STUDIES ON THE THERAPEUTIC POTENTIAL OF GANODERMA LUCIDUM. P. KARST - REISHI, OCCURRING IN KERALA

Thesis Submitted to

THE UNIVERSITY OF CALICUT

for the degree of

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

(FACULTY OF SCIENCE)

BY

SHEENA. N., M.Sc.

AMALA CANCER RESEARCH CENTRE, THRISSUR, KERALA - 680555, INDIA

SEPTEMBER 2005

CERTIFICATE

I certify that the thesis entitled " Studies on the therapeutic potential of *Ganoderma lucidum*. P. Karst -Reishi, occurring in Kerala," submitted to University Of Calicut, Calicut in partial fulfillment for the award of Doctor of Philosophy in Microbiology (Faculty of Science) is an authentic record of work carried out by Mrs. Sheena.N, under my supervision and guidance. And no part of the thesis has been presented for any other Degree, Fellowship or any other similar titles of any university or society.

Amala Nagar,
Thrissur

Professor K.K. Janardhanan
Supervising Guide.

Dr. K. K. JANARDHANAN
Professor
Amala Cancer Research Centre
Amala Nagar
Trichur 680 553, India

DECLARATION

I hereby declare that the thesis entitled " Studies on the therapeutic potential of *Ganoderma lucidum*. P. Karst -Reishi, occurring in Kerala" is a bonafied 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 thesis has been presented for any other Degree, Fellowship or any other titles of any university or society.

Amala Nagar,
Thrissur

Sheena

Sheena. N

ACKNOWLEDGEMENTS

I acknowledge my sincere gratitude and indebtedness to,

Dr.K.K.Janardhanan, Professor, Amala Cancer Research Centre, for his valuable guidance, help and constant encouragement extended to me during the course of this work.

Dr. Ramadasan Kuttan, Research Director, Amala Cancer Research Centre, for his suggestions, help and constant encouragement.

Rev. Fr. Gabriel, Founder Director, **Rev. Fr. G. Pius,** Managing Director, **Rev. Fr. Walter Thelappilly,** Joint Director, Amala Cancer Hospital and Research Centre, for their encouragements and blessings.

Dr. Girija Kuttan, Dr. Jose Padikkala, Dr. P. T. Annamala and **Dr. C. I. Jolly,** for the sincere help and support extended to me during my work.

Dr. K. M. Leelavathy (Retd Professor), **Dr. Manimohan,** Reader, Department of Botany, University of Calicut, for their help in identifying the mushroom.

Dr. Sulochana, Dr. Krishnan Nair, Dr. Mini Microbiology Department, Veterinary College, Mannuthy for their valuable suggestions.

Dr. Joseph George, Chief Pathologist, Doctors Specialty Center, Thrissur for the evaluation of the histopathology.

Dr. V. K. Mallika, Professor, Department of Horticulture, Kerala Agricultural University for providing the photographic facilities and **Sudharma Laboratory** for the photomicrography of histopathologic studies.

Dr. A. K. S. Rawath, Head, Department of Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute, Lucknow, for providing the HPTLC analysis.

Mrs. Sujatha Assistant Professor, Department of Statistics, (College of Veterinary Sciences, Mannuthy, Kerala and **Mrs. Santha**, Retd Statistical Officer, College of Veterinary Sciences, Mannuthy, Kerala for helping the Statistical analysis of the experimental data.

Dr. K. V. Sankaran, Scientist, Department of Plant Pathology, Kerala Forest Research Institute, Peechi for his constant encouragement during my work..

Dr. T. A. Ajith, Asst. Professor, Department of Biochemistry, Amala Institute of Medical Sciences for his help in experimental work and the preparation of the thesis, **Mrs. Susan Jones, Miss. Lakshmi B, Mrs. Nayana Baiju, Miss. Nitha. B, Mr. Mathew John** (Research Fellows, Department of Microbiology) and all my colleagues of Amala Cancer Research Centre for their help and support extended to me.

My **Mother, Husband, Daughter** and **Brother** for their constant help and encouragements to complete my research work.

Above all, for the Grace, Guidance and blessings of **Lord Sree Guruvayoorappan**.

Dedicated to the Ever Living Memory of my Father

M. Sivasankaran Nair

CONTENTS

LIST OF ABBREVIATIONS

Abs	:	Alveolar buds
ALP	:	Alkaline phosphatase
ARDS	:	Adult respiratory disease syndrome
CCl_4	:	Carbon tetrachloride
CDNB	:	1-Chloro-2,4-dinitrobenzene
CK	:	Creatine kinase
COX-1	:	Cyclooxygenase-1
COX-2	:	Cyclooxygenase-2
DLA	:	Dalton's lymphoma ascites tumor cells
DNA	:	Deoxyribonucleic acid
DMBA	:	7,12-dimethyl benz[*a*] anthracene
DMSO	:	Dimethyl sulphoxide
DTNB	:	5,5-dithiobis-(2-nitrobenzoic acid)
EAC	:	Ehrlich's ascites carcinoma cells
EDTA	:	Ethylene diamine tetra acetic acid
ER	:	Endoplasmic reticulum
G. lucidum	:	*Ganoderma lucidum*
GPT	:	Glutamate pyruvate transaminase
GOT	:	Glutamate oxaloacetate transaminase
GSH	:	Reduced glutathione
GST	:	Glutathione S-transferase
Hb	:	Haemoglobin
HIV	:	Human immunodeficiency virus
HPTLC	:	High performance thin layer chromatography
H_2O_2	:	Hydrogen peroxide
IBD	:	Inflammatory bowel disease

KCl	:	Potassium chloride
MAO	:	Monoamine oxidase
MDA	:	Malondialdehyde
$MgCl_2$	:	Magnesium chloride
NaCN	:	Sodium thiocyanate
NaN_3	:	Sodium azide
NBT	:	Nitroblue tetrazolium
$NO^{\bullet}$	:	Nitric oxide radical
NSAIDs	:	Nonsteroidal anti-inflammatory drugs
$O_2^{\bullet}$	:	Superoxide anion radical
OFR	:	Oxygen free radical
$OH^{\bullet}$	:	Hydroxyl radical
8-OHDG	:	8-hydroxydeoxyguanosine
$ONOO^-$	:	Peroxynitrite
PAH	:	Polycyclic aromatic hydrocarbon
PAF	:	Platelet activating factor
PARP	:	Poly (ADP-ribose) polymerase
PBS	:	Phosphate buffered saline
PGs	:	Prostaglandins
PNM	:	Polymorphonuclear monocytes
PUFA	:	Poly unsaturated fatty acids
RBC	:	Red blood cells
RNA	:	Ribonucleic acid
RNS	:	Reactive Nitrogen species
ROS	:	Reactive Oxygen species
SOD	:	Superoxide dismutase
SDS	:	Sodium dodecyl sulphate
SGPT	:	Serum glutamate pyruvate transaminase
SGOT	:	Serum glutamate oxaloacetate transaminase

TBA	:	Thiobarbituric acid
TBARS	:	Thiobarbituric acid reactive substances
TCA	:	Trichloroacetic acid
TEBs	:	Terminal end buds
TLC	:	Thin layer chromatography
TPA	:	12-*O*-tetradecanoylphorbol-13-acetate
UV	:	Ultra violet rays
WBC	:	White blood cell

INTRODUCTION

Sheena.N "Studies on the thekapeutic potential of Ganoderma lucidum P. Karst – Reishi, occurring in Kerala " Thesis. Amala Cancer Research Centre , University of Calicut, 2005

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1. INTRODUCTION

Mushrooms are macrofungi, they have been important in human history: as food, as poison, as medicine, as legends, and in folklore, and religion (Molitoris, 2001). They have recently become attractive as health beneficent foods and as sources for the development of drugs (Mizuno et al., 2001). Many higher Basidiomycetes mushrooms are known to contain a number of biologically active substances that show promising antitumor, immunemodulating, cardiovascular, hypocholesterolemic, antiviral, antibacterial, antiparasitic, hepatoprotective, and antidiabetic effects (Didukh, 2001). Species of *Ganoderma* is a highly ranked herbal medicine. The commonly used *Ganoderma* species include *G. lucidum*, *G. tsugae*, *G. caspense* and *G. applanatum* (Lai et al., 2004). *Ganoderma lucidum* (Curt.: Fr.) P. Karst. (Aphyllophoromycetideae), Reishi or Ling Zhi was recognised as a superb herb in Oriental medicine with most extensive and effective healing powers.

In Chinese folklore *Ganoderma lucidum*, Reishi has been regarded as panacea for all types of diseases, perhaps due to its demonstrated efficacy as a popular remedy to treat a large number of diseases (Jong and Birmingham, 1992a). However, the extensive range of traditional medical treatment with *Ganoderma* has not yet been fully substantiated by modern scientific standards. The fruiting bodies of *G. lucidum* contain a variety of chemical substances; the major components are terpenes and polysaccharides. A number of species of this medicinal mushroom are found in

South India (Leelavathy and Ganesh, 2000). However, no attempt has been made to investigate the therapeutic potentials of *Ganoderma* mushroom occurring in India.

Reactive oxygen species (ROS) is a collective term often used by biologists to include oxygen radicals, superoxide ($O_2^{\cdot -}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$) and alkoxyl ($RO^{\cdot}$) and certain non radicals that are either oxidizing agents and /or are easily converted into radicals, such as HOCl, ozone (O_3), peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$) and H_2O_2 (Wiseman and Halliwell, 1969). ROS can cause extensive DNA modifications including modified bases, they can trigger oxidative damage to protein, membrane lipids and DNA. Free radicals are implicated as important pathological mediators in many clinical disorders including inflammations and cancer (Conner and Grisham, 1996). Recent studies suggest that the inflammatory damages are due to the liberation of reactive oxygen species (ROS) at the inflammation sites (Murakami et al., 2000). Inflammation and platelet aggregation have been implicated in the pathophysiology of various clinical disorders including vascular diseases and cancer (Winrow et al., 1993).

In nature, a large number of chemical compounds play important roles in the natural defense of an organism. However, the amount of these protective antioxidant principles present under the normal physiological conditions is sufficient to cope up with the physiological rate of free radical generation. Thus the antioxidant status in humans reflects the dynamic balance between the antioxidant defense and pro-oxidant conditions and this has been suggested as a useful tool in estimating the risk of oxidative damage (Tiwari, 2001).

Most widely used nonsteroidal anti-inflammatory drugs (NSAID) suffer from severe side effects. Therefore search for an ideal anti-inflammatory drug, which is safe and effective, is still continuing. Antinociceptive activity is related to the analgesic activity. Anti-inflammatory drugs, muscle relaxant and antihistamines also show the analgesic activity.

The role of ROS in different stages of carcinogenesis such as tumor initiation, promotion and progression is described. (Athar, 2002). Crude drugs or natural diets that possess antioxidant or free radical scavenging activity are of significant importance to prevent or ameliorate tissue injury caused by the free radicals and subsequent disease management.

Modern oncology has made tremendous achievements in the understanding of cancer causation and preventions. The identification of molecular events involved in the multistep process of malignant transformation, signal transduction, oncogene activation, tumor suppressor genes, mechanisms and molecular targets for chemotherapeutic drugs have contributed enormously to the control of some cancers, eventhough not in all types of malignancies. Cancer chemoprevention was coined to describe the intervention with natural or synthetic agents with aims to inhibit, suppress, or reverse the onset of carcinogenesis (Sporn, 1976). Over 500 compounds have been identified with potential chemopreventive properties as a result of their ability to inhibit tumor formation or cell transformation (Smith and Gupta, 1996). Many agents have been shown to be effective for certain cancer chemoprevention, while other compounds are undergoing clinical trials.

Conventional cancer chemotherapy aims to kill or disable tumor cells while preserving the normal cells in the body by the application of synthetic compounds (Ratain, 1997). These agents have a narrow margin of safety, and the therapy may fail because of drug resistance and dose limiting toxicities. Recently, there has been an increasing interest in the use of drug for cancer prevention that may play an important role in reducing the incidence of cancer (Shureiqi et al., 2000., Sporn and Suh, 2000., Tamini et al., 2002., Young and Wilson, 2002). Many herbal medicines have direct cytotoxic effects on tumor cells, but many of them like medicinal mushrooms are used as immunostimulants, which act by modulating host immune responses, resulting in various beneficial effects in the host (Gao et al., 2004., Zhou et al., 2002).

Chemotherapy is the sheet anchor of therapy in leukemias, advanced lymphomas, choriocarcinoma and other widely disseminated malignancies. However most of the chemotherapeutants are not only cytotoxic to cancer cells, but also to healthy cells. This results in a narrow therapeutic index, with adverse events frequently limiting optimal anticancer therapy. Doxorubicin induced cardiomyopathy, at least partially, is caused by increased oxidant production in the heart and a great deal of evidence supports this hypothesis (Childs et al., 2002). Several lines of evidence indicate that free radicals are involved in the nephrotoxicity caused by cisplatin and the damage is suggested to be the consequence of decreased renal antioxidant enzyme activity with the enhanced lipid peroxidation. Any compound, natural or synthetic, with antioxidant properties

might contribute towards the partial or total alleviation of this damage (Lin et al., 1995).

The major objective of current investigation is to evaluate the various therapeutic potential of *G. lucidum* occurring in Kerala. Physiological attributes and biological properties of *Ganoderma* species have been reported to be strain specific and influenced by environmental conditions. Since the available information on the medicinal properties and uses of *Ganoderma* mushrooms available in India tropics is inadequate, studies on the therapeutic potential of this mushroom available in tropical Kerala was considered important. The current investigations were undertaken to evaluate the medicinal properties of *G. lucidum* occurring in Kerala and to demonstrate its therapeutic potentials as antioxidant, anti-inflammatory, anticarcinogenic, antitumor, hepatoprotective, nephroprotective and cardioprotective agent. The findings are presented in this dissertation.

1.2. REVIEW OF LITERATURE

1.2.1. MUSHROOMS

Edible mushrooms are globally accepted as a non-conventional, delicious food item. World production and consumption of mushrooms have increased steadily during the last 15-20 years among the people of the industrialized West and traditional consumers of South- East Asia. Almost all commercially cultivated mushrooms belong to the Basidiomycete class of fungi.

Mushrooms have played an important role in history as food, as medicine, and in folklore, legends and religion. Mushrooms are lively in folklore as "witches' eggs" and "fairy rings" (Molitoris, 2001). All of these are intimately interrelated. Mushrooms are nutritionally functional food and a source of physiologically beneficial and non-toxic medicines (Wasser and Weis, 1999). Attempts have been made in many parts of the world to explore the use of mushrooms and their metabolites for the treatment of various ailments.

1.2.2. MEDICINAL MUSHROOMS

Tonic mushrooms are nature's most potent immune system protectors. For over 7000 years, these natural allies have been treasured in Oriental medicine where they were historically used to support the immune system, reduce fatigue, and promote cardiovascular health. In the west, the health benefits of mushrooms were acknowledged in the writings of Hippocrates, the "Father of medicine", and famous herbalists Pliny, Dioscorides and Galen.

Among the medicinal mushrooms, poisonous and some inedible had a very prominent place in the folklore. Mushrooms represent a major and as yet largely untapped source of powerful new pharmaceutical products. Medicines from the poisonous mushrooms have been considered in China as 'poison as an antidote for poison' (Yang and Jong, 1989).

The most significant medicinal effect of mushrooms and their metabolites that attracted the attention of the public is their antitumor property. Polysaccharides, phenols and terpenes were isolated from mushrooms and found to be effective against various tumor models in animals. The polysaccharides like Lentinan (*Lentinus edodes*), PS-K (*Coriolus versicolor*), terpenes like illudins M and S (*Clitocybe illudens*), and lectins from *Agaricus bisporous* and *Volvariella volvaceae* were found to possess antitumor activities (Hirase et al., 1970., Nishimora et al., 1980., Anchel et al., 1950.). Antitumor polysaccharides have been obtained from a number of Basidiomycetes including *Coriolus consors, Coriolus versicolor, Flammulina velutipes, Ganoderma applanatum, Phellinus lintus, Pleurotus ostreatus* and *Schizophyllum commune* (Ikekawa et al., 1982).

The active components found in mushrooms, beta-glucan polysaccharides, wake up the immune system and encourages it to work more efficiently and effectively. Many investigators identified and isolated polysaccharides (PS) from mushrooms, such as lentinan, schizophyllan, and protein-bound PSK (Krestin) and PS-peptide (Chang, 1993., Reshetnikov et al., 2001., Wasser and Weis, 1999., Mizuno, 2000) . The antitumor activity of these polysaccharides is due to the

potentiation of the immune response, which involved lymphocyte activation. Thus, antitumor polysaccharides are focused as immunopotentiators. Mushrooms such as Shiitake, Coriolus, Reishi, and Maitake, have been shown to have antitumor and anticancer properties (Reshetnikov et al., 2001., Wasser and Weis,1999). Another group of medicinal substances is the terpenoids, which contribute mainly cytotoxic, hypolipidic, hypotensive, and hepatoprotective activities. Biologically active protein-lectins were recently discovered within the mushroom groups (Badalyan, 2001). Many higher Basidiomycete mushrooms are known to contain various biologically active substances that express promising antitumor, immune modulating, cardiovascular and antihypercholesterolemic, antiviral, antibacterial, antiparasitic, hepatoprotective, and antidiabetic effects. Anticancer drugs isolated from mushrooms such as *Lentinus edodes; Coriolus versicolor and Schizophyllum commune* are sold in Japan (Jong and Birmingham, 1992b).

In China, Reishi, *Ganoderma lucidum*, is known as the "Plant of immortality" and is the key ingredient in the treasured *elixir of life*. It is the highest class of tonics in Chinese tradition, and for over 4000 years has been thought to promote longevity and to nourish and tone the body. Reishi fortifies the body's natural immunity and supports cardiovascular health.

Shiitake, *Lentinus edodes*, is one of the most popular mushroom in Chinese cuisine. Shiitake's profound immunestimulating, antitumor properties were documented more than 25 years ago in Western medicine. Lentinan, present in shiitake are orally active and exhibit anticancer effects against numerous tumor

lines. Since the discovery, this extra ordinary fungus has been the focus of intense scientific interest.

Grifola frondosa (Maitake) has attracted the attention of researchers for its ability to support immune function and it is the most widely promoted tonic mushroom for the treatment of cancer. Polysaccharide component present in this mushroom increases many parameters of immune function including macrophages, killer cells and cytotoxic T cells.

Coriolus versicolor is the latest discovery among health-promoting fungi. The *Coriolus* extracts are found to produce an impressive enhancement of antioxidant activity and other protective qualities within body cells. One study proved it to be useful in oncological treatment as it induce the production of significant amount of interferon (Wasser and Weis, 1999). Medicinal properties of these mushrooms are listed in the (Table1.1).

Babitskaya et al., (2001) reported that in addition to polysaccharides, a higher content of polyphenols and considerably more antioxidant activity was noted in fruiting bodies compared with mycelia extracts and cultural liquid of *L. edodes* and *P. ostreatus*. The antioxidant effect of phenolic substances may be related to their ability to neutralize active oxygen forms and bind iron ions. Profound antioxidant activity was reported for *P. florida* (Jose and Janardhanan, 2000) and *Phellinus rimosus* (Ajith and Janardhanan, 2001).

1.2.3. *GANODERMA*

Species of *Ganoderma* are a famous tonic in Chinese medicine. Generally all types of *Ganoderma* species are described as beneficial to all viscera and nontoxic (Liu, 1999). *Ganoderma* belongs to the polyporaceae family of Basidiomycota. For four thousand years, enzyme rich reishi mushroom (*Ganoderma lucidum*) have been used as part of Chinese and Japanese medicine, especially in the treatment of chronic hepatitis, nephritis, hepatopathy, neurasthenia, arthritis, bronchitis, asthma, gastric ulcer, anti-inflammatory, antibacterial, antioxidant, antitumor, antiviral, blood pressure lowering, bone marrow enhancing, cardiotonic, anti – HIV and insomnia (Eo et al., 1999., Kim and Kim, 1990., Liu, 1999). *Ganoderma* species are rich in organic compounds such as polysaccharide, amino acid, triterpenes, ascorbic acid, sterols, lipids, alkaloids, and trace minerals (Ma et al., 2002., Koyama et al., 1997., Chyr and Shiao, 1991). However, the extensive range of traditional medical treatment with *Ganoderma* has not yet been fully substantiated by modern scientific standards.

Ganoderma mushrooms are widely distributed in the tropics. The mushroom is found to grow on a large number of trees in Kerala (Leelavathy and Ganesh, 2000). One of the common hosts of *G. lucidum* in Kerala is *Caesalpinia coriaria* Wild [Figure 1.1].

1.2.4. REACTIVE OXYGEN SPECIES AND 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). Oxygen

qualifies as a free radical because it possesses two unpaired electrons each in a different orbital and both spinning in the same direction. The unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non radical (Hartwig, 1994).

There has been considerable interest on the role of reactive oxygen species (ROS) as mediators of tissue injury in human disease. The oxidative properties of oxygen play a vital role in diverse biological phenomena such as utilization of nutrients, electron transports to produce ATP and the removal of xenobiotics. However, oxygen has double-edged properties. While it is essential for life, it can also provoke damaging oxidative events within the cells. Oxygen, mainly via its transformation to more reactive forms i.e. $O_2^{\cdot -}$, $OH^{\cdot}$, and H_2O_2 can nick DNA, can damage essential enzymes and structural proteins and can also provoke uncontrolled chain reactions (e.g. the polymerization of catecholamines) (Hemnani and Parihar, 1998). Two of oxides of nitrogen, NO and NO_2 possess odd number of electrons and fall into the definition of free radicals. Recent studies have shown that metals such as iron, copper, cadmium, chromium, lead, nickel and vanadium exhibit the ability to produce ROS, resulting in lipid peroxidation, DNA damage, depletion of sulphydryls and altered calcium homeostasis (Stohs and Bahega, 1995., Winston and Digiulio, 1991).

Table 1.1. Medicinal properties of various mushrooms.

Mushrooms	Medicinal properties
Lentinus edodes	Inhibition of platelet aggregation
	Antitumor
	Antibacterial
Coriolus versicolor	Antitumor
Phellinus linteus	Antiviral
Phellinus rimosus	Nephroprotective
	Anti-inflammatory
	Antitumor
Schizophyllum commune	Antibacterial
	Antitumor
Pleurotus species	Antitumor
	Hepatoprotective
Grifola frondosa	Antitumor
Ganoderma lucidum	Inhibition of platelet aggregation
	Antitumor

Figure 1.1. *Ganoderma lucidum* growing on *Caesalpinia coriaria*. Wild

Transition metals are thought to promote free radical reactions, including the Fenton reaction, which results in the formation of hydroxyl radicals. The two most commonly studied transition metals are the cations Fe^{2+} and Cu^{2+}. The basic mechanisms involving production of ROS are the same for transition metal ions (Stohs and Bahega, 1995., Aruoma et al.,1991., Geierstanger et al.,1991). Once inside the cell hydrogen peroxide can react with Fe^{2+}, Cu^{2+} to form hydroxyl radical and this may be the reason for toxic effect of H_2O_2. The $OH^{\cdot}$ can cause single stranded breaks in the DNA. Hydroxyl radical ($OH^{\cdot}$) is considered potentially the most potent oxidant encountered in biological systems and has an extremely short half-life. Under pathological conditions or during administration of certain drugs the amounts of free radicals formed can overwhelm the defense of the cell and can lead to damage or death of the cell (Hemnani and Parihar, 1998).

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. They are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. They are also found or generated through environmental pollutants, cigarette smoke, automobile exhaust fumes, radiation, air pollutants, pesticides, etc (Halliwell et al., 1992., Tiwari, 2001).

Free radical generation in living systems occurs mainly through three-electron reduction of molecular oxygen, instead of a four-electron reduction. Several redox processes (e.g., Fenton reaction) and enzymatic reactions (e.g., Xanthine oxidase) contribute to free radical production. Apart from biochemical

sources, free radicals are also generated due to exposure to radiation. Detrimental effects of ionizing radicals on living systems are mediated through these free radicals. Free radicals interact with cellular components and bring about a change in structure and function of biological molecules, which could lead to damage and death of cell. Now it is well established that free radicals also cause a number of diseases. Efforts have been made to understand the generation of free radicals, damage induced by them and the mechanism of damage (Agrawal and Kale, 2001).

Several sources of ROS in the cells are proposed. ROS are generated from leakage of electrons on to oxygen from its mitochondrial electron transport chains, microsomal cytochrome P-450 and their electron donating enzyme and other systems (Beal, 1997., Hansford, 1997). ROS are also produced from activated phagocytes (Babior and Woodman., 1990., Hemnani and Parihar, 1998) and also occurs from other normal biochemical oxidation-reductions, both enzymatic (e.g. Xanthine oxidase) and non enzymatic reactions (such as auto oxidation of catecholamines). H_2O_2 is additionally generated *in vivo* by several oxidase enzymes, viz. monoamine oxidase (MAO), tyrosine hydroxylase, and L-amino oxidase (Mc Cord, 1987). The $OH^{\cdot}$ is the most reactive form of the oxygen radical. It is highly reactive and hence no enzyme systems involving it as a substrate exist (Southern and Powis, 1998).

There is considerable current interest in the role of reactive oxygen species (ROS) as mediators of tissue injury in human disease. Peroxidation of membrane lipids is likely to lead a disturbance of the membrane integrity (Richter, 1987., Vliet

and Bast, 1992). ROS can interact with proteins directly, especially their sulfhydryl groups (Herington, 1986). ROS have been put to good use by phagocytes. ROS may sometimes function as intracellular signaling molecule (Schreck et al., 1991). ROS are known to interfere with actions of NO, which has been recognized as a messenger with wide spread actions. Interactions with $O_2^{\cdot -}$ and NO regulate vascular tone or inflammatory processes. A harmful situation might arise from the over production of $O_2^{\cdot -}$ or NO (Mathews et al., 1997., Reiter, 1997., Reiter, 1995., Hemnani and Parihar, 1998).

All aerobic organisms are continually exposed to oxidative stress. Normally there is equilibrium between free radical formation and antioxidant defense mechanisms. An imbalance leads to oxidative stress (Sies, 1993). Oxidative stress can induce apoptotic cell death, and mitochondria have a central role in this and other types of apoptosis. In many tissues, free radicals can give apoptotic signals and these may be important in many neurodegenerative diseases. Many biological effects of the free radicals result from their actions on membrane enzyme, nucleic acids and polysaccharides. A variety of critical biological molecules, including DNA, cellular protein, and membrane lipids are subjected to oxidative damage (Lee et al, 1997). The effects of these reactive species are wide ranging but these reactions are particularly relevant to cell injury [Figure 1.2]

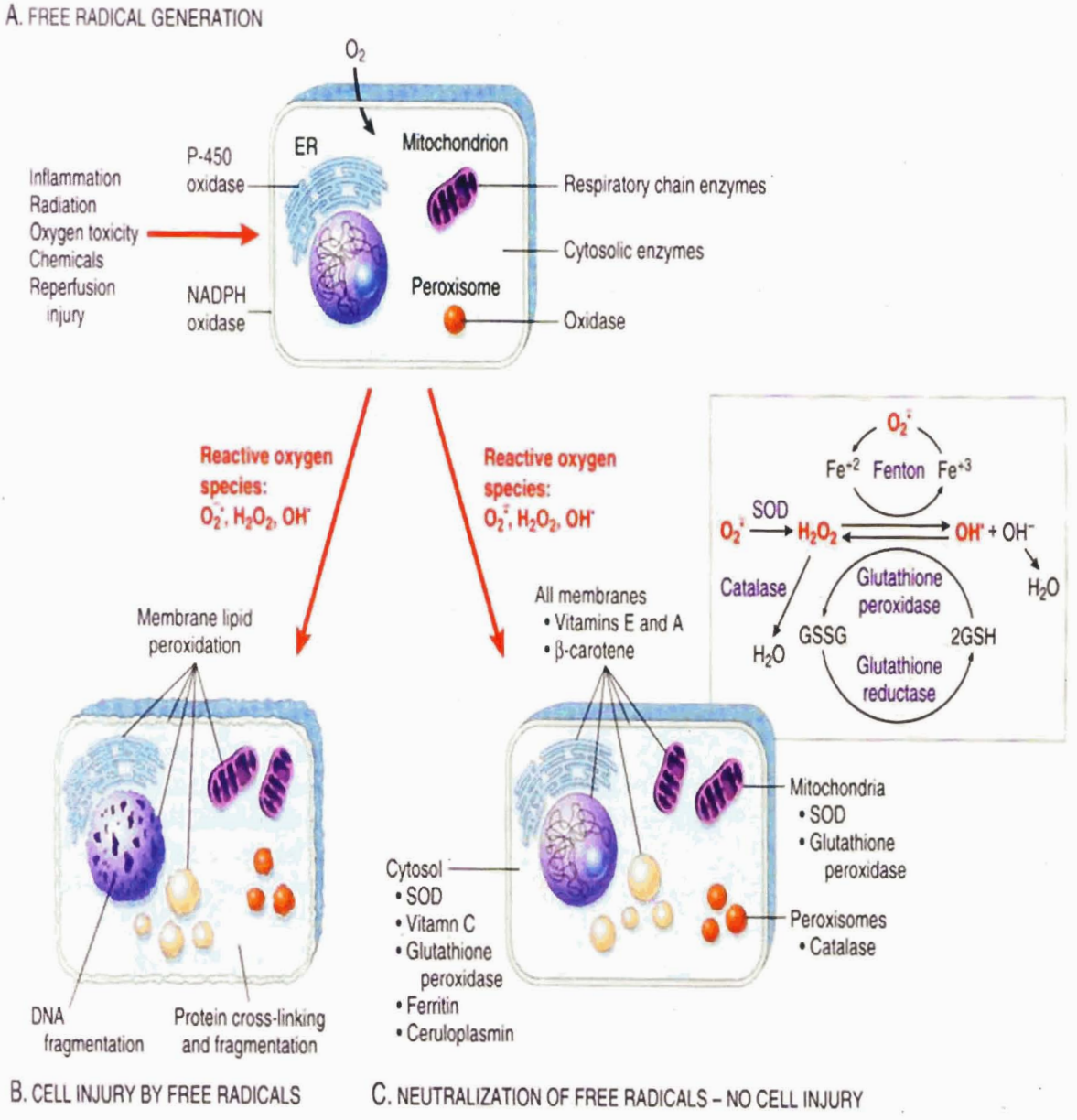


Figure 1.2. Role of free radical in cell injury. Cotran et al, 1999.

1.2.5. ROLE OF FREE RADICALS IN PATHOLOGICAL DISORDERS AND DISEASES

Evidences have shown that controlled generation of these highly reactive molecules has important roles in blastocyst implantation (Laloraya et al., 1989), disintegration of the structural elements of the sperm cells (Chatterjee et al., 1994), iodination of tyrosine in the thyroxine biosynthesis (Verma et al., 1990) and the secretion of mucous in goblet cells [Figure 1.3a] (Parihar et al., 1997). However, their uncontrolled production is generally considered to be an important factor in the etiology of pathological conditions such as myocardial infarction, rheumatoid arthritis, cardiovascular, neurodegenerative, ischemia-reperfusion and cancer. [Figure 1.3b]. Extensive research in the field of free radicals and ROS has linked them with a wide range of chronic and acute diseases, diseases such as HIV, genetic mutations, motor neuron disease, hypocuprosis, adult respiratory distress syndrome (ARDS).

A majority of disease conditions like atherosclerosis, hypertension, ischemic diseases, Alzheimere's disease, Parkinsonism, cancer, diabetes, neurodegenerative diseases and anti-inflammatory conditions are being considered primarily due to imbalance between prooxidant and antioxidant homeostasis. Oxidative stress, especially oxidation of low-density lipoproteins (LDL) has long being suspected to play a critical role in atherogenesis. There is considerable evidence to show that hypertension results in the generation of ROS, ultimately leading to increase oxidative stress in a variety of tissues. The root cause of major risk factors for

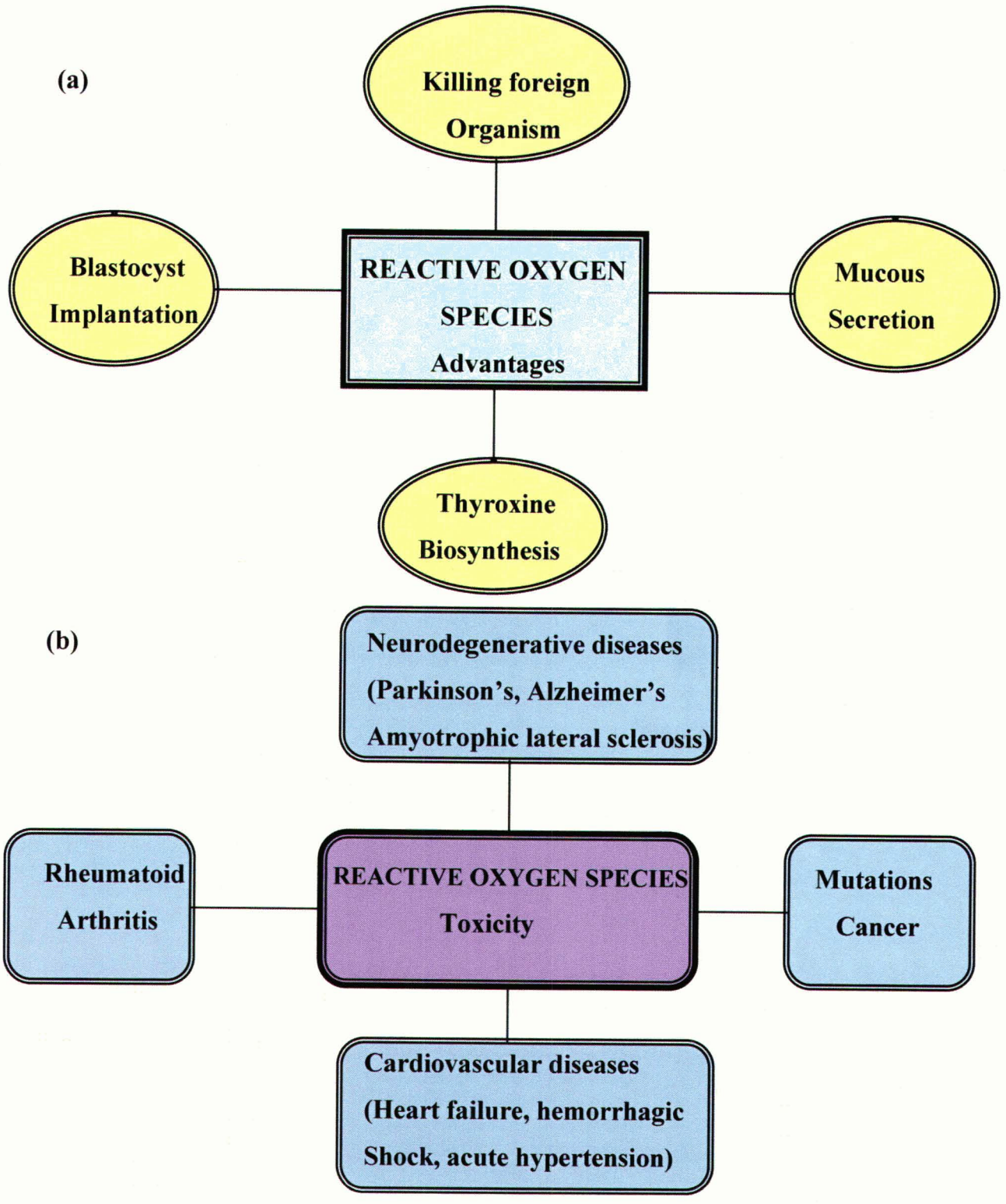


Figure 1.3 (a) Potential advantages of reactive oxygen species (ROS)., 1.3.b; Potential toxicity of reactive oxygen species (ROS). (Hemnani and Parihar, 1998).

cardiovascular diseases like hyperlipidemia and hypertension is suggested to be the effect of ROS (Tiwari, 2001).

1.2.6. ROLE OF FREE RADICALS IN CANCER

ROS/RNS can cause DNA base changes, strand breaks, damage to tumor suppressor genes and enhanced expression of protooncogenes (Cerrutti, 1994., Jackson, 1994), and oxidative stress has been shown to induce malignant transformation of cells in culture . However, the development of human cancer depends on many other factors, including the extent of DNA damage; excessive DNA damage, antioxidant defences, DNA repair systems, the efficiency of removal of oxidized nucleosides (eg. Oxo-dGTP) before they are incorporated into DNA, and the cytotoxic effects of ROS in large amounts as well as their growth promoting effects in small amounts. It is hypothesized that oxidants might contribute to carcinogenesis by causing oncogene activation or tumor suppressor gene inactivation (Wiseman and Halliwell, 1996., Wiseman et al., 1995., Ames, 1989., Cerutti, 1994).

Damage of DNA by ROS results in mutations, which are associated with initiation and progression of cancer (Hemnani and Parihar, 1998). Recent studies have attempted to identify mutations that are caused by ROS/RNS damage to DNA, with the aim of ascertaining their association with cancer. 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. 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.

Oxidant carcinogens interact with multiple cellular targets including membranes, proteins and nucleic acids. They cause structural damage to DNA and mutate cancer related genes. At the same time, oxidants activate signal transduction pathways and alter the expression of growth and differentiation related genes. One category of these genes is called DNA inducible (DDI). One group of DDI genes, gadd genes, mediate growth arrest during the late G_1 phase of cell cycle. This G_1 arrest theoretically enables the cell to undergo DNA repair before entering synthetic (S) phase (Keyse, 1993). Numerous forms of oxidative DNA damage have been identified including strand breaks, intra or inter strand cross-links, DNA-protein cross-links and various types of DNA damage. Overall studies indicate that DNA is an early target for oxidative stress, which could contribute to the cascade of pathogenesis of cells and gene mutation and cancer (Hemnani and Parihar, 1998).

1.2.7. ANTIOXIDANTS

It is increasingly being realized now that a majority of the diseases/ disorders are mainly due to the imbalance between prooxidant and antioxidant homeostatic phenomenon in the body. Prooxidant conditions dominate either due to increased generation of free radicals and /or their poor quenching/scavenging into the body. Naturally there is a dynamic balance between the amount of free radicals generated

in the body and antioxidants to quench and/or scavenge them and protect the body against their deleterious effects (Tiwari, 2001). However, the amount of these protective antioxidant principles present under the normal physiological conditions, are sufficient only to cope with the physiological rate of free radical generation. Therefore, any additional burden of free radicals either from environment or produced within the body, can tip the free radical (Pro-oxidant) and anti free radical (antioxidant) balance leading to oxidative stress, which may result in tissue injury and subsequent diseases (Davies, 1995). Antioxidants help to regulate and control the levels of free radicals at the required physiological concentrations. Antioxidants function as inhibitors at initiation, promotion, propagation and transformation stages of tumor promotion/ carcinogenesis and protect cells against oxidative damage. Considerable evidence has accumulated on the role of the antioxidant nutrients including vitamin C. E, and β carotene, in the maintenance of human health.

Cells have multiple antioxidant defenses to protect themselves against ROS. These protective mechanisms are not in excess; if oxidative damage would not occur and repair mechanisms would not be required. Instead, oxidative damage occurs continuously in the human body. Fortunately, enzymic and some other (e.g. metallothionein, caeruloplasmin and haptoglobin) antioxidant defenses are often inducible in response to oxidative damage (Halliwell and Gutteridge, 1989). According to the mode of action, antioxidants may be classified as free radical scavenger/ terminator, chelator of metal ions, capable of catalyzing lipid

peroxidation or as oxygen scavengers that react with superoxides and so on (Tiwari, 2001).

The antioxidants that prevent the formation of new ROS are called preventative antioxidants, e.g. caeruloplasmin, metallothione, albumin, transferrin, ferritin, and myoglobin. The antioxidants that remove ROS once formed, thus preventing radical chain reaction. High SOD activity has been reported in cancer patients. Changes in antioxidant defense enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione S-transferase (GST), and catalase (CAT) have been widely described in cancerous cells (Cerutti et al., 1994).

Antioxidant Enzymes

Superoxide Dismutase (SOD) $2O_2^{\cdot -} + 2H^{\cdot} \longrightarrow H_2O_2 + O_2$

Glutathione peroxidase (GPX) $ROOH + 2\,GSH \longrightarrow ROH + H_2O + GSSG$

Glutathione Reductase (GR) $NADPH + H^{\cdot} + GSSG \longrightarrow NADP^{\cdot} + 2GSH$

Antioxidant vitamins have a number of biological activities, immune stimulation, inhibition of nitrosamines formation and an alteration of metabolic activations of carcinogens. Vitamin C (ascorbic acid) readily scavenges ROS and reduces oxidative damage and mutations. Because of the antioxidant properties, vitamin E neutralizes ROS and reduces the oxidative damage and mutations (Frei, 1994).

Glutathione present in food is one of the major antioxidants and this compound protects thiol groups in protein from oxidation and serves as a reservoir

of cysteine (Moron et al., 1979). Flavonoids are phenolic compounds, present in several plants, which inhibit lipid peroxidation (Tiwari, 2001).

The consumption of fruits, grains and vegetables, which are sources of antioxidants, has been reported to protect against oxidative damage and the resulting disease (Halliwell, 1994., Block et al., 1992). Intake of fresh fruit and vegetables is considered to be inversely correlated with cancer of the stomach, pancreas, oral cavity and esophagus, and to a lesser extent of the breast, cervix, rectum and lung (Block, 1992., Block, et al., 1992). Protective effect of ascorbate has been emphasized. The ascorbate can react with, and/ or inhibit the formation of, carcinogenic *N*-nitroso compounds such as *N*-nitrosamines. Vitamin C supplementation has been reported to decrease mucosal DNA damage, as measured by a ^{32}P post labeling assay, in 28 of 43 patients attending a gastric follow- up clinic (Wiseman and Halliwell, 1996). Furthermore, dietary supplementation with moderate amounts of the relatively inexpensive and non-toxic vitamins, ascorbate and α tocopherol has been found desirable in some population groups, such as smokers (Halliwell, 1994., Block,1992., Block et al., 1992).

1.2.8. INFLAMMATION

Inflammation is fundamentally a protective response, ultimate goal of which is to rid of the organism of both the initial cause of cell injury (e.g. microbes, toxins) and the consequences of such injury, the necrotic cells and tissues. Without inflammation, infections would go unchecked, wounds would never heal, and injured organs might remain permanent festering sores. Inflammation is a complex

process in which many different mediators are involved, eg. prostaglandins, leukotrienes and platelet activating factor (PAF). By modulating the release of these mediators, overall process can be regulated (Campbell, 1990., Tunon et al., 1995).

Inflammation has been divided into acute and chronic patterns. Acute inflammation is of relatively short duration, lasting for minutes, several hours, or a few days, and its main characteristics are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, predominantly neutrophils. Chronic inflammation on the other hand is of longer duration and is associated histologically with the presence of lymphocytes and macrophages and with the proliferation of blood vessels and connective tissue. The vascular and cellular responses of both acute and chronic inflammation are mediated by chemical factors derived from plasma or cells and triggered by the inflammatory stimulus (Collins, 1999., Underwood, 2000).

Inflammation can accelerate the development of cancer (Rosin et al., 1994). Many sources of inflammation are infective, including that caused by viral, bacterial and parasitic infections. In colon cancer, predisposing sources of chronic inflammation include ulcerative colitis and infection with the parasite *Schistosoma japonicum.*

However, the link between cancer and inflammation is by no means a simple one. One chronic inflammatory disease in which patients suffer oxidative stress is rheumatoid arthritis. Indeed, there is a strong relationship between the capacity of tumor promoters to stimulate inflammatory cells to release ROS/ RNS and their

capacity to promote tumors. Genetic damage and neoplastic transformation have been demonstrated in cells co-cultured *in vitro* with activated phagocytes. The genotoxic effects observed in these cells include the formation of DNA strand breaks, sister chromatid exchange and mutations. Inflammatory cells may also increase DNA damage by activating procarcinogens to DNA-damaging species, e.g. neutrophils that can activate aromatic amines, aflatoxins, estrogens, phenols and polycyclic aromatic hydrocarbons by ROS dependent mechanisms. RNS can generate carcinogenic nitrosamines. Nitrosation of morpholine has been reported in immunostimulated rats.

Chronic hepatitis is associated with the presence of inflammatory cells, presumably generating ROS and RNS. Increased levels of 8-hydroxydeoxyguanosine (8-OhdG) have been detected in DNA from livers with chronic hepatitis. Inflammatory bowel disease (IBD) is the general name given to a series of chronic inflammatory diseases of gastrointestinal tract, including ulcerative colitis and Crohn's disease. ROS are formed in excess in IBD and are likely to play an important role not only in the pathogenesis of IBD., but also an increased risk of cancer in certain IBD. DNA damage by ROS is also implicated in inflammatory breast disease, where malignant progression can occur (Wiseman and Halliwell, 1996).

There is no strong evidence to show that antioxidant supplementation can alleviate inflammation. However, a study of vitamin E in the synovial fluid of patients suffering from rheumatoid arthritis found significantly lower levels than

those measured in healthy individuals. The antioxidant and catecholamines-sparing activities were considered to be important determinants of anti-inflammatory activity of flavonoids (Ahmadiani et al., 2000).

Nonsteroidal anti-inflammatory agents (NSAIDs) can alter the production of different metabolites of polyunsaturated fatty acids (e.g. linoleic acid and arachidonic acid) by modulating the activity of lipoxygenases and cyclooxygenases (Shureiqi et al., 2001). Indomethacin (IMN), an NSAID, is an established inhibitor of the cyclooxygenases, a component of prostaglandin synthetase, involved in prostaglandin biosynthesis. Drugs such as narcotics e.g. morphine, codeine, meperidine, peripherally acting drugs such as aspirin, oxyphenbutazone, dexamethasone and hydrocortisone are the other anti-inflammatory agents commonly used. Ficoll-bound SOD also decreases inflammation induced by injecting carrageenan into the feet of rats. Caeruloplasmin, an antioxidant is also reported to have anti-inflammatory activity. A number of coumarins have been reported to possess anti-inflammatory activity (Argaez et al., 2000).

1.2.9. HEPATOPROTECTION

The liver protects the body from potentially injurious substances absorbed from the intestinal tract as well as toxic byproducts of metabolism. A large number of chemicals are reported to be potentially hepatotoxic. Hepatotoxins may react with basic cellular constituents such as proteins, lipids, RNA and DNA and induce almost all types of toxic symptoms in the liver (Guillouzo, 1998). Control of liver diseases has attracted great attention in recent year because of the increasing

incidence of diseases affecting the liver. The main reasons for this are pollution and lifestyle. A large number of plant derived drugs have been reported beneficial to protect the liver from hepatic disorders. Hepatoprotective effect of the extracts of mushrooms, namely *Grifola frondosa*, *Dendropolyporous umbellatus*, *Schizophyllum commune* and *Tremella fuciformis* have been reported (Wasser and Weis, 1997).

1.2.10. CHEMOPREVENTION

Cancer chemoprevention, which refers to the administration of chemical agents to prevent the initiation (mutational) and promotional events that occur during the process of neoplastic development (Boone et al., 1990). The goal of cancer chemoprevention research is to identify agents, which can reverse or arrest the process of carcinogenesis. To date, >1500 structurally diverse agents have been identified with suspected anticarcinogenic activity and the potential to intervene at all stages of the carcinogenic process. These agents can be classified into three major classes based on their mechanisms. (1) blocking agents: inhibit carcinogen uptake, formation or activation, deactivate carcinogens, increase detoxification, prevent carcinogen binding to DNA and increase the level of fidelity and DNA repair (2) antiproliferatives/antiprogressives: modulate signal transduction and hormone/growth factor activity, inhibit oncogene activity and expression, and inhibit polyamine metabolism and angiogenesis; (3) antioxidants: scavenge reactive electrophiles and oxygen radicals, inhibit arachidonic acid metabolism, and induce antioxidant enzymes.

The National Cancer Research Institute has proposed four goals of chemoprevention (i) inhibition of carcinogens (ii) logical intervention for persons at genetic risk (iii) treatment of precancerous lesions., (iv) confirmation and translation of leads from dietary epidemiology to intervention strategies (Smith et al., 2001). An interesting observation that has been made repeatedly is that individuals who consume relatively large amounts of vegetables and fruits are at decreased risk of cancer of many organs.

1.2.11. CHEMOTHERAPY

Chemotherapy aims to kill or disable tumor cells while preserving the normal cells in the body. However, most of the chemotherapeutants are not only cytotoxic to cancer cells but also to healthy cells. Considering the importance of natural products in chemotherapy, development of effective agents with fewer side effects is a compelling urgency. Among the plant products the most important chemotherapeutic agents are vinca alkaloids from *Catharanthus roseus*, taxol from *Taxus brevifolia*, podophyllotoxins from *Podophyllum emodi*. Vincristine, vinblastine, taxol, cisplatin, doxorubicin etc are the most widely used chemotherapeutic agents in recent years. Most of these drugs not only kill the cancer cell but also damage healthy cells. This results in narrow therapeutic index of these drugs (De Vita, 1978).

1.2.12. TOXIC EFFECTS OF ANTICANCER DRUGS

Conventional cancer chemotherapy aims to kill or disable tumor cells which preserving the normal cells in the body by the application of synthetic compounds.

These agents have narrow margin of safety and the therapy may fail because of drug resistance and dose limiting toxicities. One of the major problems of cancer chemotherapy is the acute toxicity elaborated by anticancer drugs. This is a major limiting factor of the use of these drugs for the treatments. Cisplatin (cisplatinum II diamine dichloride) is an extensively used drug for chemotherapy particularly ovary, testes, bladder and head and neck cancer (Hamers et al., 1991). Higher doses of the drug, although more efficacious for cancer chemotherapy manifests irreversible renal dysfunction, ototoxicity and other toxicities (Bodenner et al., 1986). Doxorubicin is a powerful anthracycline antibiotic extensively used for the treatment of many human neoplasms, including acute leukemia's, lymphomas, stomach, breast and ovarian cancers, Kaposi's sarcoma, and bone tumors. However, the prolonged administration with this drug causes acute cardiotoxicity (Mott, 1997). Prevention or delimiting of the toxicities elaborated by chemotherapeutants is an important aspect of chemotherapy. Dietary supplements such as herbal extracts are now increasingly inducted to adjuvant therapy for the amelioration of toxic effects caused by anticancer drugs.

GENERAL MATERIALS AND METHODS

Sheena.N "Studies on the thekapeutic potential of Ganoderma lucidum P. Karst – Reishi, occurring in Kerala " Thesis. Amala Cancer Research Centre , University of Calicut, 2005

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. ANIMALS

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

All animal experiments were carried out according to the guidelines prescribed by Animal Welfare Board and with the approval of Animal Ethic Committee.

2.1.2. CELL LINES

Ehrlich's ascites carcinoma (EAC) and Daltons lymphoma ascites (DLA) cell lines were obtained from Cancer Institute, Adayar, Chennai. The cells were maintained in mice by frequent intraperitoneal inoculations.

2.1.3. CHEMICALS

Carbon tetrachloride (CCl_4), aniline, phenol, hydrogen peroxide (H_2O_2) and chloroform ($CHCl_3$), dimethylsulphoxide (DMSO), formaldehyde, sodium nitroprusside, sodium nitrite and thiobarbituric acid were purchased from Merck, India Ltd, Mumbai. Agar-agar, sodium azide (NaN_3), Reduced glutathione (GSH), 5,5-dithiobis-(2-nitrobenzoicacid) (DTNB), diacetylmonoxime (DAM), nitroblue tetrazolium (NBT), 1-chloro-2,4-dinitrobenzene (CDNB), riboflavin and sulphanilamide from Sisco Research Laborateries Pvt.LTD. Mumbai. Cisplatin was

purchased from Dabur India Ltd, New Delhi. 7,12-dimethylbenz [*a*] anthracene (DMBA), and carrageenan were purchased from Sigma, St. Loius, USA. All other chemicals and reagents used were analytical reagent grade.

2.2. METHODS

2.2.1. PREPARATION OF THE EXTRACT

Sporocarps of *G. lucidum* growing on the living *Caesalpinia coriaria* Wild. trees growing in different parts of Thrissur. Dt, Kerala were collected. The fruiting bodies were lignicolous, annual and mostly sessile. The pileus surface was shining, laccate and purplish brown in color. The specimen was identified and voucher specimen was deposited in the Herbarium of Centre for Advanced Studies in Botany, University of Madras, Chennai, India (HERB.MUBL.3175).

The fruiting bodies of the mushroom were cut into small pieces, dried at 40-50°C for 48 h and powdered. Samples of one hundred gram of the powdered materials were extracted with petroleum ether using Soxhlet apparatus (Suffness and Douros, 1979). The defatted materials were extracted with ethyl acetate by the same process. The ethyl acetate extract was evaporated to dryness at 40°C using a rotary vacuum evaporator. The residue was designated as ethyl acetate extract (2g/100g powder). After ethyl acetate extraction, the samples were air dried, and then extracted with methanol for 8 h in Soxhlet apparatus. The solvent was removed and the extraction repeated once again. The methanol extracts were combined, filtered through Whatman No. 1 filter paper and solvent evaporated completely at

low temperature. The residue was named methanol extract (2g/100g powder). After methanol extraction the samples were suspended in hot water and extracted at 70-80^0 C for 2hr. The hot water extract was collected by filtration, concentrated in a water bath, and finally lyophilized (2g/100g powder). The methanol extract was presolubilised in DMSO for assaying the *in vitro* activity. For the animal experiments, the extract was suspended in distilled water to form a uniform suspension.

2.2.2. PREPARATION OF TISSUE HOMOGENATES (LIVER, KIDNEYS AND HEART)

Animals after they were sacrificed, liver, kidneys and heart were excised and rinsed thoroughly in ice-cold saline to remove the blood. They were then gently blotted between the folds of a filter paper and weighed in an analytical balance. 10% of homogenate was prepared in 0.05 M phosphate buffer (pH 7) using a polytron homogeniser at 4^{0}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 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 (10%) was mixed with 0.1 ml of 25 % TCA and kept on ice for few minutes. These were then subjected to centrifugation at 3000 rpm 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 mol/mg protein.

2.2.4. DETERMINATION OF TISSUE SUPEROXIDE DISMUTASE (SOD) ACTIVITY

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

PRINCIPLE

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

PROCEDURE

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

2.2.5. DETERMINATION OF TISSUE CATALASE (CAT) ACTIVITY

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

PRINCIPLE

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

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

PROCEDURE

0.1 ml of the tissue homogenate (approximately 0.1 mg protein) was mixed with 1.9 ml of the phosphate buffer (0.5 M, pH 7). The decrease in extinction was measured at 240 nm, 1 min interval for 3 min immediately after adding 1 ml of 11 mM H_2O_2 solution in buffer. A sample control was placed in the reference cuvette containing 0.1 ml of tissue homogenate and 2.9 ml of the buffer. Activity of catalase was calculated using the mmoles extinction coefficient 40 cm^{-1}.

mmoles of H_2O_2 decomposed/ min/ mg protein

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

2.2.6. DETERMINATION OF TISSUE GLUTATHIONE PEROXIDASE (GPX) ACTIVITY

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

PRINCIPLE

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

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

PROCEDURE

Tissue homogenate (approximately 0.5 mg protein) was incubated with 0.1 ml of 5mM GSH, 0.1 ml of 1. 25 mM H_2O_2, 0.1ml of 25 mM NaN_3 and phosphate buffer (0.05 mM, pH 7) in a total volume of 2.5 ml at 37°C for 10 min. The reaction was stopped by adding 2 ml of 1.65 % HPO_3^{2-} and the reaction mixture was centrifuged at 1500 rpm for 10 min. 2 ml of the supernatant was mixed with 2 ml 0.4 M Na_2HPO_4 and 1ml of 1mM DTNB. The absorbance of the yellow colored complex was measured at 412 nm after incubation for 10 min at 37°C against distilled water. A sample without the tissue homogenate processed in the same manner was kept as nonenzymatic reaction.

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

2.2.7. DETERMINATION OF TISSUE GLUTATHIONE *S*- TRANSFERASE (GST) ACTIVITY

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

PRINCIPLE

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

PROCEDURE

The reaction mixture contained 0.05 ml of 60 mM GSH, 0.05 ml of 60 mM CDNB (in ethanol) 0.01 ml of the tissue homogenate and sodium phosphate buffer (0.1 M, pH 6.5) in total volume of 3 ml. The reaction was started by the addition of sample and the extinction was measured by 1 min interval for 3 min at 340 nm. The reference cuvette contained the complete assay mixture minus the cytosol. The activity of GST was calculated using the mmolar extinction coefficient of CDNB-GSH conjugate (9.6 $mM^{-1}cm^{-1}$) and is expressed as µmol of CDNB-GSH conjugate formed/ min/ mg protein.

µmol of CDNB-GSH conjugate-

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

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. 0.5 ml of butanol: pyridine mixture (15:1) was added to the reaction mixture, 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 of different concentrations (1-10 nmol) of 1'1'3'3'-tetramethoxypropane in 1 ml distilled water. The activity 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 absorbance of 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 and by the color developed

by the biuret reaction of the protein with the alkaline cupric tartrate were measured at 660 nm.

PROCEDURE

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

2.2.10. DETERMINATION OF SERUM GLUTAMATE OXALOACETATE TRANSAMINASE (SGOT) ACTIVITY

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

PRINCIPLE

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

PROCEDURE

0.1 ml of serum was added to 0.5 ml of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-aspartate in 100 ml phosphate buffer 0.1M, pH 7.4) at 37°C and incubated for 60 min. After the incubation, 0.5 ml of dinitrophenylhydrazine (19.8 mg/dl 1 N HCl) was added, 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, 2 mM) calibration curve. The enzyme activity (U/ml) is converted to IU/ l by multiplying with 0.483.

2.2.11. DETERMINATION OF SERUM GLUTAMATE PYRUVATE TRANSAMINASE (SGPT) ACTIVITY

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

PRINCIPLE

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

PROCEDURE

0.1 ml of serum was added to 0.5 ml of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-alanine in 100 ml phosphate buffer 0.1M, pH 7.4) at 37°C and incubated for 30 min. After the incubation, 0.5 ml of dinitrophenylhydrazine (19.8 mg/dl 1 N HCl) was added, mixed well and kept at room temperature for 20 min.0.4 ml of NaOH was added and read the absorbance after 10 min at 505 nm. 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. The enzyme activity (U/ml) is converted to IU/ l by multiplying with 0.483.

2.2.12. DETERMINATION OF SERUM ALKALINE PHOSPH ATASE (ALP) ACTIVITY

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

PRINCIPLE

ALP in the serum reacts with disodium phenyl phosphate under alkaline pH 10 releases 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 a reagent blank.

PROCEDURE

0.05ml of serum was incubated with 0.5 ml of the buffered substrate (1ml of 0.254 g of disodium phenyl phosphate dihydrate/dl water mixed with 1ml of the carbonate buffer pH 10) and 1.54 ml of distilled water at 37°C for 15 min. After the incubation, 2 ml chromogen (1ml of 0.6 g 4-aminoantipyrene/dl water and 1ml of potassium ferricyanide 2.4 g/dl water) reagent was added and measured at 510 nm. Phenol (10 mg %) was used as the standard for the calibration curve. The activity (KA/ dl) is converted to IU/ l by multiplying with 7.1.

2.2.13. DETERMINATION OF SERUM UREA

Serum urea was determined according to the method of Marshell et al., (1980)

PRINCIPLE

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

PROCEDURE

Reagents used were from Span diagnostic kit. 5 ml of diluted urea reagent (1:5 with distilled water) were mixed with 0.02 ml serum and 0.5 ml of diacetylmonoxime. Mixed well and kept in boiling water bath for 10 min. Cooled

and absorbance was measured at 525 nm against reagent blank. A standard solution of urea (30 mg%) was treated in the same way.

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

O. D_T – Optical density of test

O. D_S – Optical density of standard

2.2.14. DETERMINATION OF SERUM CREATININE

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

PRINCIPLE

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

PROCEDURE

Reagents used were from Merk diagnostic kit. 0.2 ml of serum was mixed with 0.5 ml of buffer (313 mM NaOH and 12.5 mM phosphate, pH 8), 0.5 ml of 8.73 mM picric acid. The absorbance was measured immediately after 1 min (A1) and exactly after 5 min (A2) at 500 nm. A standard creatinine solution (1 mg/dl) was treated in the same way.

$$\text{Creatinine concentration (mg/dl)} = \frac{\text{O. Dt}_2 - \text{O. Dt}_1}{\text{O. Ds}_2 - \text{O. Ds}_1}$$

O. DT_1 – Optical density of test solution 1

O. DT_2 – Optical density of test solution 2

O. DS_1 – Optical density of standard solution 1

O. DS_2 – Optical density of standard solution 2

2.2.15 DETERMINATION OF SERUM LIPID PEROXIDATION

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

PRINCIPLE

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

PROCEDURE

To 0.5 ml serum, 2.5 ml of 0.02 % TCA was added and allowed to stand for 10 min at room temperature. After centrifugation at 3500 rev./min for 10 min, the precipitate was washed once with 0.05 M H_2 SO_4. The precipitate was suspended in distilled water and estimated the TBARS by procedure given under tissue lipid peroxidation determination (section 2.2.8). The concentration was expressed as nmol/ ml of serum.

2.2.16. DETERMINATION OF SERUM CREATINE KINASE

Serum creatine kinase activity was determined by the method of Moss and Henderson (1999).

PRINCIPLE

Creatine phosphate catalyzes the conversion of creatine phosphate to creatine with a concomitant phosphorylation of ADP to ATP. The ATP produced is measured by hexokinase/ GPD coupled reactions that ultimately convert NADP to NADPH, which is monitored spectrophotometrically.

PROCEDURE

Reagents used were from Roche, diagnostics, Germany. 2.50 ml of reagent solution and 0.1 ml and sample was taken and mixed well and allow to stand for 3 min at 30^0C. Read initial absorbance and for 3 min at an interval of 1 min at 340 nm. The activity of CK in the sample was calculated by 4127x ΔA/min.

2.2.17. DETERMINATION OF HAEMOGLOBIN (HB) IN BLOOD

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

PRINCIPLE

Haemoglobin was treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogenphosphate. The ferricyanide forms methaemoglobin, which is converted to cyanmethaemoglobin by the cyanide. The

intensity of the color formed is measured at 546 nm against reagent blank. The optical density is directly proportional to the amount of haemoglobin present in the blood.

PROCEDURE

The reagents used were from Agappe diagnostic kit. 0.02 ml of fresh whole blood was mixed with 5ml of the cyanmeth reagent. The optical density was measured at 546 nm after 5 min incubation at room temperature. A standard (60 mg/dl) was treated in the same manner was used for calculating the concentration of haemoglobin in the blood.

Calculation

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

O. D_T – Optical density of test

O. D_S – Optical density of standard

2.2.18. DETERMINATION OF TOTAL RED BLOOD CELL (RBC) COUNT

Total RBC count was determined according to the method of Chaudari (2000a)

PRINCIPLE

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

PROCEDURE

0.02 ml blood was added to 3.98 ml of diluting fluid. The neubauer chamber was charged with well-mixed diluted blood and counted the total number of red cells was counted the small square in the central ruled area of neubauer counting chamber using 40X objective of the microscope.

Calculation

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

2.2.19. DETERMINATION OF TOTAL WHITE BLOOD CELL (WBC) COUNT

Total WBC count was determined according to the method of Chaudari (2000b)

PRINCIPLE

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

PROCEDURE

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

Calculation

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

2.3. STATISTICAL ANALYSIS

All experimental data were analyzed by one-way analysis of variance (ANOVA) using MSTAT soft ware package, UK. Comparisons of means for each parameter among the various experimental groups were performed using ANOVA and any two means having a common superscript are not significantly different at 5% level.

ANTIOXIDANT ACTIVITY OF GANODERMA LUCIDUM

Sheena.N "Studies on the thekapeutic potential of Ganoderma lucidum P. Karst – Reishi, occurring in Kerala " Thesis. Amala Cancer Research Centre , University of Calicut, 2005

CHAPTER 3

ANTIOXIDANT ACTIVITY OF *GANODERMA LUCIDUM*

3.1. INTRODUCTION

The biological significance of singlet oxygen (O_2), an electronically excited species of oxygen, has been realized only in the last two decades. Recent studies, using newly developed detection methods, showed that O_2 being generated in many biological systems, could significantly and quite often adversely alter several crucial biomolecules including DNA, proteins and lipids with undesirable consequences including cytotoxicity and/or disease development. Singlet oxygen has been considered as a major cytotoxic species to eukaryotic cells, bacteria and viruses. Extracellularly generated O_2 has been found to be genotoxic to mammalian cells grown in culture. On several instances, singlet oxygen has been implicated in the induction of tumor by photosensitization and in the metabolic activation of carcinogens. Besides, it has been implicated in several pathological processes like lung-oxidant injury, skin photosensitivity and erythropoetic porphyria. Some reports also showed that O_2 could play a significant role in the inactivation of cells or cellular components due to UV-A and near visible radiation (Devasagayam and Kamath, 2002).

Most of the oxygen taken up by the cells of our body is converted into H_2O during mitochondrial respiration. However, a small percentage of oxygen (less than 5%) is converted into reactive oxygen substrates (ROS) like $\cdot O_2$, H_2O_2 and $\cdot OH$ radicals. These substances are highly toxic in nature and if allowed to accumulate, they could destroy all the macromolecules of the cells like lipids, proteins,

mitochondrial and nuclear DNA molecules causing severe oxidative stress. When oxygen accepts a single electron, it forms a superoxide radical (O_2^-). Once superoxide radical is formed, it quickly undergoes dismutation to form H_2O_2 by a family of enzymes known as superoxide dismutases (SOD). H_2O_2 can also be formed by other enzymes. In dopaminergic neurons, oxidation of dopamine by the enzyme mono amine oxidase (MAO) generates hydrogen peroxide. Hydrogen peroxide is converted into a highly reactive radical ($^{\cdot}OH$). in the presence of transition metal ions like copper (Cu^{2+}), iron (Fe^{2+}) through Fenton reaction or Haber-Weiss reaction. H_2O_2 is normally disposed as H_2O and O_2 by two important antioxidative enzymes like glutathione peroxidase (GPX) and catalase. Glutathione peroxidase utilizes H_2O_2 and other hydroperoxides as substrates in the process of conversion of reduced glutathione (GSH) into its disulphide (GSSG) and thereby most of the H_2O_2 is disposed off. The hydroxyl radicals ($^{\cdot}OH$) that is formed through Fenton reaction is highly destructive in nature and can destroy lipids, proteins and DNA molecules of the cells. It also removes H^+ ions from the polyunsaturated fatty acid molecules (PUFA) of the cell membrane and generates peroxyl (LOO^-) and peroxynitrite anions, which can further substract H^+ ions causing extensive damage of the lipid molecules of the cell membrane. Thus lipid peroxidation induced by $^{\cdot}OH$ radicals will become self-propagating in nature that can eventually lead to destruction of all lipids in the cell membrane. In recent years, nitric oxide (NO) has also been shown to act as a free radical. Nitric oxide interacts with superoxide radicals causing the formation of peroxynitrite anions ($ONOO^-$)

that cause heavy damage to lipid molecules of the cell membranes (Hemnani and Parihar, 1998).

There are various compounds, mainly derived from natural sources that offer protection against damage induced by $^{\cdot}O_2$. The antioxidants include carotenoids, tocopherols, thiols and small molecular compounds such as carnosine, bilirubin etc. Several natural antioxidants play key roles in the preservation of membrane integrity including the vitamin A, C, and E. Biological antioxidants such as lipoate, methionine, flavonoids, related polyphenols, β carotene, α tocopherol and curcumin from turmeric also prevent DNA damage induced by O_2 (Devasagayam and Kamath, 2002). Many synthetic antioxidant components have shown toxic or mutagenic effects, which shifted the attention onto the naturally occurring antioxidants. A multitude of natural antioxidants have already been isolated from different kinds of plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs (Fejes et al., 2000).

Species of the genus *Ganoderma* P. Karst. (Ganodermatales) are important wood decaying polypores occurring throughout the world, mainly on tropical trees (Miller et al., 1995). Several investigations on the therapeutic effects of this medicinal mushroom have been carried out in many parts of the world. However, the information on the antioxidant activity of this mushroom is inadequate. Antioxidants are known to play a significant role in the prevention and cure of free radical mediated diseases.

3.2. MATERIALS AND METHODS

3.2.1. PREPARATION OF EXTRACTS

Ethyl acetate, methanol and aqueous extracts of *G. lucidum* were prepared as described in the section 2.2.1.

Aqueous methanol extract of *G. lucidum* employed for *in vivo* antioxidant assay was prepared as follows. The sporocarps were dried at 40-50^0 C for 48 hrs and powdered. The powdered material was defatted by extracting it with petroleum ether (60-80) for 8 hrs. The defatted material was then extracted with aqueous methanol (70%) for 12-16 hrs. The extract concentrated at low temperature and solvent completely evaporated. The residue (4g/100g powder) was named aqueous methanol extract and used in the experiments.

3.2.2 DETERMINATION OF *IN VITRO* ANTIOXIDANT ACTIVITY

3.2.2.1 SUPEROXIDE SCAVENGING ACTIVITY

Assay is based on the ability of the extract to inhibit the reduction of nitro blue tetrazolium (NBT) to deep blue colored formazan (Mc Cord and Fridovich, 1969). The reaction mixture contained, EDTA (6 mM) contained 3 μg NaCN; riboflavin (2 μg); NBT (50 μg); KH_2PO_4-Na_2HPO_4 buffer (67mM, pH 7.8) and various concentrations of the extracts in a final volume of 3ml. The tubes were illuminated under a incandescent lamp for 15 min. The optical density (O.D) at 560 nm was measured before and after illumination. The inhibition of the superoxide

radical generation was determined by comparing the absorbance values of the control with that of treatments. Quercetin was used as standard.

3.2.2.2. ASSAY OF HYDROXYL RADICAL SCAVENGING ACTIVITY

Hydroxyl radical scavenging activity was assayed by determining the competition between deoxyribose and the extracts for the hydroxyl radicals generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction). The hydroxyl radical attack deoxyribose, which eventually results in thiobarbituric acid reacting substance (TBARS). The TBARS when heated with thiobabituric acid at low pH produce a color, absorbance of which can be measured at 532 nm (Elizabeth and Rao, 1990). The reaction mixture contained deoxyribose (2.8 mM); $FeCl_3$ (0.1mM); K_2HPO_4-KOH buffer (20 mM, pH 7.4); EDTA (0.1mM); H_2O_2 (1.0 mM); ascorbic acid (0.1mM) and various concentrations of the extracts of *G. lucidum* in a final volume of 1 ml. The reaction mixture was incubated at 30°C for 60min. The TBARS formed was estimated by the method of Ohkawa et al (1979). The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treatments. Catechin was used as standard.

3.2.2.3. INHIBITION OF LIPID PEROXIDATION

Lipid peroxidation was induced by Fe^{2+}-ascorbate system (Bishayee and Balasubramanian, 1979) in the rat liver homogenate in the presence and absence of extracts to form thiobarbituric acid reacting substance (TBARS). The TBARS is measured according to the method of (Ohkawa et al., 1979). The reaction mixture contained 0.1 ml of rat liver homogenate (25 %, w/v) in Tris-Hcl buffer (20 mM,

pH 7); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4$. $6H_2O$ (0.16 mM); ascorbate (0.06 mM); and various concentrations of the extracts of *G. lucidum* in a final volume of 0.5 ml. The reaction mixture was incubated for 1 h at 37°C. After the incubation period, 0.4 ml was removed and treated with 0.2 ml SDS (8.1 %); 1.5 ml thiobarbituric acid (0.8 %); and 1.5 ml acetic acid (20 %, pH 3.5). The total volume was made up to 4ml by distilled water and then kept in a water bath at 95-100°C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1,v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The organic layer was removed and its absorbance at 532 nm was measured. Inhibition of lipid peroxidation was determined by comparing the optical density of the treatments with that of control. Catechin was used as standard.

3.2.2.4. ASSAY OF NITRIC OXIDE SCAVENGING ACTIVITY

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

3.2.3. DETERMINATION OF *IN VIVO* ANTIOXIDANT ACTIVITY

Male Swiss albino mice weighing 25 ± 2g were used for the experiment. The mice were divided into three groups of six animals each. Aqueous methanol extract of the mushroom was administered once daily for 30 days.

Group 1	Normal
Group 1I	Aqueous methanol extract 500 mg/kg bodyweight
Group III	Aqueous methanol extract 1500 mg/kg bodyweight

Group treated without any treatments was kept as normal. One day after the last dose of extract administration animals were sacrificed. Tissue homogenate (liver) was prepared in the section 2.2.2, and used for the assay of SOD, GPX, GSH and MDA.

3.3 RESULTS

3.3.1. *IN VITRO* ANTIOXIDANT ACTIVITY

3.3.1.1. SUPEROXIDE RADICAL SCAVENGING ACTIVITY

The extracts of *G. lucidum* showed significant superoxide inhibiting activity (Table 3.1). The concentration of ethyl acetate, methanol and aqueous extracts of *G. lucidum* required to scavenge 50 % superoxide anion generated from the photoreduction of riboflavin (IC_{50}) was found to be 221.8 ± 21.2, 155.5 ± 25.0 and 175. 0 ± 22.1 μg/ml respectively. The IC_{50} of quercetin was 3.70 ± 0.16.

3.3.1.2. HYDROXYL RADICAL SCAVENGING ACTIVITY

The degradation of deoxyribose to TBARS by hydroxyl radical generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system was markedly decreased by the extracts of *G. lucidum*. The IC_{50} of ethyl acetate, methanol and aqueous extracts of *G. lucidum* required to scavenge the hydroxyl radical generated was found to be 317.5 ± 14.3, 229.0 ± 16.0 and 240.0 ± 12.2 μg/ml respectively (Table 3.1). The IC_{50} of catechin was 850 ± 20 μg/ml.

3.3.1.3. INHIBITION OF LIPID PEROXIDATION

Extract of *G. lucidum* was effective in inhibiting the lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate (Table.3.1). The generation of malondialdehyde (MDA) and related substances that react with thiobarbituric acid (TBARS) was found to be inhibited by the extracts. This indicated the significant lipid peroxidation inhibiting activities of the extracts. The IC_{50} of ethyl acetate and methanol extracts of *G. lucidum* required to inhibit the lipid peroxidation was found to be 405.0 ± 13.8 and 312.2 ± 12.0 μg/ml respectively. However, the aqueous extract of *G. lucidum* was not found to inhibit the lipid peroxidation upto a concentration of 1 mg/ml. The IC_{50} of catechin was 418.0 ± 28.6 μg/ml.

3.3.1.4. NITRIC OXIDE SCAVENGING ACTIVITY

Ethyl acetate, methanol and aqueous extracts of *G. lucidum* were not found effective to scavenge nitric oxide generated from sodium nitroprusside.

3.3.2. *IN VIVO* ANTIOXIDANT ACTIVITY

The experimental results indicated that the administration of the aqueous methanol extract of *G. lucidum* at a concentration of 500 and 1500 mg/kg enhanced the antioxidant status in liver of the mice. SOD, CAT and GPX activities in the normal group of animals was 11.94 ± 1.35, 43.50 ± 1.56 and 24.60 ± 2.46 U/mg protein respectively. There is a significant increase in activities of SOD, CAT and GPX in the extract treated group at a concentration of 1500mg/kg compared to the normal group of animals (Table 3.2). The level of GSH in the normal group was 13.07 ± 0.67 nmol/mg protein. Treatment of extract (1500mg/kg) enhanced the activity to 15.79 ± 0.50 nmol/mg protein (Table 3.3). The administration of the extract of *G. lucidum* at a concentration of 1500mg/kg found to cause a decrease in the level of MDA compared to normal group of animals (Table 3.3).

Table 3.1 *In vitro* antioxidant activities of extracts of *G. lucidum* (IC_{50} µg/ml)

Activities	Extracts			Standard	
	Ethyl acetate	Methanol	Aqueous	Catechin	Quercetin
Superoxide radical scavenging	221. 8 ± 21. 2	155. 5 ± 25.0	175. 0 ± 22.1	-	3.60 ± 0. 16
Hydroxyl radical scavenging	317. 5 ± 14. 3	229. 0 ± 16.0	240.0 ± 12.2	810.0 ± 20.0	-
Lipid peroxidation inhibiting	405.0 ± 13. 8	312.2 ± 12. 0	-	408.0 ± 28.6	-

Values are mean ± S.D. n=3.

Table 3.2 Effect of aqueous methanol extract of *G. lucidum* on SOD, CAT and GPX activities in the liver after the administration of the extract.

Groups	Treatment (mg/kg)	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)
Normal	-	11.94 ± 1.35^{b}	43.50 ± 1.56^{b}	24.60 ± 2.46^{b}
G. lucidum extract	500	12.00 ± 0.79^{b}	43.24 ± 0.84^{b}	$26.7 \pm 1.30^{a\,b}$
	1500	13.41 ± 0.51^{a}	45.36 ± 0.76^{a}	30.28 ± 3.53^{a}

Values are mean ± S.D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5% level.

Table 3.3. Effect of aqueous methanol extract of *G. lucidum* on GSH and MDA activities in the liver after the administration of the extract.

Groups	Treatment (mg/kg)	GSH (nmol/mg protein)	MDA (nmol/mg protein)
Normal	-	13.07 ± 0.67^{b}	0.59 ± 0.05^{a}
G. lucidum extract	500	13.91 ± 1.55^{b}	0.45 ± 0.01^{b}
	1500	15.79 ± 0.50^{a}	0.38 ± 0.05^{c}

Values are mean ± S.D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5% level.

3.4 DISCUSSION

Free radicals have been demonstrated to be involved in the triggering of several disease conditions and the compounds that can scavenge free radicals have great potential in ameliorating these disease processes. Human body has inherent mechanisms to generate antioxidants such as superoxide dismutase, glutathione peroxidase, catalase, etc to reduce free radical induced injury. At times these natural protective mechanisms may not be sufficient. Supplementation with non-toxic antioxidants may have a protective role to prevent free radical induced tissue injury. Numerous plant constituents have been proven to show free radical scavenging or antioxidant activity. Flavonoids and other phenolic compounds (proanthocyanidins, rosmarinic acid, hydroxycinnamic derivatives, catechines, etc.) of plant origin have been demonstrated as scavengers and inhibitors of lipid peroxidation (Fejes et al., 2000). Turmeric (*Curcuma longa*), as well as its active ingredient curcumin, inhibit lipid peroxidation in *vitro* (Soudamini et al., 1992). Vitamins and other related compounds provide significant protection against oxidative damage (Uppu et al., 1996., Ischiropoulos et al., 1992., Chow, 1996., Halliwell et al., 1995). Free radical intermediates are of significant importance in the activity of many compounds that lead to tissue damages. Lipid peroxidation mediated membrane damage is one of the deleterious effects of free radicals (Jose and Janardhanan, 2000). The results of the present investigations reveal that aqueous methanol extract of *G. lucidum* have

potent superoxide radical scavenging, hydroxyl radical scavenging, and lipid peroxidation inhibiting activities.

The present investigation indicates that the *in vitro* free radical scavenging activity of *G. lucidum* is in a concentration dependent manner. The superoxide scavenging activity of the extracts may be due to the direct scavenging of superoxide anion generated from the photo illumination of riboflavin or by trapping the electron released from EDTA for the generation of superoxide anion or by reducing superoxide anion to a non- radical. None of the extract tested shows nitric oxide scavenging activity even at a concentration of 1mg/ml. The results indicate that ethyl acetate and methanol extracts possess significant *in vitro* superoxide and hydroxyl radical scavenging activity and lipid peroxidation inhibiting activities. However, aqueous extract show only superoxide and hydroxyl radical scavenging activity but no lipid peroxidation inhibiting activity. The possible interference of DMSO used as solubilizer of the ethyl acetate extract for *in vitro* antioxidant assays was also evaluated. The results showed that DMSO did not act as an antioxidant at the given concentration. The *in vitro* radical scavenging activity, mainly the superoxide, hydroxyl radical scavenging and lipid peroxidation inhibiting activity, of methanol extract was higher than the aqueous and ethyl acetate extracts. The aqueous methanol (70% methanol) extract with its easily soluble nature is found to be more effective than ethyl acetate extract, hence useful for therapeutic evaluation.

The results showed that the administration of the aqueous methanol extract of *G. lucidum* at a concentration of 500 and 1500 mg/kg enhanced the antioxidant

status in liver of the mice compared to normal group of animals. The level of MDA was reduced significantly in the extract treated group at a concentration of 1500 mg/kg.

Several epidemiological findings demonstrated that increased intake of dietary antioxidants might contribute to chemoprevention of some human cancers. Among nonenzymatic epidemiological studies on serum antioxidants and diet suggest that an elevated levels of vitamins E and β carotene reduce mortality due to cancer in the lung and colon (Menkes et al., 1986., Gey, 1987). A large number of terpenes and polyphenols are found to possess antioxidant, anti-inflammatory and antimutagenic activities (Greenwald, 1991., Wood et al., 1982). Koyama et al. (1997) identified ganoderic acids A, B, G and H, triterpene components of a commercial strain of *G. lucidum* for its antinociceptive activity. The fruiting bodies of *G. lucidum* contain a variety of chemical substances, like polysaccharides and triterpenes (Ooi and Liu, 1999., Kim and Kim, 1999). The phytochemical examination of *G. lucidum* extracts employed in the present study show the presence of polysaccharides, triterpenes and polyphenols. These compounds might be responsible for the antioxidant activities of the extracts.

ANTI-INFLAMMATORY,ANTITUMOR AND CHEMOPREVENTIVE ACTIVITIES OF GANODERMA LUCIDUM

Sheena.N "Studies on the thekapeutic potential of Ganoderma lucidum P. Karst – Reishi, occurring in Kerala " Thesis. Amala Cancer Research Centre , University of Calicut, 2005

CHAPTER 4

ANTI-INFLAMMATORY, ANTITUMOR AND CHEMOPREVENTIVE ACTIVITIES OF *GANODERMA LUCIDUM*

4.1. INTRODUCTION

A vast amount of circumstantial evidence implicates oxygen-derived free radicals such as superoxide and hydroxyl radicals and high-energy oxidants such as peroxy nitrite as mediators of inflammation, shock, and ischemia/reperfusion injury (Cuzzocrea et al., 2001). Reactive oxygen species (ROS) can initiate a wide range of oxidative reactions, and these are likely to play significant role in the inflammatory process (Ajith and Janardhanan, 2001). Several line of evidence show the production of ROS such as $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$ occurs at the site of inflammation and they contribute to tissue damage (Salvemini et al., 1996., Jose et al., 2002).

Recent studies suggest that the inflammatory tissue damages is due to the liberation of reactive oxygen species from phagocytes invading the inflammatory sites. Oxidative bursts from activated neutrophils, oxidative transformations in prostaglandin synthesis, etc contribute to the internal sources of free radicals. The free radicals generated during inflammation, a fundamental protective response, have been potentially involved in lipid peroxidation. Hence, endothelial damage and microvascular complications are the major outcome of these self-perpetuating chain reactions associated with vascular disorders and diabetes mellitus (Boyness, 1991). The degradation products of lipid peroxidation can diffuse away from the site and can give rise to cell edema, influence vascular permeability, inflammation, and chemotaxis. In addition to this, nitric oxide is also implicated in inflammation,

cancer and other pathological conditions. Interactions between superoxide and nitric oxide regulates the vascular tone or inflammation (Hemnani and Parihar, 1998).

The two known isoforms of cyclooxygenases, COX-1 and COX-2, catalyze the first committed step in the synthesis of PGs. However, COX-2 and not COX-1, is the isoform most frequently reported to have a key role in tumor development and both isoforms are biological targets of a class of medications known as NSAIDs (Tiano et al., 2002). Nonsteroidal anti-inflammatory drugs (NSAIDs) attenuate the pain by inhibition of cyclooxygenase in the arachidonic acid pathways (Ahmadiani et al., 2000) and are widely reported to inhibit carcinogenesis in humans and rodents (Tiano et al., 2002).

The study of pathogenesis of rat mammary carcinogenesis has revealed that the susceptibility of this organ to be transformed by a chemical carcinogen is modulated by specific biological conditions of the host and of the target organ (Srivastava et al, 1997). The incidence of mammary tumors in women is increased in recent years and environmental chemicals have been partially implicated for this increase (Cabello et al., 2001). Several investigations (Cabello et al., 2001., Bansal et al., 1992. and Russo and Russo, 1978) have demonstrated mammary carcinoma formation in rats can be induced by a chemical carcinogen, mainly DMBA. Breast tissue seems to be very sensitive to ionizing radiation when exposure occurs between the ages of 15 and 19 years (Russo and Russo, 1991), which suggest that events that occur during the early years of a woman's life have a significant effect on lifetime risk of breast cancer. An understanding of the mechanisms that make the

mammary glands of young women more susceptible to carcinogenesis can be achieved by using an adequate animal model system.

It is also reported that tumor initiation is obtained when DMBA was administered to rats 30-55 days of age, but the highest number of tumors per animal was observed when the carcinogen was given to animals when they were 40-46 days of age (Cabello et al., 2001., Russo and Russo., 1991), a period when terminal end buds (TEBs) were most actively differentiating into alveolar buds (Abs). DMBA induced rat mammary carcinoma closely resemble human breast cancer (Rowlands et al., 2001). Accumulating evidences derived from laboratory studies and study cohorts drawn from the general population have led to the search for chemoprotective agents to attenuate the risk of breast cancer based on observation that most human cancers are associated with a long period of latency (Banerjee et al., 2002). Evidences indicate that adenine in DNA is the major site of adduction with DMBA in rat tissue, and this might actually be responsible for its high carcinogenicity (Arif et al., 1999).

Prevention is undeniably the sensible maneuver towards the ultimate goal of cancer control (Suffness and Pezutto, 1991). Natural products and their active principles as source for new drug discovery and treatment of diseases have attracted attention in the recent years. Today, at the beginning of the third millennium, attention has recently been focused on the development of immunotherapy to target and eliminate cancer cells, as well as on substances, such as immunopotentiators,

BRM (biological response modifier) and immunoinitiators, and, that act to prevent carcinogenesis and induce carcinostasis.

Mushrooms are usually used as adaptogens and immunostimulants. The mushrooms are mainly represented by macrofungi and they represent one of the untapped sources of powerful pharmaceutical products. The fruiting bodies of *G. lucidum* (Fr.) P. Karst (Aphyllophoromycetideae), commonly known as Reishi, have long been prescribed in Chinese medicine as a tonic and sedative (Shiao et al., 1994).

Medicinal mushrooms useful against cancer are known in China, Japan, Russia, Korea and in the United States. It has been known for many years that selected mushrooms of higher Basidiomycetes origin are effective against cancer of the stomach, esophagus, lungs etc (Yang and Jong, 1989; Hobbs, 1995). Polysaccharides from mushrooms do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host. Unique anticancer preparations have been developed from mushrooms such as polysaccharides, Lentinan from *Lentinus edodes*, Krestin from *Trametes versicolor*, and Schizophyllan from *Schizophyllum commune* (Wasser et al., 2000). Investigations were carried out on the anti-inflammatory, antinociceptive, antitumor and chemopreventive activities of aqueous methanol extract of *G. lucidum*. The results are presented in this chapter.

4.2. MATERIALS AND METHODS

4.2.1. PREPARATION OF EXTRACT

Aqueous methanol extract of *G. lucidum* was prepared as described in the section 3. 2.1.

4.2.2. ANIMALS

Anti-inflammatory, antinociceptive, antiperoxidative, and antitumor studies:

Female and male Swiss albino mice 6-8 weeks of age weighing 25 ± 2 g.

DMBA-induced mammary cancer model:

Female Sprague Dawly rats 40-45 days old.

4.2.3. PREPARATION OF CROTON OIL

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

4.2.4. DETERMINATION OF ANTI-INFLAMMATORY ACTIVITY OF *G. LUCIDUM* EXTRACT

Anti-inflammatory activity of aqueous methanol extract of *G. lucidum* was determined by carrageenan induced acute and formalin induced chronic mouse paw edema models.

4.2.4.1. CARRAGEENAN INDUCED PAW EDEMA

Male Swiss albino mice were divided into four groups with six animals in each group. In all groups, acute inflammation was induced by subplantar injection of 20 µl of freshly prepared 1% suspension of carrageenan in normal saline in the right hind paw of mice. The paw thickness was measured using vernier calipers before and 3 h after carrageenan challenge in each group. Animals in control group were premedicated with vehicle (distilled water, group1), aqueous methanol extract of the mushroom (500 and 1000 mg/kg body weight, groups 2 and 3) orally, 1h before carrageenan injection. The reference drug, diclofenac (10 mg/kg body weight, group 4), was administered i.p. 30 minutes before carrageenan injection. In this model, the degree of edema formation was determined as an increase in paw thickness. The increase in paw thickness and percent inhibition were calculated using the formula,

Increase in paw thickness in control/ treatment

$$P_c / P_T = P_t - P_0$$

$$\text{Percent inhibition} = \frac{P_c - P_T}{P_c} \times 100$$

where, P_t is paw thickness at time t, Po is initial paw thickness, P_c is the increase in paw thickness of the control group, and P_T is the increase in paw thickness of the treatment groups (Ajith and Janardhanan, 2001).

4.2.4.2. FORMALIN INDUCED PAW EDEMA

Male Swiss albino mice were divided into four groups with six animals in each group. In all groups, chronic inflammation was induced by a single dose of formalin (20 µl of freshly prepared 2% formalin) was used as the edematogenic agent. Animals in control group were premedicated with vehicle (distilled water, group1), aqueous methanol extract of the mushroom (500 and 1000 mg/kg body weight, groups 2 and 3) orally, once daily for 6 consecutive days. Diclofenac (10 mg/kg body weight, group 4), was used as the reference drug. The paw thickness was measured before and 6 days after formalin injection.

The increase in paw thickness and percent inhibition were calculated using the formula,

Increase in paw thickness in control/ treatment

$$P_c / P_T = P_t - P_0$$

$$\text{Percent inhibition} = \frac{P_c - P_T}{P_c} \times 100$$

where, P_t is paw thickness at time t, Po is initial paw thickness, Pc is the increase in paw thickness of the control group, and P_T is the increase in paw thickness of the treatment groups (Ajith and Janardhanan, 2001).

4.2.5. EFFECT OF ANTI-INFLAMMATORY ACTIVITY AGAINST CROTON OIL INDUCED SKIN INFLAMMATION

Back of each female Swiss albino mouse was shaved using surgical clippers 2 days before the experiment. Animals with complete hair growth arrest were

grouped into 5 groups of six animals each and treated as follows: Group treated with vehicle (0.1 ml acetone) was kept as normal. Aqueous methanol extract of *G. lucidum* (10 and 20 mg in 0.1 ml of acetone) was applied topically to the shaved area of the dorsal skin 30 min before each application of croton oil (0.1 ml of 50% croton oil in acetone, v/v). After 24 h, the extract and croton oil treatment was repeated on the same area. Group treated with 0.1 ml of croton oil in 0.1 ml acetone was kept as control. Diclofenac (10mg) was used as the standard reference drug. 1h after the second treatment of croton oil animals were sacrificed and the skin was removed. The skin punches were obtained with an 8 mm diameter cork borer. The skin punches were weighed in an analytical balance; the percent inhibition was calculated using the formula,

% Inhibition = [1- (punch wt. of treated-punch wt. of vehicle)/ (punch wt. of control-punch wt. of vehicle)] x 100 (Lakshmi et al., 2003).

4.2.5.1. ASSAY OF LIPID PEROXIDATION IN MOUSE SKIN

Skin punches obtained from the above experiment, after double croton oil application protocol, were minced in 20mM Tris-HCL buffer (pH7) and homogenized to obtain 10% homogenate. The lipid peroxidation was measured according to the method of Ohkawa et al., (1979) using 1,1,3,3- tetra methoxy propane as the standard and percent inhibition was calculated after comparing with the control. The protein concentration was determined according to the method of Lowry et al (1951).

4.2.5.2. HISTOPATHOLOGICAL EXAMINATION

Excised skin punches was fixed in 10 % formalin and then embedded in paraffin. 3 μm microtome sections were prepared from each skin and stained with hematoxilin-eosin. The sections were evaluated for histopathological parameters for inflammation under a microscope.

4.2.6. DETERMINATION OF ANTINOCICEPTIVE ACTIVITY OF *G. LUCIDUM* EXTRACT

4.2.6.1 WRITHING TEST

Animals were divided into four groups with six animals in each group. The writhing resulting from intraperitoneal injection of 0.2 ml acetic acid (0.6%), consisting of a contraction of the abdominal muscle together with a stretching of hind limbs, was carried out according to procedures described by de Souza et al., (2000). Animals were treated with the aqueous methanol extract (500 and 1000mg/Kg) orally, 60 min prior to acetic acid injection. After acetic acid challenge, pairs of mice were placed in separate boxes. The numbers of writhing movements were counted 8 min after acetic acid injection in each mouse for 20 min. Diclofenac (10 mg/kg, i.p) was used as the reference drug. The percentage of inhibition was calculated as follows:

$$\% \text{ Protection} = \frac{\text{Control mean - Treated mean}}{\text{Control mean}} \times 100$$

4.2.7. DETERMINATION OF ANTITUMOR ACTIVITY OF *G. LUCIDUM* EXTRACT

4.2.7.1. EAC-INDUCED ASCITES TUMOR

Animals were divided into five groups of six animals in each group. All the animals were injected intraperitoneally (i.p.) with $1x10^6$ viable EAC cells in PBS (aspirated from 15 day old EAC ascites tumor in mice). After 24 hr of tumor inoculation aqueous methanol extract of *G. lucidum* was administered orally at a dose of 250, 500 and 1000 mg/kg body weight (groups 2, 3 and 4) and continued for 10 consecutive days. Group 1 injected with ascites cells alone was maintained as control. Cisplatin (2 mg/kg body weight, i .p.) was used as the standard reference drug. The mortality rate was noted in each group and the percent increase in life span (ILS) was calculated using the formula % ILS = (1-T/C) x 100 where T is mean survival time of treated group and C that of control group (Ahluwalia et al., 1984).

4.2.7.2. DLA-INDUCED SOLID TUMOR

4.2.7.2.1. Effect of extract when administered simultaneous with tumor inoculation. (preventive effect)

Animals were divided into five groups of six animals each. Viable DLA cells ($1x10^6$) in 0.1 ml PBS were transplanted subcutaneously into the right groin of mice. Aqueous methanol extract of *G. lucidum* was administered at a dose of 250, 500 and 1000 mg/kg body weight (groups 2, 3 and 4) orally 24 hr after tumor

implantation and continued for 10 consecutive days. The control group (group1) was treated with DLA cells alone and the standard reference group was treated with cisplatin (4mg/kg body weight, i.p). The tumor development on animals in each group was determined by measuring the diameter of tumor growth in two perpendicular planes using vernier calipers twice a week for 5 weeks. The tumor volume was calculated using the formula $V= 4/3\ \pi\ r_1^2 r_2$,, where r_1 and r_2 are the radii of the tumors. At the end of the fifth week, animals were sacrificed under anesthesia using diethyl ether, tumor extirpated and weighed. The percent inhibition was calculated by the formula: (1-B/A) 100, where, A is average tumor weight of the control group and B that of the treated group (Chihara et al., 1970).

4.2.7.2.2. Effect of extract when administered after tumor development. (curative effect)

Antitumor activity of aqueous methanol extract of *G. lucidum* was tested after tumor initiation in mice. Solid tumor was induced using DLA cell line in mice as described early. After 13 days, animals with tumor size around 1.1± 1cm^3 were divided into four groups of six animals each and aqueous methanol extract of *G. lucidum* (250, 500 and 1000 mg/kg body weight, p.o.) were administered for 10 consecutive days. The group injected with DLA cell line alone was maintained as control. Tumor diameter was measured using vernier calipers twice a week for a period of 3 weeks and volume was calculated (Ajith and Janardhanan, 2003). At the end of fifth week, animals were sacrificed, tumor extirpated and weighed. The percent inhibition was calculated as described earlier.

4.2.8. DETERMINATION OF THE EFFECT OF EXTRACT ON DMBA-INDUCED RAT MAMMARY CARCINOMA

Female Sprague Dawly rats were divided into five groups of ten animals each. The rats were fed with 10 mg DMBA in olive oil/ animal by gavage once in a week for three weeks. Aqueous methanol extract of *G. lucidum* was administered orally at a dose of 250, 500 and 1000-mg/kg body weight (groups 2, 3 and 4) daily for 3 weeks and thereafter 10 mg DMBA in olive oil/ animal was administered. The animals administered with DMBA alone were maintained as control group, (group 1) and the animals without any treatment were grouped as normal group (group 5). The rats were palpated for mammary tumors once/week (starting 4 weeks after DMBA treatment). This study was terminated at 120 days after DMBA administration (Bansal et al., 1992). Macroscopic tumors were histologically examined and the sections were evaluated for the pathological parameters of mammary carcinoma. The end point for data analysis included a) tumor latency period; b) the average number of animals with tumors (tumor incidence); c) the mean number of tumors/rat (tumor yield- Ratio of the total number of tumors to the number of tumor bearing rats); d) tumor volume. estimates of tumor volume was determined using the formula $V= 4/3\ \Pi r^3$, where r is half of the average diameter (in centimeters) measured with a vernier caliper at two different planes; e) tumor weights [measured at the end of 17^{th} week, animals were sacrificed, tumor extirpated and weighed]. The percent inhibition was calculated by the formula: (1-

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

4.3. RESULTS

4.3.1. ANTI-INFLAMMATORY ACTIVITY

4.3.1.1. ANTI-INFLAMMATORY ACTIVITY OF G. LUCIDUM EXTRACT AGAINST CARRAGEENAN INDUCED PAW EDEMA

The results of the investigations showed that aqueous methanol extract of *G. lucidum* possessed significant inhibitory effect on carrageenan-induced acute inflammation in mice (Table 4.1). Anti-inflammatory activity of the extract was remarkably high at a concentration of 1000 mg/kg body weight. The increase in paw thickness in the extract treated group (500 and 1000 mg/kg) after 3 h of carrageenan injection was 0.089 ± 0.006 and 0.050 ± 0.004 cm respectively. The increase in paw thickness in the control group after 3 h of carrageenan injection was 0.120 ± 0.022 cm. The percent inhibition of the paw edema in the extract treated (1000 mg/kg body wt) group was 58.3 %. The standard reference drug, diclofenac, showed 0.060 ± 0.013 cm increase in paw thickness and 50 % inhibition after the carrageenan injection. This indicated that aqueous methanol extract of *G. lucidum* showed significant activity against acute inflammation induced by carrageenan.

4.3.1.2. ANTI-INFLAMMATORY ACTIVITY OF *G. LUCIDUM* EXTRACT AGAINST FORMALIN INDUCED PAW EDEMA

Anti-inflammatory activity of extract of *G. lucidum* against formalin induced paw edema was significantly high as compared to standard reference drug (Table

4.2). Formalin induced a significant increase in paw thickness in the control group of animals on 6th day after the injection. Treatment of aqueous methanol extract of *G. lucidum* before the formalin injection significantly reduced the paw edema. The increase in paw thickness after 6 days of formalin injection in the extract treated (500 and 1000 mg/kg body wt) group of animals was 0.155 ± 0.016 and 0.100 ± 0.011 cm respectively and the percent inhibition of edema was 59.6 % in the extract (1000 mg/kg body wt) treated group of animals. The standard reference drug showed 0.145 ± 0.014 cm increase of paw thickness and 41.5% of inhibition of paw edema.

4.3.2. ANTI-INFLAMMATORY ACTIVITY OF *G. LUCIDUM* EXTRACT AGAINST CROTON OIL INDUCED SKIN INFLAMMATION

Aqueous methanol extract of *G. lucidum* showed significant anti-inflammatory activity against croton oil induced skin inflammation. Applications of the extract 30 min before the croton oil application significantly inhibited the croton oil induced skin inflammatory edema in a dose dependent manner. The double treatment of croton oil alone induced skin inflammation as evident from the skin punch weight of 62.5 ± 2.8 mg/punch in the control group of animals. Where as the application of aqueous methanol extract of *G. lucidum* (10 and 20 mg) prior to the croton oil application inhibited 35.0 and 42.4% of the inflammatory edema and the skin punch weight was 40.6 ± 2.7 and 36.0 ± 2.9 mg/punch compared to the control group of animals. The skin punch weight of standard reference drug diclofenac, 10 mg, plus

croton oil treated group was 38.22 ± 1.4 mg/punch (Figure 4.1). The malondialdehyde levels as an indicator of lipid peroxidation were elevated in the control group as compared to the extract treated group. The extract at a concentration of 20 mg showed 41.7% inhibition of lipid peroxidation (Figure 4.2).

Histopathological examination of the skin showed marked epidermal thickening and severe lymphocytes infiltration. Marked edema was also observed in the sub-epidermis. The pathological effects were markedly reduced in the extract treated group (Figure 4.3).

4.3.3. ANTINOCICEPTIVE ACTIVITY OF *G. LUCIDUM* EXTRACT AGAINST ACETIC ACID INDUCED ABDOMINAL WRITHING RESPONSE

Experimental results showed aqueous methanol extract of *G. lucidum* inhibited the acetic acid-induced abdominal constriction response in mice. The aqueous methanol extract at a concentration of 500 and 1000 mg/kg inhibited 36 and 40.6% abdominal constrictions respectively. The treatment with standard reference drug, diclofenac (10mg/kg) showed 64.4% inhibition (Figure 4.4). This indicated that the aqueous methanol extract of *G. lucidum* possessed significant antinociceptive activity and the effect was dose dependent.

4.3.4. ANTITUMOR ACTIVITY

Aqueous methanol extract of *G. lucidum* was not found to have tumor reducing activity against ascites tumor induced by EAC cells. However, the extract possessed significant antitumor activity against solid tumor models.

The extract significantly reduced the tumor development in a dose dependent manner. The extract at a concentration of 500 mg/kg prevented the tumor development by 69.9 % with respect to control and at a concentration of 1000 mg/kg prevented the tumor development by 87.1%. Tumor reducing effect of 1000 mg/kg was nearly equivalent to cisplatin at a dose of 4mg/kg (Table 4.3, Figure 4.5)

The aqueous methanol extract of *G. lucidum* was also highly effective against developed solid tumor. The extract at a concentration of 500 and 1000 mg/kg when administered for 10 consecutive days after tumor development showed 61.8 % and 72.8% tumor growth regression respectively as compared to control (Table 4.4).

4.3.5. EFFECT OF *G. LUCIDUM* EXTRACT ON DMBA-INDUCED MAMMARY CARCINOMA

The aqueous methanol extract of *G. lucidum* was also highly effective against DMBA-induced mammary carcinoma. The tumor induction was observed in the control group of animals and in the aqueous methanol extract treated groups (250 and 500mg/kg) were 73 ± 1.46, 90 ± 2.16 and 105 ± 2.44 days respectively after DMBA treatment (Figure 4.6). However, the extract treatment at a concentration of 1000 mg/kg was found to inhibit the tumor formation totally and no signs of tumor formation was observed in these animals until 120 days. All animals in the DMBA treated group developed mammary tumor and the number of animals that showed tumor incidence in the extract treated at a concentration of 250 and 500 mg/kg was 7/10 and 4/10 respectively (Tumor incidence). Tumor yield (Ratio of the total

number of tumors to the number of tumor bearing rats) in the extract treated at a concentration of 250 and 500 mg/kg was 1.4 and 1.0 compared to the control animals where the average number of tumor/animal was 2.3 (Table 4.5). The extract at a concentration of 500 mg/kg prevented the tumor growth by reducing the tumor volume by 73.8% and tumor weight by 74.7 % respectively (Table 4.6; Figure 4.7).

Histopathological evaluation indicated that the control group of animals showed atypical hyperplasia and invasive ductal carcinoma. These pathological manifestations were reduced significantly in the extract treated group of animals. The extract was also found to inhibit the necrosis of epithelial cells in a dose dependent manner (Figure 4.8).

Table 4.1 Effect of aqueous methanol extract of *G. lucidum* on carrageenan induced acute inflammation

Groups	Dose (mg/kg)	Initial paw thickness (cm).	Paw thickness after 3hr (cm).	Increase in Paw thickness (cm)	Inhibition (%)
Control		0.160 ± 0.011	0.280 ± 0.013	0.120 ± 0.022 [a]	
G. lucidum extract	500	0.161± 0.013	0.250 ± 0.013	0.089 ± 0.006 [ab]	25.8
	1000	0.170 ± 0. 011	0.220 ± 0.011	0.050 ± 0.004 [c]	58.3
Standard (Diclofenac)	10	0.177 ± 0. 012	0.237 ± 0.015	0.060 ± 0.013 [bc]	50.0

Values are mean ± S.D. n= 6 animals.
Any two means having a common superscript are not significantly different at 5% level.

Table 4.2 Effect of aqueous methanol extract of *G. lucidum* on formalin induced chronic inflammation

Groups	Dose (mg/kg)	Initial paw hickness (cm).	Paw thickness after 6 days (cm).	Increase in Paw thickness (cm)	Inhibition (%)
Control		0.162 ± 0.017	0.410 ± 0.028	0.248 ± 0.028 [a]	
G. lucidum extract	500	0.170± 0.012	0.325 ± 0.019	0.155 ± 0.016 [b]	37.5
	1000	0.160 ± 0. 014	0.260 ± 0.013	0.100 ± 0.011 [c]	59.6
Standard (Diclofenac)	10	0.170 ± 0. 017	0.315 ± 0.015	0.145 ± 0.014[d]	41.5

Values are mean ± S.D. n = 6 animals.
Any two means having a common superscript are not significantly different at 5% level.

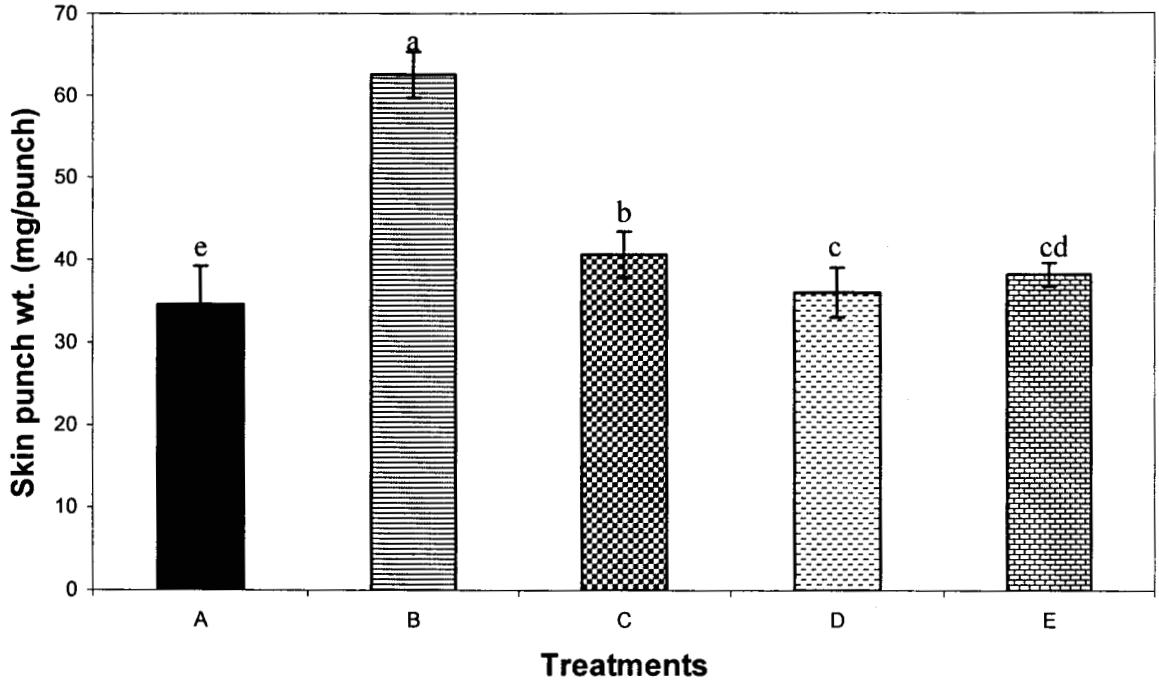


Figure 4.1 Effect of aqueous methanol extract of *G. lucidum* and diclofenac on croton oil induced skin inflammation in mice. Values are mean ± S.D. n=6 animals. Any two means having a common superscript are not significantly different at 5% level.

A : Normal

B : Control

C : 10mg/kg body weight

D : 20mg/kg body weight

E : Diclofenac 10 mg/kg body weight.

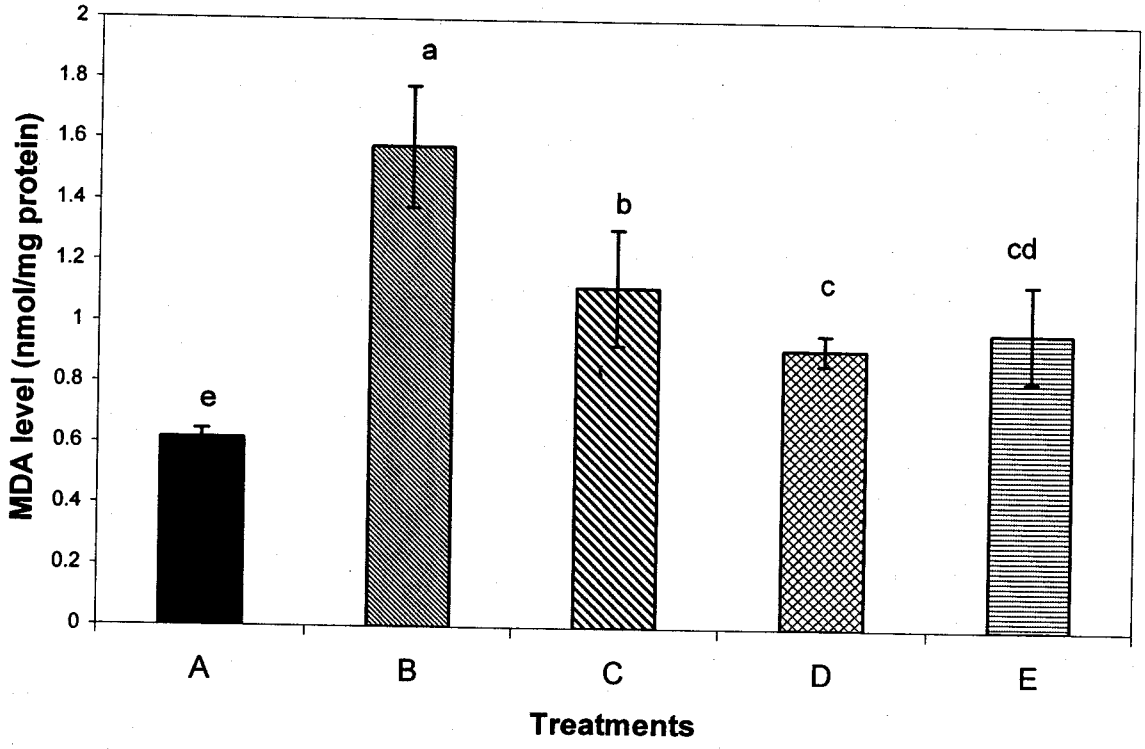


Figure 4.2 Effect of aqueous methanol extract of *G. lucidum* and Diclofenac on croton oil induced lipidperoxidation on mouse skin. Values are mean ± S. D. n=6 animals. Any two means having a common superscript are not significantly different at 5% level.

A : Normal

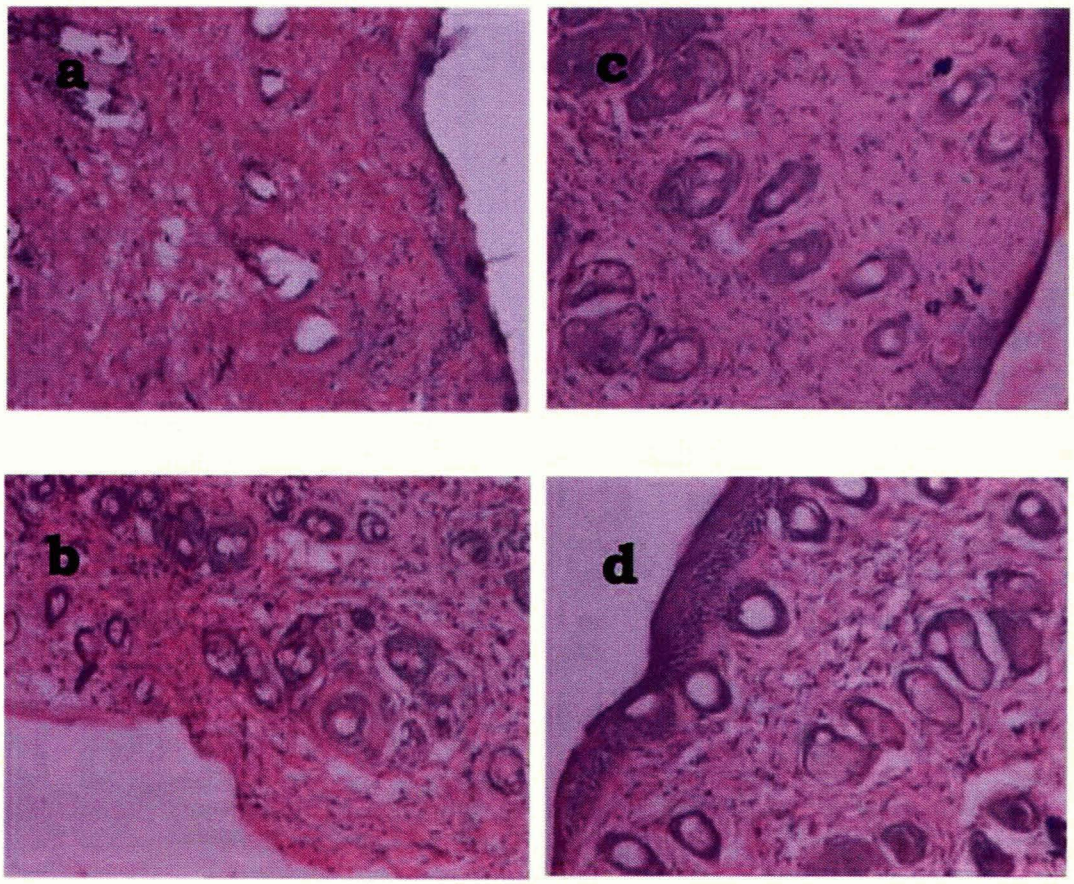
B : Control

C : 10mg/kg body weight

D : 20mg/kg body weight

E : Diclofenac 10 mg/kg body weight

Figure 4.3. Anti-inflammatory activity of aqueous methanol extract of *G. lucidum* against croton oil induced inflammation on mice skin. Skin sections stained with H&E a) Normal; b) Croton oil (0.1ml); c) *G. lucidum* extract (20 mg) + croton oil; d) Diclofenac (10 mg)+ croton oil. Magnification x 20.


a
c
b
d

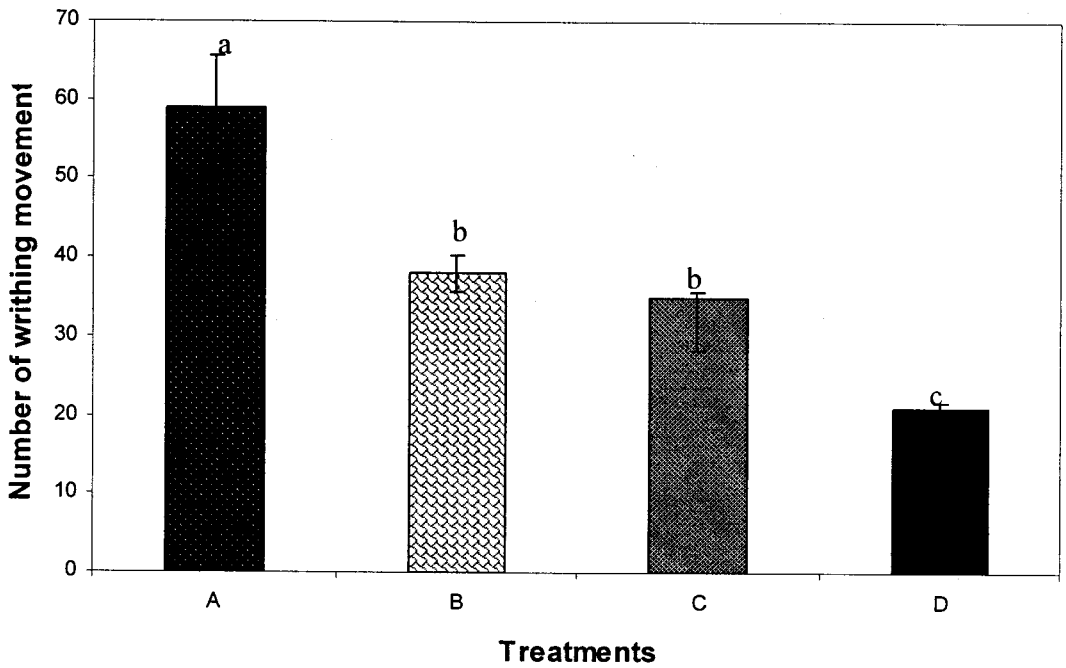


Figure 4.4 Effect of aqueous methanol extract of *G. lucidum* and diclofenac on acetic acid induced writhing response in mice. Values are mean ± S.D. n=6 animals. Any two means having a common superscript are not significantly different at 5% level.

A : Control,

B : 500mg/kg body weight

C : 1000mg/kg body weight

D : Diclofenac 10 mg/kg body weight.

Table 4.3 Effect of aqueous methanol extract of *G. lucidum* on solid tumor. Extract administered simultaneous with tumor inoculation (Preventive effect)

Groups	Treatments (mg/kg)	Volume on 5th week (cm3)	Weight of tumor (g)	% Inhibition
Control		4.48 ± 0.15^{a}	5.15 ± 0.46^{a}	
Cisplatin	4	0.14 ± 0.03^{e}	0.16 ± 0.02^{e}	96.8
G. lucidum extract	250	1.24 ± 0.25^{b}	2.29 ± 0.18^{b}	55.5
	500	0.65 ± 0.09^{c}	1.55 ± 0.14^{c}	69.9
	1000	0.43 ± 0.03^{d}	0.66 ± 0.14^{d}	87.1

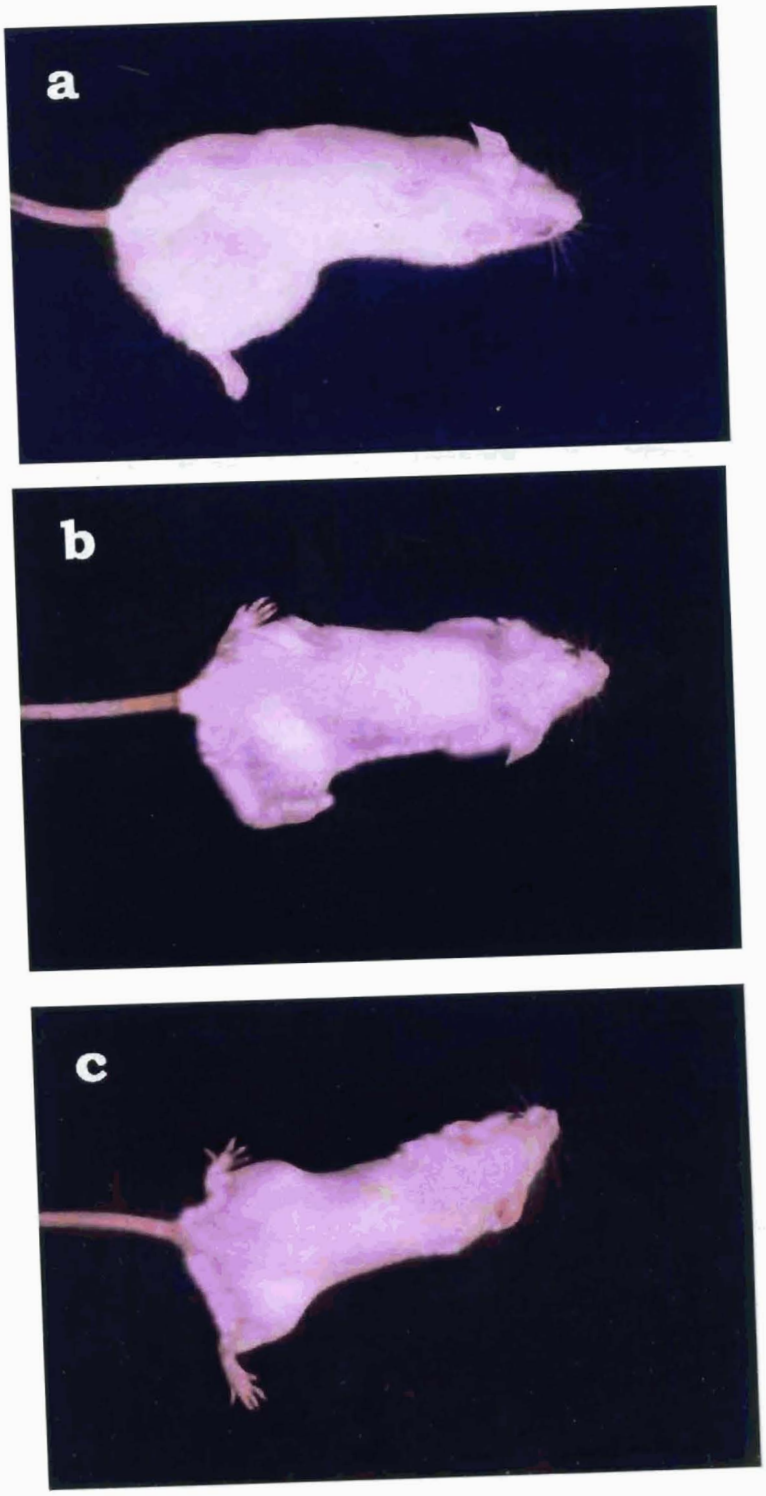
Values are mean ± S. D. n= 6 animals.
Any two means having a common superscript are not significantly different at 5% level .

Table 4.4 Effect of aqueous methanol extract of *G. lucidum* on solid tumor. Extract administered after tumor development (Curative effect)

Groups	Treatments (mg/kg)	Volume on 5th week (cm3)	Weight of tumor (g)	% Inhibition
Control		3.80 ± 0.85 [a]	4.61± 0.13 [a]	
Cisplatin	4	0.81± 0.04 [cd]	1.20 ± 0.16 [d]	73.9
G. lucidum extract	250	1.45± 0.23 [b]	3.15 ± 0.10 [b]	31.6
	500	1.11± 0.07 [bc]	1.76 ± 0.11 [c]	61.8
	1000	0.37 ± 0.04 [d]	1.25 ± 0.06 [d]	72.8

Values are mean ± S.D. n= 6 animals.
Any two means having a common superscript are not significantly different at 5% level .

Figure 4.5 Antitumor activity of aqueous methanol extract of *G. lucidum* on DLA induced solid tumor in mice. a) Control; b) *G. lucidum* extract (1000 mg/kg body weight, p.o); c) Cisplatin (4mg/kg body weight, i.p).


a
b
c

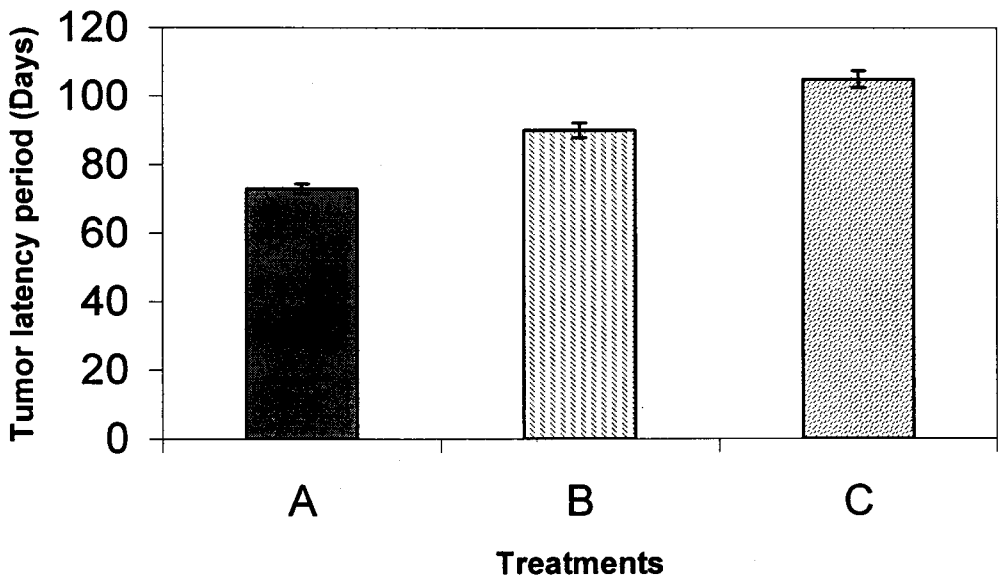


Figure 4.6 Effect of aqueous methanol extract of *G. lucidum* on DMBA induced mammary tumor in rat (Tumor latency period). n=10 animals.

A : Control

B : 250mg/kg body weight

C : 500mg/kg body weight

Table 4.5 Effect of aqueous methanol extract of *G. lucidum* on DMBA induced rat mammary tumor

Groups	Treatment (mg/kg)	Tumor incidence	Tumor Yield* On 17th week
Control	10mg/animal	10/10	2.3
G. lucidum extract	250	7/10	1.4
	500	4/10	1

*** Tumor yield- Ratio of the total number of tumors to the number of tumor bearing rats.**

Table 4.6 Effect of aqueous methanol extract of *G. lucidum* on DMBA induced rat mammary tumor

Groups	Tumor volume On 17th week (cm^3)	% decrease in Tumor volume	Tumor weight (g)	% decrease in Tumor weight
Normal	-	-	-	-
Control	9.27 ± 1.06^{a}	-	7.88 ± 1.04^{a}	-
G. lucidum extract (250 mg/kg)	4.50 ± 0.64^{b}	51.4	3.64 ± 0.57^{b}	53.8
G. lucidum extract (500 mg/kg)	2.42 ± 1.07^{c}	73.8	1.99 ± 0.15^{c}	74.7

Values are mean ± S.D. n= 6 animals.
Any two means having a common superscript are not significantly different at 5% level.

Figure 4.7. Effect of aqueous methanol extract of *G. lucidum* on DMBA induced mammary carcinoma in rats. a) Normal; b) *G. lucidum* (500 mg)+ DMBA ; c) DMBA (10mg).

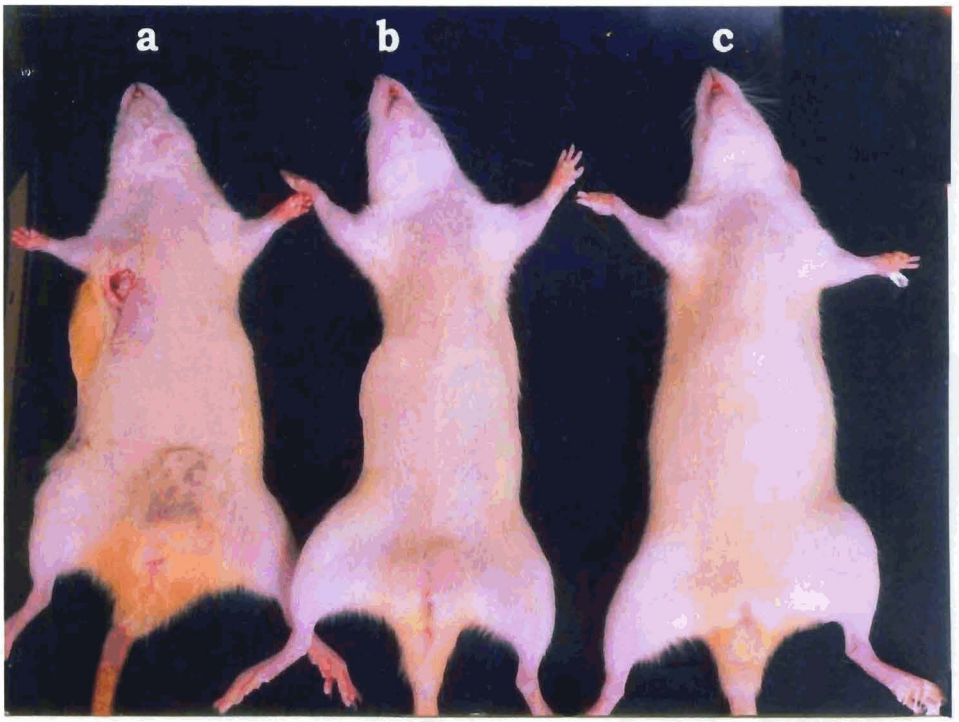
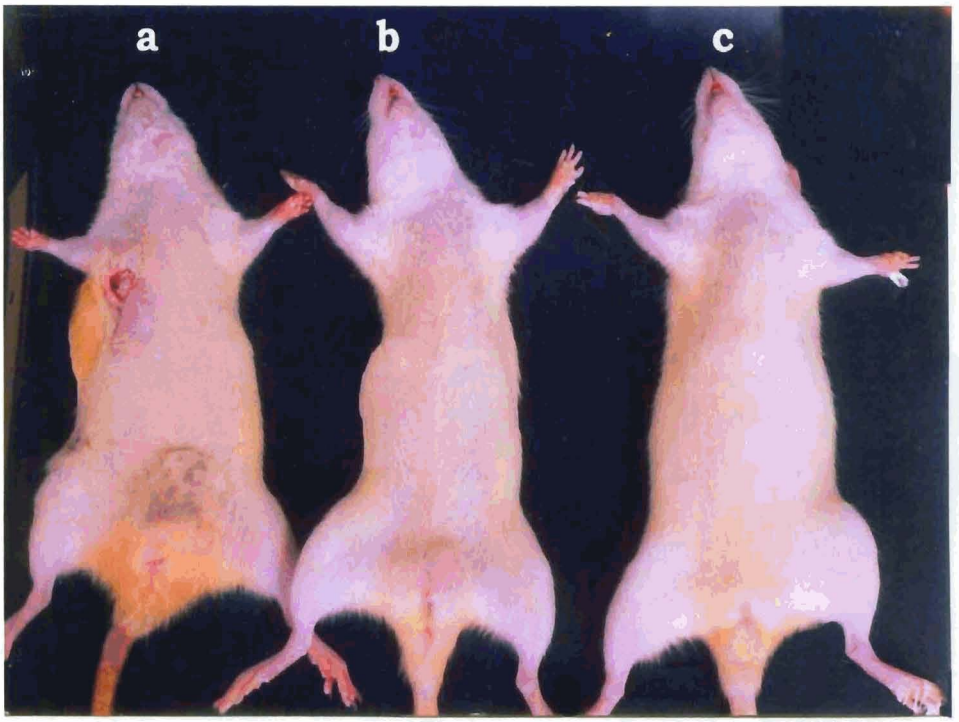


Figure 4.8. Effect of aqueous methanol extract of *G. lucidum* on DMBA induced mammary carcinoma in rats. Sections stained with H&E a) Normal; b) DMBA (10mg); c) *G. lucidum* (500 mg)+ DMBA. Magnification x 20.



4.4. DISCUSSION

The results of the investigations show that aqueous methanol extract of *G. lucidum* possessed significant effect on carrageenan-induced acute and formalin induced chronic inflammation in mice. The results also indicate that aqueous methanol extract inhibited the croton oil induced mouse skin inflammation and acetic acid-induced abdominal constriction response in mice (Figure 4.1, 4.2, 4.4).

The anti-inflammatory activity of *G. lucidum* extract is mediated in a dose-dependent manner. Carrageenan-induced acute inflammation in animals is one of the most suitable test procedures to screen anti-inflammatory agents. The carrageenan-induced edema is caused by activation of platelet activating factor (PAF), prostaglandins and other inflammatory mediators (Hwang et al., 1986). The first phase is attributed to the release of histamine, 5-HT and kinins, while the second phase is related to the release of prostaglandins (Larsen and Henson, 1983; Brooks and Day, 1991; Vane and Booting, 1987). Carrageenan also induces a protein rich exudate containing a large number of neutrophils (Lo et al., 1982). Formalin-induced paw edema is also one of the most suitable test procedures to screen chronic anti-inflammatory agents, as it closely resembles human arthritis (Greenwald, 1991). The nociceptive effect of formalin is also biphasic, an early neurogenic component followed by a later tissue mediated response (Wheeler-Aceto and Cowan, 1991).

The acetic acid-induced writhing method is used as a first screening test of analgesic activity and anti-inflammatory activity of drugs, muscle relaxant and anti-

histamines (Koyama, et al., 1997). Koyama et al. (1997) identified ganoderic acids A, B, G and H, triterpene components of a commercial strain of *G. lucidum* for its antinociceptive activity. However, aqueous methanol extract of *G. lucidum* occurring in Kerala shows higher anti-inflammatory and antinociceptive activities. Inflammation, a fundamental protective response, may be harmful in conditions such as life threatening hypersensitive reactions to insect bites, drugs, toxins, and in certain chronic diseases such as rheumatoid arthritis, atherosclerosis and lung fibrosis (Collins, 1999).

Application of croton oil on mouse skin induces inflammation. Croton oil contains TPA an inducer of inflammation. It has been found that arachidonic acid metabolites such as prostaglandin and leukotrienes play an important role in TPA-induced inflammation (Lakshmi et al., 2003). The aqueous methanol extract of *G. lucidum* appears to prevent the arachidonic acid metabolism by inhibiting epidermal cyclooxygenase or lipoxygenase activity. The extract may also be preventing the phorbol ester induced phospholipase A-2 activity resulting in the inhibition of phospholipase A-2 mediated release of arachidonic acid from the membrane. Application of TPA on skin results in the rapid accumulation of inflammatory cells such as neutrophils and macrophages (Lewis and Adams, 1987). Appropriately stimulated polymorphonuclear neutrophils (PNM) and monocytes produce a large amount of reactive oxygen intermediate (ROI) (up to 1.5 nmol of $H_2 O_2$/ 10^4 cells/ l) in a respiratory burst burn pattern. The double application of croton oil to mouse skin has been shown to stimulate the release of reactive oxygen species (ROS) (Ji and

Marnett, 1992). The free radicals thus generated might be responsible for the peroxidation of lipids in the skin epidermis. Application of aqueous methanol extract of *G. lucidum* prior to croton oil application reduced the lipid peroxidation. The effect is due to the direct anti-inflammatory and free radical scavenging properties of the extract. The significant antioxidant activity of this mushroom might be responsible for its marked anti-inflammatory activity. Histopathological examination shows a significant decrease in the lymphocyte infiltration in sub-epidermis of the mouse skin treated with the extract compared to control, this observation also supports the conclusion that *G. lucidum* extract possesses a profound anti-inflammatory activity.

The results of the present investigation demonstrate significant antitumor activity of aqueous methanol extract of *G. lucidum* against DLA induced solid tumor. However, the extract was not found to inhibit the EAC induced ascites tumor in mice.

Most of the clinically used antitumor agents possess significant cytotoxic activity. The antitumor activity of aqueous methanol extract of *G. lucidum* is in a dose dependent manner with no signs of toxicity. Preliminary chemical examination of the extract shows detectable amounts of polysaccharides. The polysaccharide-protein complex from a large number of mushrooms have been found to be effective against Ehrlich carcinoma and Sarcoma 180 cancerous tumors in mice (Jong and Donovick, 1989). The active substances exhibit neither cytotoxicity nor side effects commonly seen in connection with the use of conventional anticancer

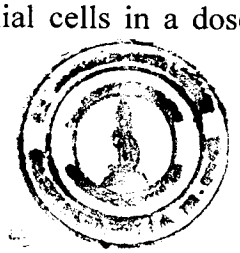
agents such as, decrease in the number of leucocytes, anemia, atrophy of the spleen, loss of body weight and loss of appetite.

DNA adduct formation is a prerequisite for carcinogenesis as shown for the majority of known carcinogens (Smith and Gupta, 1996) and a relationship between the ability of chemical carcinogens to form DNA adducts, cause mutations and induce tumorigenesis (Arif et al., 2000). DMBA treatment produces carcinogen-DNA adducts which may induce G→A transition or A→T transversion (Li et al., 2002). DMBA is a procarcinogen and requires metabolic conversion to its ultimate carcinogenic metabolite, DMBA-3,4-dihydrodiol,1-2-epoxide, a process that includes two separate oxidations by the microsomal CYP1 enzymes. The first oxidation produces the 3, 4, dihydrodiol and is catalyzed by either CYP1A1 or CYP1B1 (Christou et al., 1994). The second oxidation produces the highly mutagenic 3,4-dihydrodiol,1-2-epoxide metabolite and is catalyzed by CYP1B1. Thus, the extent to which DNA adducts occur after administration of DMBA depends on the level of oxidative metabolism of DMBA, which in turn is determined in part by the activities of CYP1A1 and CYP1B1.

Chemoprevention is an important strategy to control the process of carcinogenesis. Antioxidants are reported to act as protective agents against cancer (Huang et al., 1997., Huang et al., 1994., Kozoumbo et al., 1983., Dhawan et al., 1999., Smith et al., 2001). Inhibition of DMBA induced oral carcinogenesis in hamsters by green tea and curcumin (Li et al., 2002) supported the above statement. Lakshmi et al., (2003) reported the antimutagenicity of the ethanol extract of

Ganoderma mycelium and the effect might be mediated through the free radical scavenging activity of terpenes and polyphenols present in the extract. The inhibition of DMBA induced mammary carcinoma in female Sprague dawly rats by aqueous methanol extract of *G. lucidum*, which is an effective free radical scavenger, might be mediated through the enhancement of metabolic systems that prevent activation of carcinogens or accelerate carcinogen inactivation thereby preventing the formation of DNA adducts.

The results of the present investigation indicate that aqueous methanol extract of *G. lucidum* possessed significant anticancer activity against DMBA induced mammary adenocarcinoma in rats. The administration of the extract of *G. lucidum* at a concentration of 500 mg/kg significantly reduced the number of tumor bearing animals (4/10) when compared with control group (10/10) and the group of animals treated with the extract at a concentration of 1000 mg/kg did not produce any tumors till the termination of the experiment. Like the tumor incidence, tumor yield (ratio of the total number of tumors to the number of tumor bearing rats) also supports the significant anticancer activity of the extract. The extract of *G. lucidum* significantly reduced the tumor development in a dose dependent manner. The extract at a concentration of 500 mg/kg prevented the tumor growth by reducing the tumor volume by 73.8% and tumor weight by 74.7 % respectively. Histopathological evaluation also supports this observation. The control group of animals showed atypical hyperplasia and invasive ductal carcinoma while the extract treated group is found to inhibit the necrosis of epithelial cells in a dose



dependent manner. The experimental findings suggest the therapeutic potential of this mushroom extract as an anticancer agent.

Arachidonic acid metabolites are involved as inflammatory agents that cause paw edema. In addition to the COX-mediated arachidonic acid metabolites, kinins, histamine, and serotonin also accounted for the edema formation caused by formalin or carrageenan (Santhos et al., 2000). Inhibition of these mediators or their release might be the mechanism of the anti-inflammatory activity of the extract. The aqueous methanol extract of *G. lucidum* also possessed significant antinociceptive activity. The findings suggest the therapeutic potential of this mushroom and its metabolites for the prevention and control of diseases mediated through inflammation and nociception.

In conclusion the aqueous methanol extract of *G. lucidum* occurring in Kerala possesses profound anti-inflammatory, antinociceptive, antitumor and chemopreventive properties.

HEPATOPROTECTIVE ACTIVITY OF GANODERMA LUCIDUM

Sheena.N "Studies on the thekapeutic potential of Ganoderma lucidum P. Karst – Reishi, occurring in Kerala " Thesis. Amala Cancer Research Centre , University of Calicut, 2005

CHAPTER 5

HEPATOPROTECTIVE ACTIVITY OF *GANODERMA LUCIDUM*

5.1. INTRODUCTION

The liver protects the body from potentially injurious substances (endotoxins) absorbed from the intestinal tract as well as bye products of metabolism. The most important in the detoxification process is that of microsomal drug metabolizing system of liver. A large number of xenobiotics are reported to be potentially hepatotoxic. Hepatotoxins may react with basic cellular constituents, proteins, lipids, RNA, DNA and induce various types of pathological symptoms in liver (Wang et al., 2002). Liver diseases are of the serious health problems in the world today, mainly because of environmental pollution, life style, drugs and dietary components.

The reactive oxygen species (ROS) such as superoxide anion radical (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) have been implicated in the pathophysiology of several clinical disorders including hepatic diseases, atherosclerosis, acute hypertension, hemorrhagic shock, diabetes mellitus and cancer (Hemnani and Parihar, 1998). They play an important role in the inflammation process after intoxication by ethanol, carbontetrachloride or carrageenan (Yoshikawa, et al., 1983., Yuda et al., 1991., Halliwell and Gutteridge, 1984). These radicals and the reactive oxygen species derived from them react with cell membrane, induced lipid peroxidation and are responsible for various deleterious effects in cells and tissues where they are generated. The inhibition of free radical generation can serve as facile model for evaluating the activity of hepatoprotective agents (Ajith and Janardhanan, 2002). Lipid peroxides, produced

from unsaturated fatty acids via radicals, cause histotoxicity and promote the formation of additional free radicals in a chain reaction type manner. It is thought that, if the *in vivo* activity of enzymes or scavengers is not high enough to inhibit these radicals, various diseases such as arteriosclerosis, liver disease, diabetes, inflammation, renal failure or accelerated aging may result. It has been hypothesized that one of the principal causes of CCl_4 –induced liver injury is lipid peroxidation by free radical derivatives of CCl_4. Thus, the antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4 –induced liver lesion (Lin et al., 1998).

Testing by the U. S. National Toxicology Program (NTP) and the International Agency for Research in Cancer (IARC) has shown that only one - fourth to one-third of those substances even suspected of being carcinogenic on the basis of their chemistries actually is cancer causing agents (Perantoni, 1998). Hepatic fibrosis can be induced in rats with chronic administration of hepatotoxins such as CCl_4, ethanol or thioacetamide (Joy and Kuttan, 1999). Free radicals and oxidative stress has been implicated in disorders, resulting usually from deficient natural antioxidant defenses. Potential antioxidant therapy should, therefore include either natural free radical scavenging antioxidant enzymes or agent which are capable of augmenting the activity of the enzymes, which include SOD, CAT, GSH and GPX (Trivedi and Rawal, 2001). A number of excellent drugs have been proved useful in controlling hepatotoxicity and gastric ulceration but their long term uses are not devoid of disturbing side effects.

The attempts have been made in many part of the world to explore the use of mushrooms and their metabolites for the treatment of a variety of human ailments. The fruiting bodies of a number of fungi have long been prescribed in Chinese medicine for the treatment of neurasthenia, deficiency, fatigue, insomnia, hypertension, hepatopathy, bronchitis and carcinoma (Shiao et al., 1994). Hepatoprotective activity of methanolic extract of *G. lucidum* was evaluated using CCl_4 –induced chronic hepatotoxicity model in rats. The experimental findings are reported in this chapter.

5.2. MATERIALS AND METHODS

5.2.1. PREPARATION OF EXTRACTS

Aqueous methanol extract of *G. lucidum* was prepared as described in the section 3.2.1.

5.2.2. ANIMALS

Hepatotoxicity studies: Female Sprague Dawly rats 160 ± 20 g

5.2.3. DETERMINATION OF CCL_4 INDUCED HEPATOTOXICITY

Hepatoprotective activity against the CCl_4 induced chronic toxicity was determined by the method of Joy and Kuttan (1999), with some modifications. The animals were divided into four groups of six animals each and treated as follows. Group 1 was treated as normal. Group 2 treated with CCl_4/paraffin oil (1:5,v/v, i.p) 3 times in a week for 5 weeks (total 15 doses). Group 3 and 4 treated with *G. lucidum* extract 500, 1000mg/kg orally 1 hr before CCl_4 injection. 24 hr after the

last dose, the animals were sacrificed. Heparinized blood was collected from the heart. Serum was used for the determination of glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), alkaline phosphate (ALP) and lipidperoxidation using 1,1,3,3-tetra methoxy propane as the standard. Liver was removed and stored at -70°C until analyses could be completed. The liver was homogenized in 50 mM phosphate buffer (pH 7) to give a 10 % homogenate (W/V). The homogenate was centrifuged at 1000 rpm for 10 min in a cold centrifuge at 0°C and supernatant was used for enzyme assays and protein determinations. Remaining part of the tissue homogenate was used for assay of the activities of superoxide dismutase (SOD) by the method of Mc Cord and Fridovich, 1969 (section 2.2.4), catalase (CAT) by the method of Beers and Sizer, 1952 (section 2.2.5), glutathione peroxides (GPX) by the method of Hafemann et al., 1974 (section 2.2.6), levels of reduced glutathione (GSH) by the method of Moron et al., 1979 (section 2.2.3), Glutathione-*S*-transferase (GST) activity was determined according to the method of Habig et al., 1974 (section 2.2.7) and malondialdehyde (MDA) by the method of Ohkawa et al., 1979 (section 2.2.8) using 1,1,3,3-tetramethoxypropane as the standard. The protein content was estimated by the method of Lowry et al., 1951 (section 2.2.9) using bovine serum albumin as standard.

5.2.4. HISTOPATHOLOGICAL EXAMINATION

A portion of the liver was fixed in 10% formalin and then embedded in paraffin. Microtome sections 6μm thickness were prepared from each liver and stained with hematoxilin-eosin. The sections were evaluated for the pathological

parameters of hepatotoxicity such as necrosis, fatty infiltration, fibrosis, lymphocyte infiltration etc.

5.3. RESULT

5.3.1. CCL_4 INDUCED HEPATOTOXICITY

Serum MDA, ALP, SGOT and SGPT levels in rats were increased after administration of chronic doses of CCl_4 as compared with the normal levels. The SGPT and SGOT activities in the CCl_4 injected animal were 967 ± 24.3 and 232.3 ± 23.9 U/l respectively. Administration of aqueous methanol extract of *G.lucidum* (500, 1000 mg/kg) significantly lowered these elevated levels of SGPT and SGOT in a dose dependent manner. The activities of serum transaminases (SGPT and SGOT) in the extract treated groups of animals (1000 mg/kg) were 280.3± 25 and 84.3 ± 7.7U/l respectively (Table 5.1). Similarly the serum ALP, level was elevated significantly in the CCl_4 treated control animals and level was reduced in the extract treated groups (Table 5.1) MDA levels in both the serum and tissue were elevated significantly in the CCl_4 treated control animals and level was reduced in the extract treated groups (Figure 5.1, 5.2).

CCl_4 administration caused drastic decline of GST levels. Administration of aqueous methanol extract of *G.lucidum* (500 and1000 mg/kg) caused significant increase in hepatic GST (939.4 ± 56.6 and 1409.8 ± 44.3 μmol of CDNB conjugate formed /min/mg protein) activity in CCl_4 intoxicated animals (Figure 5.3). GPX activity was also found to be increased in the extract treated groups of animals. The

activity of SOD, CAT and GPX were 20.3 ± 1.4, 60.3 ± 1.7, 21.4 ± 0.6 U/mg protein respectively in the extract (1000 mg/kg) treated group of animals (Table 5.2).

CCl_4 administration caused drastic decline of GSH levels. The GSH antioxidant system consist of an array of non-enzymatic and enzymatic reaction pathways involved in the neutralization of reaction free radicals. The aqueous methanol extract of *G. lucidum* (500 and1000 mg/kg) caused significant increases in hepatic GSH (8.960 ± 0.39 and 11.23 ± 1.32 nmol/mg protein) activities in CCl_4 intoxicated animals (Figure 5.4)

5.3.2. HISTOPATHOLOGICAL EXAMINATION

Histopathological examination of the liver challenged with chronic doses of CCL_4 showed severe necrosis, fatty infiltration, fibrosis, ballooning degeneration and lymphocytes infiltration. The effects were moderate to low in the liver of animals administered with the extract (500 and 1000 mg/kg body weight) and challenged with CCl_4 (Figure 5.5).

Table 5.1. Effect of aqueous methanol extract of *G. lucidum* on serum GOT, GPT and ALP activities in rats administered with chronic doses of CCl_4.

Groups	Treatment (mg/kg)	GOT (IU/l)	GPT (IU/l)	ALP (IU/l)
Normal	-	67.28 ± 3.8^{d}	123.4 ± 12.3^{d}	165.5 ± 18.4^{d}
Control CCl_4/paraffin oil, 1:5)	1.5 ml/kg	232.3 ± 23.9^{a}	967 ± 24.3^{a}	438.4 ± 35.4^{a}
CCl_4/paraffin oil,+ *G. lucidum* extract	500	114.4 ± 11.3^{b}	526.8 ± 34.4^{b}	247.4 ± 13.8^{b}
CCl_4/paraffin oil,+ *G. lucidum* extract	1000	84.3 ± 7.7^{c}	280.3 ± 25^{c}	212.6 ± 7.6^{c}

Values are mean ± S.D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5% level.

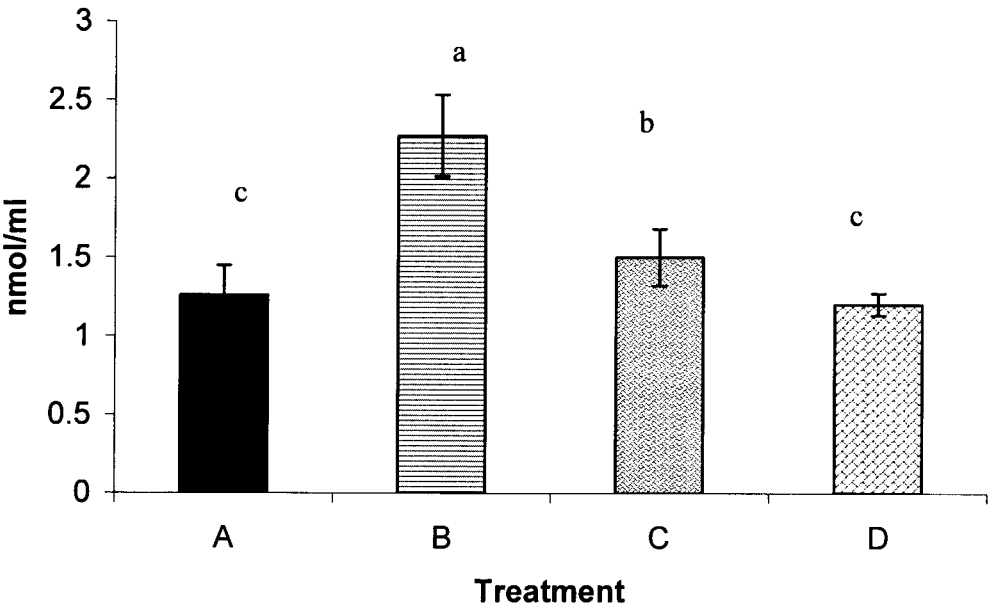


Figure 5.1. Effect of aqueous methanol extract of *G. lucidum* on serum MDA level in rats, administered with chronic doses of CCl_4. Values are expressed as mean ± SD. n=6. Any two means having a common superscript are not significantly different at 5% level.

A : normal

B : control (CCl_4/paraffin oil, 1.5ml/kg)

C : *G. lucidum* extract 500 mg/kg body weight

D : *G. lucidum* extract 1000 mg/kg body weight

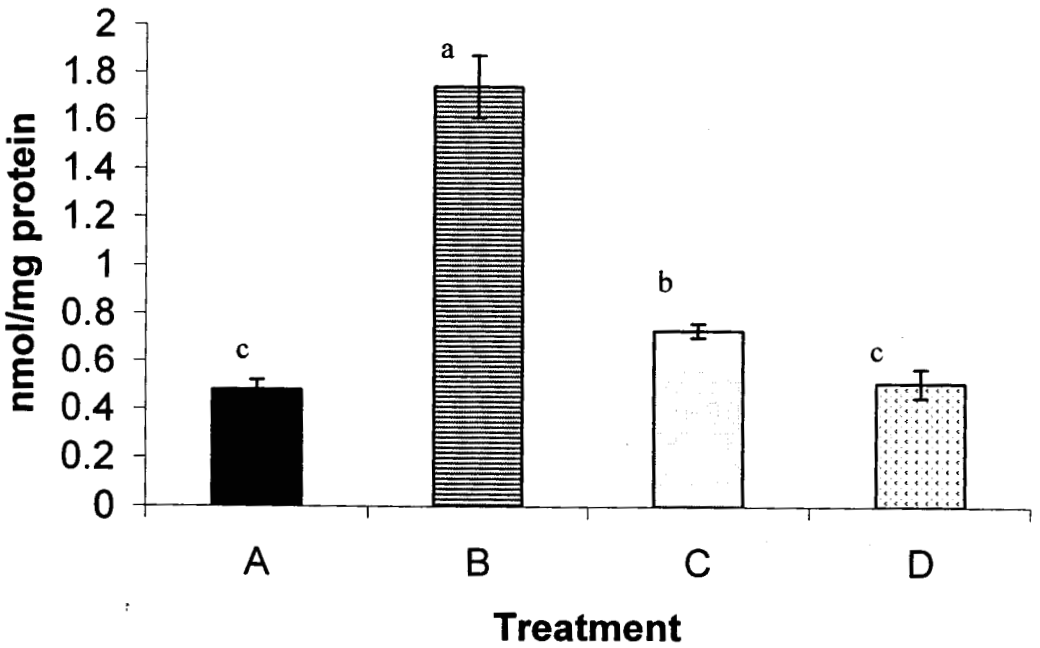


Figure 5.2. Effect of aqueous methanol extract of *G. lucidum* on tissue MDA level in rats, administered with chronic doses of CCl_4. Values are expressed as mean ± SD. n=6. Any two means having a common superscript are not significantly different at 5% level.

A : normal,

B : control (CCl_4/paraffin oil, 1.5ml/kg),

C : *G. lucidum* extract 500 mg/kg body weight

D : *G. lucidum* extract 1000 mg/kg body weight

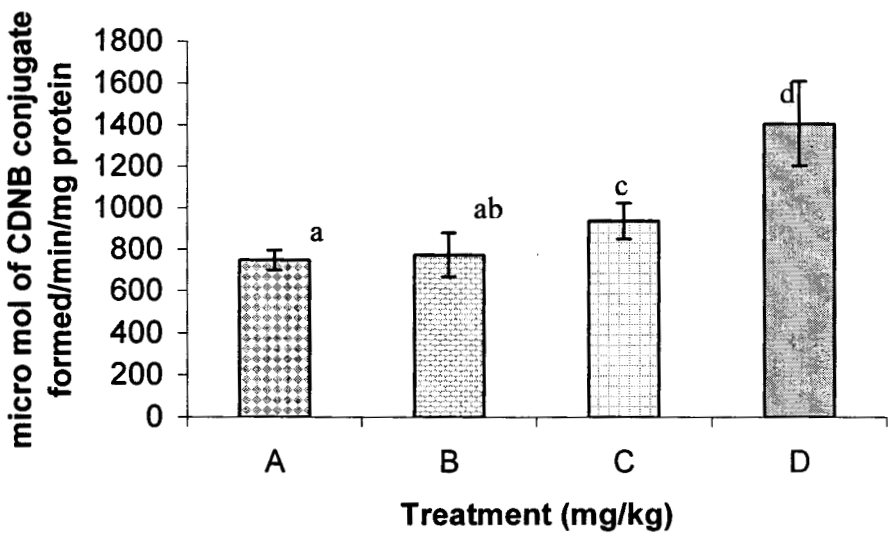


Figure 5.3. Effect of aqueous methanol extract of *G. lucidum* on GST level in in rats, administered with chronic doses of CCl_4. Values are expressed as mean ± SD. n=6. Any two means having a common superscript are not significantly different at 5% level.

A : control (CCl_4/paraffin oil, 1.5ml/kg)

B : normal,

C : *G. lucidum* extract 500 mg/kg body weight

D : *G. lucidum* extract 1000 mg/kg body weight

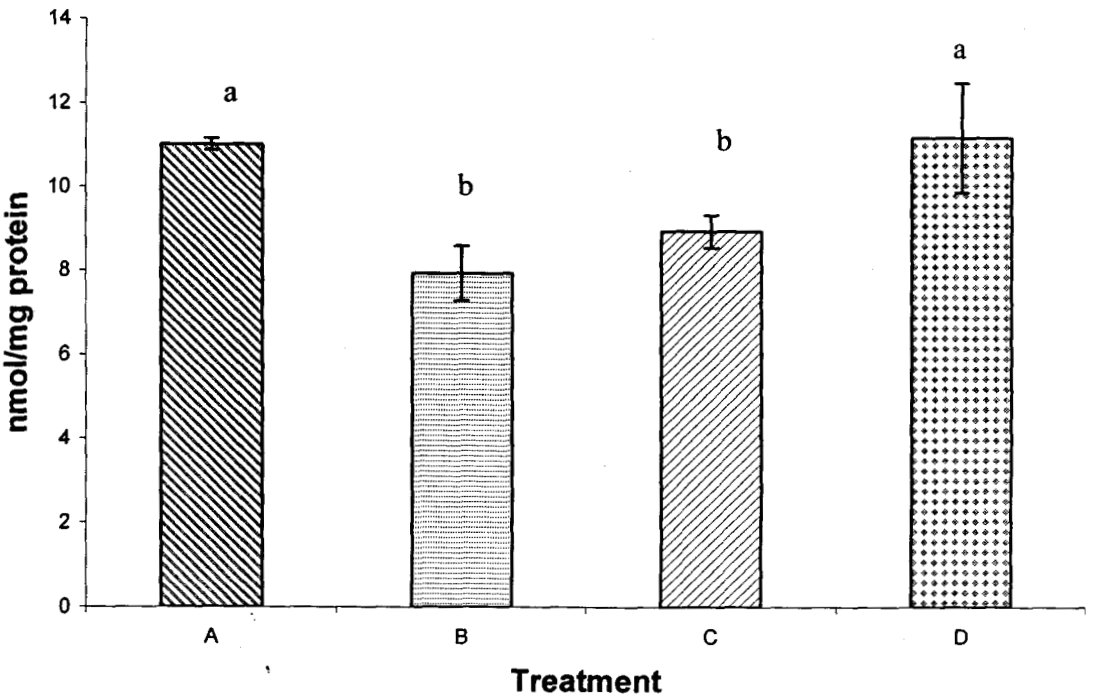


Figure 5.4. Effect of aqueous methanol extract of *G. lucidum* on serum GSH level in rats, administered with chronic doses of CCl_4. Values are expressed as mean ± SD. n=6. Any two means having a common superscript are not significantly different at 5% level.

A : normal

B : control (CCl_4/paraffin oil, 1.5ml/kg)

C : *G. lucidum* extract 500 mg/kg body weight

D : *G. lucidum* extract 1000 mg/kg body weight

Table 5.2. Effect of aqueous methanol extract of *G. lucidum* on hepatic SOD, CAT and GPX activities in rats administered with chronic doses of CCl_4.

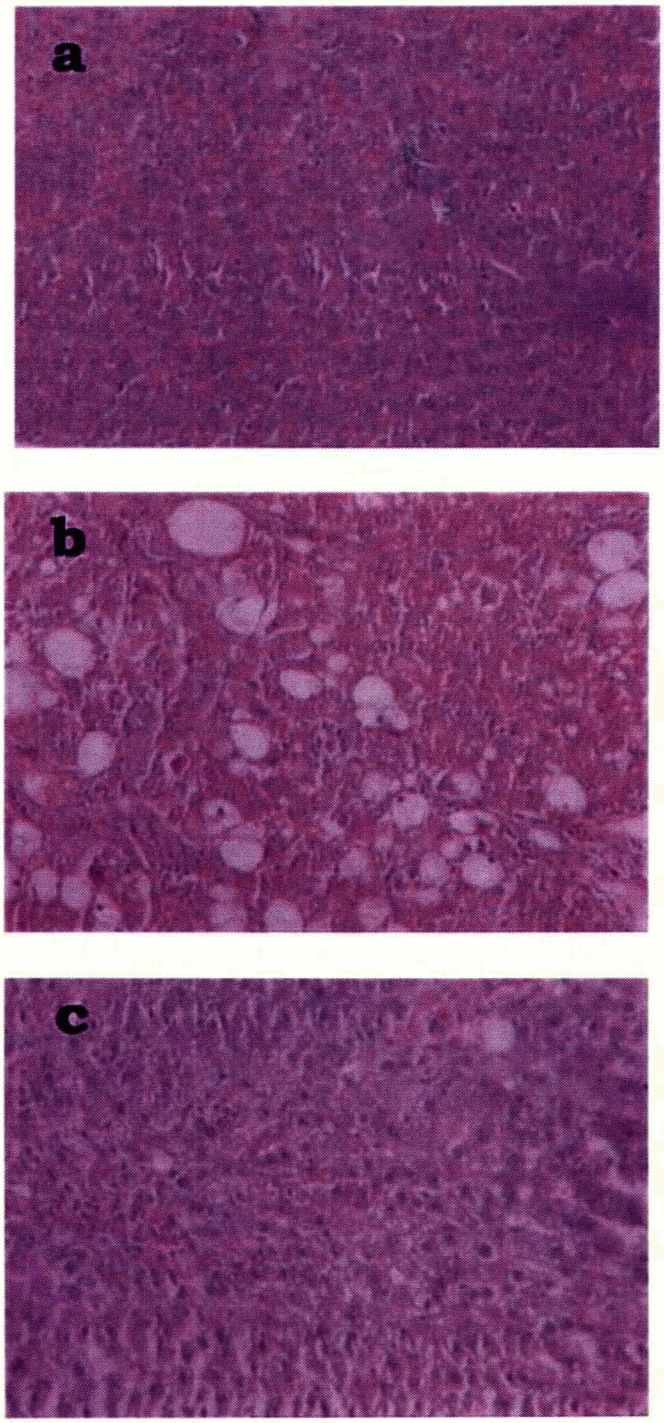
Groups	Treatment (mg/kg)	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)
Normal	-	21.2 ± 2.6^{c}	64.5 ± 4.5^{c}	23.2 ± 1.5^{d}
Control CCl_4/paraffin oil, 1:5)	1.5 ml/kg	14.6 ± 2.2^{a}	46.3 ± 4.8^{a}	17.1 ± 0.7^{a}
CCl_4/paraffin oil, + *G.lucidum* extract	500	17.9 ± 1.3^{b}	53.1 ± 3.8^{b}	19.3 ± 0.5^{b}
CCl_4/paraffin oil, + *G.lucidum* extract	1000	20.3 ± 1.04^{c}	60.3 ± 1.7^{c}	21.4 ± 0.6^{c}

Values are mean ± S.D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5% level.

Figure 5.5 Hepatoprotective effect of aqueous methanol extract of *G. lucidum* against CCl_4 induced chronic hepatotoxicity in rats. Liver sections stained with H&E. a) Normal; b) CCl_4/paraffin oil (1:5, 1.5 ml/kg. body wt, i.p); c) GLME (1000 mg/kg body wt, p.o) + CCl_4/paraffin oil (1.5 ml/kg. body wt, i.p). Magnification x 20.

116A



5.4. DISCUSSION

The results of the investigation indicate that aqueous methanol extract of *G.lucidum* significantly rendered protection against CCl_4 induced toxicity. It has been hypothesized that one of the principal causes of CCl_4–induced liver injury is due to lipid peroxidation by free radical derivatives of CCl_4. Thus, the antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4–induced liver injury. Studies on the hepatoprotective experimental model indicated that CCl_4 is first metabolized by cytochrome P450 in the liver endoplasmic reticulum to the highly reactive CCl_3 radical. The free radical, in the presence of oxygen, leads to auto oxidation of the fatty acids present in the cytoplasmic membrane phospholipid and causes functional and morphological changes in the cell membrane. Furthermore, influx of extra cellular Ca-ions into cell is claimed to be an important step leading to cell death. Therefore, the examination of the preventive action in liver damage, caused by CCl_4, may give an indication of the liver-protective action of drugs in general. The results of this study show that the methanolic extract of *G.lucidum* has preventive action on CCl_4 induced hepatotoxicity. This hypothesis was also confirmed by histological observation.

The character of a free radical is that it is able to attack any cell in the body by oxidation reaction. The oxidation and antioxidation system are in dynamic equilibrium in the normal body (Wang et al., 2002). The organism has some substances, such as superoxide dismutase (SOD), catalase (CAT), glutathione

peroxidase (GPX), glutathione reductase (GR), and glucose6- phosphate dehydrogenase. These antioxidant enzymes protect against toxic oxygen radicals produced during normal metabolism and after oxidative insult. Liver contains high concentrations of CAT and GPX. The treatment by CCl_4 decreased the activity of SOD, CAT, GSH and GPX levels in the liver. In fact there is direct correlation between GSH depletion and enhanced lipid peroxidation. Histopathological observation also confirms this hypothesis.

Lipid peroxidation is a complex natural deleterious process. The effect of free radicals on human beings have been considered as their close relation to toxicity, disease and aeging. The lipid solubility of CCl_4 allows it to cross cell membranes, and the CCL_4 injected is distributed to all organs. However, its main toxic effects are shown on the liver due to a blockage in synthesis of the lipoproteins that carry triglyceride away from this organ. The structure of the liver endoplasmic reticulum becomes distorted, hepatic protein synthesis slows down, and the activity of enzymes located in the ER, such as glucose-6-phosphate and cytochrome P450, rapidly declines, as does the ability of the ER to sequester Ca^{2+} ions by the Ca^{2+}-ATPase. Hence rises in intra cellular Ca^{2+} concentrations occur in the liver parenchymal cells (Grun et al., 1977). The nuclear membrane is attacked more slowly. Eventually there is necrosis of liver cells in the central areas of the lobes. Administration of wide range of antioxidants, or inhibitors of P450, decreases CCl_4 toxicity is in parallel with decreased lipid peroxidation in animals. Present result also supports the significant decrease in activity of lipid peroxidation level in

G.lucidum extract treated group of animals. This clearly indicates that *G.lucidum* contain certain substances, which are capable of preventing lipid peroxidation, a natural deleterious process.

The experimental results reveal that aqueous methanol extract of *G. lucidum* is capable to prevent hepatotoxicity induced by carbontetrachloride administration. The effect is mainly due to the capacity of the extract to restore hepatic antioxidant defense system. The experimental findings thus suggest the potential therapeutic use of *G. lucidum* occurring in Kerala for preventing hepatic disorders.

PREVENTION OF ANTICANCER DRUG INDUCED ORGAN TOXICITY BY GANODERMA LUCIDUM

Sheena.N "Studies on the thekapeutic potential of Ganoderma lucidum P. Karst – Reishi, occurring in Kerala " Thesis. Amala Cancer Research Centre , University of Calicut, 2005

CHAPTER 6

PREVENTION OF ANTICANCER DRUG INDUCED ORGAN TOXICITY BY *GANODEMA LUCIDUM*

6.1. INTRODUCTION

As the great threat to human life by neoplastic diseases continues to increase, the pursuit for discovering new anticancer drugs has been considered as a compelling urgency. A number of chemotherapeutic agents, either of natural products origin or synthetics have been developed during recent years (Ajith and Janardhanan, 2002). A number of therapeutic agents have been evaluated experimentally and clinically and none of the compounds tested was found clinically efficacious as a complete protective agent in patients. Cisplatinum (II) diamine dichloride (cisplatin) is extensively used for the management of oncological disorders particularly of the ovary, testis, bladder and head and neck (Hamers et al., 1991). Higher doses of cisplatin, although more efficacious for cancer chemotherapy manifests irreversible renal dysfunction, ototoxicity and other toxicities (Bodenner et al., 1986). Similar case is reported with doxorubicin, which is a powerful anthracycline antibiotic, originally isolated from *Streptomyces peucetius* and used for the treatment of many human neoplasia, including acute leukemia's, lymphomas, stomach, breast and ovarian cancers, Kaposi's sarcoma, and bone tumors. This when used for a prolonged period has been found to cause cardiotoxicity thereby limiting its clinical use (Mott, 1997). Recent evidences have suggested that the free radicals and ROS are involved in cisplatin induced and doxorubicin induced oxidative stress (Ajith et al., 2002., Naidu et al., 2002).

Attempts have been made in many part of the world to explore the use of mushrooms and their metabolites for the treatment of a variety of human ailments.

The fruiting bodies of a number of fungi have long been prescribed in Chinese medicine for the treatment of a large number of diseases (Shiao et al., 1994). Fruiting bodies of *Ganoderma lucidum* have been considered as panacea in Chinese medicine. Investigations were carried out to evaluate the effect of methanolic extract of *G. lucidum* to prevent nephrotoxicity caused by cisplatin and cardiotoxicity by doxorubicin. The findings are reported in this chapter.

6.2. MATERIALS AND METHODS

6.2.1. PREPARATION OF THE EXTRACT

Aqueous methanol extract of *G. lucidum* was prepared as described in the section 3.2.1.

6.2.2. ANIMALS

Nephrotoxicity studies: Male Swiss albino mice of 6-8 weeks of age weighing 25 ± 2 g

Cardiotoxicity studies: Male Sprague dawly rats weighing 180 ± 20 g

6.2.3. TUMOR CELL LINE

DLA cell line was originally obtained from Cancer Institute, Adayar, Chennai and maintained in our laboratory.

6.2.4. DETERMINATION OF CISPLATIN INDUCED NEPHROTOXICITY

Animals were divided into 4 groups of 6 animals each. Group without any treatment was kept as normal (Group 1). Group 2 was given cisplatin (16 mg/kg body weight, i.p) (Somani et al., 2000) Group 3 and 4 were given aqueous

methanolic extract of *G. lucidum* (250 and 500 mg/kg body wt; p.o) 1h before the cisplatin injection (16 mg/kg body weight, i.p). Mice in all groups were sacrificed 72 h after the treatment. The blood was collected directly from heart; serum was separated for creatinine and urea analyses. The kidneys were dissected and stored at -70°C until analyses could be completed. The kidneys were homogenized in 50 mM phosphate buffer (pH 7) to give a 10 % homogenate (W/V). The homogenate was centrifuged at 1000 rpm for 10 min in a cold centrifuge at 0°C and supernatant was used for enzyme assays and protein determinations. Serum creatinine and urea were estimated by the method of Brod, 1980 (section 2.2.14) and Marshell et al., 1980 (section 2.2.13) respectively. Tissue homogenate was used for assay the activities of superoxide dismutase (SOD) by the method of Mc Cord and Fridovich, 1969 (section 2.2.4), catalase (CAT) by the method of Beers and Sizer, 1952 (section 2.2.5), glutathione peroxidase (GPX) by the method of Hafemann et al., 1974 (section 2.2.6), levels of reduced glutathione (GSH) by the method of Moron et al., 1979 (section 2.2.3), and malondialdehyde (MDA) by the method of (Ohkawa et al., 1979 (section 2.2.8). The protein content was estimated by the method of Lowry et al., 1951 (section 2.2.9).

6.2.5. DETERMINATION OF THE EFFECT OF PRETREATMENT OF THE EXTRACT ON THE EFFICACY OF ANTITUMOR ACTIVITY OF CISPLATIN

Animals were divided into 3 groups of 6 animals each. Animals in all groups were injected with $1x10^6$ viable cells of Dalton's lymphoma ascites (DLA) in

phosphate buffered saline. Twenty-four hour after tumor implantation, animals were treated as follows. Group 1 administered with vehicle (distilled water, po) was kept as control. Group 2 was administered with aqueous methanol extract of *G. lucidum* (500 mg/kg body weight) orally and then with cisplatin (3 mg/kg body weight, i.p) after one hour, once daily for 10 consecutive days. Group 3 was given cisplatin (3 mg/kg body wt, i.p) alone once daily. The treatments in all groups were continued for 10 consecutive days. At the end of the 5th week, animals were sacrificed, extirpated the tumor and weighed. The percent inhibition was calculated using the formula (I-B/A) x100, where A is average tumor weight of the control group and B that of the treated group (Ajith et al., 2002).

6.2.6. HISTOPATHOLOGICAL EXAMINATION

A portion of the kidney was fixed in 10% formalin and then embedded in paraffin. Microtome sections 6μm thickness were prepared from each kidney and stained with hematoxilin-eosin. The sections were evaluated for the pathological parameters of nephrotoxicity such as necrosis, protein ultrafiltrate in the proximal convoluted tubules, lymphocyte infiltration etc.

6.2.7. DETERMINATION OF DOXORUBICIN INDUCED CARDIOTO-XICITY

Animals were divided into 4 groups of 6 animals each. Group 1 treated with distilled water was kept as normal. Group 2 treated with doxorubicin (6 mg/kg body weight, i.p) was kept as control (Liu, 1999). Group 3 and 4 treated with *G. lucidum* extract 500,1000 mg/kg orally 1 hr before each doxorubicin injection. The

doxorubicin injection repeated at 48 and 72 hrs. The animals were sacrificed after the last dose of doxorubicin; blood was collected directly from the heart. Serum was used for the determination of CK by the method of Moss and Henderson 1999 (section 2.2.16). Heart was removed and stored at -70°C until analyses could be completed. The heart was homogenized in 50 mM phosphate buffer (pH 7) to give a 10 % homogenate (W/V). Supernatant was used for the determinations of superoxide dismutase (SOD) by the method of Mc Cord and Fridovich 1969, (section 2.2.4), glutathione peroxides (GPX) by the method of Hafemann et al., 1974 (section 2.2.6), levels of reduced glutathione (GSH) by the method of Moron et al., 1979 (section 2.2.3), and malondialdehyde (MDA) by the method of Ohkawa et al., 1979 (section 2.2.8). The protein content was estimated by the method of Lowry et al., 1951 (section 2.2.9).

6.2.8. HISTOPATHOLOGICAL EXAMINATION

A portion of the heart was fixed in 10 %formalin and then embedded in paraffin. Microtome sections 6 µm thickness was prepared from each heart and stained with hematoxilin-eosin. The sections were evaluated for the pathological parameters of cardiotoxicity such as elongation of cardiomycetes, increase intercellular spaces, myocyte nuclear swellings etc.

6.3. RESULT

6.3.1. CISPLATIN INDUCED NEPHROTOXICITY

Serum creatinine and urea levels were significantly elevated in the cisplatin treated animals compared to the normal group. The increase of serum creatinine and urea levels was 7 and 5.7 fold respectively. Treatment of animals with aqueous methanol extract of *G. lucidum* significantly reduced the elevated levels of serum creatinine and urea. The extract treatment was able to lower the serum creatinine and urea to almost normal level (Table 6.1).

Renal SOD activity decreased significantly in the cisplatin treated animals compared to the normal group (Table 6.2). Administration of aqueous methanol extract of *G. lucidum* prior to cisplatin treatment restored the level of SOD in a dose dependent manner in groups of animals treated with cisplatin. Renal CAT and GPX activities were also decreased significantly after the administration of cisplatin (Table 6.2). Administration of aqueous methanol extract of *G. lucidum* prior to cisplatin treatment restored the activities of CAT and GPX to normal level.

The renal GSH concentration was decreased over 40 % in cisplatin treated animals as compared to the normal. However, the administration of aqueous methanol extract *G. lucidum* at a concentration of 500 mg/Kg body weight prior to cisplatin treatment increased the renal GSH concentration to the normal level (Figure 6.1).

The concentration of malondialdehyde increased over 50% in the cisplatin treated animals as compared to the normal, indicating the enhanced activity of lipid peroxidation. Administration of the methanolic extract of *G. lucidum* at a concentration of 500 mg/kg body weight decreased the MDA to the normal level (Figure 6.2).

6.3.2. THE EFFICACY OF ANTITUMOR ACTIVITY OF CISPLATIN WITH THE PRETREATMENT OF THE EXTRACT

The treatment of aqueous methanol extract of *G. lucidum* prior to the cisplatin injection did not interfere with the antitumor efficacy of cisplatin (Table 6.3). The average weight of tumor in cisplatin and the extract (500 mg/kg body wt) plus cisplatin treated animals were 0.48 ± 0.06 and 0.24 ± 0.02 g respectively. The tumor weight in the control animals was 10.2 ± 0.18 g. The percent inhibition of tumor growth by cisplatin and cisplatin plus *G. lucidum* extract (500 mg/kg body wt) was 95 and 97 % respectively. This indicated that the extract did not interfere with the antitumor activity of cisplatin.

6.3.3. HISTOPATHOLOGICAL EXAMINATION

Sections of the kidney of cisplatin alone treated animals showed increased accumulation of plasma ultra filtrate in the proximal convoluted tubule and renal cell necrosis. Treatment of G. *lucidum* extract before the cisplatin injection decreased these toxic manifestations to a moderate level (Figure 6.3).

6.3.4. DOXORUBICIN INDUCED CARDIOTOXICITY

The results of the present study indicated that intraperitoneal administration of doxorubicin (6 mg/kg body weight, i.p) 3 doses on alternate days induced clear signs of cardiac toxicity in rats. The animals in the control group are more sick, weaker and suffered from diarrhea as compared to the normal and extract pretreated group of animals. Administration of *G. lucidum* extract (500 and1000 mg/Kg) prior to doxorubicin challenge abruptly increased the heart GSH activity compared to doxorubicin alone injected animals (Table 6.4). The product of lipid peroxidation measured as MDA in heart tissue was higher in doxorubicin treated group (1.006 ± 0.13) compared to normal (0.34 ± 0.02). The MDA level was found to be almost normalized on extract treated group of animals (Table 6.4).

Doxorubicin treatment produced significant decrease in SOD (4.3 ± 0.3 U/mg protein), GPX (25.7 ± 3.6 U/mg protein) in the control animals, compared to the activities of SOD (6.2 ± 1.4 U/mg protein) and GPX (48.5 ± 7.2 U/mg protein) in the normal group (Table 6.5). The activity of SOD and GPX in the *G. lucidum* extract treated group was comparable to normal. Administration of aqueous methanol extract of *G.lucidum* (500,1000 mg/Kg) significantly lowered the levels of CK activity in a dose dependent manner. The activities of serum CK in the treated groups (500 and 1000 mg/Kg) were 423.3 ± 79.6 and 186.5 ± 36.5 IU/L respectively. The activity was reduced by 68.3 % in the 1000 mg/kg extract treated group of animals (Table 6.6).

6.3.5. HISTOPATHOLGICAL EXAMINATION

Histopathological examination of heart of doxorubicin alone treated animals showed elongation of cardiomyocytes, increased intercellular spaces and myocyte nuclear swellings while in the pretreatment of *G. lucidum* extract retained the almost normal cardiac texture (Figure 6.4).

Table 6.1 Effect of aqueous methanol extract of *G. lucidum* on the increase in serum urea and creatinine levels induced by cisplatin administration.

Groups	Treatments (mg/kg)	Urea (mg/dl)	Creatinine (mg/dl)
Normal	-	52.5 ± 3.8^{d}	0.40 ± 0.03^{c}
Cisplatin	16	300.4 ± 28.5^{a}	2.98 ± 0.29^{a}
Cisplatin + *G. lucidum* extract	500	148.6 ± 5.6^{b}	1.08 ± 0.04^{b}
	1000	99.1 ± 11.1^{c}	0.44 ± 0.06^{c}

Values are mean ± S. D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5 % level.

Table 6.2 Effect of aqueous methanol extract of *G. lucidum* on renal SOD, CAT and GPX activities induced by cisplatin administration.

Groups	Treatment (mg/kg)	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)
Normal	-	11.96 ± 1.42^{d}	47.10 ± 1.40^{d}	24.5 ± 2.8^{c}
Cisplatin	16	6.45 ± 0.85^{a}	13.41 ± 2.84^{a}	5.25 ± 1.1^{a}
Cisplatin + *G. lucidum* extract	250	8.88 ± 0.80^{b}	30.42 ± 1.80^{b}	11.5 ± 3.2^{b}
	500	13.33 ± 0.88^{c}	42.08 ± 3.11^{c}	22.5 ± 2.9^{c}

Values are mean ± S. D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5 % level.

Table 6.1 Effect of aqueous methanol extract of *G. lucidum* on the increase in serum urea and creatinine levels induced by cisplatin administration.

Groups	Treatments (mg/kg)	Urea (mg/dl)	Creatinine (mg/dl)
Normal	-	52.5 ± 3.8^{d}	0.40 ± 0.03^{c}
Cisplatin	16	300.4 ± 28.5^{a}	2.98 ± 0.29^{a}
Cisplatin + *G. lucidum* extract	500	148.6 ± 5.6^{b}	1.08 ± 0.04^{b}
	1000	99.1 ± 11.1^{c}	0.44 ± 0.06^{c}

Values are mean ± S. D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5 % level.

Table 6.2 Effect of aqueous methanol extract of *G. lucidum* on renal SOD, CAT and GPX activities induced by cisplatin administration.

Groups	Treatment (mg/kg)	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)
Normal	-	11.96 ± 1.42 [d]	47.10 ± 1.40[d]	24.5 ± 2.8 [c]
Cisplatin	16	6. 45 ± 0.85[a]	13.41 ± 2.84[a]	5.25 ± 1.1 [a]
Cisplatin + *G. lucidum* extract	250	8.88 ± 0.80[b]	30.42 ± 1.80[b]	11.5 ± 3.2 [b]
	500	13.33 ± 0.88 [c]	42.08 ± 3.11[c]	22.5 ± 2.9 [c]

Values are mean ± S. D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5 % level.

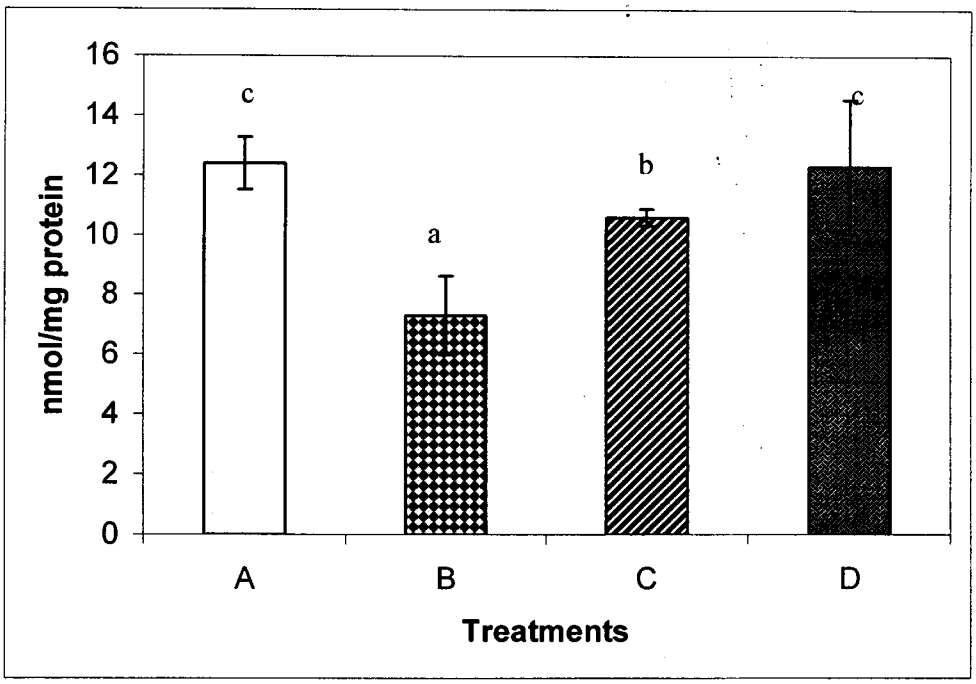


Figure 6.1. Renal GSH level in A) normal; B) cisplatin (16mg/kg); C) cisplatin plus aqueous methanol extract of *G. lucidum* (250 mg/kg body weight); D) cisplatin plus aqueous methanol extract of *G. lucidum* (500 mg/kg body weight) treated mice in the kidney, three days after cisplatin administration. Values are expressed as mean ± S. D. n=6. Any two means having a common superscript are not significantly different at 5 % level.

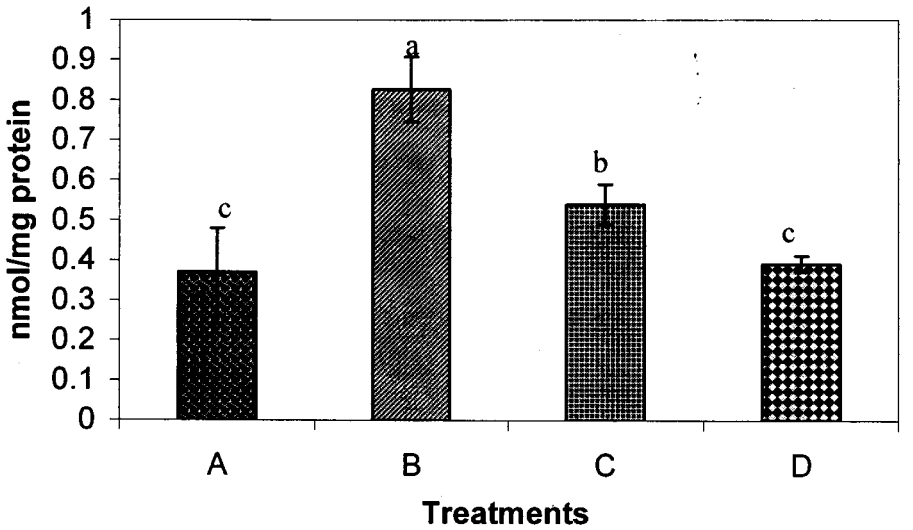


Figure 6.2 Renal MDA level in A) normal; B) cisplatin (16mg/kg); C) cisplatin plus aqueous methanol extract of *G. lucidum* (250 mg/kg body weight); D) cisplatin plus aqueous methanol extract of *G. lucidum* (500 mg/kg body weight) treated mice in the kidney, three days after cisplatin administration. Values are expressed as mean ± S. D. n=6. Any two means having a common superscript are not significantly different at 5 % level.

Table 6.3 Antitumor activity of cisplatin (3 mg/kg body wt (i.p) and cisplatin + aqueous methanol extract of *G. lucidum* (500 mg/kg body wt, p.o) on DLA induced solid tumor in mice.

Groups	Treatment (mg/kg)	Tumor wt on 5th week (g)	Inhibition %
Control		10.2 ± 0.18^{a}	
Cisplatin	3	0.48 ± 0.06^{b}	95.3
Cisplatin + *G. lucidum* extract	500	0.24 ± 0.02^{c}	97.6

Values are mean ± S. D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5 % level.

Figure 6.3. Nephroprotective effect of aqueous methanol extract of *G. lucidum* against cisplatin induced nephrotoxicity in rats. Kidney sections stained with H&E a) Normal; b) Cisplatin (16 mg/kg. body wt, i.p); c) *G. lucidum* extract (500 mg/kg body wt, p.o) + Cisplatin (16 mg/kg. body wt, i.p). Magnification x 20.

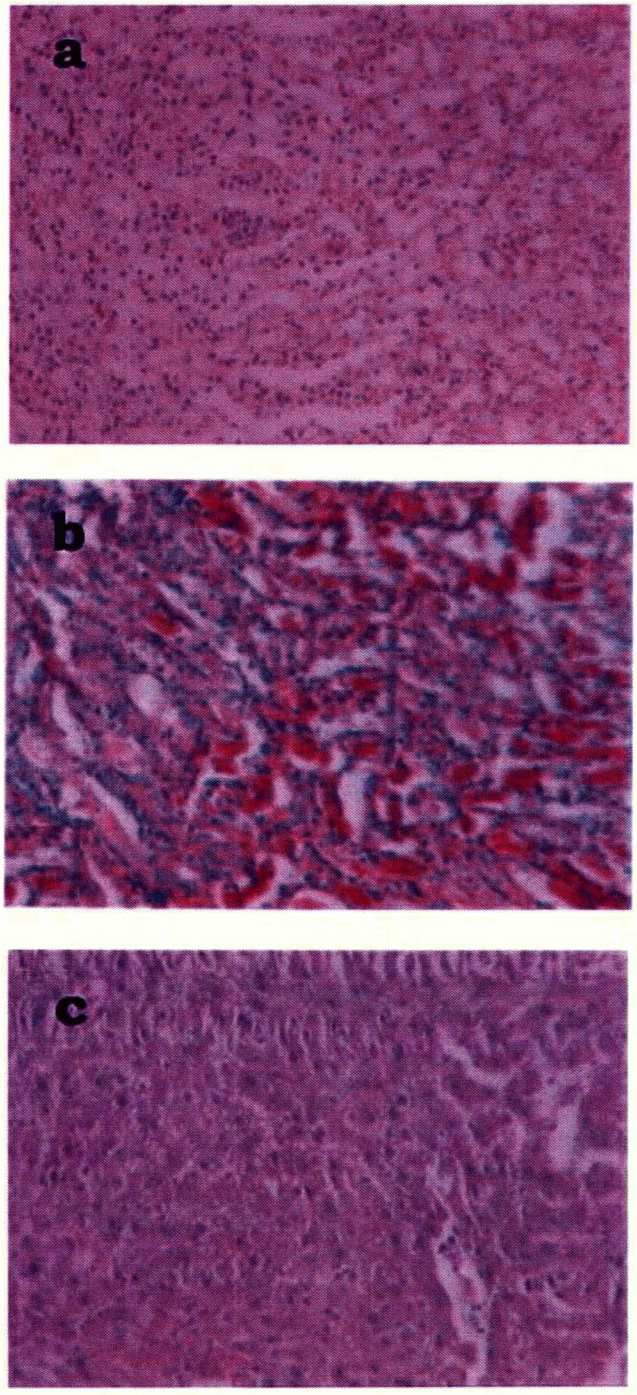

a
b
c

Table 6.4 Levels of GSH and lipid peroxidation (MDA) in the heart of rats treated with doxorubicin (6mg/kg body wt., i.p), doxorubicin + aqueous methanol extract of *G. lucidum* (500 mg/kg and 1000 mg/kg body wt., p.o).

Groups	Treatments (mg/kg)	GSH (nmoles/mg protein)	MDA (nmoles/mg protein)
Normal	-	4.18 ± 0.37^{c}	0.34 ± 0.02^{bc}
Doxorubicin	6	2.37 ± 0.14^{a}	1.00 ± 0.13^{a}
Doxorubicin + *G. lucidum* extract	500	3.48 ± 0.27^{b}	0.42 ± 0.06^{b}
	1000	4.43 ± 0.25^{c}	0.28 ± 0.03^{c}

Values are mean ±S.D, n=6 animals.

Any two means having a common superscript are not significantly different at 5 % level.

Table 6.5 Levels of SOD and GPX in the heart of rats treated with doxorubicin (6mg/kg body wt., i.p), doxorubicin + aqueous methanol extract of *G. lucidum* (500 mg/kg and 1000 mg/kg body wt., p.o).

Groups	Treatments (mg/kg)	SOD (U/mg protein)	GPX (U/mg protein)
Normal	-	6.2 ± 1.4^{c}	48.5 ± 7.2^{d}
Control (Doxorubicin)	6	4.3 ± 0.3^{a}	25.7 ± 3.6^{a}
Doxorubicin+ *G. lucidum* extract	500	4.8 ± 0.1^{a}	41.1 ± 1.7^{b}
	1000	7.8 ± 0.3^{b}	50.2 ± 0.6^{c}

Values are mean ± S. D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5 % level.

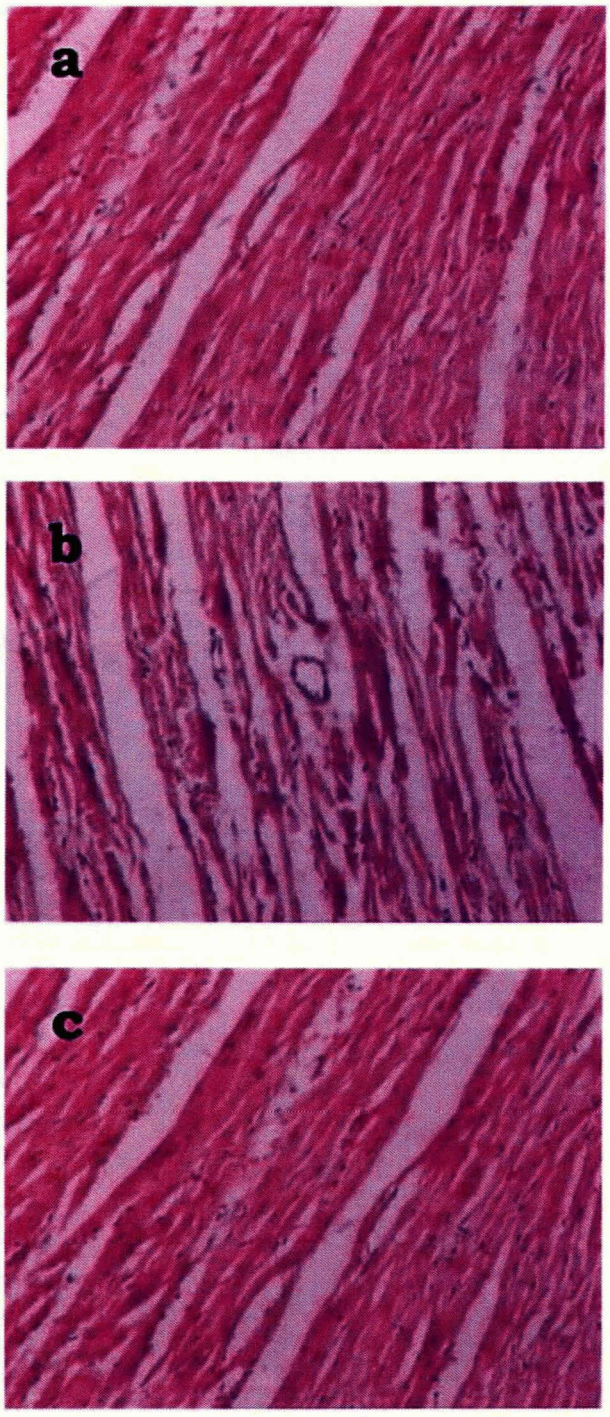
Table 6.6 Serum CK in the heart of rats treated with doxorubicin (6mg/kg body wt., i.p), doxorubicin + aqueous methanol extract of *G. lucidum* (500 mg/kg and 1000 mg/kg body wt., p.o).

Groups	Treatments (mg/kg)	CK (IU/l)
Normal	-	131.7 ± 39.4^{d}
Doxorubicin	6	588.3 ± 194.0^{a}
Doxorubicin + *G. lucidum* extract	500	423.3 ± 79.6^{b}
	1000	186.5 ± 36.5^{c}

Values are mean ±S. D. n= 6 animals.

Any two means having a common superscript are not significantly different at 5 % level.

Figure 6.4. Cardioprotective effect of aqueous methanol extract of *G. lucidum* against doxorubicin induced cardiotoxicity in rats. Heart sections stained with H&E a) Normal; b) doxorubicin (6 mg/kg body weight, i.p); c) *G. lucidum* extract (500 mg/kg body wt, p.o) + doxorubicin (6 mg/kg body weight, i.p). Magnification x 20.


a
b
c

6.4. DISCUSSION

Several lines of evidence indicate that free radicals are involved in the nephrotoxicity caused by cisplatin and the damage is suggested to be the consequence of decreased renal antioxidant enzyme activity with the enhanced lipid peroxidation. However, administration of antioxidants has been shown to ameliorate cisplatin- induced nephrotoxicity in animals (Babu et al., 1995). The results of the present study show that renal SOD, CAT, GPX activities and reduced GSH level significantly decreased in the cisplatin treated animals compared to normal. These observations support the hypothesis that the mechanism of nephrotoxicity in cisplatin treated animals is related to depletion of antioxidant defense system. Treatment of *G. lucidum* extract (250 and 500 mg/kg body weight, p.o) prior to cisplatin administration prevents the depletion of renal antioxidants.

The activity of CAT and GPX is also found to decrease after cisplatin administration. This resulted in the decreased ability of the kidney to scavenge toxic H_2O_2 and lipid peroxides. The restoration of renal SOD, CAT and GPX activities by the pretreatment of *G. lucidum* extract suggests that the extract is capable of protecting the enzymes even three days after cisplatin administration. GSH depletion can markedly increase the toxicity of cisplatin. The increased GSH levels render protection, which is evident from the treatment of extract prior to cisplatin administration.

The concentration of malondialdehyde is observed significantly increased in the cisplatin treated animals compared to the normal group. Administration of *G. lucidum* extract to animals along with cisplatin decreased the MDA to the normal level compared to animals treated with cisplatin alone. Depletion of renal GSH can markedly increase the toxicity of cisplatin, probably due to the augmentation of lipid peroxidation. The present investigation also indicates that pretreatment of *G. lucidum* extract does not interfere with the antitumor efficacy of cisplatin. The tested doses of methanol extract of *G. lucidum* show no signs of toxicity in mice. Survival rate of animals treated with *G. lucidum* extract prior to cisplatin administration supports the nephroprotective effect of this mushroom.

The free radicals and reactive oxygen species induce oxidative stress in kidneys (Hannemann and Banumann, 1988., Uslu and Bonavida, 1966). Consequent to cisplatin administration platinum- sulfhydryl group complexes formed are taken up by renal cells and stabilized by intracellular GSH for several hours. In case of intracellular GSH depletion, the complexes undergo rapid transformation to reactive metabolites (Ban et al., 1994). Thus GSH depletion results in increased toxicity of cisplatin. GSH depletion also results in lipid peroxidation and this seems to be the prime factor that permits lipid peroxidation and impaired antioxidant enzyme activities. These observations support the conclusion that mechanism of nephrotoxicity in cisplatin treated mice is related to depletion of antioxidant systems. Thus nephroprotection by *G. lucidum* extract might be directly related to the free radical scavenging property of the extracts.

The experimental results reveal that aqueous methanol extract of *G. lucidum* could help to prevent nephrotoxicity manifested consequent to cisplatin chemotherapy. The effect is mainly due to the capacity of the extract to restore renal antioxidant defense system. Treatment of mice at a dose of 500 mg/kg body wt for a period of ten days after the implantation of tumor was found to reduce 97.7 % of tumor load. This indicates that the antioxidant activity of the mushroom extract does not interfere with its antitumor property of cisplatin. The experimental findings thus suggest the potential therapeutic use of *G. lucidum* mushroom occurring in Kerala in cancer chemotherapy.

It is believed that doxorubicin-induced cardiomyopathy, at least partially, is caused by increased oxidative stress in the heart and a great deal of evidence supports this hypothesis. Doxorubicin generates oxygen radicals in several ways. During redox cycling of doxorubicin superoxide anion radical (O_2) is formed which is then dismutated into H_2O_2 and O_2 by superoxide dismutase. Further more, doxorubicin is a very strong chelator of transition metal ions. The doxorubicin-iron complex is an even better radical generator than doxorubicin itself because doxorubicin potentiates the catalytic activity of ferrous irons in the production of HO· radicals or of reactive oxygen species that are able to initiate lipid peroxides. Doxorubicin may even release bound non-heme iron eg: from ferrous by the reduction of the iron (Minotti, 1989). In mice and rats, doxorubicin significantly increased MDA levels in myocardial tissues (Naidu et al., 2002). In this study, doxorubicin treated rats showed increase in heart tissue MDA levels with decrease in levels of SOD and glutathione peroxidase. Aqueous methanol extract of *G. lucidum* prevented the doxorubicin-induced changes in MDA and enzyme levels in the drug treated

groups. In fact there is direct correlation between GSH depletion and enhanced lipid peroxidation. Serum CK, which is used as a marker for the assessment of cardiotoxicity was higher in control animals (588.3 ± 194.0 IU/L) administered with doxorubicin alone than the group of animals pretreated with the extract (1000 mg/kg) was (186.5 ± 36.5 IU/L).

The experimental results also show the significant decrease in the activity of lipid peroxidation when animals were pretreated with the extract prior to doxorubicin administration. This indicates the capacity of the extract to restore cardiac antioxidant defense system during the onset of doxorubicin induced cardiotoxicity. The findings thus suggest potential therapeutic use of *G. lucidum* extract in the prevention of nephrotoxicity and cardiotoxicity caused by the administration of anticancer drugs.

EVALUATION OF ACUTE AND SUB-ACUTE TOXICITIES OF GANODERMA LUCIDUM

Sheena.N "Studies on the thekapeutic potential of Ganoderma lucidum P. Karst – Reishi, occurring in Kerala " Thesis. Amala Cancer Research Centre , University of Calicut, 2005

CHAPTER 7

EVALUATION OF ACUTE AND SUB-ACUTE TOXICITIES OF *GANODERMA LUCIDUM*

7.1. INTRODUCTION

Medicinal mushrooms and mushroom metabolites have notable place in the folklore throughout the world. They are traditionally used in China and Japan for medicinal and tonic properties (Wasser et al., 2000., Mizuno, 2000., Begell and Wasser, 2001., Cimerman, 1999). Species of genus *Ganoderma* P. Karst. is used in folk medicine for thousands of years in the folklore in China and persistent use of medicinal mushrooms among people of China could be considered a good evidence of their efficacy. Although there are several reports regarding the frequent use of mushrooms in the treatment of a number of disease conditions. Experimental studies, which validate the toxicity studies of mushrooms are rare. Mushrooms are promising resources of physiologically functional food and materials for the development of medicines, pharmaceutical products such as new drugs, dietary supplements and healthy beverages, cosmetic products, among others (Lomderg, et al., 2001). In the last decade medicinal mushrooms were intensively investigated for medicinal effects in *in vivo* and *in vitro* model systems, and pharmacologically active substances were identified.

Ganoderma lucidum (W. Curt.: Fr.) Karst. most commonly known as reishi, is a wood rotting fungus generally found growing on trees and stumps is prescribed by practitioners of Traditional Chinese Medicine for the treatment of neurasthenia, debility from prolonged illness, insomnia, anorexia, dizziness, chronic hepatitis, hypercholesterolemia, coronary heart disease, hypertension, altitude sickness,

fatigue, carcinoma, and bronchial cough (Ma et al., 2002). Investigations were carried out on the acute and sub acute toxicity of aqueous methanol extract of *G. lucidum* occurring in Kerala. The findings are reported in this chapter.

7.2. MATERIALS AND METHODS

7.2.1. PREPARATION OF THE EXTRACT

Aqueous methanol extract of *G. lucidum* was prepared as described in the section 3.2.1.

7.2.2. ANIMALS

Toxicity studies : Male Swiss albino mice weighing 25 ± 2 g

7.2.3. ACUTE TOXICITY STUDY

Mice were divided into three groups of six animals each. The drug was administered orally as single dose as follows (Walum, 1998).

Group 1	Normal
Group 1I	Aqueous methanol extract 500 mg/kg bodyweight
Group III	Aqueous methanol extract 2500 mg/kg bodyweight

The animals were observed for toxic symptoms and mortality for 72 h.

7.2.4. SUB-ACUTE TOXICITY STUDY

The mice were divided into three groups of six animals each. The drug was administered to animals once daily for 30 days (Parchment, 1998).

Group 1	Normal
Group II	Aqueous methanol extract 500 mg/kg bodyweight

Group III Aqueous methanol extract 1500 mg/kg bodyweight

The animals were observed for toxic symptoms and mortality. Group 1 treated with distilled water was kept as normal. One day after the last dose of extract administration animals were sacrificed. The blood was collected from the heart. Hematological parameters for total erythrocytes count (section 2.2.18), total leukocytes count (section 2.2.19) and haemoglobin (section 2.2.17) were determined. Serum was used for the determination of liver function test, transaminases, SGOT (section 2.2.10) and SGPT (section 2.2.11), alkaline phosphatase (section 2.2.12), and renal function tests such as urea (section 2.2.13) and creatinine (section 2.2.14).

7.3. RESULTS

7.3.1. ASSAY FOR ACUTE TOXICITY STUDY

The animals administered with aqueous methanol extract of *G.lucidum* did not produce any external symptoms of toxicity or mortality up to the dose of 2500 mg/kg bodyweight orally.

7.3.2. ASSAY FOR SUB-ACUTE TOXICITY STUDY

In sub acute toxicity studies, treatment of extract also did not produce any statistically significant change in the hematological or biochemical parameters when compared to the normal group of animals. However, treatment of aqueous methanolic extract (1500 mg/kg bodyweight) for 30 days produces a slight increase

in the WBC, RBC and Hb count (Table 7.1). Similarly the administration of extract did not produce any significant changes in the liver function tests (SGOT, SGPT) and renal function tests (urea and creatinine). The SGOT and SGPT activities in the normal and the extract treated (1500 mg/kg body wt) were (35. 2 ± 1.8 IU/l and 37.5 ± 2.7 IU/l) and (100.2 ± 6 IU/l and 102.0 ± 3.2 IU/l) (Table 7.2). The ALP level in the normal and the extract treated (1500 mg/kg body wt) was 70.7 ± 16.4 IU/l and 74.5 ± 6.7 IU/l (Table 7.2). The normal animals showed serum urea level 69.4 ± 8.7 mg/dl and creatinine level 0.43 ± 0.02 mg/dl, while the extract (1500 mg/kg body wt) treated group showed 78.0 ± 8.1 mg/dl and 0.44 ± 0.01 mg/dl (Table 7.3).

Table 7.1. Effect of aqueous methanol extract of *G. lucidum* on total WBC, RBC counts and haemoglobin concentration.

Groups	Treatments (mg/kg)	WBC (Cells/ µl)	RBC (Cells/ µl))	Hb (g/dl)
Normal	-	10680 ± 514	71.4 ± 4.5	16.6 ± 0.9
G. lucidum Extract	500	10744 ± 330	71.6 ± 2.3	16.4 ± 0.8
	1500	11228± 366	76.2 ± 3. 0	16.8± 0.54

Values are mean ± S. D. n= 6 animals.

Treatments are not significantly different

Table 7.2. Effect of aqueous methanol extract of *G. lucidum* on serum SGOT, SGPT and ALP levels.

Groups	Treatments (mg/kg)	SGOT (IU/l)	SGPT (IU/l)	ALP (IU/l)
Normal	-	35.2 ± 1.8	100. 2 ± 6.0	70.7 ± 16.4
G. lucidum Extract	500	35.8 ± 2.3	98.1 ± 7.0	70.1± 14.3
	1500	37.5 ± 2.7	102.0 ± 3.2	74.5± 6.7

Values are mean ± S. D. n= 6 animals.

Treatments are not significantly different

Table 7.3. Effect of aqueous methanol extract of *G. lucidum* on serum urea and creatinine concentration

Groups	Treatments (mg/kg)	Urea (mg/dl)	Creatinine (mg/dl)
Normal	-	69.4 ± 8.7	0.43 ± 0.02
G. lucidum Extract	500	73.8 ± 5.6	0.44 ± 0.03
	1500	78.0± 8.1	0.44 ± 0.01

Values are mean ± S. D. n= 6 animals.

Treatments are not significantly different

7.4. DISCUSSION

Results of the study reveal that aqueous methanolic extract of *G. lucidum* showed no signs of toxicity. The extract administered up to 1500 mg/kg body wt. was not lethal to animals. Similarly the administration of the extract is not found to produce any hematological toxicity. Liver function and renal function tests show no significant change in the serum SGOT, SGPT, ALP and also serum urea and creatinine levels in the extract treated group from the normal group support the safety of the extract for therapeutic use.

Many pharmaceutical substances with potent and unique valuable properties have been isolated recently from mushrooms and distributed worldwide (Wasser et al., 2000). Biologically active polysaccharides are the best-known mushroom derived substances with antitumor properties (Reshetnikov et al., 2001). *G. lucidum* is most commonly used in traditional Chinese medicine for the treatment of various diseases (Ma et al., 2002). But a clear picture of its toxicokinetics is still obscure and the present study is an attempt to validate the safety of *G. lucidum* extract. The current experimental findings reveal non- toxic nature of *G. lucidum* occurring in Kerala and its potential therapeutic use.

PRELIMINARY PHYTOCHEMICAL EXAMINATION OF GANODERMA LUCIDUM

Sheena.N "Studies on the thekapeutic potential of Ganoderma lucidum P. Karst – Reishi, occurring in Kerala " Thesis. Amala Cancer Research Centre , University of Calicut, 2005

CHAPTER 8

PRELIMINARY PHYTOCHEMICAL ANALYSIS OF *GANODERMA LUCIDUM*

8.1. INTRODUCTION

Plant kingdom represents a virtually untapped reservoir of new chemical compounds, some with extraordinary biodynamics, some providing novel bases on which synthetic chemists may build even more interesting structures (Schultes, 1974). Plants have been particularly valuable in the search for drugs. They have been exploited in the discovery of many classes of drugs. They have provided important leads which led to the development of novel classes of compounds. Fungi have been one of the most significant groups of plants in human affairs. They have been source of large number of life saving drugs. Mushrooms are macro fungi. Recent investigations have shown that mushrooms are important source of novel pharmaceutical products (Wasser et al., 2000). *Ganoderma* species were demonstrated to possess many interesting biological activities. *Ganoderma lucidum* has been used as a traditional herbal medicine in China for the treatment of diseases including chronic hepatopathy, hypertension, hypoglycemia, cancer etc. Accumulated evidences support that these biological activities of *G. lucidum* are attributed mainly to the triterpenes and polysaccharide constituents of this mushroom (Gao et al., 2004).

Investigations were thus carried out to examine the major constituents of the fruiting bodies of *G. lucidum* occurring in Kerala. The chloroform, methanol and aqueous extract of the mushroom were analyzed by various phytochemical methods. The results of the investigations are reported in this chapter.

8.2. MATERIALS AND METHODS

8.2.1. PREPARATION OF THE EXTRACTS

The sporocarps were cut into small pieces, dried at 40-50^0 C for 48 hr and powdered. Two hundred gram samples of the powdered material were extracted with chloroform using a Soxhlet apparatus for 8-10 hrs. The extraction was repeated again for the same period of time. The chloroform extracts were combined and concentrated to 100ml at low temperature under vacuum. The extract was then dried with anhydrous sodium sulphate. The solvent was completely evaporated at low temperature under vacuum. The residue thus obtained was named as chloroform extract (1.6g).

The powder after chloroform extract was air dried and then extracted with methanol using a Soxhlet apparatus for 8-10 hrs. The extraction was repeated again. The methanol extracts were combined and the solvent was completely evaporated at low temperature. The residue obtained was named as methanol extract (3.0g).

The powder after methanol extraction was air dried and then extracted with hot water (70-80^0 C) for 8-10 hrs twice. The extracts combined and evaporated on a water bath. The residue obtained was named as aqueous extract (4.08g).

8.2.2. PREPARATION OF TLC PLATE, SAMPLE APPLICATION AND ANALYSIS

25 g of silica gel G was suspended in 50ml distilled water. The slurry thus formed was poured uniformly on to a clean TLC plates, air dried and then kept in hot air oven for 1hr at 110^0 C for activation. The samples (chloroform extract, methanol extract and aqueous extract) were dissolved in a small amount of chloroform- methanol and spotted on the TLC plates 2 cm above the base of the plates. Solvent system (chloroform: methanol, 90: 10) was poured into a TLC jar and left undisturbed for half an hour for saturation with vapours. The plate was then placed in the solvent at 45^0 angles and allowed the solvent to run.

After complete solvent run the plate was removed from the jar, the solvent front was marked and allowed to dry at room temperature. TLC analyses of the sample were also carried out using toluene: ethyl acetate (93:7) and ethyl acetate: methanol: water (50:45:5). n-butanol: aetic acid: water (4:1:5) as solvent systems.

8.2.3. EXAMINATION OF TLC PLATES

The plates were first examined under UV for detecting fluorescent spots. The plates were then sprayed with specific spray reagents to detect components such as polysaccharides, terpenes and flavonoids.

8.2.4. SPRAY REAGENTS

8.2.4.1. ANISALDEHYDE-SULPHURIC ACID REAGENT (AS)

0.5 ml anisaldehyde is mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5ml concentrated sulphuric acid in that order. The reagent has only limited stability, and is no longer usable when the color has turned to red violet. The plate is sprayed with about 10 ml, heated at 100^0 C for 5-10 min, then evaluated in visible or UV-365nm for the detection of terpenoid (Wagner et al., 1984).

8.2.4.2. ANTIMONY (III) CHLORIDE REAGENT ($SbCl_3$)

20% solution of antimony trichloride is chloroform. The plate was sprayed with 15-20 ml reagent and then heated for 5-6 min at 100° C. Evaluation in visible or UV-365nm for the detection of glycosides (Wagner et al., 1984).

8.2.4.3. IODINE VAPOUR

About 10g solid iodine was placed in the bottom of a jar and allowed to get saturated with iodine vapour. The plate was then placed in the jar. Most of the organic compound developed yellow to brownish yellow color when exposed to iodine vapour.

8.2.4.4. VANILLIN-SULPHURIC ACID REAGENT (VS)

10 % Ethanolic sulphuric acid (solution 1)

1% Ethanolic vanillin (solution II)

The plate was sprayed vigorously with 10 ml solution 1, followed immediately by 5-10 ml solution II. After heating at 110° C for 5-10 minutes under

observation, the plate was evaluated for the detection of terpenes, polysaccharides and other organic compounds (Wagner et al., 1984).

8.2.5. SPOT TEST FOR THE DETECTION OF TERPENES

The extracts were dissolved in methanol and a drop of extract was placed on a clean glass slide and a drop of acetic anhydride followed by a drop of concentrated sulphuric acid was placed on the extract. The changes in the color were noted.

8.2.6. ANTHRONE REAGENT

Polysaccharide component of the extract was detected by anthrone reagent. The polysaccharides react with concentrated sulphuric acid to give a derivative of furfural, which on reaction with anthrone produce a deep bluish-green color (Yemn and Wills, 1954).

PREPARATION OF ANTHRONE REAGENT

Anthrone	200mg
95% sulphuric acid	100 ml

Dissolve 200mg anthrone in 100 ml 95% ice-cold sulphuric acid

ANTHRONE TEST

To a small amount of extracts dissolved in 1 ml distilled water and 4ml anthrone reagent was added. The reaction mixture in a test tube was heated on a boiling water bath for 10 minutes. The tube was cooled rapidly and the absorbance of dark green color was measured at 630 nm.

8.2.7. HPTLC ANALYSIS

HPTLC analysis of the chloroform, methanol and aqueous extracts was carried out. HPTLC was performed using silica gel F254 plates. The extracts (5µl) were applied by means of micro syringes in the form of bands. The plates after application of the samples were developed in twin trough chamber. Chloroform: methanol was used as solvent system. The plates were air dried after development and scanned under UV (254 nm) or sprayed with vanillin sulphuric acid reagent and then scanned. Camag TLC scanner was used for scanning. The HPTLC profile was obtained with Desaga video documentation unit III.

8.3. RESULT

TLC analysis of the chloroform extract showed three spots under UV and four spots when sprayed with vanillin sulphuric acid. Three spots each under UV and vanillin sulphuric acid was detected in methanol extract and only two spots each under UV and spraying with vanillin sulphuric acid were detected in aqueous extract (Table 10.1). TLC analysis showed the presence of terpenes with chloroform extract. The examination of TLC plates showed blue colored spots with vanillin sulphuric acid reagent and this indicated the probable presence of terpenes in chloroform extract. HPTLC analysis also showed a similar blue colored band with identical Rf value in the chromatogram under UV and with vanillin sulphuric acid reagent in the chloroform extract. The Rf values of the spots detected by TLC and HPTLC analyses are given in the tables (8.1and 8.2).

The spot test of terpene also confirmed the result that on reacting with acetic anhydride and concentrated sulphuric acid gave green color, which immediately changed into blue. This reaction of play of colors is characteristic to terpenes. The reaction thus indicated that one of the constituent of the extract might be terpenes. However, the major constituent of the extract was polysaccharides. This observation was based on the reaction of the extract with anthrone reagent. The anthrone test showed typical green color of the reaction mixture.

The results of the present investigation indicated the major constituents of chloroform extract might be terpenoids. However, the major constituent of the aqueous extract appears to be polysaccharides. HPTLC analyses confirm this conclusion.

Table 8.1. Thin Layer Chromatography (TLC) analysis of the extracts on silica gel G using chloroform: methanol (90: 10) as solvent system.

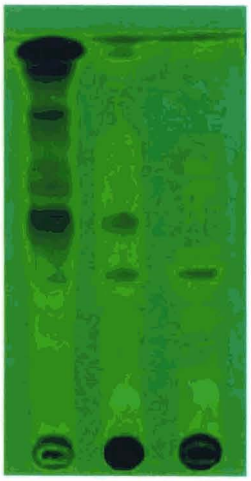
Extract	Detection under UV Rf value of the spot	Detection by Vanillin-H_2SO_4 spray reagent Rf value of the spot
Chloroform extract	0.05, 0.54, 9.6	0.05, 0.54, 0.69, 0.90
Methanol extract	0.05, 0.47,0.54,	0.05, 0.47, 0.54
Aqueous extract	0.05, 0.45	0.05, 0.47

Table 8.2. High Performance Thin Layer Chromatography (HPTLC) of the extracts using silica gel G as stationary phase and chloroform: methanol (90: 10) as mobile phase.

Extract	Detection under UV Rf value of the spot	Detection by Vanillin-H_2SO_4 spray reagent Rf value of the spot
Chloroform extract	0.06, 0.45, 0.54, 0.8, 0. 96	0.06, 0.47, 0.54, 0.61, 0.69, 0.8, 0.90
Methanol extract	0.06, 0.45, 0.56,	0.06, 0.36, 0.54
Aqueous extract	0.06, 0.45	0.06, 0,36

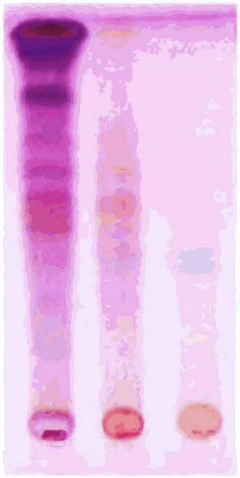
Figure 8.1 HPTLC analysis of *G. lucidum* extracts.

A- chloroform, B- methanol, C- aqueous



A B C

Under UV 254 nm



A B C

After spraying with vanillin sulphuric acid

8.4. DISCUSSION

The results of the present investigation indicate that fruiting bodies of *G. lucidum* occurring in Kerala contain several chemical constituents. The TLC and HPTLC analyses confirm this conclusion. However, the major constituents appear to be terpenoids and polysaccharides. Over 300 reports have been published on the chemical constituents of *Ganoderma*. More than 180 chemical constituents have been isolated from this mushroom, which includes polysaccharides, triterpenes, ergosteroids, fatty acids, proteins/peptides, polyphenols, steroids, ligans, ganomycin, vitamins, trace elements etc. Polysaccharides and triterpenes are the major constituents (Gao and Zhou, 2003). Polysaccharides of *G. lucidum* are among the major sources of its pharmacologically active constituents. More than 100 types of polysaccharides have been isolated from *G. lucidum* and many of them have molecular weight ranging from $4x\ 10^5$ to $1x\ 10^6$ (Gao et al., 2004). The polysaccharides in particular $\alpha\ \beta$. D- glucans are considered to be major component that contributes to its bioactivity. Triterpenoids are the second major constituent of G. *lucidum.* Currently 130 triterpenoids have been isolated from the fruiting body and mycelium of *G. lucidum*. They are highly oxygenated compounds. Many triterpenoids for *G. lucidum* have been shown to have antioxidant, antitumor, immunomodulating and hepatoprotective activities.

SUMMARY AND CONCLUSION

Sheena.N "Studies on the thekapeutic potential of Ganoderma lucidum P. Karst – Reishi, occurring in Kerala " Thesis. Amala Cancer Research Centre , University of Calicut, 2005

SUMMARY AND CONCLUSION

The relationship between mushroom and man can be traced far back into antiquity. Mushrooms have played an important role in human history: as food as poison, as medicine, and in folklore, as legends and in religion (Molitoris, 2001). Mushrooms are nutritionally functional food and a source of physiologically beneficial and non-toxic medicines (Wasser and Weis, 1999). They represent a major and as yet largely untapped source of powerful new pharmaceutical products. Mushrooms such as Shiitake, Coriolus, Reishi, and Maitake, have been found to possess pharmaceutical substances with potential and unique properties. (Reshetnikov et al., 2001., Wasser and Weis,1999). The active components found in mushrooms, beta-glucan polysaccharides, wake up the immune system and encourages it to work more efficiently and effectively. Many investigators identified and isolated polysaccharides from mushrooms, such as lentinan, schizophyllan, and protein-bound PSK (Krestin) and PS-peptide (Chang, 1993., Reshetnikov et al., 2001., Wasser and Weis,1999., Mizuno, 2000) .

Ganoderma lucidum commonly known as Reishi or Ling Zhi grows in many parts of the world and it has been used in folk medicine in China and Japan for more than 4000 years for a wide range of human ailments. In China, Reishi is known as the "Plant of immortality" and is the key ingredient in the treasured *elixir of life*. Reishi mushroom has been used as part of Chinese and Japanese medicine, especially in the treatment of chronic hepatitis, nephritis, hepatopathy, neurasthenia, arthritis, bronchitis, asthma, gastric ulcer, anti-inflammatory, antibacterial, antioxidant, antitumor, antiviral, blood pressure lowering, bone marrow enhancing,

cardiotonic, anti – HIV and prevention of insomnia (Eo et al., 1999., Kim and Kim, 1990., Liu, 1999). *Ganoderma* species are rich in organic compounds such as polysaccharide, amino acids, triterpenes, ascorbic acid, sterols, lipids, alkaloids, and trace minerals (Ma et al., 2002., Koyama et al., 1997., Jong and Birmingham, 1992., Chyr and Shiao, 1991). However, the extensive range of traditional medicinal treatments with *Ganoderma* has not yet been fully substantiated by modern scientific standards.

A number of species of this medicinal mushroom are found in Kerala (Leelavathy and Ganesh, 2000). The main objective of the present investigation was to evaluate the therapeutic potential of *Ganoderma lucidum*. P. Karst. Reishi, occurring in Kerala. Investigations were undertaken to evaluate the antioxidant, anti-inflammatory, antitumor, chemopreventive and hepatotoprotective activities of the extracts of *G. lucidum*. In addition to these studies, protective effect of *G. lucidum* extract on, prevention of anticancer drug induced organ toxicity was also investigated. The evaluation of acute and sub acute toxicities of the extract and its phytochemical analysis were also under taken.

There has been considerable interest on the role of reactive oxygen species (ROS) as mediators of tissue injury in pathophysiology of human diseases. Free radicals trigger oxidative damage to protein, membrane lipids and DNA, thereby destroying neurons. Epidemiological studies have shown that low antioxidant levels are associated with increased incidence of several diseases. Hence antioxidants have significant importance in prevention of a number of human ailments. The *in vitro*

antioxidant activities of ethyl acetate, methanol and aqueous extracts of *G. lucidum* were studied. Ethyl acetate and methanol extracts showed significant scavenging activity of superoxide and hydroxyl radical and inhibition of lipid peroxidation activities. However, aqueous extract showed only superoxide and hydroxyl radical scavenging activity, but not lipid peroxidation inhibiting activity. Nevertheless, none of the extract showed nitric oxide scavenging activity. The concentration of methanol extract of *G. lucidum* required to scavenge 50 % superoxide anion and hydroxyl radical generated (IC_{50}) was found to be 155.5 ± 25.0 and 229.0 ± 16.0 µg/ml respectively. The methanol extract of *G. lucidum* was effective in inhibiting the lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate. The lipid peroxidation inhibiting activity (IC_{50}) of the extract 312.22 ± 12 µg/ml was higher than ethyl acetate extract. The methanol extract of the mushroom when administered to animals for a prolonged period was found to manifests or enhance *in vivo* antioxidant defense without causing any apparent toxicity.

Although inflammation is a protective response intended to eliminate initial cause of all injury, it has considerable potential to cause harm. The anti-inflammatory activity of methanol extract of *G. lucidum* was evaluated using various experimental models. Application of croton oil, which contains TPA, induced inflammation on skin and the effect of extract to inhibit the croton oil induced inflammation on mouse skin was studied. The application of the extract at a concentration of 20 mg showed 42.4% inhibition of skin edema and 41.7% inhibition of lipid peroxidation. Histopathological observation also supported the

effectiveness to inhibit mouse skin edema by the extract. The extract also showed significant antinociceptive activity. Antinociceptive activity is correlated with the analgesic activity. The methanol extract at a concentration of 1000 mg/kg inhibited 40.6% abdominal constrictions compared to the standard reference drug, diclofenac (10mg/kg i.p) which showed 64.4% inhibition. Anti-inflammatory activity of the extract against carrageenan induced acute and formalin induced chronic inflammatory models in mice was also studied. The extract at a dose of (1000 mg/kg body wt) inhibited 58.3 % acute inflammation induced by carrageenan and 59.6 % chronic inflammation induced by formalin. This indicated that extract of *G. lucidum* showed significant activity against both acute and chronic inflammations induced by carrageenan and formalin respectively.

Cancer is the second largest cause of death in the world today. Breast cancer is one of the most serious and widespread cancer in both developing and developed countries. Chemical carcinogens are considered to be a major cause of mammary carcinoma. Investigations were thus carried out to find out the antitumor and chemopreventive activities of *G. lucidum*. Antitumor activity of the methanolic extract of the mushroom was determined using EAC induced ascites and DLA induced solid tumor model in mice. The chemopreventive activity of the extract was determined by Dimethyl benz (*a*) anthracene (DMBA) induced mammary tumor model in rats. Methanol extract of *G. lucidum* was not found to show tumor reducing activity against ascites tumor induced by EAC cells at a dose of 1000 mg/kg body weight. However, the extract when administered prior to tumor

induction possessed significant antitumor activity against solid tumor. At a dose of 1000 mg/kg the extract prevented the tumor development by 87.1%. The extract at a concentration of 500 and 1000 mg/kg when administered for 10 consecutive days after tumor development showed 61.8 % and 72.8% tumor growth regression respectively as compared to control. The aqueous methanol extract of *G. lucidum* was also highly effective against DMBA-induced mammary carcinoma. Number of animals with tumor (tumor incidence) in control group was 10/10, and the extract treatment at a concentration of 250 and 500 mg/kg reduced the incidence to 7/10 and 4/10 respectively. The treatment with the extract also reduced tumor multiplicity (tumor/animal). Tumor yield of 2.3 tumors were observed in DMBA treated animals and the tumor yield was reduced to 1.4 and 1 respectively in animals treated with the extract at a dose of 250 and 500 mg/kg body weight. The extract at a concentration of 500 mg/kg prevented the increase in tumor volume by 73.8% and tumor weight by 74.7 % respectively. The results of the current investigations indicate methanol extract of *G. lucidum* occurring in Kerala possessed significant antitumor and chemopreventive activities.

The liver protects the body from potentially injurious substances absorbed from the intestinal tract and also from bye products of metabolism. Liver diseases are one of the serious health problems affecting a vast majority of population in the world. Studies on the hepatoprotective experimental models indicate that the principal cause of CCl_4 –induced liver injury is due to lipid peroxidation by free radical derivatives of CCl_4 (Recknagel, 1967., Noguchi et al., 1982). CCl_4 is first

metabolized by cytochrome P-450 in the liver endoplasmic reticulum to higher reactive CCl_3 radical. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this damage (Lin et al ., 1995). Administration of methanol extract of *G. lucidum* at a dose of (500, 1000 mg/kg body weight) significantly lowered the elevated levels of SGPT, SGOT and ALP by CCl_4 in a dose dependent manner. The extract also significantly enhanced hepatic antioxidant defense in CCl_4 intoxicated animals. The depleted levels of GSH and activities of GST, GPX and CAT consequent to the intoxication of CCl_4 were found to increase consequent to the extract treatment. The investigation reveals that methanol extract of *G. lucidum* is capable to prevent CCl_4 induced hepatotoxicity in a dose dependent manner. The amelioration of liver toxicity by the extract is evident from its effect on serum transaminases. Histopathological observations also support this conclusion.

Chemotherapy is one of the most effective treatment modalities of cancers. A large number of chemotherapeutic agents developed from synthetic or natural sources are clinically used for the treatment of cancer. Cisplatin (cisplatinum II diamine dichloride) and doxorubicin are extensively used drugs for the treatment of cancer. However, higher doses therapy of cisplatin have been found to cause severe nephrotoxicity and prolonged use of doxorubicin resulted in cardiotoxicity. The investigations were carried out to find out the effect of *G. lucidum* on the prevention of toxicities induced by anticancer drugs. The nephrotoxicity and cardiotoxicity induced by these drugs are reported to mediate through free radicals. Treatment of

G. lucidum extract (250 and 500 mg/kg body weight, p.o) prior to cisplatin administration prevented the depletion of renal antioxidants and elevation of serum creatinine and urea. The restoration of renal SOD, CAT and GPX activities by the pretreatment of *G. lucidum* extract suggested that the extract was capable of preventing the depletion of antioxidant defense. Administration of *G. lucidum* extract decreased the elevated levels of MDA in animals treated with cisplatin. The extract also increased GSH levels in animals treated with cisplatin. The present investigation indicated that pretreatment of *G. lucidum* extract was able to ameliorate the nephrotoxicity caused by cisplatin without interfering with the antitumor efficacy of the drug. The methanol extract of *G. lucidum* also rendered protection against cardiotoxicity induced by doxorubicin. The extract restored the decreased activity of SOD and GPX and inhibited the enhanced lipid peroxidation caused by doxorubicin treatment. Administration of extract (500 and 1000 mg/Kg) significantly lowered the levels of CK activity in a dose dependent manner. The results thus indicated that methanol extract of *G. lucidum* significantly protected from the toxicities caused by cisplatin and doxorubicin treatments. The findings suggest the potential use of *G. lucidum* extract in complementary cancer therapy.

The acute and sub-acute toxicities of the aqueous methanol extract of *G. lucidum* were also studied. The acute and sub-acute toxicity studies indicated that the extract did not show any toxic effect at a dose of 2500 mg/kg body weight and 1500 mg/kg body weight respectively. The liver function and renal function tests and haematological parameters after 30 days of the administration of the extract

supported this conclusion. The results thus revealed the safety of *G. lucidum* extract for therapeutic use.

CONCLUSION

The experimental findings indicate that the methanol extract of *G. lucidum* occurring in Kerala possessed significant medicinal properties such as antioxidant, anti-inflammatory, antinociceptive, antitumor, nephroprotective, cardioprotective, hepatoprotective and chemopreventive activities. The profound antioxidant activity of *G. lucidum* extract explains at least in part the mechanism of its anti-inflammatory, antitumor, hepatoprotective, nephroprotective, and cardioprotective properties.

The preliminary phytochemical examination indicates that major constituents of *G. lucidum* extract are terpenes and polysaccharides. The presence of immune stimulating polysaccharides might be responsible for the significant anticancer activity of this mushroom. The extract of *G. lucidum* appears to be safe for oral administration as it did not display any toxic effect at comparatively high dose.

In conclusion, the present investigations reveal that methanol extract of *G. lucidum* from Kerala possessed significant medicinal and pharmacological properties. The findings suggest the potential therapeutic use of this mushroom and its metabolites for the prevention and control of several diseases including cancer.

BIBLIOGRAPHY

Agrawal, A. and Kale, R. K. (2001). Radiation induced peroxidative damage: Mechanism and significance. *Ind. J. Exp. Biol.* 39, 291-309.

Ahluwalia, G. S., Jayaram, H. N., Plowhan, J. P., Cooney, D. A. and Johns, D. G. (1984). Studies on the mechanism of activity of 2-β-D- ribofuranosyl thiazol-4 carboxamide. *Biochem. Pharmacol.* 33, 1195-1203.

Ahmadiani, A., Hosseiny, J., Semmanian, S., Javan, M., Saeedi, F., Kamalinejad, M. and Saremi, S. (2000). Antinociceptive and anti-inflammatory effects of *Flaeagnus angustifolia* fruit extract. *J. Ethnopharmacol.* 72, 287-292.

Ajith, T. A. and Janardhanan, K. K. (2001). Antioxidant and Anti-inflammatory activities of methanol extract of *Phellinus rimosus* (Berk) Pilat. *Ind. J. Exp. Biol.* 39, 1166-1169.

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

Ajith, T. A., Jose, N. and Janardhanan, K. K. (2002). Amelioration of cisplatin induced nephrotoxicity in mice by ethyl acetate extract of a polypore fungus, *Phellinus rimosus*. *J. Exp. Clin. Cancer. Res.* 21, 213-217.

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

Ames, B. N. (1989). Endogenous oxidative DNA damage, aeging and cancer. *Free Radical. Res. Commun.* 7, 121-128.

Anchel, M., Harvey, M. A. and Robbins, W. J. (1950). Antibiotic substance from Basidiomycetes. VII. *Clitocybe illudens. Proc. Natl. Acad. Sci.* U. S. A. 36, 300-305.

Argaez, A. N. G., Apan, T. O. R., Delgado, H. P., Velazquez, G. and Vazquez, M. M. (2000). Anti-inflammatory activity of Coumarins from *Decatropis bicolor* on TPA ear mice model. *Planta. Med.* 66, 279-281.

Arif, J. M., Smith, W. A. and Gupta, R. C. (1999). DNA adduct formation and persistence in rat tissues following exposure to the mammary carcinogen dibenzo [*a,l*] pyrene. *Carcinogenesis.* 20, 1147-1150.

Arif, J. M., Gairola, C. G., Kelloff, G. J., Lubet, R. A. and Gupta, R. C. (2000). Inhibition of cigarette smoke related DNA adducts in rat tissues by indole-3-carbinol. *Mutat. Res.* 452, 11-18.

Aruoma, O. I., Halliwell, B., Gajewski, E. and Dizdaroghe, M. (1991). Copper- ion dependent damage to the bases in DNA in the presence of hydrogen peroxide. *Biochem. J.* 273, 601-604.

Athar, M. (2002). Oxidative stress and experimental carcinogenesis. *Ind. J. Exp. Biol.* 40, 656-667

Babior, B. M. and Woodman, R. C. (1990). Chronic granulomatous disease. *Semin. Hematol.* 27, 247-259.

Babitskaya, V. G., Puchukova, T. A., Scherba, V. V. and Osadchaya, O. V. (2001). Biologically active substances of mycelia and fruiting bodies of mushrooms *Lentinus* Fr. And *Pleurotus* (Fr.) P. Karst. *Int. J. Med. Mushr.* 3, 106.

Babu, E., Gopalkrishnan, V. K., Sriganth, N. P., Gopalkrishnan, R. and Sakthisekhran, D. (1995). Cisplatin induced nephrotoxicity and the modulating effect of glutathione ester. *Mol. Cell. Biochem.* 144, 7-11.

Badalyan, S. M. (2001). Higher Basidiomycetes as prospective objects for mycopharmacological research. *Int. J. Med. Mushr.* 3, 108.

Ban, M., Hettich, D. and Huguet, N. (1994). Nephrotoxicity mechanism of cisplatinum (II) diamine dichloride in mice. *Toxicol. Lett.* 71, 161-168.

Banerjee, S., Ramos, C. B. and Aggarwal, B. (2002). Suppression of 7,12-Dimethylbenz(*a*)anthracene induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-κB, cyclogenase 2, and matrix metalloprotease 9. *Cancer. Res.* 62, 4945-4954.

Bansal, M. R., Jain, P. K., Gupta, K. G. and Khanna, D. (1992). Protective and therapeutic efficacies of protein A on 7, 12-Dimethylbenz(*a*)anthracene induced rat mammary adenocarcinoma. *J. Envtal. Pathol. Toxicol. Oncol.* 11(1), 43-46.

Beal, M. F. (1997). Oxidative damage in neurodegenerative diseases. *Neuroscientist.* 3, 21-27.

Beers, R. F. and Sizer, I. W. (1952). A spectrophotometric method for measuring the break down of hydrogen peroxide by catalase. *J. Biol. Chem.* 195, 133- 140.

Begell, W. and Wasser, S. P. (2001). The first international Journal of medicinal mushrooms. *Int. J. Med. Mushr.* 3, 115.

Berenblum, I. (1941). The cocarcinogenic action of croton resin. *Cancer. Res.* 1, 44-48.

Bishayee, S. and Balasubramanian, A. S. (1979). Lipid peroxide formation in rat brain. *J. Neurchem.* 18, 909-920.

Block, G. (1992). The data support a role for antioxidants in reducing cancer risk. *Nutr. Rev.* 50, 207-213.

Block, G., Patterson, B. and Subar, A. (1992). Fruit, Vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer.* 18, 1-29.

Bodenner, D. L., Dedon, P. C., Keng, P. C., Katz, J. C. and Borch, R. F. (1986). Selective protection against cisplatin induced toxicity in kidney, gut and bone marrow DDTC. *Cancer. Res.* 46, 2751-2755.

Boone, C. W., Kelloff, G. J. and Malone, W. E. (1990). Identification of candidate cancer chemopreventive agents and their evaluation in animal models and human clinical trials: A review. *Cancer. Res.* 50, 2-9.

Boyness, J. W. (1991). Role of oxidative stress in development of complications in diabetes. *Diabetes.* 40, 405-411.

Brod, J. and Sirota, J. H. (1980). Non-protein nitrogen, urea, urate, creatine and creatinine. In: *Practical clinical biochemistry.* Varley, H., Gowenlock, A. H., Bell, M. (eds). Vol 1. Vth edn,William Heinmann Medical Books Ltd. London, pp 478-480.

Brooks, P. M. and Day, R. O. (1991). Nonsteroidal anti-inflammatory drugs: differences and similarities. *N. Engl. J. Med.* 324, 1716-1719

Cabello, G., Valenzuela, M., Vilaxa, A., Duran, V., Rudolph, I., Hrepic, N. and Calaf, G. (2001). A rat mammary tumor model induced by the organophosphorous pesticides parathion and malathion, possibly through acetylcholinesterase inhibition. *Envtal. Health. Perspect.* 109, 471-479.

Campbell, W. B. (1990). Lipid-derived autocoids: eicosanoids and platelet activating factor. In: *Goodman and Gillman's The Pharmacological basis of therapeutics*. Goodman Gillman, A., Rall, T. W., Nies, A. S., Taylor, P. (eds). 8 th edn. Pergamon Press, New york, pp 600-617.

Cerutti, P. A. (1994). Oxy-radicals and cancer. *Lancet.* 344, 862-863.

Cerutti, P., Ghosh, R., Oya, Y. and Amstad, P. (1994). The role of the cellular antioxidant defense in oxidant carcinogenesis. *Environ. Health. Perspect.* 102, 123-130.

Chang, S. T. (1993). Mushroom biology: the impact on mushroom production and mushroom products. Keynote lecture. The first International conference on mushroom biology and mushroom products, 23-26 August, Hong Kong.

Chatterjee, S., Laloraya, M. and Kumar, G. P. (1994). Free radical bombing of spermatozoa in spermatic granulosa: an attempt to prevent autoimmune switch on. *Biochem. Biophys. Res. Commun.* 201, 472-477.

Chaudari, A. R. (2000a). RBC count. In: *A Text Book of Practical Physiology.* Chaudari, A. R. (ed). Paras Medical Publisher, Hyderabad, India. 97-103.

Chaudari, A. R. (2000b). Total leukocyte count. In: *A Text Book of Practical Physiology*. Chaudari, A. R. (ed). Paras Medical Publisher, Hyderabad, India. 97-103.

Chihara, G., Hamuro, J., Maeda, Y. Y., Arai, Y., Fukoka, F. (1970). Fractionation and purification of the polysaccharides with marked antitumor activity, especially Lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom). *Cancer. Res.* 30, 2776-2781.

Childs, A. C., Sharon, P. L., Dirks, A. J., Philips, T. and Leeuwenburgh, C. (2002). Doxorubicin treatment *in vivo* causes cytochrome *c* release and cardiomyocytes apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl_2: Bax ratio. *Cancer. Res.* 62, 4592-4598.

Chow, C. K. (1996). Vitamins and related compounds with increased intake provide greater protection against oxidative damage. *Nutrition.* 12, 822-823.

Christou, M., Savas, U., Spink, D. C., Gierthy, J. F. and Jefcoate, C. R. (1994). Co expression of human CYP1A1 and a human analog of cytochrome P450-EF in response to 2, 3, 7, 8- tetrachloro-dibenzo-p-dioxin in the human mammary carcinoma-derived MCF-7 cells. *Carcinogenesis.* 15: 725-732.

Chyr, R. and Shiao, M. S. (1991). Liquid chromatographic characterization of the terpenoids patterns in *Ganoderma lucidum* and related species. *J. Chromat.* 542, 327-336.

Cimerman, G. N. (1999). Medicinal value of the genus *Pleurotus* (Fr.) P. Karst. (Agaricales S. I., Basidiomycetes). *Int. J. Med. Mushr.* 1, 69-80.

Collins, T. (1999). Acute and chronic inflammation. In: *Text Book of Robbins Pathologic Basis of Diseases*. Cotran, R. S., Kumar, V. and Collins, T. (eds). 6 th edn, W. B. Sounders Company, Philadelphia, pp 50-51.

Conner, E. M. and Grisham, M. B. (1996). Inflammation, free radicals and antioxidants. *Nutritions*. 12, 274

Cotran, R. S., Kumar, V. and Collins, T. (1999). Neoplasia. In: *Text Book of Robbins Pathologic basis of Diseases*. Cotran, R. S., Kumar, V. and Collins, T. (eds). 6 th edn, W. B. Sounders Company, Philadelphia, pp 270-310.

Cuzzocrea, S., Riley, D. P., Caputi, A. P. and Salvemini, D. (2001). Antioxidant therapy: a new pharmacological approach in shock, inflammation and ischemia/reperfusion injury. *Pharmacol. Rev.* 53, 135-139

Davies, K. J. A. (1995). In free radicals and oxidative stress: *Environment, Drugs and food additives*. Portland Press, London, pp 1-31.

Dhawan, D., Balasubramanian, S., Amonkar, A. S. and Singh, N. (1999). Chemopreventive effect of 4- dimethyl epidophyllotoxin on DMBA/TPA- induced mouse skin. *Carcinogenesis*. 20 (6), 997-1003.

de Souza, M. M., Madeira, A., Berti, C., Krough, R., Yunes, R. A. and Cechinel Filho, V. (2000). Antinociceptive properties of the methanolic extract obtained from *Ipomoea pes-caprae* (L.) R. Br. *J. Ethnopharmacol.* 69, 85-90.

Devasagayam, T. P. A. and Kamath, J. P. (2002). Biological significance of singlet oxygen. *Ind. J. Exp. Biol.* 40, 680-692.

De Vita, V. T. (1978). The evaluation of therapeutic research in cancer. *N. Eng. J. Med.* 298, 907-910.

Didukh, M. Y. (2001). Species diversity and ecology of medicinal and edible mushrooms (Agaricales S.I.) of the Kiev green zone (Ukraine). *Int. J. Med. Mushr.* 3, 133.

Drabkin, D. L. and Austin, J. M. (1932). Spectrophotometric studies, spectrometric constants for common haemoglobin derivatives in human, dog, and rabbit blood. *J. Biol. Chem.* 98, 719-733.

Elizabeth, K. and Rao, M. N. A. (1990). Oxygen radical scavenging activity of curcumin. *Int. J. Pharmacol.* 58, 237-240.

Eo, S. K., Kim, Y. S., Lee, C. K. and Han, S. S. (1999). Antiherpetic activities of various protein bound polysaccharides isolated from *Ganoderma lucidum*. 68, 175-181.

Feig, D. I., Reid, T. M. and Loeb, L. A. (1994). Reactive oxygen species in tumorigenesis. *Cancer. Res.* 54 (Suppl). 1890-1894.

Fejes, S., Blazovics, A., Lugasi, A., Lembetkovics, E., Petri, G. and Kery, A. (2000). *In vitro* antioxidant activity of *Anthriscus cerefolium L.* (Hoffm.) extracts. *J. Ethnopharmacol.* 69, 259-265.

Frei, B. (1994). ROS and antioxidant vitamins: mechanism of action. *A. J. med.* 97, 55-58.

Gao, Y. and Zhou, S. (2003). Cancer prevention and treatment by *Ganoderma*, a mushroom with medicinal properties. *Food. Rev. Inter.* 19, 275-325.

Gao, Y. H., Chen, G. L., Dai, X. H., Gao, H. and Zhou, S. F. (2004). Phases I/II study of a *Ganoderma lucidum* extract in patients with type II diabetes. *Int. J. Med. Mushr.* 16 (1), 33-40.

Geierstanger, B. H., kagawa, T. F., Chen, S. L., Quigley, G. J. and Hops. (1991). Base-specific binding of copper (II) to Z-DNA. *J. Biol Chem.* 266, 20185-20191.

Gey, F. (1987). Plasma levels of antioxidant vitamins in relation to ischemic heart disease and cancer. *Ann. J. Nutr.* 45, 1368-1377.

Greenwald, R. A. (1991). Animal model for evaluation of arthritic drugs. *Meth. Find. Clin. Pharmacol.* 13, 75-83.

Grun, M., Liehr, H. and Rasenack, V. (1997). Significance of endotoxemia in experimental Galactosamine hepatitis in the rat. *Acta. Hepatogastroenterol.* 24, 64-81.

Guillouzo, A. (1998). Liver cell models in *in vitro* toxicology. *Environ. Health. Perspect.* 106 (2) 511-532.

Habig, W. H., Pabst, M. J. and Jacobky, W. R. (1974). Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130-7139.

Hafemann, D. G., Sunde, R. A. and Houestra, W. G. (1974). Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. *J. Nutr.* 104, 580-584.

Halliwell, B. (1994). Free radicals, antioxidants, and human disease: curiosity, cause or consequence. *Lancet.* 344, 721-724.

Halliwell, B. and Gutteridge, J. M. C. (1984). Lipid peroxidation. Oxygen radicals: cell damage and antioxidant therapy. *Lancet.* 1396-1397.

Halliwell, B. and Gutteridge, J. M. C. (1989). Free radicals, aeging and diseases. In: *Free radicals in Biology and Medicine.* 2nd edn Oxford, Clarendron Press. pp 416.

Halliwell, B., Aeschbach, R., Loliger, J. and Aruoma, O. I. (1995). The characterization of antioxidants. *Fd. Chem. Toxicol.* 33, 401-617.

Halliwell, B., Gutteridge, J. M. C. and Cross, C. E. (1992). Free radicals, antioxidants, and human disease: Where are we now?. *Lab. Clin. Med.* 199, 598-620.

Hamers, F. P.T., Gispen, W. H. and Neijt, J. P. (1991). Neurotoxic side effects of cisplatin. *Eur. J. Cancer.* 27, 372-376.

Hannemann, J. and Baumann, K. (1988). Cisplatin induced lipid peroxidation and decrease of gluconeogenesis in rat kidney cortex: differential effects and radical scavengers. *Toxicol.* 51,119-132.

Hansford, R. G., Hogue, B. A. and Mildaziene, V. (1997). Dependence of H_2O_2 formation by rat heart mitochondria on substrate availability and donor age. *Bioener. Biophys.* 29 (1), 89-97.

Hartwig, A. (1994). Role of DNA repair inhibition in lead and cadmium induced genotoxicity: a review. *Environ. Health. Perspect.* 102, 45-50.

Hemnani, T. and Parihar, M. S. (1998). Reactive oxygen species and oxidative DNA damage. *Ind. J. Physiol. Pharmacol.* 42 (4), 440-452.

Herington, A. C. (1986). Effect of disulfide bond reducing agents on the specific binding of growth hormone to microsomal membrane preparation from rabbit liver. *Biochem. Pharmacol.* 35, 1359-1364.

Hirase, S., Nakae, Y., Otsuka, S., Ueno, S., Kumi, Y. C. (1970). Studies on antitumor activity of polysaccharides. *I. Proc. Jpn. Cancer Assoc*, 29 th Annual. Meet. 288.

Hobbs, C. H. (1995). Medicinal mushrooms: an exploration of tradition, healing and culture. Botanica Press, Santa Cruz, CA. 251.

Huang, M. T., Smart, R. C., Wong, C. O. and Coney, A. H. (1988). Inhibitory effect of curcumin, chlorogenic acid, caffeic acid and ferulic acid on tumor promotion in mice skin by 12-*O*-tetradecanoylphorbol-13-acetate. *Cancer. Res.* 48, 5941-5946.

Huang, M. T., Ho, C. T., Wang, Z. Y., ferraro, T., Lou, Y. R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J. D. and Conney, A. H. (1994). Inhibition of skin

tumorigenesis by rosemary and its constituents carnosol and ursolic acid. *Cancer. Res.* 54, 701-708.

Huang, M. T., Ma, W., Yen, P., Xie, J. G., Han, J., Frenkel, K., Grunber, D. and Conney, A. H. (1997). Inhibitory effects of topical application of low doses of curcumin on 12-*O*- tetradeconyl phorbol- 13 –acetate induced tumor promotion and oxidized DNA bases in mouse epidermis. *Carcinogenesis.* 18, 83-88.

Hwang, S., Lam, M., Li, C. and Shen, T. (1986). Release of platelet activating factor and its involvement in the first phase of carrageenan rat foot edema. *Eur. J. Pharmacol.* 120, 33- 41.

Ikekawa, T., Ikeda, Y., Yoshioka, Y., Nakanishi, K., Koyama, E. and Yamazaki, E. (1982). Studies on the antitumor polysaccharides of *Flammulina Velutipes.* Sing, II. The structure of EA3 and further purification of EA5. *J. Pharm. Dyn.* 5, 576-581.

Ischiropoulos, H., Zhu, L. and Chen, J. (1992). Peroxynitrite mediated tyrosine nitration catalysed by superoxide dismutase. *Arch. Biochem. Biophys.* 298, 431-437.

Jackson, J. H. (1994). Potential molecular mechanisms of oxidant-induced carcinogenesis. *Environ. Health. Perspect.* 102,155-158.

Ji, C. and Marnett, L. J. (1992). Oxygen radical–dependent epoxydation of (7S, 8S)-dihydroxy-7,8-dihydrobenzo[a]-pyrene in mouse skin *in vivo*. Stimulation by phorbol esters and inhibition by anti-inflammatory steroids. *J. Biol. Chem.* 267, 17842-17878.

Jong, S. C. and Birmingham, J. M. (1992a). Medicinal benefits of the mushroom *Ganoderma. Adv. Appl. Microbiol.* 37, 101-134.

Jong, S. C. and Birmingham, J. M. (1992b). Edible mushrooms in biotechnology. *Proc. Asian. Mycol. Symp.* Seoul. 18-35.

Jong, S. C. and Donovick, R. (1989). Antitumor substance from fungi. *Adv. Applied. Microbiol.* 34, 183-261.

Jose, N. and Janardhanan, K. K. (2000). Antioxidant and antitumor activity of *Pleurotus florida. Curr. Sci.* 79, 941-943.

Jose, N., Ajith, T. A. and Janardhanan, K. K. (2002). Antioxidant, anti-inflammatory, and antitumor activities of Culinary-Medicinal mushroom *Pleurotus Pulmonarius* (Fr.) Quel. (Agaricomycetideae). *Int. J. Med. Mushr.* 4, 329-335.

Joy, K. L. and Kuttan, R. (1999). Inhibition by *Picorrhiza Kurroa* extract of oxygen free radical reactions and hepatic fibrosis in rats. *J. Clin. Biochem. Nutr.* 27, 9-17.

Kensler, T., Bush, D. and Kozumbo, W. (1983). Inhibition of tumor promotion by a biomimetic superoxide dismutase. *Science*. 221,75-77.

Keyse, S. M. (1993). The induction of gene expression in mammalian cells by radiation. *Seminars. Cancer. Biol.* 4, 119-128.

Kim, H. W. and Kim, B. K. (1999). Biomedical triterpenoids of *G. lucidum* (Curt.: Fr.) P. Karst. (Aphyllophoromycetideae). *Int. J. Med. Mushr.* 1, 121-138.

Kim, S. S. and Kim, Y. S. (1990). Korean mushrooms. *Yupoong Pub*, Seoul, 298-299.

Kind, P. R. N. and King, E. J. (1954). Estimation of plasma phosphatase by determination of hydrolyzed phenol with antipyrene. *J. Clin. Pathol.* 7, 322-326.

Koyama, K., Imaizumi, T., Akiba, M., Kinoshita, K. and Takahashi, K. (1997). Antinociceptive components of *Ganoderma lucidum. Planta. Medica.* 63, 224-227.

Kozoumbo, W. J., Seed, J. L. and Kensler, T. W. (1983). Inhibition of 2 (3) tert butyl-4-hydroxyanisole and other antioxidants of epidermal ornithine decarboxylase activity by quercetin: possible involvement of lipoxygenases inhibition. *Carcinogenesis.* 4, 1301-1305.

Lai, T., Gao, Y. and Zhou, S. (2004). Global marketing of medicinal Ling Zhi mushroom *Ganoderma lucidum* (W. Curt.: FR.) Lioyd (Aphyllophoromycetideae) products and safety concerns. *Int. J. Med. Mushr.* 6, 189-194.

Lakshmi, B., Ajith, T. A., Sheena, N., Gunapalan, N. and Janardhanan, K. K. (2003). Antiperoxidative, anti-inflammatory, and antimutagenic activities of ethanol extract of the mycelium of *Ganoderma lucidum* occurring in South India. *Terato. Carcino. Mutagen.* Supple 1, 85-97.

Laloraya, M., Kumar, G. P. and Laloraya, M. M. (1989). A possible role of superoxide anion radical in the process of blastocyst implantation in *Mus musculus. Biochem. Biophys. Res. Commun.* 161, 762-770.

Larsen, G. L. and Henson, P. M. (1983). Mediators of inflammation. *Ann. Rev. Immunol.* 1, 335-339.

Lee, C. M., Veindruch, R. and Aiken, J. M. (1997). Age associated alterations of the mitochondrial genome. *Free. Radic. Biol. Med.* 22 (7), 1259-1256.

Kind, P. R. N. and King, E. J. (1954). Estimation of plasma phosphatase by determination of hydrolyzed phenol with antipyrene. *J. Clin. Pathol.* 7, 322-326.

Koyama, K., Imaizumi, T., Akiba, M., Kinoshita, K. and Takahashi, K. (1997). Antinociceptive components of *Ganoderma lucidum. Planta. Medica.* 63, 224-227.

Kozoumbo, W. J., Seed, J. L. and Kensler, T. W. (1983). Inhibition of 2 (3) tert butyl-4-hydroxyanisole and other antioxidants of epidermal ornithine decarboxylase activity by quercetin: possible involvement of lipoxygenases inhibition. *Carcinogenesis.* 4, 1301-1305.

Lai, T., Gao, Y. and Zhou, S. (2004). Global marketing of medicinal Ling Zhi mushroom *Ganoderma lucidum* (W. Curt.: FR.) Lioyd (Aphyllophoromycetideae) products and safety concerns. *Int. J. Med. Mushr.* 6, 189-194.

Lakshmi, B., Ajith, T. A., Sheena, N., Gunapalan, N. and Janardhanan, K. K. (2003). Antiperoxidative, anti-inflammatory, and antimutagenic activities of ethanol extract of the mycelium of *Ganoderma lucidum* occurring in South India. *Terato. Carcino. Mutagen.* Supple 1, 85-97.

Laloraya, M., Kumar, G. P. and Laloraya, M. M. (1989). A possible role of superoxide anion radical in the process of blastocyst implantation in *Mus musculus. Biochem. Biophys. Res. Commun.* 161, 762-770.

Larsen, G. L. and Henson, P. M. (1983). Mediators of inflammation. *Ann. Rev. Immunol.* 1, 335-339.

Lee, C. M., Veindruch, R. and Aiken, J. M. (1997). Age associated alterations of the mitochondrial genome. *Free. Radic. Biol. Med.* 22 (7), 1259-1256.

Leelavathy, K. M. and Ganesh, P. N. (2000). In: *Polypores of Kerala*, Daya Publishing House, Delhi. 166.

Levin, D. A. (1976). Nature Biotechnology. *Amer. Natural. 110*: 261-284.

Lewis, J. G. and Adams, D. O. (1987). Early inflammatory changes in the skin of SENCAR and C57BL/6 mice following exposure to 12-*O*- tetradecanoyl-phorbol-13-acetate. *Carcinogenesis.* 8, 889-898.

Li, N., Chen, X., Liao, J., Yang, G., Wang, S., Josephson, Y., Han, C., Junshi, C., Huang, M. T. and Yang, C. S. (2002). Inhibition of 7, 12-dimethylbenz [*a*] anthracene (DMBA)- induced oral carcinogenesis in hamsters by tea and curcumin. *Carcinogenesis.* 23 (8), 1307-1313.

Lin, J. M., Lin, C. C., Chen, M. F., Ujiie, T., Takata, A. (1995). Radical scavenging and hepatotoxic activity of *Ganoderma formosanum*, *G. lucidum* and *G. neo-japonicum. J. Ethnopharmacol.* 47, 33-41.

Lin, C. C., Yen, M. H., Lo, T. S. and Lin, J. M. (1998). Evaluation of the hepatoprotective and antioxidant activity of *Boehmeria nivea* var. nivea and *B. nivea* Var. *tenacissima. J. Ethnopharmacol.* 60, 9-17.

Liu, G. T. (1999). Recent advances in research of pharmacology and clinical applications of *Ganoderma P. Karst.* Species (Aphyllophoromycetideae) in China. *Int. J. Med. Mushr.* 1, 63-67.

Lo, T. N., Almeida, A. P. and Beaven, M. A. (1982): Dextran and carrageenan evoke different inflammatory response in rat with respect to composition of infiltrates and effect of indometacin. *J. Pharmacol. Exp Ther.* 221, 261-267.

Lomderg, M. L., Solomko, E. F., Oksana, V. and Kleschenko, P. H. (2001). Some medicinal mushrooms in submerged culture. *Int. J. Med. Mushr.* 3, 176.

Lowry, H. D., Rosenberg, N. J., Farr, A. L. and Randa, R. J. (1951). Protein measurement with folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

Ma, J., Ye, Q., Hua, Y., Zhang, D., Cooper, R., Chang, M. N., Chang, J. Y. and Sun, H. H. (2002). New lanostanoids from the mushroom *Ganoderma lucidum. J. Nat. Prod.* 65, 72-75.

Marshell, M. H., Fingerhut, B. and Miller, H. (1980). In: *Practical Clinical Biochemistry* (Varley, H., Gowenlock, A. H., Bell, M., eds). William Heinmann Medical Books Ltd, London. 546-460.

Mathews, R. T., Yang, L., Beal, M. F. (1997). s-methylthiocitrulline, a neuronal nitric oxide synthase inhibition protects, against malonate and MPTP neurotoxicity. *Exp. Neurol.* 143, 282-286.

Mc Cord, J. M. and Fridovich, I. (1969). Superoxide dismutase, an enzymatic function for erythrocuprein. *J. Biol. Chem.* 244, 6049-6055.

Mc Cord, J. M. (1987). Oxygen derived radicals a look between reperfusion injury and inflammation. *Biol. Proc.* 46, 2402-2406.

Menkes, M., Comstock, G., Vulleumier, J., Helsing, K. and Brookmeyer, R. (1986). Serum-carotene, vitamins A and E, selenium and the risk of lung cancer. *N. Eng. J. Med.* 45, 1250-1254.

Miller, R. N. G., Holderness, M., Bridge, P. D., Paterson, R. R. M., Sariah, M., Hussain, M. Z. and Hilsley, E. J. (1995). A multidisciplinary approach to the characterization of *Ganoderma* in oil palm cropping system. In: *Proc. Contr. Sym.* 59 A. B, 5th International Mycological Congress. Buchnan, P. K., Hseu, R. S. and Moncalvo, J. M. (eds). Taipei, China, pp 57-66.

Minotti, G. (1989). Adriamycin-dependent release of iron from microsomal membranes. *Arch. Biochem. Biophys*, 268, 398-403.

Mizuno, M., Kawakimi, S., Hashioto, T., Ashida, H. and Minato, K. (2001). Antitumor polysaccharides from edible mushrooms and immunomodulating action against murine macrophages. *Int. J. Med. Mushr.* 3, 89.

Mizuno, T. (2000). Development of an antitumor biological response modifier from *Phellinus linteus* (Berk. et Curt.) Teng (Aphyllophoromycetideae) (review). *Int. J. Med. Mushr.* 2, 21-33.

Molitoris, H. P. (2001). Mushrooms and man in medicine, myth, and religion. *Int. J. Med. Mushr.* 3, 97.

Moron, M. A., Depierre, J. W. and Mannervik, B. (1979). Levels of glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochem. et. Biophys. Acta*, 582, 67-68.

Moss, D. W. and Henderson, A. R. (1999). Determination of creatine kinase activity. In: *Tietz textbook of clinical Chemistry*. Brutis, C. A. and Ashwood, E. (eds). W. B. Sounders Company, Singapore, 662.

Mott, M. G. (1997). Anthracycline cardiotoxicity and its prevention. *Ann. N. Y. Acad. Sci.* 824, 221-228.

Murakami, A., Nakamura, Y., Torikai, K., Ohto, Y. and Tanaka, T. (2000). Inhibitory effect of *citrus nobiletin* on phorbol ester-induced skin inflammation, oxidative stress and tumor promotion in mice. *Cancer. Res.* 60, 5059-5066

Naidu, M. U. R., Kumar, V. K., Mohan. K. I., Sundaram C. and Sashi Singh (2002). Protective effect of *Gingko biloba* extract against doxorubicin- induced cardiotoxicity in mice. *Ind. J. Exp. Biol.* 40, 894-900.

Nishimora, Y., Koyama, Y., Umezawa, S., Takeuchi, T., Ishizuka, M. and Umezawa, H. (1980). Synthesis of corilin, 1-deoxy-1-Ketocoriolin and 1,8-diketocoriolin from coriolin B. *J. Antibiot.* 33, 404 -407.

Noguchi, T., Fong, K.L., Lai, E. K. Olson, L., Mc Gay, P. B., (1982). Selective early loss of polypeptides in liver microsomes of CCl_4 treated rats. *Biochem. Pharmacol.* 31, 615-624.

Ohkawa, H., Ohishi, N. and Yagi, K. (1979). Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. *Annal. Biochem.* 95, 351-358.

Ooi, V. E. C. and Liu, F. (1999). A review of pharmacological activities of mushroom polysaccharides. *Int. J. Med.* Mushr. 1,195-206.

Parchment, R. E. (1998). Alternative testing system for evaluating non carcinogenic hematological toxicity. *Envtal. Health. Perspect.* 106 (2) 541-557.

Parihar, M. S., Manjula, Y., Banu, S., Hemnani, T., Javeri, T. and Prakash, P. (1997). Nicotinamide and α- tocopherol combination partially protects tert-butyl hydroperoxide neurotoxicity: implication for neurodegenerative disease. *Curr. Sci.* 73 (3), 290-293.

Perantoni, A. O. (1998). Carcinogenesis. In: *The Biological basis of cancer.* Mc Kinnell, R. G., Parchment, R. E., Perantoni, A. O. and Pierce, G. B. (eds), Cambridge University Press, London. 79-114.

Ratain, M. J. (1997). Pharmacology of cancer chemotherapy. In: *Principles and Practice of Oncology.* Rosenberg, S. A. (ed). Lippincott-Raven Publishers, Philadelphia, pp 375-509.

Recknagel, R. O. (1967). Carbon tetrachloride hepatotoxicity. *Pharmacol. Rev.* 19, 145-208.

Reiter, R. J. (1995). The role of the neurohormone melatonin as a buffer macromolecular oxidative damage. *Neurochem. Int.* 27, 453-460.

Reiter, R. J. (1997). Oxidative processes and antioxidative defense mechanisms in the aeging brain. *FASEB. J.* 9, 526-533.

Reitman, S. and Frankel, A. S. (1957). A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *Am. J. Clin. Pathol.*, 28, 56-63.

Reshetnikov, S. V., Wasser, S. P. and Tan, K. K. (2001). Higher Basidiomycota as a source of antitumor and immunestimulating polysaccharides (Review). 3, 361-394.

Richter, C. (1987). Biophysical consequences of lipid peroxidation in membranes. *Chem. Phys. Lipids.* 44, 175-179.

Rosin, M. P., Anwar, W. A. and Ward, A. J. (1994). Inflammation, chromosomal instability and cancer: The Schistosomiasis model. *Cancer. Res.* (Suppl) 54, 1929-1933.

Rowlands, J. C., He, L., Hakkak, R., Ronis, M. J. J. and Badger, T. M. (2001). Soy and whey proteins downregulate DMBA-induced liver and mammary gland CYP1 expression in female rats. *J. Nutr.* 131, 3281-3287.

Russo, J. and Russo, I. H. (1978). DNA-labeling index and structure of the rat mammary gland as determinants of its susceptibility to carcinogenesis. *J. Natl. Cancer. Inst.* 61, 1451-1460.

Russo, J. and Russo, I. H. (1991). Mammary tumorigenesis. In: Modification of tumor development in rodents. *Prog. Exp. Tumor. Res.* 33, 175-191.

Salvemini, D., Wang, Z. Q., Bourdon, D. M., Ster, M. K., Currie, M. C. and Manning, P. T. (1996). Evidence of peroxynitrite involvement in the carrageenan induced rat paw edema. *Eur. J. Pharmacol.* 303, 217.

Santhos, A. R. S., De Campos, R. O. P., Miguel, O. G., Filho, V. C., Siani, A. C., Yunes, R. A., Calix, J. B. (2000). Antinociceptive properties of extracts of new species of plants of the genus *Phyllanthus* (Euphorbiaceae). *J. Ethnopharmacol.* 72, 229-238.

Satoh, K. (1987). Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. *Clin. Chem. Acta.* 90, 37-43.

Schreck, R., Reiber, P. and Baeverte, P. A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of NE-KB transcription factor and HIV-1. *EMBO. J.* 10, 2247-2258.

Schultes, R. V. (1974). The future of plants as source of new biodynamic compounds. In: *plants in the development of modern medicine*. Tang Swain (ed). Harward Press, pp 103-124.

Shiao, M. S., Lee, K. K., Lin, L. J. and Wang, C. T (1994): Natural products and biological activities of the Chinese medicinal fungus *Ganoderma lucidum*. In: *Food Phyto chemicals II. Teas, Spices and Herbs.* (Ho, C. T., Osawa, T., Huang, M. T., Roem, R. T.(eds). Washington DC, American Chemical Society, pp 342-354.

Shureiqi, I., Reddy, P. and Brenner, D. E. (2000). Chemoprevention: general perspective. *Crit. Rev. Oncol. Hematol.* 33, 157-167.

Shureiqi, I., Xu, X., Chen, D., Lotan, R., Morris, J. S., Fischer, S. M. and Lippman, S. M. (2001). Nonsteroidal anti-inflammatory drugs induce apoptosis in esophageal cancer cells by restoring 15-Lipoxygenase –1 expression. *Cancer. Res.* 61, 4879-4880.

Sies, H. (1993). Strategies of antioxidant defense. *Eur. J. Biochem.* 215, 213-221.

Smith, W. A. and Gupta, R. C. (1996). Use of a microsome-mediated test system to assess efficacy and mechanisms of cancer chemopreventive agents. *Carcinogenesis*. 17, 1285-1290.

Smith, W. A., Freeman, J. W. and Gupta, R. C. (2001). Effect of chemopreventive agents on DNA adduction induced by the potent mammary carcinogen dibenzo [a, *l*] pyrene in the human breast cells MCF-7. *Mut Res*. 480-481, 97-108.

Somani, S. M., Husain, K. and Whitworth, C. (2000). Dose dependent protection by lipoic acid against nephrotoxicity in rats: antioxidant defense system. *Pharmacol.Toxicol.* 86, 234-241.

Soudamini, K. K., Unnikrishnan, M. C., Soni, K. B. and Kuttan, R. (1992). Inhibition of lipid peroxidation and cholesterol levels in mice by curcumin. *Ind. J. Physiol. Pharmacol.* 36, 239-243.

Southern, P. and Powis, G. (1998). Free radicals in Medicine. II involvement in human disease. *Mayo. Clin. Proc.* 63, 390-408.

Sporn, M. B. (1976). Approaches to prevention of epithelial cancer during the preneoplastic period. *Cancer. Res.* 36, 2699-2702.

Sporn, M. B. and Suh, N. (2000). Chemoprevention of cancer. *Carcinogenesis*. 21, 525-530.

Sreejayan, M. and Rao, M. N. A. (1997). Nitric oxide scavenging by curcuminoids. *J. Pharm. Pharmacol.* 49, 105-107.

Srivastava, P., Russo, J. and Russo, I. H. (1997). Chorionic gonadotropin inhibits rat mammary carcinogenesis through activation of programmed cell death. *Carcinogenesis.* 18 (9), 1799-1808.

Stohs, S. J. and Bageha, D. (1995). Oxidative mechanism in the toxicity of metal ions. *Free. Radic. Biol. Med.* 18, 321-326.

Suffness, M. and Douros, J. (1979). In: *Methods in Cancer Research.* Academic Press, New York, 16, 116.

Suffness, M. and Pezutto, M. (1991). Assays related to cancer drug discovery, In: *Methods in plant Biochemistry.* Academic Press, New York. 6, 71.

Tamini, R. M., Lagiou, P., Adami, H. O. and Trichopoulos, D. (2002). Prospects for chemoprevention of cancer. *J. Int. Med.* 251, 286-300.

Tiano, H. F., Loftin, C. D., Akunda, J., lee, C. A., Splading, J., Sessoms, A., Dunson, D. B., Rogan, E. G., Morhan, S. G., Smart, R. C. and Langenbach, R. (2002). Deficiency of either cyclooxygenase (COX)-1 or COX-2 alters epidermal differentiation and reduces skin tumorigenesis. *Cancer. Res.* 62, 3395-3401.

Tiwari, A. K. (2001). Imbalance in antioxidant defense and human diseases: multiple approach of natural antioxidants therapy. *Curr. Sci.* 81 (9), 1179-1186.

Trivedi, N. P. and Rawal, U. M. (2001). Hepatoprotective and antioxidant property of *Andrographis paniculata* (Nees) in BHC induced liver damage in mice. *Ind. J. Exp. Biol.* 39, 41-46.

Tunon, H., Olavsdotter, C. and Bohlin, L. (1995). Evaluation of anti-inflammatory activity of some Swedish medicinal plants. Inhibition of prostaglandin biosynthesis and PAF-induced exocytosis. *J. Ethnopharmacol.* 48, 61-76.

Underwood, J. C. E. (2000). In: *General and Systemic pathology*. Harcourt Publishers Limited, Churchill Living Stone, pp 201-221.

Uppu, R. M., Squadrito, G. L. and Pryor, W. A. (1996). Acceleration of peroxynitrite oxidants by carbon dioxide. *Arch. Biochem. Biophys.*327, 335-343.

Uslu, R. and Bonavida, B. (1996). Involvment of the mitochondrion respiratory chain in the synergy achieved by the treatment of human ovarian carcinoma cell lines with tumor necrosis factor –alpha and cis-diamine dichloroplatinum (II). *Cancer*. 77, 725-732.

Vane, J. and Booting, R. (1987): Inflammation and the mechanism of action of anti-inflammatory drugs. *FASEB J.* 1, 89-96.

Verma, S., Kumar, G. P., Laloraya, M. and Singh, A. (1990). Activation of iodine into a free radical intermediate by superoxide: a physiologically significant step in the iodination of tyrosine. *Biochem. Biophys. Res. Commun.* 170, 1026-1034.

Vliet, A. V. D. and Bast, A. (1992). Effect of oxidative stress on receptors and signal transmission. *Chem. Biol. Interactions*. 85, 95-116.

Wagner, H., Bladt, S. and Zgairski, E. M. (1984). In: *Plant drug analysis*, Springer-Verlag, Berling, New York, Part E, pp 107-116.

Walum, E. (1998). Acute oral toxicity. *Envtal. Health. Perspect.* 106 (2) 497-503.

Wang, M. Y., Liu, Q., Che, Q. M., and Lin, Z. B. (2002). Effects of total triterpenoids extract from *Ganoderma lucidum* (Curt.:Fr.) P. Karst. (Reishi Mushroom) on experimental liver injury models induced by carbon tetrachloride or D- Galactosamine in mice. *Int. J. Med. Mushr.* 4, 337-342.

Wasser, S. P. and Weis, A. L. (1999). Medicinal properties of substances occurring in higher Basidiomycetes mushrooms: Current perspectives (review). *Int. J. Med. Mushr.* 1, 31-62.

Wasser, S. P., Nevo, E., Sokolov, D., Reshetnikov, S. V., and Timor- Tismenetsky, M. (2000). Dietary supplements from medicinal mushrooms: diversity of types and variety of regulations. *Int. J. Med. Mushr.* 2, 1-19.

Wheeler-Aceto, H. and Cowan, A. (1991): Neurogenic and tissue mediated components of formalin-induced edema. *Agents. Actions.* 34, 264-268.

Winrow, V. R., Winyard, P. G., Morris, C. J. and Blake, D. R. (1993). Free radicals in inflammation, second messengers and mediators of tissue destruction. *Br. Med. Bull.* 49, (3), 506.

Winston, G. W. and Digiulio, R. T. (1991). Peroxidant and antioxidant mechanisms in aquatic organisms. *Aquat. Toxicol.* 19, 137-161.

Wiseman, H. and Halliwell, B. (1996). Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem. J.* 313, 17-29.

Wiseman, H., Kaur, H. and Halliwell, B. (1995). DNA damage and cancer: Measurement and mechanism. *Cancer. Lett.* 93, 113-120.

Wood, A. W., Huang, M. T., Chang, R. L., Newmark, H. L., Lehr, R. E., Yagi, H., Sayer, J. M., Jerina, D. M. and Conney, A. H. (1982). Inhibition of the mutagenicity of bay region diol epoxides of polycyclic aromatic hydrocarbons by naturally occurring plant phenols: exceptional activity of ellagic acid. *Proc. Natl. Acad. Sci* of the USA. 79, 5513-5517.

Yang, Q. Y. and Jong, S. C. (1989). Medicinal mushrooms in China. *Mushr. Sci.* 9 (1), 631-643.

Yemn, E.W. and Wills, A. J. (1954). The estimation of carbohydrate in plant extract by anthrone. *Biochem. J.* 57, 508-514.

Yoshikawa, T., Tanaka, H., Yoshida, N., Seto, O., Sugino, N., Kondo, M. (1983). Adjuvant arthritis and lipid peroxide protection by superoxide dismutase. *Lipid. Perox. Res.* 7, 108-110.

Young, R. C. and Wilson, C. M. (2002). Cancer prevention: Past, present, and future. *Clin. Cancer. Res.* 8, 11-16.

Yuda, Y., Tanaka, J., Hirano, F., Igarani, K., Satch, T. (1991). Participation of lipid peroxidation in rat pertusis vaccine pleurisy. *Chem. Pharma. Bull.* 39, 505-506.

Zhou, S. F., Kestell, P., Baguley, B. C. and Paxton, J. W. (2002). 5,6-Dimethylxanthenone-4-acetic acid: a novel biological response modifier for cancer therapy. *Invest. New. Drugs*, 20, 281-295.

LIST OF PUBLICATIONS

N. Sheena, T. A. Ajith. and K. K. Janardhanan. (2003). Antiinflammatory and antinociceptive activities of *Ganoderma lucidum* occurring in South India. *Pharm. Biol.* 41, 301-304.

N. Sheena, T. A. Ajith. and K. K. Janardhanan. (2003). Prevention of nephrotoxicity induced by the anticancer drug cisplatin, using *Ganoderma lucidum* occurring in South India. *Curr.* Sci. 85 (4), 478-482.

N. Sheena, T. A. Ajith., A. T. Mathew. and K. K. Janardhanan. (2003). Antibacterial activities of three macrofungi, *Ganoderma lucidum, Navesporus floccosa and Phellinus rimosus* occurring in South India. *Pharm. Biol.* 41, (8) 564-567.

N. Sheena, T. A. Ajith. and K. K. Janardhanan. (2005). Protective effect of *Ganoderma lucidum* P. Karst. Reishi from South India against doxorubicin induced cardiotoxicity in rats. *O. P. E. M.* 5 (1), 62-68.

LIST OF PUBLICATIONS

N. Sheena, T. A. Ajith. and K. K. Janardhanan. (2003). Antiinflammatory and antinociceptive activities of *Ganoderma lucidum* occurring in South India. *Pharm. Biol.* 41, 301-304.

N. Sheena, T. A. Ajith. and K. K. Janardhanan. (2003). Prevention of nephrotoxicity induced by the anticancer drug cisplatin, using *Ganoderma lucidum* occurring in South India. *Curr.* Sci. 85 (4), 478-482.

N. Sheena, T. A. Ajith., A. T. Mathew. and K. K. Janardhanan. (2003). Antibacterial activities of three macrofungi, *Ganoderma lucidum, Navesporus floccosa and Phellinus rimosus* occurring in South India. *Pharm. Biol.* 41, (8) 564-567.

N. Sheena, T. A. Ajith. and K. K. Janardhanan. (2005). Protective effect of *Ganoderma lucidum* P. Karst. Reishi from South India against doxorubicin induced cardiotoxicity in rats. *O. P. E. M.* 5 (1), 62-68.