FREE RADICAL SCAVENGING ACTIVITY OF ANTITUMOR POLYSACCHARIDE FRACTIONS ISOLATED FROM *GANODERMA* spp.

A PROJECT REPORT

Submitted by

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DEDICATED TO

OUR BELOVED

PARENTS

ABSTRACT

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Mushroom represents a major and as yet largely untapped source of potent pharmacological products.*Ganoderma lucidum* (Fr.) Karst. (*Polyporaceae*), a fungus, also known as Reishi is an edible Chinese mushroom. The most significant constitutents of mushrooms are long chain polysaccharides known as beta-glucans. Various polysaccharides (i.e., ß-D-glucans and glycoproteins) and triterpenoids are known to be major active constituents present in *Ganoderma*. In our study, the fruiting body of the mushroom (fresh and dried) was powdered and used for enzymatic and nonenzymatic antioxidant determination. It was found that the fresh mushroom sample exhibited significantly increased antioxidant activities compared to dried sample. Further four different polysaccharide fractions (hot water extract, ammonium oxalate extract, acetic acid-NaOH extract and ethanol-NaOH extract) were isolated and free radical scavenging ability was determined using different antioxidant capacity assays. The results are tabulated and found that fraction 3 comparitively showed potent free radical scavenging activity. Thus, the antitumor activities of the polysaccharides are due to antioxidants present in *G.lucidum* as evidenced by the free radical scavenging ability of the polysaccharide fractions.

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ABBREVIATIONS

µg	microgram
µl	microlitre
µM	micromoles
ABTS	2,2-azobis-3-ethylbenzthiazoline-6-sulfonic acid
AEAC	Ascorbic acid equivalent Antioxidant Capacity
AIDS	Acquired Immuno Deficiency Syndrome
ALP	Alkaline Phosphatase
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxy toulene
BRMs	Biological Reactive Molecules
CAT	Catalase
CDNB	1-Chloro-2,4-dinitro phenyl hydrazine
CNS	Central Nervous System
CTL	Cytotoxic Lymphocyte
DBMA	7,12-dimethylbenz[a] anthracene
DNA	Deoxy Nucleic acid
DNPH	2,4 dinitro phenyl hydrazine
DPPH	1,1-Diphenyl-2-Picryl hydrazyl
EDTA	Ethylene Diamine Tetraacetic acid
EtOH	Ethanol
FAD	Flavin adenine dinucleotide
FDA	Food and Drug Administration
$FeCl_3$	Ferric chloride
FRAP	Ferric Reducing Antioxidant Potential

g	gram
G6PD	Glucose-6- phosphate dehydrogenase
GGT	Gamma Glutamyl Transpeptidase
GOT	Glutamate Oxaloacetate Transaminase
GPT	Glutamate Pyruvate Transaminase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Total Reduced Glutathione
GSSG	Oxidized Glutathione
GST	Glutathione-S-Transferase
H_2O_2	Hydrogen peroxide
HCC	Hepatocarcinoma
HIV	Human Immunodeficiency Virus
hr	hour
l	litre
LDL	Low Density lipprotein
LPO	Lipid Peroxidation
M	moles
MDA	Malondialdehyde
MDP	Murayl dipeptide
mg	milligram
min	minute
ml	millilitre
mM	millimoles
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium

NDEA	N-nitrosodiethylamine
NH_4	ammonium
NK	Natural Killer cells
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
O_2	Oxygen
OD	Optical Density
PG	Propyl Gallate
PUFA	Poly Unsaturated Fatty Acids
ROM	Reactive Oxygen Metabolites
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	Terbutyl hydroquinone
TCA	Trichloro Acetic acid
TEAC	Trolox Equilavent Antioxidant Capacity
TNF	Tumor Necrosis Factor

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INTRODUCTION

1.0 INTRODUCTION

Mushroom represents a major and as yet largely untapped source of potent pharmacological products (Janardhanan, et *al.*, 2003). Of the approximately 10,000 known species of mushrooms, 2000 are safe for people's health and about 300 of them possess medicinal properties (Wasser and Weis, 1999a). The edible nature of mushroom is now well known. Usually *Agaricus*, *Pleurotus* and *Volvariella* spp. are eaten all over the world in general and tropical countries in particular. However, the medicinal uses of the mushroom still need to be worked out for their biological activities due to a fast increasing number of multi-drug resistances in pathogenic microbes like *Candida* spp., *Staphylococcus aureus*, *Streptococcus* spp., *Enterococcus* spp. and *Escherichia coli* (Ishikawa *et al.*, 2001). The use of mushrooms as medicine was first mentioned by Berkeley (1857). In the second half of twentieth century, the mushroom producing technologies have grown enormously and the value of world mushroom production was estimated to be worth about eighteen billion US dollar. Many pharmaceutical substances with potent and unique properties were recently extracted from mushrooms and made their way all over the world.

Hot water extracts of many mushrooms used in traditional Chinese medicine and other folk medicines have long been set to be efficacious in the treatment of various diseases including many forms of cancer. The use of medicinal mushroom extracts in the fight against diseases is well known and documented in China, Japan, Korea, Russia and now increasingly in the USA (Mizuno *et al.*, 1995). However, it is only within the last three decades that chemical technology has been able to isolate the relevant compounds and use them in controlled experiments. They have been extensively

screened for medical properties especially for anticancer applications (Mizuno, 1999). Many species of mushrooms have been found to be highly potent immune system enhancers, potentiating animal and human immunity against cancer (Wasser and Weis, 1999a, Borchers *et al.*, 1999, Kidd, 2000; Ikikawa, 2000; Feng *et al.*, 2001). While atleast 30 mushroom species have yielded compounds with pronounced anticancer actions in xenographs, only a small number have taken the next step, viz objective of clinical assessment for anticancer potential in humans.

Ganoderma is one such member of the *Polyporus*, a group of fungi characterized by the presence of pores, instead of gills on the underside of the fruiting body. *G. lucidum*, considered by many mycophiles being one of the most beautiful shelf fungi, it is distinguished by its varnished, red surface. When it is young, it also has white and yellow shades on the varnished surface, differing from the dull surface of *Ganoderma applanatum*. *G. lucidum* is a saprophytic fungus that tends to grow more prolifically in warm climates on decaying hardwood logs and stumps (Kathleen Engelbrecht and Tom Volk, 2005). For thousands of years, *Ganoderma lucidum,* a kind of medicinal fungi has been highly regarded by the Chinese as the miraculous king of herbs. China is the hometown of *Ganoderma lucidum*. In the ancient times, *Ganoderma lucidum* was regarded as the elixir, it had high value for treatment and nutrition.Under the attentive research done by both Chinese and foreign scholars in recent years, and the cooperative analysis and clinical experiments done by hospitals, colleges and pharmaceutical manufacturers, its extensive efficacy has finally been discovered. It is supposed to be as detoxifier, diuretic, liver protector, intestine regulator, cardio tonic, blood pressure adjuster, a cold tonic, anti-tussis and expectorant, a tranquilizer and anti-tumor drug.

Ganoderma lucidum (reishi) is a medicinal fungus with a variety of biological activities. Reishi has long been used as a folk remedy for promotion of health and longevity in China and other oriental countries. The most attractive character of this kind of medicinal fungus is its effect on the immune system and anti-tumor activities. Large numbers of studies have shown that Reishi modulates many components of the immune system such as the antigen-presenting cells, NK cells, T and B lymphocytes. The water extract and the polysaccharides fraction of Reishi exhibited significant anti-tumor effect in several tumor-bearing animals mainly through its immune system enhancing activity. Recent studies also showed that the alcohol extract or the triterpene fraction of Reishi possessed anti-tumor effect, which seemed to be related to the cytotoxic activity against tumor cells directly. Preliminary study indicated that antiangiogenic effect might be involved antitumor activity of Reishi (Lin and Zhang, 2004).

Free radicals are substances with one or more unpaired electrons, which are formed as a result of many physiological and pathological cellular metabolic processes especially, in mitochondria. Free radical mediated genetic instability is widely thought to be a major etiological factor for initiation of carcinogenesis. Investigations into the anti-cancer activity of Reishi have been performed in both *in vitro* and *in vivo* studies, supporting its application for cancer treatment and prevention. The proposed anti-cancer activity of Reishi has prompted its usage by cancer patients. It remains debatable as to whether Reishi is a food supplement for health maintenance or actually a therapeutic "drug" for medical proposes. Thus, far there has been no report of human trials using Reishi as a direct anti-cancer agent, despite some evidence showing the usage of Reishi as a potential supplement to cancer patients. Cellular immune responses and mitogenic

reactivity of cancer patients have been enhanced by Reshi, as reported in two randomized and one nonrandomized trials, and the quality of life of 65% of lung cancer patients improved in one study. The direct cytotoxic and anti-angiogenesis mechanisms of Reishi have been established by *in vitro* studies; however, clinical studies should not be neglected to define the applicable dosage *in vivo*.

Many, if not all, Basidiomycete mushrooms have been shown to contain biologically active antitumour and immunostimulative polysaccharides. In a review, Reshetnikov *et al.*, (2001) have listed 650 species and 7 intraspecific taxa from 182 genera of higher hetero- and homo-basidiomycetes that contain pharmacologically active polysaccharides that can be derived from fruiting bodies, culture mycelium and culture broths. In general, there is normally a higher level and number of different polysaccharides extracted from fruiting bodies than from the other cultural sources (Wasser and Weis, 1999b).

The first definitive studies on these anticancer substances came in the late 1960s with the reports by Ikekawa *et al.*, (1968, 1969) and Chihara *et al.*, (1969,1970). They demonstrated that extracts of several different mushroom species exhibited remarkable host-mediating antitumour activities against Xenographs, e.g. Sarcoma 180. In the species xenograph, suitable dosage and schedule are essential to achieve the anti-tumour effects (Jong and Donovick, 1989, Jong *et al.*, 1991).

Antitumour polysaccharides isolated from mushrooms are either water-soluble beta-D-glucans, beta-D-glucans with heterosaccharide chains of xylose, mannose, galactose and uronic acid or beta-D-glucans- protein complexes- proteoglycans. As a general rule, the protein- linked glucans have a greater immunopotential activity than the corresponding glucans.

Levels of anticancer activity are related to their molecular weight, branching and solubility in water. The study of their steric structures by NMR analyses and X-Ray diffractions clarified that active beta-D-glucans shows a triple-stranded right- winding helix structure (Bluhm and Sarco, 1977).

The basic beta-D-glucan is a repeating structure with the D-glucose units joined together in linear chains by beta-bonds. These can extend from carbon 1 of one saccharide ring to carbon 3 of the next (beta 1-3), from carbon 1 to carbon 4 (beta 1-4) or from carbon 1 to carbon 6 (beta 1-6). Mostly there is a main chain which is beta 1-3, beta 1-4 or mixed beta 1-3, beta 1-4 with beta 1-6 side chains.

Currently available data from numerous *in vitro* and *in vivo* studies suggest that the cancer-preventive and tumoricidal properties of *Ganoderma* might be ascribed to its antioxidative and radical-scavenging effects, enhancement of host immune function, induction of cell-cycle arrest and apoptosis, and other biological effects (Lin *et al.*, 1995; Watchel-Galor *et al.*, 2004). The antitumor activity of the polysaccharide, Ganopoly may be suggested to operate through free radical mediated mechanisms. However, the antioxidant nature of the polysaccharide fraction, relating to its anti-tumor activity has not been yet elucidated.

Hence, the main objective of this study has been aimed at isolating and investigating the free radical scavenging activity of the anti-tumor polysaccharides, suggesting its probable mode of action in cancer treatment.

The main objectives of the project are categorized into three phases -

1. **Phase I** - Preliminary antioxidant studies in fruiting body of *Ganoderma lucidum* mushroom extracts by determining endogenous antioxidants - enzymatic (SOD, catalase, glutathione peroxidase, glutathione-S-transferase) and non-enzymatic (reduced glutathione, ascorbic acid).

2. **Phase II** -Isolation of polysaccharides fractions from fruiting body of *Ganoderma lucidum.*
3. **Phase III** -Assessment of free radical scavenging activity in polysaccharide fractions by different *in vitro* antioxidant assays.

LITERATURE REVIEW

2.0 LITERATURE REVIEW

Many of the bioactive substances recently isolated from higher Basidiomycetes, known as medicinal mushrooms show antitumor, immunomodulating, antiviral and other promising effects. The inhibition of growth of different tumors was detected in about 200 species(Wasser and Weiss, 1999a).Several antitumor polysaccharides such as hetero-ß- glucans ant their protein complexes (e.g., xyloglucans and acidic ß-glucan containing uronic acid) as well as dietary fibers, lectins and terpenoids have been isolated from medicinal mushrooms. One of the medicinal mushrooms with all these properties is *G.lucidum* and recent studies have indicated that components extracted from *Ganoderma* have a wide range of pharmacological actions including suppressing inflammation and scavenging free radicals (Kar Neng Lai *et al.*, 2006).

2.1 *Ganoderma lucidum*

Lingzhi (traditional Chinese) is the name for one form of the mushroom *Ganoderma (G). lucidum*. This fungal species has a worldwide distribution in both tropical and temperate geographical regions, including North and South America, Africa, Europe, and Asia, growing as a parasite or saprophyte on a wide variety of trees . *G.lucidum* enjoys special veneration in Asia, where it has been used in traditional Chinese medicine as an herbal medicine for more than 4,000 years, making it one of the oldest mushrooms known to have been used in medicine. The word *lingzhi*, in Chinese, means "herb of spiritual potency" and has also been described as "mushroom of immortality". Because of its presumed health benefits and apparent absence of side-effects, it has attained a reputation in the East as the ultimate herbal

substance. Lingzhi has now been added to the American Herbal Pharmacopoeia and Therapeutic Compendium.

2.1.1 Distribution

In nature, Lingzhi grows at the base and stumps of deciduous trees, especially maple (National Audubon Society; Field guide to Mushrooms, 1993). Only two or three out of 10,000 such aged trees will have Lingzhi growth, and therefore its wild form is generally rare. Today, Lingzhi is effectively cultivated both indoors under sterile conditions and outdoors on either logs or woodchip beds.

Lingzhi can be found for sale in many Asian markets as well as Western health shops. Extracts of 'lingzhi,' which may also be called 'reishi', are also available. In general, a hot water extract is best at concentrating the polysaccharides in lingzhi and alcohol extracts are best at concentrating the triterpenoids in lingzhi but an extract can also be made with a blend of both extracts. There are multiple species of lingzhi, scientifically known to be within the *G.lucidum* species complex and mycologists are still researching the differences between species within this complex of species.

2.1.2 Morphology

Lingzhi is a polypore mushroom that is soft (when fresh), corky, and flat, with a conspicuous red-varnished, kidney-shaped cap and, depending on specimen age, white to dull brown pores underneath. It lacks gills on its underside and releases its spores through fine pores, leading to its morphological classification as a polypore.

Ganoderma lucidum

The naturally produced Basidiocarp of *G.lucidum* shows various morphological characteristics; sessile, stipitate, imbricate and non-imbricate (Shin *et al.,* 1986). The morphological variation appears to be affected by the environmental conditions during Basidiocarp development. The size and color of the basidiocarp shows significant differences between the specimens, but the pore sizes are similar. The size and shape of pores are useful character for species classification.

2.1.3 Chemical constituents

Pharmacologically active compounds from *G.lucidum* include triterpenoids, proteins, steroids, alkaloids, nucleotides, lactones and fatty acids. Polysaccharides (especially ß- D-glucans) have been recognized as an effective anti-cancer drug (Kim *et al*, 1999;Mizushina *et al.,* 1999). Polysaccharide fractions containing (1 → 3) - ß- D-glucans, branched mainly at the C-6 position demonstrate high antitumor activity (Sone *et al*, 1985). Terpenoids are cytotoxic to hepatoma cells (Mizuno *et al,* 1995), they inhibit the response of platelets to various aggregating agonists (Su *et al*, 2000) and inhibit eukaryotic DNA polymerase and HIV type 1 reverse transcriptase.

G. lucidum is the only known source of a group of triterpenes, known as ganoderic acids, which have a molecular structure similar to steroid. It is a source of biologically active polysaccharides with presumed medicinal properties, and it also contains:

- ergosterol,
- coumarin,
- mannitol,

- lactones,
- alkaloids,
- unsaturated fatty acids,
- vitamins and minerals.

2.1.4 Pharmacological Actions

G.lucidum (Fr.) Karst. (*Polyporaceae*), a fungus, also known as Reishi is an edible Chinese mushroom. Its fruiting bodies have been used for their medicinal properties in traditional Chinese medicine for over 2000 years to promote vitality and longevity. In modern Chinese medicine, *G.lucidum* is used in the treatment of debility and weakness, insomnia, hepatitis, bronchitis and asthma, diabetes, altitude sickness, cardiovascular disease, AIDS and cancer (Yun, 1999; Shiao, 2003; Zhang and Lin, 2004). *G.lucidum* and its isolates have been known as a traditionally remedy, used in Chinese and Japanese traditional medicine for treatment of several diseases such as hepatitis, hypertension, hyperglycemia, chronic bronchitis, bronchial asthma, cancer and others(Yun,1999).

Scientific studies on the extracts and substances from *G.lucidum* have demonstrated their anti-aging, sedative, hepatoprotective, anti-allergic, hypoglycemic, cardiovascular, immunomodulatory and anti-viral effects (Lee and Rhee, 1990; Min *et al.*, 1998; Bao *et al*, 2001). Of particular significance among the reported biological / pharmacological properties of *G.lucidum* are its antitumor activities. *In vitro* and animal studies have indicated that *Ganoderma* exhibits cancer-preventive and anticancer activity. Recent clinical and pharmacological research substantiating benefits and effects of *Ganoderma lucidum* has focused further on cancer prevention as

well as treatments (Hu *et al.*, 2002; Gao, *et al.*, 2003). Current investigations carried out in *G.lucidum* occurring in South India has been shown to possess significant antioxidant, anti-inflammatory, antimutagenic, anti-carcinogenic, antitumor, hepatoprotective, nephroprotective and cardio protective activities (Sheena *et al.*, 2005).

2.1.5 Antitumor Polysaccharides

Polysaccharides are polymers of sugars (monosaccharides) joined to each other by glycosidic linkages. These are very complex molecules because sometimes covalent bonds occur between many pairs of carbon atoms. Consequently one sugar unit can be joined to more than two other sugars, which results in the formation of highly branched enormous macromolecules. Polysaccharides are a structurally diverse class of macromolecules able to offer the highest capacity for carrying biological information due to a high potential for structural variability (Wasser *et al.*, 2002). Whereas the nucleotides and amino acids in nucleic acids and proteins effectively, interconnect in only one way, the monosaccharide units in polysaccharides can interconnect at several points to form a wide variety of branched or linear structures (Sharon and Lis, 1993).

This high potential for structural variability in polysaccharides gives the necessary flexibility to the precise regulatory mechanisms of various cell-cell interactions in higher organisms. The polysaccharides of mushrooms occur mostly as glucans. Some of which are linked by β-(1-3), (1-6) glycosidic bonds and α-(1-3) glycosidic bonds but many are true heteroglycans. Most often there is a main chain, which is either β (1-3), β (1-4) or mixed β (1-3), β (1-4) with β (1-6) side chains. Hetero-β-D-glucans, which are linear polymers of glucose with other D-monosaccharides, can

have anticancer activity but α-D-glucans from mushroom usually lack anticancer activity (Wasser, 2002). Heteroglucan side chains contain glucuronic acid, galactose, mannose, arabinose or xylose as a main component or in different combinations. Glycans are polysaccharides containing units other than glucose in their backbone. A wide range of antitumor or immunostimulating polysaccharides of different chemical structure from higher Basidiomycetes mushrooms has been investigated (Wasser, 2002). Some correlation has been drawn between the chemical structure and antitumor activities of mushroom polysaccharides. A wide range of glycans extending from homopolymers to highly complex heteropolymers (Ooi and Liu, 1999) exhibits antitumor activity. Differences in activity can all be correlated with ability of the polysaccharide molecule to solubilize in water, size of the molecules, branching rate and form. Such structural features as β-(1-3) linkages in the backbone (main chain) of the glucan and additional β-(1-6)-branch points are needed for antitumor activity (Wasser, 2002). β-glucans with only (1-6) glycosidic linkages have little or no activity. Higher molecular weight glucans have been reported by Mizuno et al. (1996) and Mizuno *et al.*, (1999a) to be more effective than those of low molecular weight against tumors.

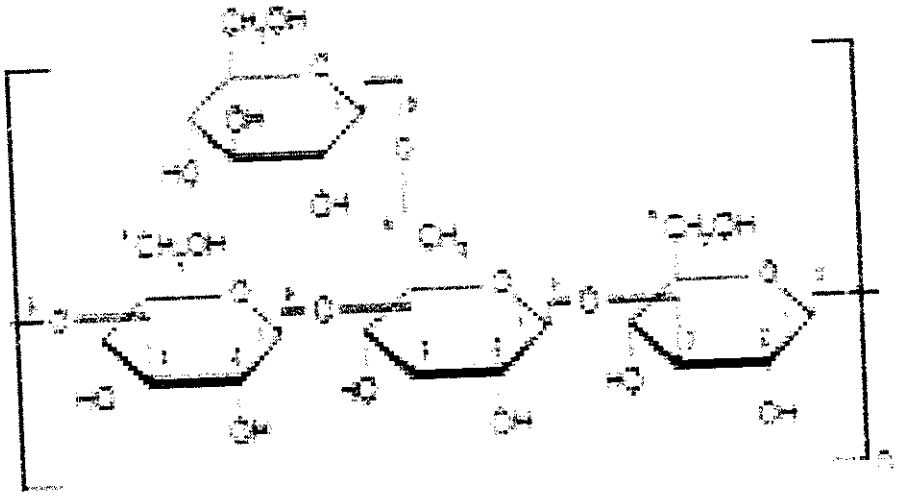


Fig 2.1.5: Structure of ß- D-glucan

G. lucidum is a mushroom traditionally used in oriental medicine because of its complex polysaccharides which are known to stimulate the immune response. Several polysaccharides obtained from fruiting bodies of *G. lucidum* were reported to have anti-tumor activity and consist of glucose, mannose, galactose and fucose (Mizuho *et al*, 1985; Bao *et al.*, 2002). This mushroom produces a peptidoglycan with hypoglycemic activity. Its glycan is composed of D-glucose and D-galactose and physico-chemical and chemical studies demonstrated that the backbone and side chains of peptidoglycan contained D-glucopyranosyl ß-1-3 and ß-1-6 linkages and a D-galactopyranosyl α-1-6 linkages (Tomoda.*et al*, 1986).

While water soluble ß- D-glucans are widely distributed in mushroom species, many species also contain ß- D-glucans with heterosaccharide chains of xylose, mannose, galactose and uronic acid which can be extracted by salt and alkali treatments. Other species can contain polysaccharide-peptide or glycoproteins which are polypeptide chains or small proteins to which polysaccharide ß- D-glucan chains are stably attached (Boldizsar *et al.*, 1998).

Beta-glucans found in certain fungi are thought to have anticancer properties. In Japan, mushroom-derived extracts rich in beta-glucans have been used for over 20 years in intravenous forms and are approved for use as adjuncts to chemotherapy.

2.2 *Ganoderma lucidum* in cancer:

The following are Indications and Evidence supporting the use of *Ganoderma* Supplementation in Cancer.

A. As a supplement during chemotherapy or radiotherapy to reduce side-effects such as fatigue, loss of appetite, hair loss, bone marrow suppression and risk of infection. There are studies demonstrating *Ganoderma* efficacy against fatigue (Yang 1994), hair loss (Miyamoto *et al.*, 1985), and bone marrow suppression (Jia 1993) and the presence of similar clinical evidence for other glucan BRMs applied in the setting of cancer chemotherapy or radiotherapy (Shi 1993) lends further support to the supplementation of *Ganoderma* in combination with cytotoxic cancer therapies.

B. As a supplement for cancer patients to enhance survival and reduce likelihood of metastasis. While, only data exists that *Ganoderma* supplementation may enhance survival of cancer patients, this survival advantage has been demonstrated for a number of comparable glucan BRMs.More appropriate for comparison to *Ganoderma* is perhaps PSK or PSP, which are orally administered. Mitomi *et al.*, (1994) found significantly improved survival and disease-free survival in resected colorectal cancer given PSK supplementation over three years when compared to control in a multi-center randomized controlled trial. In an animal model, *Ganoderma* has been demonstrated to effectively prevent metastasis (Lee,1984). Other

glucan BRMs have been demonstrated to effectively prevent or suppress pulmonary metastasis of methylcholanthrene-induced sarcomas, human prostate cancer DU145M, and lymphatic metastasis of mouse leukemia P388 (Kobayashi *et al,* 1995).

C. As a supplement for cancer patients to improve quality of life. Again, only anecdotal information exists for *Ganoderma* in this situation but other oral glucan derivatives such as PSP has been found to be useful in improving quality of life in cancer patients (Yao, 1993). Significantly, *Ganoderma* supplementation was noted to decrease pain in cancer patients (Kupin, 1994). The recommended dose would be five to ten grams of fruiting body or equivalent per day (Chang, 1994).

D. As a supplement for the prevention of occurrence or recurrence of cancer. Since immune stimulation, especially Natural Killer (NK) and Cytotoxic Lymphocyte (CTL) activation may be effective in the immune prevention of cancer by enhanced immune surveillance (Lotzova 1985), and *Ganoderma* has been demonstrated to enhance NK and CTL activity when administered orally (Won *et al,* 1989), it is thus a candidate for prevention of the occurrence or recurrence of cancer. Stavinoha et al. demonstrated the efficacy of *Ganoderma* in preventing the progression of microadenomatous growths in animals (Stavinoha 1993), and the efficacy of other glucan BRMs in primary and secondary cancer prevention have been similarly demonstrated in vitro, in vivo and in clinical trials.

The products of *G.lucidum* are prescribed in various forms; it can be injected as a solution of powdered spores or given as syrup. It can be taken as tea, soup, capsules, tinctures or bolus. The dried mushroom is prepared in water and given as a drink.

In Japan, *G.lucidum* is used for the treatment of the cancer (Willard,

1990). The results obtained after application shows that the patient sleeps well with a healthier feeling and has an increased appetite; Reishi also provides relief from angina pectoris. Injection of spore powder is effective in curing progressive deterioration, atrophy and muscles stiffness. The effect of elevation changes has been prevented and cured by tablets of mushroom spores (Mahendra Rai *et al* , 2005).

In an experimentally study for therapeutic application of *G.lucidum* 143 patients with advanced previously treated cancer were given an oral *G.lucidum* polysaccharide extract three times daily for 12 weeks. 27 patients were not assessable for response and toxicity, because they were unable to track for follow-up or refused further therapy before 12 weeks of treatment were up. Of the 100 fully assessable patients, 46 (32.2%) had progressive disease before or at the six weeks evaluation point. There was no significant change in the functional assessment of cancer Therapy-G (FACT-G) scores in 85 assessable patients. In the group with stable disease, FACT-G scores improved in 23 patients, remained unchanged in 5, and declined in one. This indicates that Ganopoly may have an adjunct role in the treatment of patients with advanced cancer although objective responses were not observed in the study (Wasser and Weis, 1997a).

2.3 MEDICINAL PROPERTIES of *G.Lucidum*

2.3.1 Antioxidant

The antioxidant activity of the extract of Reishi were assayed by FRAP (Ferric Reducing Power), DPPH (1, 1- diphenyl-2-picryl hydrazyl) assay and ABTS (2, 2-azobis-3-ethylbenzthiazoline-6-sulfonic acid) spectrophotometric assay using TEAC (Trolox Equivalent antioxidant capacity) and AEAC (Ascorbic Acid equivalent antioxidant capacity) as

standards. The antioxidant assays also showed that aqueous extract of the mushrooms possessed significant superoxide radical and hydroxyl radical scavenging and lipid peroxidation inhibiting activities. The FRAP assay indicated the first line defense (preventive antioxidant) activity of the extract which suppressed the formation of the free radicals. The ABTS assay showed the second line defense activity of the extract against free radicals (suppression of chain initiation and/or break of chain propagation reactions). The DPPH assay showed significant antioxidant activity of the extract to scavenge the primary free radicals (Sheena, *et al.* , 2005).

2.3.2 Anticarcinogenic

The anticarcinogenic activity of the extract of *G.lucidum* was determined by N-nitrosodiethylamine (NDEA) induced hepatocarcinoma (HCC) and 7; 12-dimethylbenz[a] anthracene (DBMA) induced rat mammary tumor models. Experimental results showed that all animals in the NDEA treated group developed liver tumors by the end of 32 weeks. The number of tumors and percent of incidence was reduced significantly in the group of animals administered with *G.lucidum* extract. The NDEA treatment drastically elevated gamma glutamyl transpeptidase (GGT), glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT), alkaline phosphatase (ALP) activities and lipid peroxidation. The treatment of the animals with the extract significantly reduced the enhanced activities of GGT, GPT, GOT and ALP and the melondialdehyde (MDA) level (lipid peroxidation). This indicated the preventive effect of *G.lucidum* extract against hepatocarcinoma caused by NDEA.

Mammary tumors were first observed in animals 73 days after the administration of DMBA. The treatment with *G.lucidum* extract delayed the

induction of the tumor by 25 days. The incidence of tumor was 100% in animals treated with DMBA alone while treatment with the extract reduced the incidence by 33.3%. DBMA treatment enhanced ALP level and the mushroom extract significantly reduced the activity of this enzyme (Sheena *et al.*, 2005).

2.3.3 Antitumor

Antitumor activity of the aqueous extract of the mushroom was determined by implanted tumor model in mice. Twenty-four hours after the implantation of tumor cells, the animals were administered with either aqueous extract of *G.lucidum* once daily for a period of 10 days. The tumor development was monitored for a period of one month and tumor volumes were measured. At the end of the experiment animals were sacrificed, tumors were excised and weighed. From these data tumor inhibition was calculated. The extracts inhibited >80% tumor volume and weight and results indicated significant antitumor property (Sheena *et al.*, 2005).

2.3.4 Prevention of anticancer drug toxicity

Cisplatin, diamine dichloride and Doxorubicin are extensively used as anticancer drugs. However, cisplatin chemotherapy is found to manifest dose-dependent nephrotoxicity and long-term administration of doxorubicin results in cumulative dose related cardiotoxicity in cancer patients.

The effect of extract of *G.lucidum* was examined to ameliorate the toxicity associated with these drugs. Extract of *G.lucidum* was administered orally 1hour before cisplatin administration. The results indicated that the extract significantly reduced the elevated serum creatinine and urea levels. Renal antioxidant defense systems, such as SOD, Catalase, GPx activities

and GSH levels depleted by cisplatin therapy were restored to normal with the treatment of the mushroom extract. Cisplatin- induced increased lipid peroxidation was also found markedly reduced by the treatment with the extract. The experimental findings indicated that the extract rendered significant protection against cisplatin- induced nephrotoxicity (Sheena *et al.*, 2005).

2.3.5 Hepatoprotective effect

Hepatoprocective effect of the extract of *G.lucidum* was evaluated using carbon tetrachloride induced chronic hepatotoxicity in rats. Hepatotoxicity was induced by the administration of 1.5 ml of carbon tetrachloride 3 times in a wee for 5 weeks. Two groups of animals were administered with the extract orally once daily for 5 weeks. 24 hours after the last dose of the treatments, animals were sacrificed, blood was collected and serun GPT, GOT and ALP were determined. Histopathological examination of the liver sections was also made. Carbon tetrachloride injection caused drastic increase in the activities of GPT, GOT and ALP indicating hepatic damages. The administration of the extract lowered the elevated levels of these enzymes in a dose dependent manner. The liver sections of rats treated with the mushroom extract showed well preserved architecture (Sheena *et al.*, 2005).

2.3.6 Immunomodulating effect

One of the important approaches to evaluate potential immunomodulating activity is the assessment of the capacity of particular substance to influence immune functions *in vivo, ex vivo* and *in vitro*, among them also cytokine synthesis. Many potential immunomodulating substances

have been synthesized ant tested for modulation of cytokine response, such as synthetic muramyl dipeptide (MDP) analogues (Parant, 1994; Simcic *et al.*, 2000; Gobec *et al.*, 2001), including a MDP analogue romurtide, which is an example of a registered immunomodulatory drug (Azuma, 1992; Kino *et al.*, 1991). In recent years, attention has been focused also on immunomodulatory active isolates from natural resources, well known in different national healing traditions. An example is potentially immunomodulatory active substances from *G.lucidum.* In 1997, Wang reported an increased interleukin (IL-1, IL-6), TNF and INF production by human macrophages and T-lymphocytes after incubation with polysaccharides from fruiting bodies of *G.lucidum* (Wang *et al.*, 1997).

2.3.7 Antithrombotic Compounds (Platelet Aggregation Inhibitors)

Some active compounds have been isolated from mushrooms as platelet aggregation inhibitor. They have also isolated and identified adenosin, guanosin, and their derivatives as potent inhibitors from the 80% ethanol extract of Reishi. A novel substance showing a higher activity than those of the nucleotides was also obtained. Its structure has been identified as both epimers of 5'-deoxy-5'methylsulphinyl adenosine (Mizuno, 1994).

2.3.8 Antimicrobial properties

In recent years Basidiomycetes and other higher fungi including some recognised medicinal mushrooms have been re-investigated as sources of novel antibiotics mainly as a result of increasing difficulty and the cost of isolating novael bioactive compounds from the *Actinomycetes* and *Streptomycetes* (Mahendra Rai *et al.*, 2005).The current practice of ingesting

phytochemicals to support the immune system or to fight infections is based on centuries-old tradition. While some of the herbaceous plants have a direct inhibitory effect on microbial organisms, they observed that each plant has at least one compound that selectively modulates cells of the immune system. The successful derivation of pure bioactive compounds from *G.lucidum*, ginseng and *Zingiber officinale* supports the traditional practice of using these plants to stimulate the immune system. As many modern drugs are often patterned after phytochemicals, studying the influence of each compound on immune cells as well as microbes can provide useful insights to the development of potentially useful new pharmacological agent.

2.3.9 Antiperoxidative and Anti-inflammatory effect

Free radical mediated genetic instability is widely thought to be a major etiological factor for initiation of carcinogenesis.Antiperoxidative activity was evaluated using Fe^{2+}-ascorbate-induced lipid peroxidation in rat liver homogenate and a phorbol ester induced lipid peroxidation in mouse skin. Anti-inflammatory activity was evaluated against carrageenan-induced acute and formalin-induced chronic inflammatory paw edema in mouse and phorbol ester-induced mouse skin inflammation. The results of this study revealed that extract of *G.lucidum* possessed significant antiperoxidative and antiinflammatory (Lakshmi, et *al.*, 2003).

2.4 FREE RADICALS

Free radicals can be defined as chemical species possessing an unpaired electron, which is formed by homolytic cleavage of a covalent bond of a molecule by the loss of a single electron from a normal molecule or by the addition of a single electron to a normal molecule. Free Radicals

are a group of active molecules with unpaired electrons that react with biological membrane, resulting in cell damage. Antioxidants are capable of taking up these free radicals. In doing so, they protect healthy cells from damage and abnormal growth. A number of antioxidant activity assays have been published, and its antioxidant activity is often expressed as Trolox Equivalent.

Most of the molecular oxygen consumed by aerobic cells during metabolism is reduced to water by using cytochrome oxidases in mitochondria. However, when oxygen is partially reduced, it becomes activated and reaches readily with a variety of biomolecules. This partial reduction occurs in one step, by addition of one, two and four electrons to oxygen, which leads to successive formation of reactive oxygen metabolites (ROMs). These ROMs include superoxide anion, hydroperoxy radical, peroxide ion, hydrogen peroxide and hydroxyl radical (Green and Hill, 1984).

The oxygen and hydrogen peroxide so formed may lead to the formation of the most reactive OH. This hydroxyl radical oxidizes lipids giving rise to lipid peroxidation. Hydrogen peroxide is known to cause DNA breaks in intact cells and purification DNA. Malondialdehyde (MDA) is the major reactive aldehyde resulting from the peroxidation of biological membranes of polyunsaturated fatty acid (PUFA) (Vaca *et al.,* 1998). MDA is the secondary product of LPO and used as an indicator of tissue damage (Ohkawa *et al.,* 1979). MDA can modify xanthine oxidoreductase activity through interaction with xanthine oxide and for xanthine dehydrogenase (XDH) (Cighetti *et al.,* 2001). Lipid hydroperoxides may directly induce DNA chain breaking (Cochrane, 1991), and lipid peroxyl and alkoxyl

radicals may cause base oxidation in DNA (Park, 1992).

The reactive oxygen metabolites produced during cell metabolism are believed to be involved in various disease processes including cancer. Their main targets are membrane lipids and enzymatic proteins, where they cause cell injury and death (Nadkarni *et al.*, 1991). The damage induced by activated oxygen species leads to fluid loss, protein modification, ion leaks and eventually cell lysis (Paller et *al.*, 1991). However, these problems are overruled by administering the antioxidant compounds.

2.5 Reactive oxygen species

2.5.1 Singlet oxygen

When energy is supplied to the ground state ($3O_2$) molecule, the spin of the unpaired electrons is reversed, producing high energy singlet, which is usually unstable and delays energy singlet state. Singlet oxygen can directly attack PUFA (Poly Unsaturated Fatty Acids), forming lipid hydroperoxides.

2.5.2 Superoxide ($O_2^{\cdot}$)

It is a single electron is accepted by the ground stsate oxygen molecules, superoxide radical is formed. The interior of the biological membrane is of the biological membrane is hydrophobic and oxygen produced in this environment colud be harmful, much of the oxygen generated within the cells is from the membrane bound systems (Halliwell *et al.*, 1987) and gets dismuted to hydrogen peroxide. The interaction of oxygen and hydrogen peroxide give rise to ($^{\cdot}OH$) which can damage membrane (Fridovich, 1975).

2.5.3 Perhydroxy radical ($OOH^{\cdot}$)

Protonation of O_2 gives rise to OOH radical (Perhydroxy radical) which can initiate peroxidation.

2.5.4 Hydrogen peroxide (H_2O_2)

Addition of an electron to oxygen generates peroxide ion, O_2 that is protonated immediately to give hydrogen peroxide.

2.5.5 Hydroxy radical ($OH^{\cdot}$)

Addition of an electron to H_2O_2 generates $OH^{\cdot}$. Hydroxy radical is very reactive and interacts immediately with all biological molecules in its vicinity producing secondary radicals of variable reactivity.

2.6 DEFENSE MECHANISMS

The human body although continuously produce free radicals, it possess several defense system which includes enzymes and radical scavengers. These are called first line antioxidant defense system. The second line defense system consists of repair system for biomolecules which are damaged by the attack of free radicals. Specific enzymes are known to be involved in the context and several of them have been identified in prokaryotes and in eukaryotes. The function of enzymes are involved in the repairing, directly damaged biomolecules such as lipids, polysaccharides, proteins, nucleic acids etc., or in eliminating oxidized compounds.

2.7 ANTIOXIDANTS

Oxygen is essential for aerobic life process. However, cells under

aerobic conditions are threatened with the reactive oxygen metabolites and are efficiently taken care by the powerful antioxidant system. Aerobic life is characterized as continuous production of oxidants balanced by equivalent synthesis of antioxidants (Rice Evans and Diplock, 1993).

A shift of the balance on the oxidant side may trigger a cascade of reaction leading to the formation of highly reactive cytotoxic compounds such as Reactive Oxygen Metabolites, ROMs (Halliwell and Gutteridge, 1989). The improper balance between ROMs production and antioxidant defense results in oxidative stress, which deregulates the cellular functions leading to various pathological conditions including cancer (Bandyopathyay *et al.,* 1999). In order to counteract the lethal effects of oxidative damage of DNA, normal living cells have developed multiple antioxidative defenses (Demple and Harrison, 1994).

2.7.1 ANTIOXIDANT AS SCAVENGERS

To deal with the free radicals or so called ROS the body is equipped with an effective defense system which includes various enzymes and high and low molecular weight antioxidants. Antioxidants neutralize free radicals by donating one of their own electrons, ending the electron stealing reaction. The antioxidants do not themselves become free radical by donating electrons because they are stable in other form. These act as scavenger and play the housekeeper's role by mopping up free radicals before they get a chance to create havoc in a body. Thus they may be well defined as the substances that are capable of quenching or stabilizing free radicals.

Antioxidants have also been suggested to have a well-defined role as preservatives. These have been defined by the US Food and Drug Administration (FDA) as substance used to preserve food by retarding

deteriortation, rancidity or discoloration caused by oxidation (Dziezak, 1986). Lipid peroxidation is an important deteriorative reaction of foods during processing and storage. Toxic substance formed by lipid peroxidation may lead to adverse effects such as carcinogenesis, cell DNA mutagenesis and aging. Antioxidants therefore, according to their mode of action, have also been classified as the compounds that terminate the free radical chain in lipid peroxidation by donating electrons or hydrogen to fat containing a free radical and to the formation of a complex between the chain and a free radical.Antioxidants stop the reactions by contributing hydrogen from the phenolic hydroxyl hydrogen from the phenolic hydroxyl groups, themselves forming stable free radicals that do not initiate or propagate further oxidation of lipids (free radical terminators). Some of the important synthetic antioxidants of this class are butylated hydroxyanisole (BHA), butylated hydroxy toluene, terbutyl hydroquinone (TBHQ), propyl gallate (PG) and tocopherols.

Antioxidants also referred to as chelators which bind metal ions such as copper and iron that catalyze lipid peroxidation; oxygen scavengers are those compounds that react with oxygen in closed systems and secondary antioxidants which function by breading down the hydroperoxides (Shahidi and Wanasundra, 1992).

In recent years, the use of some synthetic antioxidants has been restricred because of their possible toxic and carcinogenic effects (Frankel, 1991). This concern has resulted in an increased interest in the investigation of the effectivess of naturally occurring compounds with antioxidant properties. A number of plant and medicinal mushrooms constituents have been recognized to have positive effects when tested against the oxygen reactive compounds. Foods rich in antioxidants have been shown to play an

essential role in the prevention of cardiovascular diseases; cancers, neurodegenerative diseases, the most well known of which are Parkinson's and Alzheimer's diseases, inflammation and problem caused by cell and cutaneous aging.

2.7.2 TYPES OF ANTIOXIDANTS

Antioxidants are of different types such as natural or enzymic antioxidants, non-enzymic antioxidants, scavenging or chain breaking antioxidants, pharmacologic antioxidants and others (Irwin Fridovich and Joe Mc Cord, 1976).

2.7.3 ENZYMATIC ANTIOXIDANTS

The first line of defense against O_2 and H_2O_2 mediated injury are antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). The term antioxidant has been defined as any substance that delays or inhibits oxidative damage to a target molecule (Halliwell and Gutteridge, 1990). Antioxidant enzymes, together with the substances that are capable of either reducing ROMs or preventing their formation, form a powerful reducing buffer which affects the ability of the cell to counteract the actions of oxygen metabolites and which maintain the lowest possible levels of ROMs inside the cells (Sies, 1997).

2.7.3.1 Superoxide dismutase (SOD)

SODs are a family of metalloenzymes that convert O_2 to H_2O_2. SOD is the most important enzyme, because it is found virtually in all aerobic organisms. The transition metal of the enzyme reacts with O_2 taking its electron. SOD is considered to be a stress protein, which is synthesized in

response to oxidative stress (Mc Cord, 1990).

2.7.3.2 Glutathione peroxidase (GPx)

Glutathione peroxidase is well known first line of defense against oxidative stress, which in turn requires glutathione as a cofactor. GPx catalyses the oxidation of GSH to GSSG at the expense of H_2O_2. By its selenium dependency, GPx can be divided into two forms namely selenium dependent GPx and selenium independent GPx.

2.7.3.3 Catalase

Catalase is an enzyme, which is present in most cells and catalyzes the decomposition of hydrogen peroxide to water and hydrogen. It is a heme-containing protein and reacts 104 times faster than peroxidase.

2.7.3.4 Glutathione-S- transferase

GSTs catalyze the conjugation of reduced glutathione via the sulfhydryl group, to electrophilic centers on a wide variety of substrates. This activity is useful in the detoxification of endogenous compounds such as peroxidised lipids as well as the metabolism of xenobiotics.

2.7.4 NON-ENZYMATIC ANTIOXIDANTS

2.7.4.1 Total reduced glutathione

Reduced glutathione, most commonly called glutathione or GSH, is a relatively small molecule ubiquitous in living systems (Kidd, 2000; Sen, 1997). Occurring naturally in all human cells, GSH is a water-phase orthomolecule. Its intracellular depletion ultimately results in cell death and

its clinical relevance has been researched for decades. Technically N-L-gamma-glutamyl-cysteinyl glycine or L-glutathione, the molecule has a sulfhydryl (SH) group on the cysteinyl portion, which accounts for its strong electron-donating character. As electrons are lost the molecule becomes oxidized, and two such molecules become linked (dimerized) by a disulfide bridge to form glutathione disulfide or oxidized glutathione (GSSG). GSH is an extremely important cell protectant. It directly quenches reactive hydroxyl free radicals, other oxygen-centered free radicals, and radical centers on DNA and other biomolecules. It contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain. Glutathione, an antioxidant, protects cells from toxins such as free radicals.

2.7.4.2 Antioxidative vitamins

The major protective functions of the vitamins are the scavenging of ROMs (Torn *et al.*, 1995). Antioxidative vitamin A, E and C have a number of biological activities such as immune stimulation, alteration of metabolic activations of carcinogens, mopping up free radicals, etc., other natural body antioxidant compounds include cysteine, glutathione and transferrin. Although free radicals are implicated in many diseases, it is not possible that free radicals are not the cause, but consequence of the disease atleast in some situations.

2.7.4.2.1 Vitamin C

An important non-enzymatic antioxidant in blood plasma is ascorbic acid (vitamin c). It functions mainly by donating an electron to a lipid radical. Vitamin c is known to be a potential antioxidant and is essential for

the functioning of the central nervous systems (CNS) and helps in fighting infectious diseases.

2.8 ANTIOXIDANT CAPACITY ASSAYS

2.8.1 DPPH radical scavenging activity

The 1, 1-diphenyl -2-picryl hydroxyl (DPPH) radical was widely used as the model system to investigate the scavenging activities of several natural compounds such as phenolic and anthocyanins or crude mixtures such as the ethanol extract of plants. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH.When DPPH is placed in an assay system containing free radical scavengers such as flavonoids, the color vanishes. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition.

2.8.2 Trolox Equivalent Antioxidant capacity Assay

In this improved version, $ABTS^{\cdot}$, the oxidant is generated by persulfate oxidation of 2, 2,-azinobis (3-ethylbenzoline-6- sulfonic acid) - ($ABTS^{2-}$). Specifically, 7 mmol of ABTS ammonium was dissolved in water and treated with 2.45 mmol of ammonium persulphate and the mixture was then allowed to stand at room temperature for 12-16 h to give a dark blue solution. This solution was diluted with ethanol or buffer (pH 7.4) until the absorbance reached 0.7 at 734 nm. One milliliter of the resulting solution was mixed with 10µl of sample. The absorbance was read at 30°C at 1, 4, and 6 min after mixing at 30°C. The difference of the absorbance reading is plotted versus the antioxidant concentrations to give a straight line.

2.8.3 Hydroxy radical and Nitric oxide scavenging assay

The hydroxy radical scavenging activity was measured as the percentage of inhibition of hydroxy radicals generated in the Fenton's reaction mixture.Hydroxy radical scavenging activity was measured by studying the competition between deoxy ribose and the extract for hydroxy radicals generated from the Fe^{3+}/Ascorbate/EDTA/H_2O_2 system. The hydroxy radicals attack deoxyribose, which eventually results in TBARS formation.

Nitric oxide is a potent phototropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity.

2.8.4 Lipid peroxidation inhibition assay

Initiation of lipid peroxidation by ferrous sulphate takes place either through hydroxy radical by Fenton's reaction. The inhibition could be caused by absence of ferryl-perferryl complex or by scavenging the hydroxy radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself.

Iron catalyses the generation of hydroxy radicals from hydrogen peroxide and superoxide radicals. The hydroxy radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane. Lipid hydroperoxides can be decomposed to produce apoxy and peroxy radical they eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damages, generation of cancer and aging related

disease (Okhawa *et al.*, 1979). Thus, decrease in MDA level with increase in the concentration of the extracts indicates the role of the extracts as an antioxidant.

2.8.5 Reducing power

The reducing capacity was investigated by measuring Fe^{3+}-Fe^{2+} conversion. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activities of putative antioxidants have been attributed to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued proton abstraction and radical scavenging.

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 Materials

All chemicals and solvents used were of analytical grade and were obtained from Sigma chemicals, USA and Loba chemicals.

3.2 Mushroom

The mushroom *Ganoderma lucidum* were collected from Top Slip, Pollachi, Tamilnadu. The mushroom was authenticated by Plant Pathologist, TamilNadu Agricultural University (TNAU), Coimbatore.

3.3 Methods

3.3.1 Phase I

3.3.1.1 Preparation of mushroom extract:

The fruiting body of *Ganoderma lucidum* was cut into small pieces and dried at room temperature for 48 hours and then powdered. The powdered sample was homogenized with 50 mM phosphate buffer (pH 7.4) in mortar and pestle and centrifuged at 10000 rpm for 15 minutes. The supernatant obtained was used for the estimation of enzymatic and non-enzymatic antioxidants.The fresh mushroom sample processed in the similar way was also used for experimental studies.

3.3.1.2 Estimation of superoxide dismutase (SOD)

The activity of SOD was estimated by the method as illustrated in Appendix 1.

3.3.1.3 Estimation of catalase

The activity of Catalase was assayed by the method as presented in Appendix 2.

3.3.1.4 Estimation of glutathione peroxidase (GPx)

Glutathione peroxidase was determined by the method as in Appendix 3.

3.3.1.5 Estimation of glutathione S- transferase (GST)

The activity of glutathione S- transferase was determined according to the method as represented in Appendix 4.

3.3.1.6 Estimation of total reduced glutathione (GSH)

GSH was estimated by the method as described in Appendix 5.

3.3.1.7 Estimation of Vitamin C (Ascorbic acid)

The ascorbic acid levels were determined as given in Appendix 6.

3.3.1.8 Estimation of protein

The protein levels in the sample were estimated by Lowry method as explained in Appendix 7.

3.3.2 Phase II

The procedure for extraction of polysaccharide fractions from fruiting body of *Ganoderma lucidum* was described in Appendix 8.

3.3.3 Phase III

The following assays are used to estimate the free radical scavenging activity present in fruiting body of *Ganoderma lucidum.*

3.3.3.1 Estimation of DPPH scavenging activity

DPPH scavenging activity was measured spectrophotometrically as summarized in Appendix 9.

3.3.3.2 Estimation of ABTS radical cation scavenging activity

ABTS was assayed by spectrophotometric method as depicted in Appendix 10.

3.3.3.3 Estimation of nitric oxide scavenging activity

The nitric oxide scavenging activity was measured spectrophotometrically as summarized in Appendix 11.

3.3.3.4 Estimation of hydroxy radical scavenging activity

The level of hydroxy radical scavenging activity was measured by spectrophotometric method as depicted in Appendix 12.

3.3.3.5 Estimation of super oxide radical scavenging activity

The super oxide radical scavenging activity was measured spectrophotometrically as described in Appendix 13.

3.3.3.6 Determination of lipid peroxidation inhibition activity

The lipid peroxidation was assayed spectrophotometrically as explained in Appendix 14.

3.3.3.7 Determination of ferric reducing antioxidant potential

The reducing power was measured by the spectrophotometric method as described in Appendix 15.

3.4 Statistical Analysis

The results were statistically analysed using F- test and comparison was made by DMRT analysis. Statistical comparisons were made using student T-test, the significance of difference between means of control of treated groups were determined by DMRT analysis.

RESULTS AND DISCUSSION

4.0 RESULTS AND DISCUSSION

Oxidative stress has been implicated in the pathology of many diseases, inflammatory conditions, cancer and aging (Marx, 1987). Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by many other mechanisms and thus prevent disease (Youdim *et al.*, 2001).

"Lingzhi" (*G.lucidum*), a popular medicinal mushroom, has been used in China for longevity and health promotion since ancient times. Investigations into the anticancer activity of lingzhi have been performed in both *in vitro* and *in vivo* studies, supporting its application for cancer treatment and prevention. The proposed anticancer activity of lingzhi has prompted its usage by cancer patients. It remains debatable as to whether lingzhi is a food supplement for health maintenance or actually a therapeutic "drug" for medical proposes. Thus far there has been no report of human trials using lingzhi as a direct anticancer agent, despite some evidence showing the usage of lingzhi as a potential supplement to cancer patients. Cellular immune responses and mitogenic reactivity of cancer patients have been enhanced by lingzhi, as reported in two randomized and one nonrandomized trials, and the quality of life of 65% of lung cancer patients improved in one study. The direct cytotoxic and anti-angiogenesis mechanisms of lingzhi have been established by in vitro studies; however, clinical studies should not be neglected to define the applicable dosage in vivo. At present, lingzhi is a health food supplement to support cancer patients, yet the evidence supporting the potential of direct *in vivo* anticancer effects should not be underestimated. Lingzhi or its products can be

classified as an anticancer agent when current and more direct scientific evidence becomes available (John Yuen *et al.*, 2005).

Biologically active substances from higher Basidiomycetes possess antifungal, antibacterial, and antiviral properties; they can be used as insecticidal and nematocidal agents. In medicine they are used to immunomodulate both humoral and cellular immune factors in the body. Polyfunctional acidic glucuronoxylomannan isolated from jelly mushrooms (*Tremella* spp., Tremellaceae), for instance, stimulates vascular endothelial cells, possesses pronounced antiradiating effects, stimulates hematogenesis, demonstrates antidiabetic, anti-inflammatory, hypocholesterolemic, anti-allergic activities, and shows hepatoprotective effects. It can be recommended to improve immunodeficiency, including that induced by AIDS, physical stress or aging, and it prevents senile degeneration of microvessels, maintaining better blood perfusion conditions in vital organs(Wasser *et al.*,2000).

Reactive oxygen species (ROS), such as superoxide anions and hydroxyl radicals, are associated with carcinogenesis and other pathophysiological conditions. Therefore, elimination or inactivation of ROS or inhibition of their excess generation may be beneficial in terms of reducing the risk for cancer and other diseases.*G. lucidum* has been used in traditional oriental medicine and has potential antiinflammatory and antioxidant activities (Jong-Min Lee *et al.*, 2005).

Antioxidants are known to protect the body against free radical mediated toxicities, a large number of plants and mushrooms have shown potent antioxidant activities (Badami *et al.*, 2003). The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of

sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases. There are a number of clinical studies suggesting that the antioxidants in fruits, vegetables, tea and red wine are the main factors for the observed efficacy of these foods in reducing the incidence of chronic diseases including heart disease and some cancers. The free radical scavenging activity of antioxidants in foods have been substantially investigated and reported in the literature by Miller and Rigelhof *et al.*, (2000).

Although *Ganoderma* and its derivatives are not pharmaceuticals and have not undergone rigorous clinical trials to be tested against cancer, there is abundant use *in vitro*, animal and indirect clinical evidence to support its supplemental use in cancer. Standardization in bioactive polysaccharide content and dosages will be necessary to assure its rational use, and clinical trials in select cancers with defined endpoints will confirm its efficacy (Raymond Chang, 1994).

Phase I

4.1 Enzymatic antioxidants

The enzymatic antioxidants defence systems are the nature protector against lipid peroxidation. SOD, Catalase and GPx enzymes are important scavengers of superoxide ion and hydrogen peroxide. These enzymes prevent the generation of hydroxyl radical and protect the cellular constituents from oxidative damage (Osawa *et al.*, 1995).SOD and catalase

are the two major scavenging enzymes that remove the toxic free radicals *in vivo*.

Mushroom	SOD	Catalase
Group 1	11.36 ± 1.58 a**	499.60 ± 31.53 a**
Group 2	6.79 ± 1.13	21.36 ± 3.36

Table 4.1.1: Activities of hydrogen peroxide decomposing enzymes in fresh and dried fruiting body of *Ganoderma lucidum*.

Values are expressed as mean ± SD of 3 replicates.

SOD- 50% inhibition of nitrite/min/mg protein

Catalase- n moles of H_2O_2 decomposed/min/mg protein

Treatment of Groups:

Group 1: Fresh mushroom.

Group 2 : Dried mushroom.

Comparisons made between the groups

a. Group 1 and Group 2.

Statistical representation

* $p< 0.05$ significance at 5% level.

** $p<0.01$ significance at 1% level

4.1.1 Peroxide metabolising enzymes

From table 1 it can be inferred that the superoxide dismutase activity was found to be significantly higher in fresh mushroom than dried one. Also from table 1 it was inferred that the catalase activity was found to be significantly higher in fresh. Hence the fresh one serves as a rich source of these free radicals scavenging enzymes.

Mushroom	GPx	GST
Group 1	274.28 ± 31.54 a*	241.28 ± 30.77 a**
Group 2	218.26 ± 36.80	74.65 ± 17.78

Table 4.1.2: Activities of Glutathione metabolizing enzymes in fresh and dried fruiting body of *Ganoderma lucidum*.

Values are expressed as mean ± SD of 3 replicates.

GPx-n moles of GSH consumed/min/mg protein

GST-μmoles of CDNB-GSH conjugate formed/min/mg protein.

Treatment of Groups:

Group 1: Fresh mushroom.

Group 2 : Dried mushroom.

Comparisons made between the groups

a. Group 1 and Group 2.

Statistical representation

* $p< 0.05$ significance at 5% level.

** $p<0.01$ significance at 1% level

4.1.2 Glutathione metabolizing enzymes

4.1.2.1 Glutathione peroxidase

Glutathione peroxidase is a cytosolic enzyme involoved in glutathione oxidation. The cells maintain the cellular level of glutathione predominantly in the reduced state by fast reduction of oxidised glutathione to reduced glutathione using the nicotinamide adenine dinucleotide phosphate (NADPH) dependent glutathione reductase. NADPH is regenerated from $NADP^+$ by the pentose phosphate pathway enzyme G6PD.The efficiency of

the glutathione defence system depends on the endogenous synthesis of glutathione and its replenishment from its oxidised form in tissues.

GR is a family of homologous proteins whose members are dimeric NADPH dependent and FAD containing enzymes. Significantly GR maintains the cellular level of GSH (by the reduction of GSSG), which protects the cellular membranes from peroxides (Zarida *et al.*, 1993).

4.1.2.2 Glutathione-S- Transferase

Glutathione-S- Transferase is a group of selenium independent enzyme that exhibits glutathione peroxidase activity with fatty acid peroxides, utilised as hydrogen donor (Jakoby and Habig, 1980).

From the table 2 it is evident that fresh mushroom exhibited an increased glutathione -S- transferase activity. In addition it is also demonstrated that fresh mushroom exhibited increased activity of glutathione peroxidase.

From the above observations it was concluded that fresh mushroom showed higher glutathione metabolising enzyme activities.

4.2 Nonenzymatic antioxidants

Nonenzymatic antioxidants such as reduced glutathione, vitamin C and vitamin E, play an excellent role in protecting the cells from oxidative damage (Koranda 1981; Magwere *et al.*, 1997). It is well estabilshed that the GSH in blood keeps up the cellular levels of the active forms of Vitamin C and Vitamin E by neutralising the free radical when there is a reduction in the GSH. The cellular levels of Vitamin C are also lowered, indicating that GSH, Vitamin C and Vitamin E are closely interlinked to each other (Murugavel *et al.*, 2004).

Mushroom	GSH	Vitamin C
Group 1	10.30 ± 0.30 a**	0.39 ± 0.03 a*
Group 2	3.00 ± 0.30	0.24 ± 0.02

Table 4.2: Levels of nonenzymatic antioxidants in fresh and dried fruiting body of *Ganoderma lucidum*.

Values are expressed as mean ± SD of 3 replicates

GSH- µg/g tissue.

Vitamin C-mg/g tissue.

Treatment of Groups:

Group 1: Fresh mushroom.

Group 2 : Dried mushroom.

Comparisons made between the groups

a. Group 1 and Group 2.

Statistical representation

* $p< 0.05$ significance at 5% level.

** $p<0.01$ significance at 1% level

4.2.1 Total reduced glutathione

Glutathione is an important consitutent of detoxification mechanism operating in biological system. Glutathione reduced the formation of toxic lipid peroxide and hydrogen peroxide in biological system by acting as substrate for glutathione peroxidase (Banumathi *et al.*, 1992). Synergistic action of glutathione-S-transferase detoxifies a wide range of xenobiotics and hence exhibits important functions in cellular protection (Zheng and Kenny, 1993). Dietary GSH has been shown to be an effective anticarcinogen against a wide range of carcinogen (Novi, 1981; Lotlikar *et al.*, 1981).

Glutathione can fuction as an antioxidant in many ways. It can react chemically with singlet oxygen, superoxides and hydroxyl radicals and therefore, function directly as free radical scavenger. GSH may stabilise membrane structure by removing acyl peroxides formed by lipid peroxidation reaction (Prince *et al.*, 1990). GSH is the reducing agent that reduces anti-inflammatory and oxidative effects (Gaulejac *et al.*, 1999).

From table 3 it was concluded that fresh mushroom showed higher reduced glutathione activity compared to dried mushroom.

4.2.2 Antioxidant Vitamins

Vitamin C occurs abduntantly in fruits and vegetables especially citrus, berries, tomatoes, leafy vegetables and cabbage heavily. Water soluble Vitamin C neutralises free radicals that oxidise LDL cholesterol thereby preventing artery wall damage that precipitates atherosclerosis.Table 3 shows that fresh mushroom has higher level of ascorbic acid compared to dried sample.

Phase II

4.3 Extraction of polysaccharide fractions from fruiting body of *G. lucidum.*

Many of the biological effects of *Ganoderma* have recently been reported in scientific literature. Many of these suggest that *Ganoderma* has both antioxidant and free radical scavenging property (Lai *et al.*, 2006). During the past two decades, modern research has revealed that *Ganoderma* contains variety of chemical ingredients, including triterpenes, polysaccharides, nucleosides, steroids, fatty acids, alkaloids, proteins, peptides, amino acids and inorganic elements (Huie and Di, 2004). Among

these ingredients, triterpenes and polysaccharides have attractive considerable attention as they have been shown to possess diverse and potentially significant pharmacological activities. The water soluble preparations of *Ganoderma* extract contains many bioactive ingredients (mainly polysaccharides and some triterpenes). Four fractions of *Ganoderma* extract were isolated according to the procedure reported by Mizuno, (1999). *Ganoderma* extract contains different low molecular weight components including monosaccharides, oligosaccharides, triterpenes, Vitamins and selenium. Of particular interest, the polysaccharides have been reported to possess significant antioxidant activity. The polysaccharide component with the branched (1-3) - beta-D- glucan moiety from *G.lucidum* has shown evidence of enhancement of immune responses and of eliciting anti-tumor effects.

Phase III

4.4 Antioxidant capacity assays

4.4.1 DPPH scavenging activity

DPPH is a relatively stable free radical. The assay is based on the measurements of the scavenging ability of antioxidants towards the stable radical DPPH. DPPH radicals react with suitable reducing agents. The electrons become paired off and the solution loses colour stiochiometrically depending on the number of electrons taken up (Blois 1958).

The proton radical scavenging action is known as an important mechanism of auto-oxidation. DPPH was used to determine the proton radical scavenging action of fractions 1, 2, 3 and 4, because it possesses a proton free radical and shows a characteristic absorbance at 517nm. The

purple colour of DPPH solution fades rapidly when it encounters proton radical scavengers (Yamaguchi *et al.*, 1998).

Concentration (µg/ml)	% inhibition of fractions			
	1	2	3	4
50	42.70	31.00	22.70	13.10
100	52.80	35.10	37.15	29.70
150	57.40	47.50	42.60	35.21
200	59.60	54.70	54.30	56.01
250	67.00	59.40	60.30	58.90

Table 4.4.1: DPPH scavenging activity of polysaccharide fractions isolated from fruiting body of *G.lucidum*

Values are expressed as mean of 3 replicates.

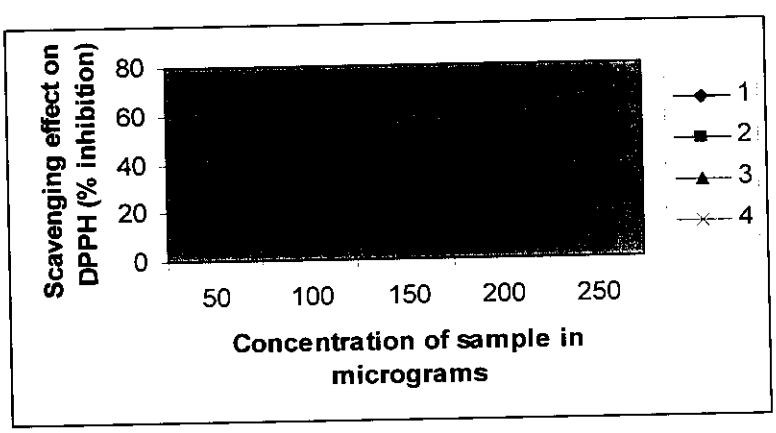


Figure 4.4.1.1: Dose response curves of DPPH radical scavenging activity of polysaccharide fractions isolated from *G.lucidum*

Fraction 1: Hot water extract.

Fraction 2: Ammonium oxalate extract.

Fraction 3: Acetic acid- NaOH extract.

Fraction 4: Ethanol-NaOH extract.

Fig 2 and table 4 shows the dose response curve and percentage inhibition values for the radical scavenging activity of fractions of 1, 2, 3 and 4 respectively.

At a dose of 50-250µg/ml, the methanolic extract of fraction 1 showed 42.70% to 67.00% inhibition with IC_{50} of 85µg/ml. At a dose of 50-250µg/ml, the methanolic extract of fraction 2 showed 31.00% to 35.10% inhibition with IC_{50} of 170µg/ml. Similarly the methanolic extract of fraction 3 and fraction 4 showed 22.70% to 60.30% and 13.10% to 58.90% inhibition with IC_{50} of 185µg/ml. From the present results, it may be postulated that fraction 1 reduces the radical to corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principle (Sanchez-Moreno, 2002). Studies by Gezer *et al* (2006) showed that ethanolic extracts of *Ramaria flava* was capable of quenching DPPH radical with IC_{50} value of 276µg/ml.

4.4.2 ABTS radical cation decolorization assay

ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{+}$, which has a characteristic long wavelength absorption spectrum (Sanchez-Moreno, 2002).

Concentration (µg/ml)	% Inhibition of fractions			
	1	2	3	4
50	24.89	32.13	36.19	31.03
100	32.58	36.65	44.14	32.31
150	38.00	38.91	46.38	38.62
200	41.63	40.27	47.51	38.46
250	49.77	47.06	54.29	41.54

Table 4.4.2: ABTS cation scavenging activity of polysaccaharide fractions isolated from fruiting body of *G.lucidum*

Values are expressed as mean of 3 replicates.

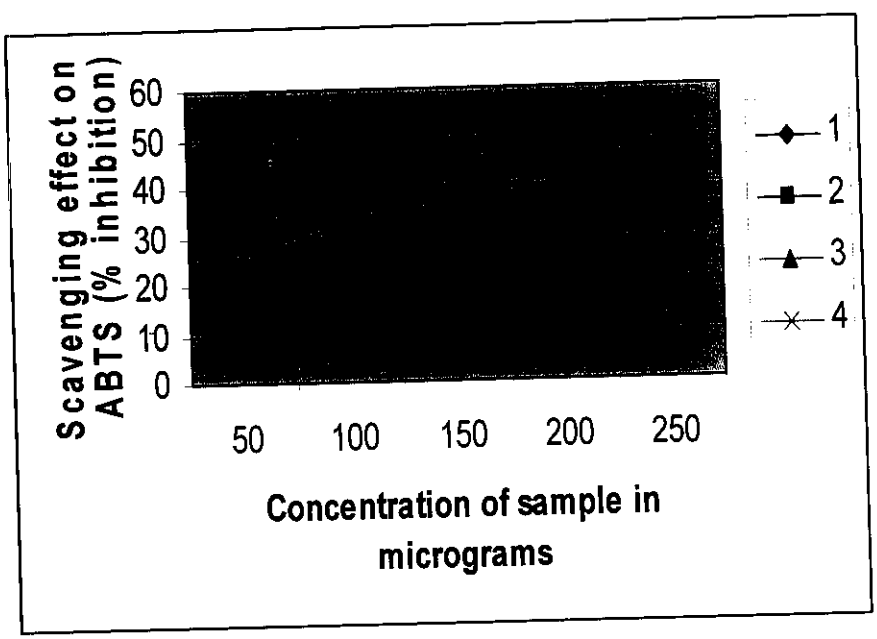


Figure 4.4.2.1: Dose response curves of ABTS cation scavenging activity of polysaccharide fractions isolated from *G.lucidum*

Fraction 1: Hot water extract.

Fraction 2: Ammonium oxalate extract.

Fraction 3: Acetic acid- NaOH extract.

Fraction 4: Ethanol-NaOH extract.

Fig 3 and table 5 shows the dose response curve and percentage inhibition values for the radical scavenging activity of fractions of 1, 2, 3 and 4 respectively. At a dose of 50-250µg/ml, the water extract of fraction 1 showed 24.89% to 49.77% inhibition with IC_{50} of 173µg/ml. At a dose of 50-250µg/ml, the water extract of fraction 2 showed 32.13% to 47.06% inhibition with IC_{50} of 183µg/ml. Similarly the water extract of fraction 3 and fraction 4 showed 36.19% to 54.29% and 31.03% to 41.54% inhibition with IC_{50} of 73µg/ml and 228µg/ml respectively. From the present results, it may be postulated that fraction 3 inhibit or scavenge the $ABTS^{+}$ radicals. Studies conducted by Akanksha Mishra *et al.*(2006) reported that the aqueous extract of Indian spice *Garcinia indica* possessed highest antioxidant potential with the value of 1.005 ± 0.08.

4.4.3 Nitric oxide scavenging activity

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases (Ross, 1993; Ialenti *et al.*, 1993).

Concentration (µg/ml)	% inhibition of fractions			
	1	2	3	4
50	68.74	58.12	68.17	67.42
100	70.32	60.65	70.65	69.11
150	74.62	65.22	74.81	70.32
200	78.71	68.02	79.35	71.27
250	80.32	72.58	89.03	73.87

Table 4.4.3: Nitric oxide radical scavenging activity of polysaccaharide fractions isolated from fruiting body of *G.lucidum*

Values are expressed as mean of 3 replicates.

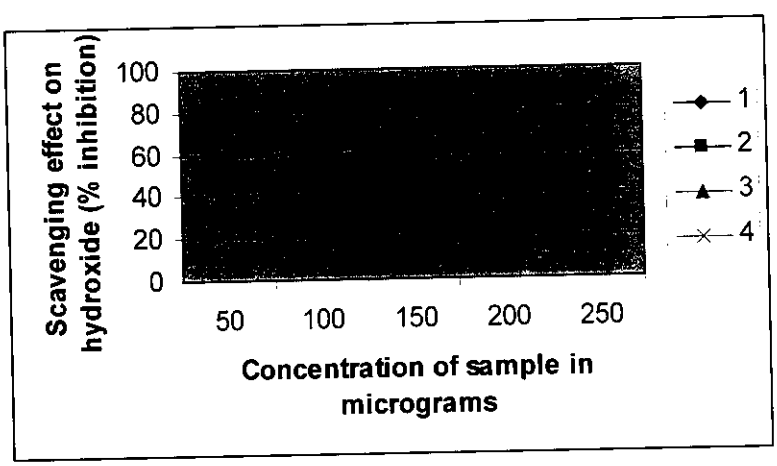


Figure 4.4.3.1: Dose response curves of nitric oxide radical scavenging activity of polysaccharide fractions isolated from *G.lucidum*

Fraction 1: Hot water extract.

Fraction 2: Ammonium oxalate extract.

Fraction 3: Acetic acid- NaOH extract.

Fraction 4: Ethanol-NaOH extract.

Fig 4 and table 6 shows the dose response curve and percentage inhibition values for the radical scavenging activity of fractions of 1, 2, 3 and 4 respectively. At a dose of 50-250µg/ml, the water extract of fraction 1 showed 68.74% to 80.32% inhibition with IC_{50} of 155µg/ml. At a dose of 50-250µg/ml, the water extract of fraction 2 showed 58.12% to 72.58% inhibition with IC_{50} of 285µg/ml. Similarly the water extract of fraction 3 and fraction 4 showed 68.17% to 89.03% and 67.42% to 73.87% inhibition with IC_{50} of 145µg/ml and 243µg/ml respectively. In the present study, the nitrite produced by the incubation of solution of sodium nitroprusside in standard phosphate buffer at 25°C was reduced by the fraction 3. This may be due to the antioxidant principles in the extract which compete with oxygen to react with nitric oxide (Marcoccil *et al.,* 1994), thereby initiating the generation of nitrite. Similar results were observed by Madan Mohan Pandey *et al.,* (2005) in *Saussarea costus* with the maximum inhibition of 58.4% achieved with 1mg/ml ethanolic extract concentration.

4.4.4 Hydroxy radical scavenging activity

The hydroxy radical scavenging activity of different fractions was measured as the percentage of inhibition of hydroxy radicals generated in the Fenton's reaction mixture. Hydroxy radical scavenging activity was measured by studying the competition between deoxy ribose and the extract for hydroxy radicals generated from the Fe^{3+}/Ascorbate/EDTA/H_2O_2 system. The hydroxy radicals attack deoxyribose which eventually results in TBARS formation.

Concentration (µg/ml)	% inhibition of fractions			
	1	2	3	4
50	48.80	45.07	45.18	54.32
100	51.19	48.02	45.63	54.71
150	51.97	48.80	46.96	56.82
200	53.93	53.54	49.97	58.22
250	54.54	55.93	55.59	61.00

Table 4.4.4: Hydroxy radical scavenging activity of polysaccaharide fractions isolated from fruiting body of *G.lucidum*

Values are expressed as mean of 3 replicates.

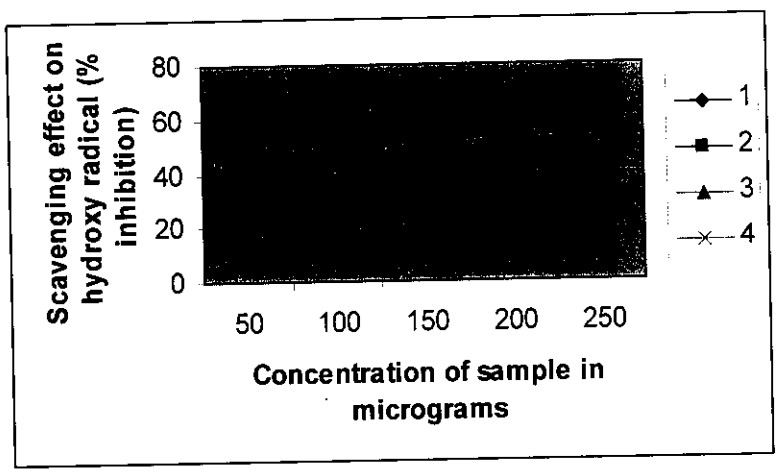


Figure 4.4.4.1: Dose response curves of hydroxy radical scavenging activity of polysaccharide fractions isolated from *G.lucidum*

Fraction 1: Hot water extract.

Fraction 2: Ammonium oxalate extract.

Fraction 3: Acetic acid- NaOH extract.

Fraction 4: Ethanol-NaOH extract.

Fig 5 and table 7 shows the dose response curve and percentage inhibition values for the radical scavenging activity of fractions of 1, 2, 3 and 4 respectively. At a dose of 50-250µg/ml, the water extract of fraction 1 showed 48.80% to 54.54% inhibition with IC_{50} of 70µg/ml. At a dose of 50-250µg/ml, the water extract of fraction 2 showed 45.07% to 55.93% inhibition with IC_{50} of 165µg/ml. Similarly the water extract of fraction 3 and fraction 4 showed 45.18% to 55.59% and 54.32% to 61.00% inhibition with IC_{50} of 205µg/ml and 113µg/ml respectively. From the present result, it may be suggested that fraction 2 have better hydroxy radical scavenging activity as reflected in terms of inhibition (%).Studies conducted by Janardhanan, (2000) reported that ethyl acetate extract of *Pleurotus florida* exhibited potent antioxidant activity of hydroxy radical with IC_{50} value of 530µg/ml.

4.4.5 Superoxide radical scavenging activity

Superoxide Dismutase (SOD) is the cellular antioxidant enzyme which removes this ubiquitous superoxide metabolic product by converting it into H_2O_2 and oxygen (Kamala kannan *et al.*, 2003).

Concentration (µg/ml)	% inhibition of fractions			
	1	2	3	4
50	66.67	25.00	54.17	26.25
100	77.08	45.83	66.67	39.58
150	83.33	62.50	77.08	43.75
200	89.58	85.42	85.42	58.33
250	93.75	87.50	87.50	83.33

Table 4.4.5: Superoxide radical scavenging activity of polysaccaharide fractions isolated from fruiting body of *G.lucidum*

Values are expressed as mean of 3 replicates.

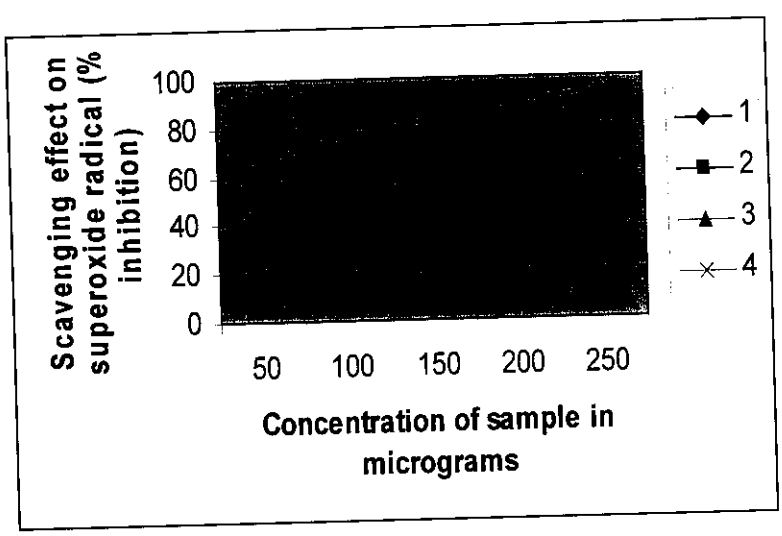


Figure 4.4.5.1: Dose response curves of Superoxide radical scavenging activity of polysaccharide fractions isolated from *G.lucidum*

Fraction 1: Hot water extract.

Fraction 2: Ammonium oxalate extract.

Fraction 3: Acetic acid- NaOH extract.

Fraction 4: Ethanol-NaOH extract.

Fig 6 and table 8 shows the dose response curve and percentage inhibition values for the radical scavenging activity of fractions of 1, 2, 3 and 4 respectively. At a dose of 50-250µg/ml, the water extract of fraction 1 showed 66.67% to 93.75% inhibition with IC_{50} of 90µg/ml. At a dose of 50-250µg/ml, the water extract of fraction 2 showed 25.00% to 87.50% inhibition with IC_{50} of 83µg/ml. Similarly the water extract of fraction 3 and fraction 4 showed 54.17% to 87.50% and 06.25% to 83.33% inhibition with IC_{50} of 140µg/ml and 105µg/ml respectively. Superoxide anion is the first reduction product of oxygen (Re *et al.*, 1999) which is measured in terms of inhibition of generation of $O_2^{\cdot-}$. The result shows that fraction 1 has a potent activity with the increasing (%) inhibition. A similar trend in superoxide radical scavenging activity was observed by Madan Mohan Pandey *et al.*,(2005) in *Saussarea costus* with a maximum inhibition being 66% at 1mg/ml ethanolic extract concentration.

4.4.6 Lipid Peroxidation inhibition assay

Lipid peroxidation (LPO) is the oxidative degradation of polyunsaturated fatty acids and involves lipid radicals. This is a basic membrane damage process results in deleterious effects.

Concentration (µg/ml)	% inhibition of fractions			
	1	2	3	4
50	15.86	12.45	10.52	17.51
100	26.31	41.37	33.13	30.12
150	48.59	48.39	37.35	38.76
200	53.01	51.41	39.16	42.17
250	54.82	56.63	42.37	44.38

Table 4.4.6: Lipid peroxidation inhibition activity of polysaccaharide fractions isolated from fruiting body of *G.lucidum*

Values are expressed as mean of 3 replicates.

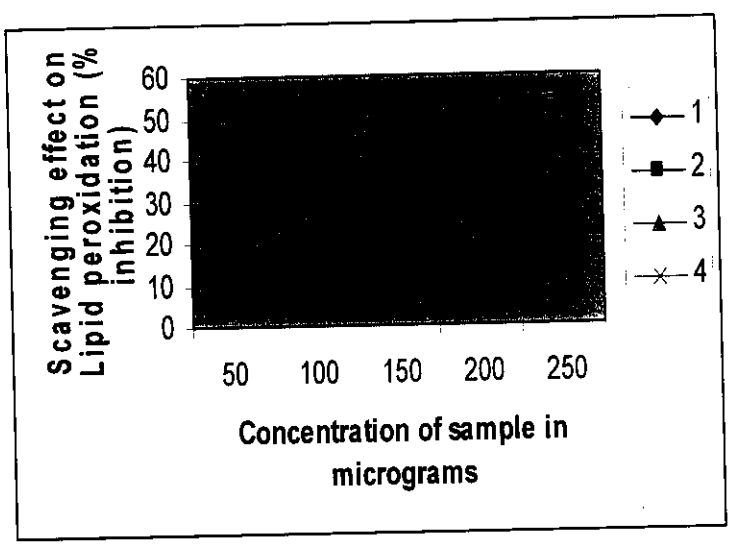


Figure 4.4.6: Dose response curves of Lipid peroxidation activity of polysaccharide fractions isolated from *G.lucidum*

Fraction 1: Hot water extract.

Fraction 2: Ammonium oxalate extract.

Fraction 3: Acetic acid- NaOH extract.

Fraction 4: Ethanol-NaOH extract.

Fig 7 and table 9 shows the dose response curve and percentage inhibition values for the lipid peroxide inhibition activity of fractions of 1, 2, 3 and 4 respectively. At a dose of 50-250µg/ml, the water extract of fraction 1 showed 15.86% to 54.82% inhibition with IC_{50} of 113µg/ml. At a dose of 50-250µg/ml, the water extract of fraction 2 showed 12.45% to 56.63% inhibition with IC_{50} of 88µg/ml. Similarly the water extract of fraction 3 and fraction 4 showed 10.52% to 42.37% and 17.51% to 44.38% inhibition with IC_{50} of 88µg/ml and 100µg/ml respectively. The result shows that fraction 2 have better peroxidation scavenging activity as reflected in terms of inhibition (%). Studies conducted by Janardhanan, (2000) reported that ethyl acetate extract of *Pleurotus florida* exhibited potent inhibition of lipid peroxidation with IC_{50} value of 496µg/ml.

4.4.7 Reducing power

The reducing power increased as the concentration increased indicating some compounds in water extract of fraction 1, 2, 3 and 4 were electron donors and could react with free radicals to convert them into more stable products, terminating the radical chain reactions.

Concentration (µg/ml)	Absorbance of fractions			
	1	2	3	4
50	0.0155	0.0265	0.0375	0.0380
100	0.0190	0.0280	0.0380	0.0460
150	0.0270	0.0335	0.0395	0.0515
200	0.0330	0.0355	0.0525	0.0625
250	0.0350	0.0424	0.0905	0.0685

Table 4.4.7: Reducing power of polysaccaharide fractions isolated from fruiting body of *G.lucidum*

Values are expressed as mean of 3 replicates.

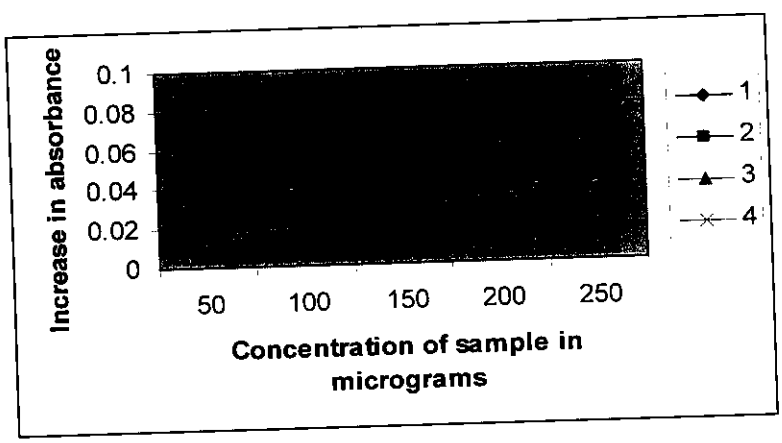


Figure 4.4.7: Dose response curves of reducing power of polysaccharide fractions isolated from *G.lucidum*

Fraction 1: Hot water extract.

Fraction 2: Ammonium oxalate extract.

Fraction 3: Acetic acid- NaOH extract.

Fraction 4: Ethanol-NaOH extract.

Fig 8 and table 10 shows the dose response curve and absorbance of water extracts of fractions of 1, 2, 3 and 4 respectively. At a dose of 50-250µg/ml, the increase in absorbance observed in fraction 1 is from 0.0155 to 0.0350, fraction 2 is 0.0265 to 0.0425, fraction 3 is 0.0037 to 0.0905 and that of fraction 4 is 0.0380 to 0.0685. This result indicates that reducing power of fraction 3 was higher than the other fractions. Studies conducted by Mau *et al.,*(2002) reported that methanolic extract of *Coriolus versicolor* exhibited potent reducing power activity of 0.79 at 4mg/ml concentration.

CONCLUSION

5.0 CONCLUSION

Antioxidants are compounds in fruits and vegetables, which help in avoiding chronic disease. They act as a defense system against oxidative damage in our bodies and may help in avoiding chronic diseases. The present work has been undertaken to study about the free radical scavenging ability of the Chinese traditional medicinal mushroom *G.lucidum.* The preliminary study carried out in our work has confirmed the presence of both enzymatic and non-enzymatic antioxidants in the mushroom extract. Further, we have extracted polysaccharide fractions from *G. lucidum* and assessed its free radical scavenging ability by different antioxidant assays. Thus from our study, we conclude that the antitumor activity of the polysaccharides are due to antioxidants present in *G.lucidum* as evidenced by the free radical scavenging ability of the polysaccharide fractions.

The present study was mainly aimed in studying free radical scavenging ability *in vitro.* The future perspectives of this study were to extend the work to *in vivo* studies in animals and assessing the capability of inhibition of tumor by polysaccharide fractions

APPENDICES

APPENDICES

Appendix 1

Estimation of superoxide dismutase

Principle:

The method involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound which subsequently reacts with naphthylamine to produce a red azo compound whose absorbance is measured at 540nm.

Reagents:

1) 50mM phosphate buffer, pH 7.4.
2) 20mM L- methionine.
3) 1% (v/v) Triton X-100.
4) 10mM Hydroxylamine hydrochloride.
5) 50 µM EDTA.
6) 50 µM Riboflavin.
7) Griess reagent: 1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride.

Procedure:

Pipetted 1.39 ml aliquot of reaction mixture (1.1 ml of phosphate buffer, 75 µl of methionine, 40 µl of Triton X-100, 75 µl of hydroxylamine hydrochloride and 100 µl of EDTA) in a test tube. 100 µl of the sample was added followed by preincubation at 37 degree celsius for 5 min. 80 µl of Riboflavin was added and the tubes were exposed to UV lamp for 10 min. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the

exposure time, 1.0 ml 0f Griess reagent was added to each tube and the absorbance of the colour formed was measured at 540nm.

One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay conditions.

Appendix 2

Estimation of Catalase

Principle:

Catalase cause rapid decomposition of hydrogen peroxide to water.

$$H_2O_2 \longrightarrow H_2O + O_2$$

The method is based on the fact that dichromate in acetic acid reduces to chromic acetate when heated in the presence of Hydrogen peroxide with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically at 610nm. Since dichromate has no absorbency in this region, the presence of the compound in the assay mixture does not interfere with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split Hydrogen peroxide for different periods of time. The reaction is stopped at specific time intervals by the addition of dichromate/acetic acid mixture and the remaining Hydrogen peroxide is determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

Reagents:

1) 0.01 M phosphate buffer, pH 7.0.
2) 0.2 M Hydrogen peroxide.
3) Stock dichromate/acetic acid solution: Mixed 5% potassium dichromate with glacial acetic acid.

4) Working dichromate/acetic acid solution: The stock was diluted to 1:5 with water to make the working dichromate/acetic acid solution.

Procedure:

The assay mixture contained 1.0 ml of buffer, 0.5 ml of hydrogen peroxide and 0.4 ml of water. 0.2 ml of sample was added to initiate the reaction. 2.0 ml of the dichromate/acetic acid reagent was added after 0,30,60,90 seconds of incubation. To the control tube the sample was added after the addition of the acid reagent. The tubes were then kept in boiling water bath for 10 min and the colour developed was read at 610nm. The activity of catalase was expressed as mole of hydrogen peroxide decomposed/min/mg protein.

Appendix 3

Estimation of glutathione peroxidase

Reagents:

1) 0.4 M sodium phosphate buffer, pH 7.0.
2) 10mM sodium azide.
3) 2.5mM Hydrogen peroxide.
4) 4mM reduced glutathione.
5) 10% TCA.
6) 0.3M phosphate solution.
7) 0.04% DTNB in 1% sodium citrate.
8) Reduced glutathione: 20 mg reduced glutathione was dissolved in 100 ml of distilled water.

Procedure:

0.4 ml of buffer, 0.1 ml of sodium azide, 0.2 ml of reduced glutathione, 0.1 ml of hydrogen peroxide, 0.2 ml of sample and 1.0 ml of water were added to a final incubation volume of 2.0 ml. The tubes were incubated for 0, 30, 60, 90 seconds. The reaction was then terminated by the addition of 0.5 ml of TCA. To determine the glutathione content, 2.0 ml of the supernatant was removed by centrifugation and 3.0 ml of disodium hydrogen phosphate solution and 1.0 ml of DTNB reagent was added. The colour developed was read at 412nm. Standards in the range of 40-200 µg were taken and treated in the similar manner.

The activity was expressed in terms of µg of glutathione utilized/min/mg protein.

Appendix 4

Estimation of Glutathione S- transferase

Principle:

Glutathione S- transferase catalyses the reaction of 1- chloro 2, 4 dinitrobenzene (CDNB) with the sulphydryl group of glutathione.

CDNB + GSH $\longrightarrow$ CDNB-S-glutathione.

The conjugate, CDNB-glutathione, absorbs light at 340nm and the activity of the enzyme can therefore be estimated by measuring the increase in absorbance.

Reagents:

1) 0.5 M phosphate buffer, pH 6.5.

2) 30mM CDNB in 95% ethanol.

3) 30mM reduced glutathione.

Procedure:

To 1.0 ml of buffer, 1.7 ml of water, 0.1 ml of CDNB and 0.1 ml of sample were added and incubated at 37°C for 5 min. After incubation, 0.1 ml of reduced glutathione was added. The increase in OD was measured against that of the blank at 340nm.

The enzyme activity was calculated in terms of mole of CDNB conjugate formed/min/mg protein.

Appendix 5

Estimation of Total reduced glutathione

Principle:

Glutathione (GSH) was measured by its reaction with DTNB to give a compound that absorbs at 412nm.

Reagents:

1) 0.2M phosphate buffer, pH 8.0.
2) 0.4M disodium hydrogen phosphate.
3) 0.04% DTNB in 1% sodium citrate.
4) 5% TCA.
5) Standard glutathione: 20 mg reduced glutathione was dissolved in 100 ml of distilled water.

Procedure:

1.0 ml of 10% homogenate was mixed with 1.0 ml of TCA. The solution was centrifuged at 1000 rpm for 10 min. To 1.0 ml of supernatant, 1.0 ml of disodium hydrogen phosphate and 2.0 ml of DTNB reagent was added. The absorbance was read within 2 min at 412nm against a reagent

blank. A set of standards was also treated in the above manner. The amount of glutathione was expressed as µg/mg protein.

Appendix 6

Estimation of Vitamin C (Ascorbic acid)

Principle:

Ascorbic acid is oxidized by copper to form dehydroascorbic acid and diketoglutaric acid. These products are treated with 2,4 dinitrophenyl hydrazine to form the derivative of bis 2,4 dinitrophenyl hydrazine. This compound in strong sulphuric acid undergoes a rearrangement to form a product with an absorption that is measured at 540nm. The reaction is run in the presence of thiourea to provide a mildly reducing medium, which helps to prevent interference from non-ascorbic acid chromogens.

Reagents:

1) 4% oxalic acid.
2) 0.5N sulphuric acid.
3) 2% DNPH in 0.5N sulphuric acid.
4) 10% thiourea.
5) 80% sulphuric acid.
6) Standard solution: 100 mg of ascorbic acid was dissolved in 100 ml of 4% oxalic acid.
7) Working standard: The stock was diluted to 1:10 with 4% oxalic acid.

Procedure:

1.0 g of sample was grounded in 5-10 ml of 4% oxalic acid in pestle and mortar and centrifuged at 10000 rpm for 20 min. 1.0 ml of supernatant

was transferred to test tube and activated charcoal was added in drop wise. Then the solution was made upto known volume of 5-10 ml with 4% oxalic acid.

1.0 ml of sample (from the above), 3.0 ml of water, 1.0 ml of DNPH and 1 to 2 drops of thiourea was added and incubated at 37 degree celsius for 3 hours. Then, 7.0 ml of ice-cold 80% sulphuric acid was added mixed well and the solutions were allowed to stand at room temperature for an additional 30 min. The absorbance was read at 540nm. The results were expressed as µg/mg protein.

Appendix 7

Estimation of Protein

Principle:

The blue color developed by the reduction of phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the aminoacids tyrosine and tryptophan present in the protein plus the color developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured at 660nm.

Reagents:

1) 2% sodium carbonate in 0.1% NaOH (Reagent A).
2) 0.5% copper sulphate in 1% potassium sodium tartarate (Reagent B).
3) Alkaline copper reagent: Mixed 50ml of A and 1.0 ml of B prior to use.
4) Folin-Ciocalteau reagent: Mixed 1 part of reagent with 2 parts of water.

5) Stock standard: Weighed 50 mg of bovine serum albumin and made upto 50 ml in a standard flask with saline.

6) Working standard: Diluted 10 ml of the stock to 50 ml with distilled water. 1.0 ml of this solution contains 200 µg of protein.

Procedure:

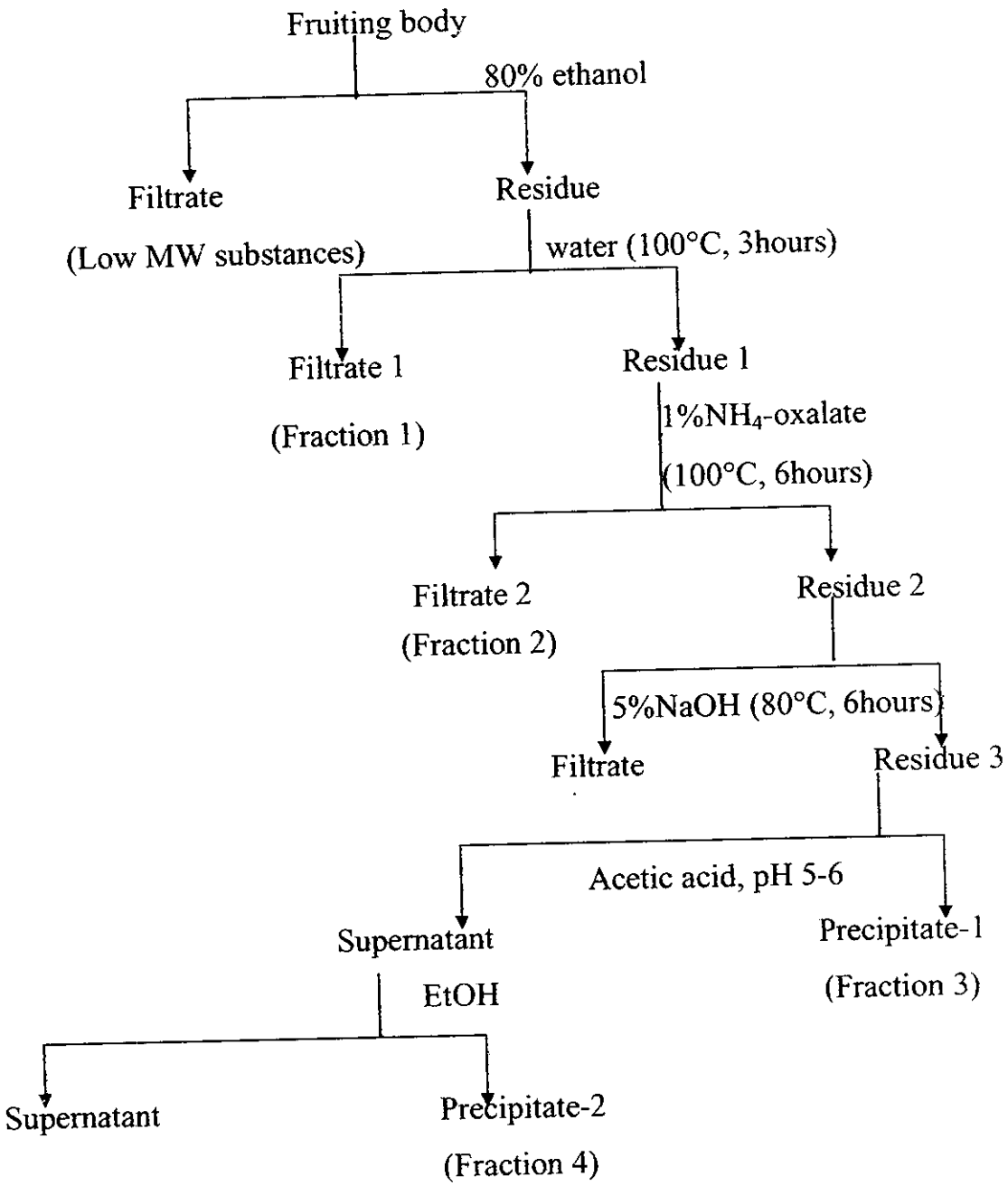
Pipetted out 0.2 ml to 1.0 ml of working standard solution. 0.1 ml of the sample was taken. The volumes in all the tubes were made upto 1.0 ml with distilled water. Added 5.0 ml of alkaline copper reagent to each tube. Mixed well and allowed to stand for 10 min. Then added 0.5 ml of Folin-Ciocalteau reagent. Mixed well and incubated at room temperature for 30 min. A reagent blank was also prepared. After 30 min, the blue color developed was read at 660nm. The results were expressed as mg protein.

Appendix 8

Extraction of Antitumour polysaccharides from fruiting body of *Ganoderma lucidum*

There is a broad similarity in the various methods that have been developed to extract the anticancer polysaccharides from fruiting body of mushroom (Mizuno, 1999).

In the initial step, mushroom was cut into small pieces and is repeatedly heated in 80% ethanol to extract and eliminate low molecular weight substances. Crude fractions are obtained from the remaining ethanol extract residue by extraction with water (100°C, 3hours), 1% ammonium oxalate (100°C, 6hours) and 5% sodium hydroxide (80°C, 6hours) in the same order described above. The flowchart is given below.


Fruiting body
80% ethanol
Filtrate
(Low MW substances)
Residue
water (100°C, 3hours)
Filtrate 1
(Fraction 1)
Residue 1
1%NH4-oxalate
(100°C, 6hours)
Filtrate 2
(Fraction 2)
Residue 2
5%NaOH (80°C, 6hours)
Filtrate
Residue 3
Acetic acid, pH 5-6
Supernatant
Precipitate-1
(Fraction 3)
EtOH
Supernatant
Precipitate-2
(Fraction 4)

Appendix 9

Estimation of DPPH radical Scavenging activity

DPPH scavenging activity was measured by the spectrophotometric method (Sreejayam. and Rao, 1996). To 2.0 ml of methanolic solution of DPPH (0.2mM), 0.5 ml of the sample dissolved in methanol were added at different concentration (50-250µg/ml). An equal amount of methanol was added to the blank and 2.0 ml of methanolic solution of DPPH with 0.5 ml of methanol serves as control. The reaction mixtures were allowed to stand in dark at room temperature for 30 min. After 30 minutes, the decrease in the absorbance of test mixture (due to quenching of DPPH free radicals) read at 517 nm and the percentage inhibition was calculated (Prashanth Kumar *et al.*, 2000).

Appendix 10

Estimation of ABTS radical cation scavenging activity

ABTS radical cation (ABTS+) was produced by reacting ABTS solution (7mM) with 2.45mM ammonium per sulphate and the mixture were allowed to stand in dark at room temperature for 12-16 hours to give a dark blue color solution before use. After 16 hours, this solution was diluted with ethanol until the absorbance reaches 0.7 ± 0.02 at 734nm. For our study, different concentrations (50-250µg/ml) of sample (0.5 ml) were added to 0.3 ml of ABTS solution (which was adjusted to above said absorbance) and the final volume was made up with ethanol to make 1.0 ml. The absorbance was read at 734nm against control with ethanol instead of sample and 1.0 ml of ethanol serves as blank and the % inhibition was calculated using the formula.

$$\text{Inhibition (\%)} = \frac{\text{(Control - Test)}}{\text{Control}} * 100$$

Appendix 11

Estimation of Nitric oxide radical scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction (Marcocci *et al.,* 1994). 1.0 ml of Sodium nitroprusside (5 mM) in standard phosphate buffer solution pH 7.4 was incubated with different concentration (50-250µg/ml) of the 1.0 ml of sample and the tubes were incubated at 25°C for 5 hr. Control experiments without the test compounds but with equivalent amounts of sodium nitroprusside and blank with phosphate buffer were conducted in an identical manner. After 5 hr, 0.5 ml of incubation solution was removed and 0.5 ml of Griess reagent (1% Sulphanilamide, 2% o-phosphoric acid and 0.1% Napthyl ethylene diamine dihydrochloride) was added. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm.

Appendix 12

Estimation of hydroxy radical scavenging activity

The reaction mixture contained 1.5 ml of phosphate buffer (0.2M, pH 7.4), 0.5 ml of deoxyribose (10mM), 0.25 ml of EDTA (20 mM), 0.25 ml of $FeCl_3$ (20 mM), various concentrations (50-250µg/ml) of the 0.1 ml of sample extracts, 1.9 ml of water, 0.5 ml of H_2O_2 (10 mM) and the reaction mixture was incubated for 4 hour at 37°C. After incubation 2.5 ml of 2.8% TCA and 2.5 ml of 1% TBA were added and test tubes were kept in boiling water bath for 10 min. Deoxyribose degradation was measured as TBARS at

520nm against a control with buffer instead of sample and buffer as reaction mixture in blank and the percentage inhibition was calculated.

Appendix 13

Estimation of Super oxide radical scavenging activity

The scavenging activity towards the superoxide radical ($O_2^{\cdot-}$) was measured in terms of inhibition of generation of $O_2^{\cdot-}$(Sanchez-Moreno, 2002). The reaction mixture consisted of 2.0 ml of phosphate buffer (50 mM, pH 7.6), 0.2 ml of riboflavin (20 µg / 0.2 ml), 0.2 ml of EDTA (12 mM), 0.2 ml of NBT (0.1 mg / 3ml) and 0.2 ml of sodium cyanide (3µg / 0.2 ml) Test compounds of various concentrations of 50-250µg / ml were added to make a total volume of 3.0 ml. The absorbance was read at 530 nm before and after illumination under UV lamp for 15 minutes against a control with buffer instead of sample and 3.0 ml of buffer as blank.

Appendix 14

Determination of lipid peroxidation inhibition activity

Rat liver was processed to get 10 % homogenate in cold phosphate buffered saline, pH 7.4 using pestle and mortar and filtered to get a clear homogenate. The degree of lipid peroxidation was assayed by estimating the thiobarbituric acid reactive substances (TBARS) by using the standard methods with minor modifications. Lipid peroxidation was initiated by adding 100 µl of 15 mM ferrous sulphate solution to 3.0 ml of the tissue homogenate. Different concentrations of the sample (50-250µg/ml) in water were added to the liver homogenate. The tubes were incubated at room temperature for 30 min. After 30 minutes, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 10 % TCA. After 10 minutes tubes

were centrifuged and supernatant was separated and mixed with 1.5 ml of 0.67% TBA in 50 % acetic acid. The mixture was heated for 30 minutes in a boiling water bath. The intensity of the ink colored complex formed was measured at 530 nm. The results were expressed as the % inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls not treated with the sample and buffer serves as blank.

Appendix 15

Determination of Ferric Reducing Antioxidant potential

Various concentrations (50-250µg/ml) of 2.5 ml of sample was mixed with 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of 1 % potassium ferricyanide and the mixture was boiled at 50° C for 20 minutes. After 20 min, 2.5 ml of 10% TCA was added and the mixture was centrifuged at 650 g for 10 minutes. 5.0 ml of supernatant was mixed with 1.0 ml of 0.1 % ferric chloride and absorbance was measured at 700nm. A higher absorbance indicated a higher reducing power.

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