. The findings suggest the potential therapeutic use of the extracts of these mushrooms in radiation protection.

Carcinogenesis may result from the action of any one or a combination of chemical, physical, biological and/or genetic insult to cells. Chemical carcinogenesis and co- carcinogens are considered responsible for many of the cancers of humans (Higginson, 1976). Prevention of carcinogenesis is one of the major strategies for cancer control. Anticarcinogenic potential of methanolic extracts of *G.lucidum* and *P.florida* were investigated. N-nitrosodiethylamine induced hepatocarcinogenesis; DMBA initiated and croton oil promoted mouse skin pappiloma and DMBA induced rat mammary tumor were employed as experimental models. The animals treated with NDEA alone-induced large number of tumors in the liver. However, administration of the extracts of *P.florida* and *G.lucidum* decreased the tumor development. NDEA treatment elevated the activities of serum GOT, GPT and ALP. Marked decline of the activities of these enzymes was observed with administration of mushroom extracts indicating prevention of hepatic damage. The activities of

GST, GPX and GSH level in the liver homogenate of the extract treated animals showed significant decrease compared to the NDEA alone treated group of animals. The serum GGT activity was significantly elevated in the NDEA alone treated group of animals indicating the induction of hepatocellular carcinoma. However, treatment with 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. 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 the extracts prior to NDEA treatments. 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 expression of both γGT and GPx has 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. *P.florida* and *G.lucidum* extract showed significant activity against DMBA induced mammary tumors and mouse skin pappiloma in a dose dependent manner. The administration of mushroom extracts for the prevention of tumor formation in both DMBA initiated mammary tumor and skin pappiloma promoted by croton oil open up new approach in chemoprevention. The experimental results indicate that methanolic extracts of *P.florida* and *G.lucidum* possess significant protective and antipromotional effect against hepatocellular carcinoma induced by NDEA and DMBA induced mammary tumors and skin papilloma.

CONCLUSION

The results of the investigations indicate that aqueous methanolic extracts of *P.florida* and *G.lucidum* possessed profound antioxidant, antimutagenic, radioprotective and anticarcinogenic activities. The extract of both the mushroom show significant protective effect against hepatocarcinoma induced by NDEA,

mouse mammary tumor induced by DMBA and promotion of mouse skin pappiloma. The experimental findings suggest the potential cancer chemopreventive properties of these mushrooms. The findings are of significant therapeutic significance indicating the potential use of these mushrooms in chemoprevention.

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