MEDICINAL BIO-ACTIVITIES OF *GANODERMA LUCIDUM* AND EMPIRICAL TOXICITY ANALYSES ON ITS PRODUCTS

A

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By

Krishna Prasad Subedi

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Supervisor

***Associate Prof*. Dr. Giri Raj Tripathi**

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Acronyms

a.m.u. …….. Atomic Mass Unit

AAS…… Atomic Absorption Spectrometry

ATCC…… American Type Culture Collection

BHA…… Butylated Hydroxy Anisole

BHT…… Butylated Hydroxy Toluene

CAM…… Complementary and Alternative Medicine

CAT…… catalase

CEGS …… (β)-carboxyethylgermanium sesquioxide

CVD…….. Cardiovascular Disease

D…….. Dextrorotatory

DPPH…….. 1, 1- Diphenyl-2-picrylhydrazyl

EPS……Extracellular Polysaccharide

et al……*et alia*

EXP……Exponential Function

FITC…… Fluorescein Isothiocyanate

FT-IR…… Fourior Transform Infrared spectroscopy

GA ……Ganoderic Acid

GAIN…… Ganoderic Acid Infused Nano-particles

GL…… *Ganoderma lucidum*

Gl…… Ganocelium

GLPP …….. *G. lucidum* polysaccharide preparation

GLEEX…….. *G lucidum* Ethanolic Extract

GMP…… Good Manufacturing Practices

GOT…… Glutamic-Oxaloacetic Transaminase

GPT…… Glutamate Pyruvate Transaminase

ICAM…… Intercellular Adhesion Molecule

IC_{50}…… $Conc^{n}$ that inhibits 50% population

IDDM…… Insulin Dependent Diabetes Mellitus

IFN…… Interferon

IPSS …… International Prostate Symptom Score

ISO …… international standard operation

kPa…… kilo Pascal

LC_{50}…… Concentration that kills 50% population

LN…… Natural Logarithm

LZ-8…… Ling Zhi-8 protein

MEA…… Malt Extract Agar

MEP…… Methyl Erythritol Phosphate

MHA…… Mueller Hinton Agar

µL …… Microliter

NIDD…… Non Insulin Dependent Diabetes

NP…… Nano-Particles

OD…… Optical Density

PBS…… Phosphate Buffer Saline

PDA…… Potato Dextrose Agar

PI…… Percentage Inhibition

PPM…… Parts Per Million

PVC…… Polyvinyl Chloride

RNS…… Reactive Nitrogen Species

ROS…… Reactive Oxygen Species

SDA…….. Saboraud Dextrose Agar

SOD……. Superoxide Dismutase

SOP…… Standard Operation Procedure

TCM ……. Traditional Chinese Medicine

t.i.d. …… *Ter In Die*

TNF …….. Tumor Necrosis Factor

Upa…… Urokinase Plasminogen Activator

Abstract

Precious bioactive compounds: β D-glucan and Organic Germanium, antioxidants, immune boosters and triterpenoids made *Ganoderma lucidum* the miraculous herbal king being used in Medicines and supplements worldwide. Scientific basis for such potent multidrug mechanism is being explored worldwide along with advancement in culture techniques and metabolic engineering. This research focuses particularly on its growth parameters, medicinal activities and empirical toxicities. Growth parameters were optimized in semisolid media. Extracts were received in ethanol through Soxhlet Extraction system and were used for antibacterial activity by disc diffusion method, antioxidant activity by DPPH method, total antioxidant potential by phosphomolybdenum method and reducing activity by FRAP method *in vitro* and polyphenolics and flavonoids were quantified compared to their respective standards: gallic acid and quercetin. Heavy metals Cadmium (Cd) and Plumbum (Pb) were analyzed on its supplement products: capsules, coffee and wild and cultivated powder. Lastly, brine shrimp bioassay was performed on above supplements to assess their pharmacopotency. Growth optimization predicted that at 30°C, at pH 5±0.25 and with sugars viz; Sorbose, Trehalose, Glucose and Maltose it grows better with maximum rate 1.08 mm per day than with cellulose and lactose. *Bacillus subtillis* was most susceptible with all extracts and *Staphylococcus aureus* and *Pseudomonas aerogenosa* were mildly inhibited and *Salmonella typhii* was inhibited with cloudy inhibition zone. Mycelia was more potent in antioxidant activity having IC_{50} 157.49 µg/ml than carpophores having 176.77 µg/ml compared to standard ascorbic acid having IC_{50} only 30.60 µg/ml. Total antioxidants for carpophore extract showed 144.28 ±81.72 mg whereas mycelia extract 150.6 ±56.92 mg eqv to ascorbic acid per gram extract. Average reducing power value was 104.08 ± 7.59 mg eqv BHT/g for carpophores extract and 13.58 ± 3.18 mg equivalent to BHT/g for mycelia extract. Immature carpophore was found to contain 70.068 mg phenolics, mature carpophore56 mg, submerged mycelia 25.24 mg and lawn mycelia 9.448 mg phenolics eqv to gallic acid per gram dry biomass. Next, lawn mycelia contained 34.375 mg flavonoids, mature carpophore 45.875 mg, immature carpophore 74.125 mg and submerged mycelia 26.875 mg flavonoids eqv to quercetin per gram dried biomass. Heavy metal quantification by AAS on four supplements viz; capsules, coffee, lab grown chips and wild Reishi powder revealed Lead and Cadmium 7 times and 26 times below Codex Alimentarius Commission (1993). Above four supplements revealed their pharmacopotential having LC_{50} below 1000 mg/l highly cytotoxic being coffee with LC_{50} 73.37 µg/ml and weakly cytotoxic being capsules with LC_{50} 776.24 µg/ml (ppm) in Brine shrimp bioassay. Medicinal activities and Empirical toxicity studied *in vitro* assured that *Ganoderma lucidum* is medicinally valuable supplement and it is safe and acceptable as per WHO standards.

Key words: *Ganoderma lucidum*, DPPH, IC_{50}, Flavonoids, AAS, LC_{50}, Bioassay.

CHAPTER ONE

INTRODUCTION

1.1 Background

Since modernization and industrialization non-communicable human diseases are loading pressure on medical civilization and research to search for reliable approaches better suited for their prevention than treatment. Modern allopathic drugs and approaches are more curative than preventive based on specific symptoms seen as disease progresses. Recently pharmacologists and health and nutrition scientists have given their attention on searching natural products that enhance immune system and better defend against non-communicable and chronic diseases. These products should protect body against age related progressively degenerative non communicable diseases and reduce susceptibility to opportunistic diseases even under high pathogenic titer. Traditional oriental medicine practitioners have proved that *Ganoderma lucidum* has such multiple utilities as well as constituents capable for preventing these diseases and their progression. So international companies are producing its various products and exporting them even to Nepal. Only by testing its medicinal as well as noxious effects can it be recommended to public consumers assuring its safety and reliability. On the other hand *Ganoderma lucidum* with so many potent medicinal characteristics should be grown on commercial scale in Nepal. Neighboring world's trade leading countries having biopharmaceutical based industries demand huge raw phytochemicals. Cultivating *Ganoderma lucidum* and other similar medicinal plants and processing them has immense commercial scope in Nepal as it can export them and earn foreign currency in addition reduce poverty, unemployment and brain drain.

1.2 Current studies

Engineered nano-particles have higher molecular activities than their bulk materials and currently been used in targeted drug therapy embedding them in other composites to enhance certain biological characteristics. Their capacity to interact with other particles, cells and tissues at a molecular level provides them with a distinct advantage over other polymeric macromolecular substances (Jombo *et al*., 2012). Their enhanced solubility together with bioactivity caused a revolution in classical therapeautics and pharmaceauticals (Nel *et al*., 2006). Treating extracellular filtrate from ganocelia with silver nitrate reduces the metal ions to nanoparticles. Optical detection and spectroscopic confirmation suggests that *Ganoderma lucidum* can be used in synthesing silver nanoparticles, demand for which is growing in

modern medicine. LM-20 analysis reveals their polydispersity and distribution in the range: 10-70 nm with an average size: 45 nm and a concentration: 0.37 x 10^8 particles/mL. FT-IR spectrum confirms their stability due to amide linkages and protein capping. These nanoparticles have shown strong bactericidal activity against test pathogens *Staphylococcus aureus* and *Escherichia coli* which also have exhibited their efficiency in enhancing synthetic tetracycline's activity. Li *et al*. (2010) reported that nanoparticles loaded with *G. lucidum* polysaccharide at 6 µg/ml and chitosan: sodium tripolyphosphate (mass ratio 5.5) had significantly greater cytotoxic effects on tumour cells and growth promotion effects on mouse spleen cells than empty nanoparticles. Radwan *et al*. (2011) explained that Ganoderic Acid-Infused Nanoparticles (GAINs) have both diagnostic and therapeutic functions. Both GA- A and Imaging-dye are conjugated to a biocompatible polymer bonded to a folate ligand to trigger internalization. GAINs preferentially target the folate receptor-rich cancer cells, where linkages are hydrolyzed and the nanoparticles release the drug and dye allowing drug delivery as well as their imaging. Santra *et al*. (2010) reported Cytochrome C encapsulating theranostic nanoparticle system which is a novel bifunctional system for targeted delivery of therapeutic membrane-impermeable proteins to tumors and imaging of cancer therapy.

1.3 Hypothesis

Claiming research fundamentals about *Ganoderma lucidum*, health supplements, tonics, and cosmetics are blooming worldwide along with few scandals on against. However, report on its heavy metal content and developmental toxicity has not been released yet along with its medicinal claims to assure its medicinal safety and efficacy. This thesis was designed to erase any skepticism about *Ganoderma lucidum* and hypothesizes that worldwide trusts established about it are true i.e. it is medicinally potent and its commercial derivatives are nutritionally harmless for human consumption.

1.4 Medicinal effects

Ganoderma lucidum is a white rot macrofungus, a decomposer ecologically. In Latin gan means shining, derm means bark and lucida means light and thus this binomial profile descibes its distinguishing morphology having brightly colored carpophore growing on wood bark. *Ganoderma lucidum,* a medicinal mushroom commonly called 'Red mushroom', belongs to Polyporaceae (Basidiomycetes). Orientals have long been using it as traditional herbal medicine in several illnesses especially in Cancer , CVDs , diabetes etc and as tonic in immune empowerment. Recently it has even been proved to have anti HIV potential. *Ganoderma lucidum* has no known mycotoxins so it can be consumed either raw as dried

slices or capsules or coffee but it is not cooked for dishes. Recently *Ganoderma lucidum* is recognized as a complementary and alternative medicine (CAM) as an adjuvant in treating leukemia, carcinoma, hepatitis and diabetes. Many countries are producing its herbal capsules to use in Ganotherapy (Sullivan *et al*., 2006) assisting conventional chemo and radiotherapy for cancer. So it is cultivated all over the world as to harvest its fruiting bodies in solid substrates and to harvest mycelium in submerged stationary or shaking culture in controlled and enriched conditions. Increasing desire for self-immune-empowerment amongst people and patients has led scientists to explore complementary and alternative medicines (Sze *et al*., 2009). Thus *Ganoderma* has received their attention as multi drug pharmacopotent mushroom (Sanodiya *et al*., 2009). Carpophores or fruiting bodies are used for preparing herbal capsule or particular extract can be used in formulating drugs. Gano-coffee is also produced from it as it is bitter and deep brown, similar to tea and coffee, enriched with tannins and terpenoids. Tea formulation with *Stevia* can also be produced for diabetic patients since this non saccharic constituent doesn't release glucose and bitter principles in *Ganoderma* provides medicines for diabetic patients. But this formulation can equally be preventive and health supplement for healthy beings as well.

Bioactive compounds: Bioactive compounds are those having functional role in empowering basal physiology and immune system and in pathogen defense. Sporophore, mycelia and spores contain approximately 400 different bioactive compounds, which mainly include triterpenoids, ganoderic acids, polysaccharides: β (1, 3) or (1, 6) D-Glucan, nucleosides, steroids, phenyl propanoids, flavonoids, cardiac glycosides (Jombo *et al*., 2012), immune modulating proteins-peptides, selenocysteine and Germanium sesquioxide (800 to 2000ppm). Terpenoids called bitter principles which along with flavonoids and β D-glucans are anti-cancerous. Ganoderic acids are both anti-cancerous and anti- HIV. LZ-8, immune-modulating proteins and seleno-cysteine empower body defense system. *Ganoderma lucidum* contains organic Germanium (Ge-132) much higher than in garlic, Gin seng and *Aloe vera* with the 5th highest metal concentration (489 µg/g) which is the most common medicine used against cancer. Spores contain abundant Ganosporeric acid, lanostane triterpenes: ganoderic acids (γ, δ, ε, ζ, η and θ), lucidenic acids and triterpene lactones. Bioactive compounds have various medicinal roles: anticancerous, antioxidant, tumoricidal and several immune modulating functions that strengthen human immune system and free radicals scavenging capacity enabling us to fight against aging and age related degenerative diseases.

Medicinal actions: Pharmacological effects are based on immune-modulating, radical scavenging, tumoricidal and immune potentiating function that enhance the overall immune system to combat non communicable degenerative and opportunistic diseases. Pharmacomedicinal effects include immune modulation, anti-atherosclerotic, antitumor, chemo and radio protective, antibacterial, antiviral (including anti-HIV), hepatoprotective, anti-diabetic, anti-androgenic, anti-angiogenic, antioxidative and radical-scavenging, anti-aging and anti-ulcer properties (Sanodiya *et al*., 2009). Bioactive compounds in *Ganoderma lucidum* destroy the telomerase in tumor cells and kill them, enhance immunoglobulin and immune effecter cells and components. Organic Germanium seizes electrons from tumor cells, decreases their potential and inhibits their growth and spread (up to 83.9%). Sequencing studies on LZ-8 shows it to be similar to variable region in immunoglobulin heavy chain and its predicted secondary structure resembles lectins with mitogenic capacity.

1.5 Empirical toxicity

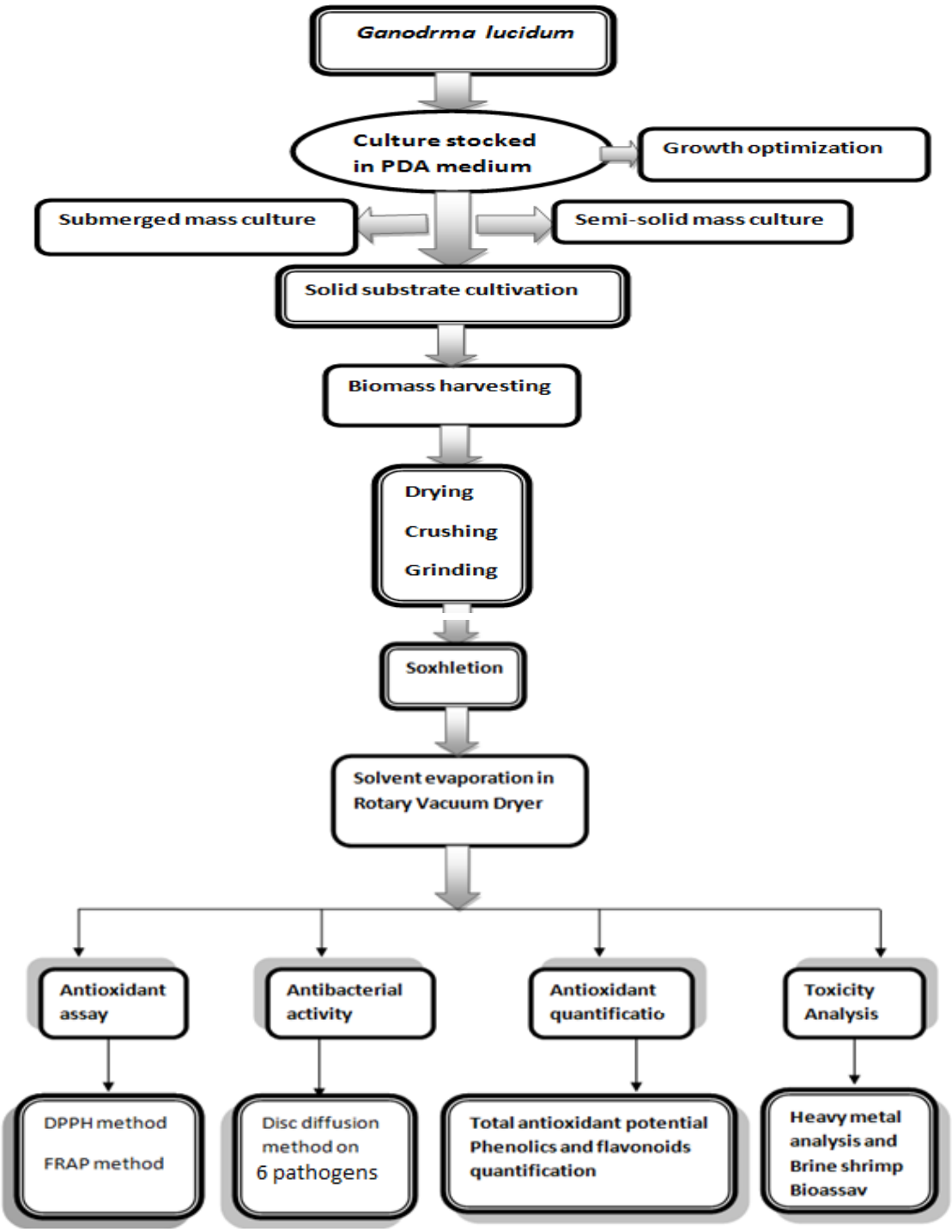
Public skepticism is still spread on herbal medicines about their reliability and safety. Many herbal formulations and production are based on traditional methods so unavoidable associated hazards persist. Eastern herbal medicines are returned from westerner's quarantine blaming them polluted with heavy metals, microbes and teratogens. Toxins are those that disturb vital development by funneling electrons, inhibiting enzyme on life rating metabolism etc. Toxin can be heavy metals, mutagen, carcinogen, teratogen, receptor analogs or enzyme inhibitor, electron funneller etc. To establish health food business and export it as organic products, pharmaceuticals and coffee, preliminary screening their presence in crude *Ganoderma lucidum* product is essential and contextual.

Heavy metal content: Many drugs, herbal formulations, food products and supplements are contaminated with heavy metals. Heavy metals are toxic, radioactive and hence ulcerogenic and obviously carcinogenic. Their quantification should be regularly monitored in order to assure public consumers. Heavy metals in human consuming products should be null or below WHO and FAO standard. For their quantification, samples are digested with conc. nitric acid completely liberating heavy metals bound or intercalated in organic compounds. Then thus digested suspension is filtered through special ash-less filter paper. Metal quantification is done by Flame Atomic Absorption Spectrometer (FAAS) with particular wavelength detecting particular metal.

***In vivo* Brine shrimp lethality bioassay:** Brine shrimp bioassay method is simple inexpensive and rapid to evaluate tentative drug toxicity. This lethality test involves treating the newly

hatched naupalii with the crude plant extracts or fractions or isolated compounds. In this test LC_{50} value is determined for that introduced extracts or drugs. Compounds or extracts having LC_{50} value less than 1000 ppm are considered pharmacologically potent.

1.6 Experimental overview



1.7 Objectives

1.7.1 Broad objectives

- To establish public trust on *Ganoderma lucidum* as versatile pharmacopotent mushroom so that its consumption as versatile products could be expanded.
- To establish a base in order to run medicinal mushroom industry in Nepal.
- To assist in therapy against cancer using it as CAM.
- To spread knowledge about *Ganoderma lucidum* as nontoxic mushroom and to command its processing should be in WHO-GMP standard, SOP, ISO 9001-2000 etc standard without toxins, metals and xenobiotics.
- To use local low grade substrates in order to recover valuable products viz; cosmetics, neutraceauticals and laccase to alleviate immune compromised diseases, assist in bioremediation and bioethanol production.

1.7.2 Specific objectives

- To cultivate *Ganoderma lucidum* in various media.
- To determine optimum growth parameters: temperature, pH and carbon sources.
- To extract its bioactive compounds.
- To perform antibacterial tests on opportunistic pathogens.
- To determine total antioxidants, phenolics and flavonoids.
- To determine *in vitro* antioxidant potential.
- To measure heavy metal contents in *Ganoderma lucidum* products sold in Nepal.
- To perform brine shrimp lethality assay to measure crude toxicity.

1.8 Rationale

Nature has diverse and tremendous resources to feed people with food, supplements and herbal medicines. Researches on screening natural bioactive compounds, their importance on immune empowerment and disease treatment promote their judicious utilization to decrease poverty and malnutrition and increase healthier lives. Curative approach is not so effective and allopathic drugs are almost unsuccessful to treat cancer, diabetes and cardiovascular problems, so their preventive approach is the best way. Neutraceauticals, Prebiotics and nutritional supplements are highly desired in daily foods. *Ganoderma lucidum* has become a popular dietary supplement ingredient in Western countries (Stanley *et al*., 2005) with an annual global market value over $1.5 billion for its extracts (Sullivan *et al*., 2006). *G. lucidum* is

very important economic crop in China and is used to prepare commercial bioactive extracts including the water-soluble health *G. lucidum* polysaccharide preparation (GLPP) (Pang *et al*., 2007). Bioactive constituents in *Ganoderma lucidum* will enable people at risk live expected life and get rid of those side effects, costly and unguaranteed treatments. Moreover it can be used daily as sugar free gano coffee or capsule. Establishing its biotech industries for production and processing in Nepal leads to alleviate poverty, increase employment, lengthen expected life and even earn foreign currency. Researches on its medicinal values are countless. So testing its medicinal efficacy and empirical toxicity can recommend public patients its reliable and safe consumption in Nepal neglecting whatever its opponent's hearsay and scandalize. Utilizing local substrate and requiring no sophisticated technology for its production has made it the best way to improve living standard and income. International researches on *Ganoderma lucidum* have reached a peak and nevertheless even its small inauguration in Nepal is contextual even though it is too late already.

1.9 Scope

Ganoderma lucidum, being an easily growing medicinal mushroom, biotech industry can be established to grow it in large scale in order to produce its coffee and capsules to consume in Nepal and crude powder to export to neighboring countries. *Ganoderma lucidum* being a wood rotting fungus, it can degrade both lignin and cellulose. This capacity can be exploited to degrade biomass to increase lower and fermentable sugars for bio ethanol production so that world's heavy reliance on fossil fuel can be minimized. Such industries lead a nation from simple agricultural practices towards sustainable industrial development and prosperity. Moreover, screening natural bioactive compounds can help to find new biochemical pathways and produce those compounds in large scale ultimately assisting pharmaceutical and metabolite industries to formulate new approaches for cancer, diabetes, immune deficiencies etc. Molecular biologists will be stimulated to design new metabolic engineering in order to over-express the responsible gene or modify the concerned pathway to enhance that particular product. Immune modulator protein gene i.e. LZ-8 gene can be transformed into efficient expression system ultimately its gross production can be established. Nowadays targeted drug delivery is used to kill particular cell or tissue however mutation in target site and resistance development are major problems so emerging "multi-targeted mechanism" and "multi drug therapy" is an alternative approach and *Ganoderma lucidum* is vital and versatile hero (Sze & Chan, 2009).

CHAPTER TWO

LITERATURE REVIEW

2.1. Neutraceauticals from Mushrooms

Mushrooms are "macrofungi" with prominent reproductive fruiting bodies belonging to Basidiomycetes or Ascomycetes large enough to be seen and picked. Mushrooms are spore bearing upright (sometimes subsoil) massive structures enriched with nutrients and comprising intermingled secondary mycelia responsible for spore dispersal. There are approximately 1.5 million fungal species in the world among which nearly 70,000 species are described. About 10,000 known species belong to the macro fungi. Among which about 5,000 species are edible and over 1,800 species are considered to have medicinal properties (Prasad & Wesely, 2008). The macro fungi can be divided into four groups: 1. Edible flesh e.g. *Agaricus bisporus, Tuber* spp, 2. Medicinal e.g. *Ganoderma lucidum*; 3. Poisonous e.g. *Amanita muscaria*; and 4. Miscellaneous, which are less studied and defined. Among medicinal species, *Ganoderma lucidum* is the leader in production (Prasad & Wesely, 2008).

Mushroom "neutraceauticals" are bioactive compounds that are extractable from mushrooms, and have prophylactic as well as anaphylactic medicinal features (Chang & Buswell, 1996). Several neutraceautical products have been isolated from medicinal mushrooms and three carcinostatic polysaccharide drugs have been developed from mushrooms in Japan. These are "Krestin" from *Trametes versicolor,* "Lentinan" from *Lentinula edodes* and "Schizophyllan" or “Sonifilan” from *Schizophyllum commune.* Lentinan and schizophyllan are pure p-glucans where as Krestin is a protein bound polysaccharide (Ooi & Liu, 2000). Their biological activity is related to immune-modulating properties, which enhance the host's defence system against various infectious diseases. These immune-potentiators or immune-initiators are also referred to as "biological response modifiers" (BRM). Several other fungi are used as pro-drug or health supplements directly or as their products. *Ophiocordyceps sinensis, Antrodia camphorata, Tramates versicolor, Lentinula edodes, Morchella esculenta* etc are long being used as immune potentiator health supplements or tonics. Several bioactive compounds and dietary fibres (Prebiotics) allocated in mushroom make them versatile medicine. *Saccharomyces cerevisae* is currently being used as ‘Probiotics’ for intestinal maintenance.

2.2. *Ganoderma* species available in Nepal

Compiled literature about following *Ganoderma* spp from Nepal can be obtained in Adhikari, 2000, 2012, '2006a b', Hattori *et al*., 2002

***Ganoderma applanatum* (Schaeff.)With.**

On rotten tree, Tarai forest, Dharan (Balfour-Browne, 1968); on rotten tree trunk, Bakhri Kharka; Taglung (Kaligandaki) (Balfour-Browne, 1968); Hetauda (Pandey, 1976); on *Quercus* sp., Daman (Singh, 1976); on stump, Phulchowki (Lalitpur Dist.) (Singh & Nisha, 1976c); on tree trunk, Namrung (Gorkha Dist.) (2460m) (Adhikari, 1988a) and on *Betula utilis* wood, Nilgatti Odar (Bajhang Dist.) (3500m) (Adhikari, 1988a); Chakale pani, Jumla (Hattori *et al*., 2002). Common in tropical to temperate belts (Adhikari, 1996a); growing on dead log of *Abies lausas* (3570m) (Rana & Giri, 2006; Giri & Rana, 2007) and Matatirtha, Maitidevi, Kirtipur (Pandey, 2008).

***Ganoderma australe* (Fr.) Pat.**

On wood (place not mentioned) (Llyod, 1808; Berkeley, 1854).

***Ganoderma carnosum* Pat.**

Growing on *Populus* tree trunk Harisidhhi (Adhikari & Manandhar, 2004b) stump root, Matatirtha and growing on wood, in Subtropical forest, Chitawan and growing on wood, in moist shady place, Matatirtha (Adhikari, 2011)

***Ganoderma lucidum* (Curt.:Fr.) Karst.**

Kenja Likhu khola (Ryv., 1977); on rotten trunk, Bakhri Kharka (Pokhara) (Balfour-Browne, 1968); on tree trunk, Lele (Kathmandu valley) (Singh & Nisha, 1976c) and on *Rhododendron arboreum* trunk and *Quercus*, Manichur (Adhikari, 1988); on stump, between Seti khola Bagar and Agra goan (Bajhang Dist.) (1700m) (Adhikari, 1988); in stump root crevices, Phulchowki (1800m); on tree stump (Thapa, 1990); Suryvinayak (1540m) (Adhikari *et al*., 1996) and very common in *Dalbergia sissoo* and *Acacia catechu* plantations in Tarai belts (Hetauda, Chitwan, Bara, Parsa, Rautahat, Siraha, Saptari, Dhanusha, Mahotari, Udayapur, Rajbiraj etc. (between 70 and 500m), found infecting mango plantations. Daman (Adhikari & Manandhar, 2004) and on *Quercus semecarpifolia* trunk, Thulakharka, Lumle (2190m) (Devkota et al 2005) and on dead stumps and logs of *Dalbergia s*issoo and *Acaccia* sp., Lumbini (Adhikari *et al.,* 2006); listed as ornamental in Pandey *et al.,* (2006a); Nagarjoon (Pandey et al., 2009); Saljhandi to Peepaldanda community forest Lumbini, Aryal, Budathoki & Adhikari (2012); on rotten trunk, Karhiya Community Forest, western Terai, Nepal (Aryal & Budathoki, 2012)

***Ganoderma tsugae* Murr.**

Place not mentioned (Pandey *et al*, 2009)

***Ganoderma endochroum* Steyaert:**

On tree stump, Adhobar, Parsa (Thapa, 1990).

***Ganoderma* sp.**

On tree trunk, Sundarijal (Kathmandu Dist.) (Singh & Nisha, 1976c); Nagarjun and Kakani (Kathmandu Dist.) (Pandey, 1976) and substrate unknown, Muse forest, Lukla (2783m) (Rana & Giri, 2006)

2.3. *Ganoderma* in nature

2.3.1. As recycler

Ganoderma lucidum is a wood rotting fungi that grows on barks and hard woods in temperate regions being involved in the fundamental lignino-cellulosic degradation in nature. Not only is it economically important as medicinal and nutritional supplement but also ecologically as dead wood decomposer in nutrient recycling. There is significant commercial interest in harnessing their powerful wood-degrading enzymes for industrial applications such as bio-pulping or bioremediation. Several species like *G. lucidum, G. appalantum, G. tsugae* etc are being used in medicine and nutraceauticals all over the world, *G lucidum* being the major.

G. lucidum being wood decomposer grows on both coniferous and hardwood species. Commonly growing on deciduous trees like oak, maple, elm, willow, sweet gum, magnolia and locust, *G. lucidum* is less frequently found on coniferous trees like *Larix, Picea* and *Pinus* in Europe, Asia and North and South America (in temperate and subtropical regions). In the Orient, it grows primarily on plum trees. *G. lucidum* generally occurs in two growth forms one, found in North America is sessile and rather large with only a small or no stalk, while the other is smaller and has a long narrow stalk and is found mainly in the tropics (Stamets, 2000).

Being wood rotting fungi *Ganoderma lucidum* have enzymes that allow them to break down wood components such as lignin and cellulose simultaneously (Schlegel, 2004). Wood contains about 50% cellulose. Since cellulose contains β-D-Glucose units in β 1, 4- glycosidic linkage, cellobiose is considered as its monomer. High mechanical tensile strength and insolubility is due to inter- and intra-molecular hydrogen bridges and strong cohesion due to Van der Waal's forces. Cellulase system brings its enzymatic digestion which comprise

three enzymes: endo β 1,4- glucanase attacks β 1,4 bonds in the centre and produces long chain fragments with free ends, exo β 1, 4-glucanase removes disaccharide cellobioses from cellulose ends and β glucosidase hydrolyses cellobiose into glucose (Schlegel, 2004). Wood contains 18-30% lignin which is embedded in the plant tissues and within secondary lamellae. Lignin is the most slowly degraded component in plants. Structurally it contains phenyl - propane derivatives as building blocks like coniferyl alcohol, sinapyl alcohol and coumaryl alcohol. Methoxy group (C-O-C) in phenyl propene makes them different building derivatives. Bonds in lignin are extremely resistant to enzymatic attack.

But some fungi can degrade lignin even in the living plants. Three groups have been distinguished as wood rotting fungi; brown rot converts wood into a reddish brown mass degrading cellulose leaving lignin, white rot converts into white mass degrading lignin and some degrade both lignin and cellulose like *Ganoderma, Pleurotus, Armillaria* etc. Lignin degradation occurs slowly but requires oxygen and glucose i.e. no anaerobic breakdown (Schlegel, 2004). Enzyme system includes ligninase, laccase and peroxidases (lignin peroxidase and manganese dependent peroxidase). Peroxidase requires H_2O_2 for their function. Glucose oxidase oxidizes glucose releasing H_2O_2 and peroxidase uses the later to cleave β O-4 ether bonds and C-C skeleton in lignin. Nitrogen limitation promotes peroxidase formation i.e. lignin breakdown is not to obtain metabolic energy but to build its structure and to quest nitrogenous components from biomass which would otherwise be inaccessible. Lignin breakdown starts with one electron transfer to produce stable aromatic cation radical in lignin skeletons which can further function as one electron oxidants and act at a distance from enzyme producing numerous radicals in a snowball reaction. These radicals ease the ether bonds (C-O-C) cleavage and then lignin skeletons collapse into low molecular phenolics compounds which are further oxidized by phenolic oxidases (Schlegel, 2004).

Figure 2.1: *Ganoderma lucidum* growing in forest wood

2.3.2. Systematics

Kingdom: Mycota
Division**:** Basidiomycota
Class: Basidiomycetes
Order: Polyporales
Family: Ganodermataceae
Genus: *Ganoderma*
Species: *lucidum* (Curtis, Fr.) Karst

2.3.4. Conventional applications

Ganoderma lucidum commonly known as Ling zhi, Reishi, Mannentake etc is a traditional Chinese medicinal mushroom used to increase immune modulation and longevity and against several diseases like diabetes, hypertension, cancer etc. Chinese name Ling zhi means "spiritual potency". Recently it has also shown anti HIV protease -1 activity. ‘‘Lingzhi’’ has been used in treating migraine, hypertension, arthritis, bronchitis, asthma, anorexia, gastritis, hemorrhoids, hypercholesterolemia, nephritis, dysmenorrhoea, constipation, lupus, hepatitis, and cardiovascular problems. Additionally neuro active compounds in its extracts mediating neuronal differentiation and neuro-protection in rat PC12 cells have also been demonstrated recently (Huie & Di, 2004). Extraordinary claims such as this need to have equivalent evidence to prove them factual.

Studies on medicinal mushrooms in the west began around thirty years ago. From that time until nowadays, it could be demonstrated interesting biological activities for *Ganoderma lucidum*, including antitumor, anti inflammatory effects and cyto-toxicity to hepatoma cells. Antitumor effects were associated with triterpenes (Min *et al*., 2000), polysaccharides (Kuo *et al*., 2006) or immune-modulating proteins. AIDS is a worldwide problem, particularly in Africa and Asia. Best known as an immune system enhancer and modulator with health benefits, it is generally safe for long-term use. The LD_{50} (lethal dose to kill 50% studied subjects) for a single intra peritoneal injection dose extract in rodents was as high as 38 g/kg. The LD_{50} of a water-soluble polysaccharide fraction in rodent was higher than 5 g/kg. Since the toxic/lethal doses in rodents are quite high relative to conventional human dosages, they do not indicate significant limitations for clinical dosages.

2.3.5. Bioactive compounds in *Ganoderma lucidum*

Basidiocarp, mycelia and spores in *Ganoderma lucidum* contain approximately 400 different bioactive compounds which mainly include terpenoids, polysaccharides (β D- glucans), nucleosides, steroids, ganoderans, Seleno-cysteine, organic Germanium, Ling zhi-8 and some immune modulating proteins make it marvelous medicinal mushroom often called 'Anti aging mushroom' and 'versatile pharmaco-potent macro fungus'. Ganoderic acids (GAs) are highly oxygenated lanostane-triterpenoids. Furthermore, it possesses dietary fibers (quitin and polysaccharides) having high molecular weight which are not absorbed or transformed in the digestive tract and hence directly excreted. These compounds exhibit carcinostatic activity due to their capacity to absorb and excrete carcinogenic substances. Bioactive compounds in *Ganoderma* have been reported to have numerous pharmacological effects including immune modulation, antitumor, chemo and radio protective, antibacterial, anti-HIV, anti-diabetic, anti-androgenic, anti-angiogenic, radical-scavenging, and anti-ulcer activities (Sanodiya *et al*., 2009). *Ganoderma lucidum* has now become recognized as an alternative adjuvant in the treating leukemia, carcinoma and diabetes.

Polysaccharides:

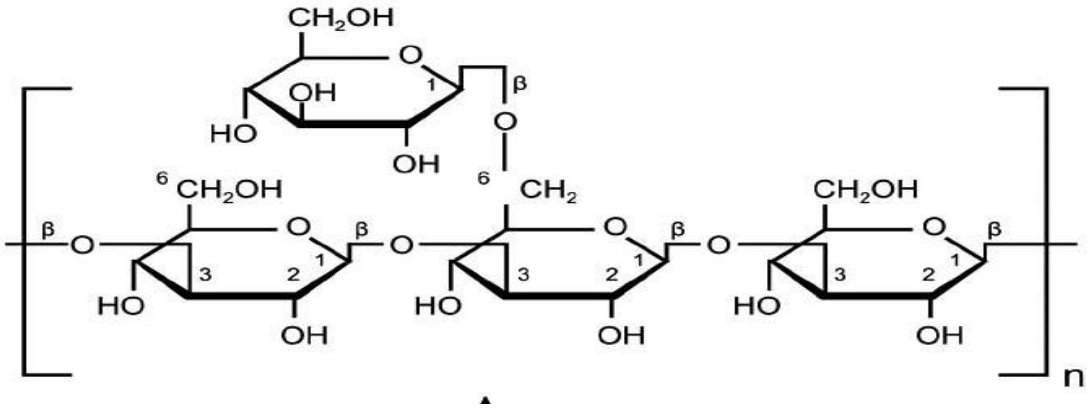


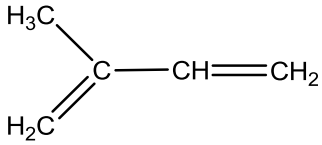
Figure 2.2: β D Glucan's molecular structure

Ganoderma contains about 20 type polysaccharides which have an average molecular weight 10^{4-6} Da. *Ganoderma*-derived β D -glucans are mainly glucose polymers with a β 1→3 backbone and side branches extended with either 1→4 or 1→6 linkages. Studies have found that β-D glucans can be taken up in the upper gastrointestinal tract and trigger various immune responses via membrane receptors such as Dectin-1, CR-3, SIGNR1, TLR- 2/6 and 4 (Bohn and BeMiller, 1995). Other studies have shown that β-

glucans can enhance monocyte, macrophage, dendritic cell, neutrophil and NK cell functions (Brown *et al*., 2003). *Ganoderma*-derived β-glucans might induce selected monocyte leukemic cell lines to differentiate into leukemic-derived dendritic cells (Chan *et al*., 2008). In addition, β-glucan a biological response modifier (BRM) has been reported to alleviate chemotherapeutic agent induced immune depression. β -glucan can be expected to exhibit synergistic anti-tumor activity and suppress adverse effects in combination with CY (Nonaka *et al*., 2008). Since β (1-3) D-glucan content in *G. lucidum* is high it can thus be expected to act as a good BRM in combination with chemo therapeutic agents for cancer therapy (Kohguchi *et al*., 2004). Based on different cellwall broken methods, the extraction methods can be divided into three ways: ultrasonic extraction method (Zhang *et al*., 2010), micro wave extraction methods, and enzymatic method (Huang & Ning, 2010).

Terpenoids

Terpenoids are bioactive bitter principle. Terpenoids are generally insoluble in water built up from terpene building blocks biosynthesized from acetyl-CoA or glycolytic or photosynthetic intermediates through Mevalonic acid pathway and Methyl erythritol phosphate (MEP) pathway. Each terpene contains at least two paired isoprene units (five carbon unsaturated 2 methyl but-1, 3 ene). More than 200 oxygenated lanostane triterpenoids have been found in *Ganoderma lucidum*. Triterpenes could be divided into three groups: C30, C27 and C24 compounds demonstrated as GAS: R, T, U, V, W, X, Y and Z lucidimol A and B, ganodermanondiol and ganoderiol F showed inhibitory activities against human HeLa Cervical cancer cell lines (Cheng *et al*., 2010).



Isoprene unit

The names monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}) polyterpenoids $\{(C_5)_n: n>8\}$ are based on the isoprene number. Gibberlines are diterpenes. Sterols are triterpenes. The carotenoids produce red, yellow and orange colors in fruits respectively are tetraterpenes. *Ganoderma lucidum* contains abundant triterpenoids and lanostane terpenoids. Terpenoids exert high antimicrobial and insecticidal activity sometimes almost toxic enough to higher organisms in higher concentration like cardenolides and limonoids. Cardenolides like Limonoids are bitter glycosides and extremely toxic enough to repel herbivore animals. In humans, these

have dramatic effects on the heart muscle through their influence on Na^+-K^+activated ATPases. Well regulated doses slow and strengthen the heartbeat. Saponins are steroid and triterpene glycosides, so named due to their soap-like properties. Both lipid-soluble (the steroid or triterpene) and water soluble (the sugar) elements in one molecule give saponins their detergent properties. Their toxicity is due to their ability to form complexes with sterols which interfere with sterol uptake from the digestive system or disrupt cell membranes after being absorbed into the bloodstream. *Ganoderma lucidum* contains more than 200 tri-terpenoid compounds.

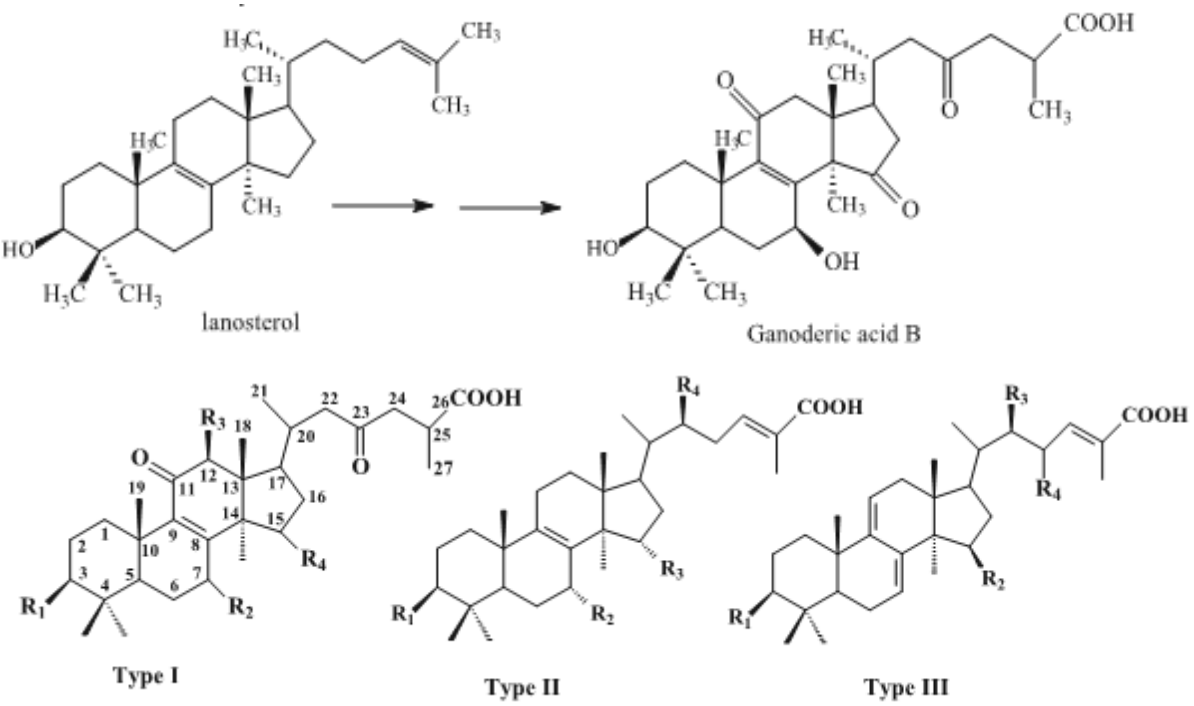
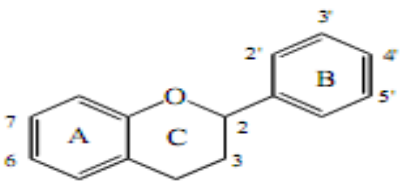


Figure 2.3: Pathway for ganoderic acids synthesis through lanosterol and their types

Phenolics or **phenylpropanoids**: **Phenolics** are derived from phenylalanine produced through shikkimic acid and or malonic acid pathways using carbohydrate precursors contain phenol rings. Phenyl propanoids, phenyl lactones, benzoic acid derivatives and flavonoids are collectively called phenolics. *Ganoderma* contains abundant polyphenolics: coniferyl alcohol, coumaryl alcohol and sinapyl alcohol are precursors for phenolics and flavonoids responsible for antioxidant, antimicrobial, anti aging and anti cancerous activity.

Flavonoids: Flavonoids are soluble in water and some are soluble only in non-polar solvents. Their roles are defence and pigmentation. Coumarines are



Flavonoids' mother structure

carcinostatic. The anthocyanins are flavonoid glycosides that give grapes their purple color, the isoflavones and the phytoestrogens from soy and the tannins that give tea its astringency are phenolics. Isofavonoids give strong antimicrobial actions. Flavonoids are the most significant compounds for the antioxidant properties. Their antioxidant activity is mainly due to their redox properties, which allow them to act as hydrogen donors, singlet oxygen quenchers, metal chelators and reductants (Rice-Evans *et al.*, 1997; Prior *et al.*, 2005; Lopez *et al.*, 2007; Ciz *et al.*, 2010; Gebicka & Banasiak, 2009).

Nucleosides

Nucleosides include adenosine and 50-deoxy-50 methyl sulphinyl adinosine. Yu and Zhai in an obscure journal were the first to report adenine, adenosine, uracil and uridine from *Ganoderma capense*. Among them, nucleosides, uridine and uracil were found to be able to lower the serum aldolase level in mice suffering from experimental myotonia. Adenosine has also been shown to inhibit platelet aggregation (Shimizu *et al*., 1985). In contrast, Gau *et al*. (1990) found that administrating crude *Ganoderma* extracts, known to have a high adenosine, had no effect on platelet aggregation in hemophiliac patients who were HIV positive. Kawagishi *et al*. (1993) reported that 50-deoxy-50-methyl sulphinyl adenosine inhibits platelet aggregation *in vitro*. Qian *et al*. (2012) reported 19 components in *Ganoderma lucidum* (two sugars, three nucleosides, and 14 triterpenes), and four components (two sugars and two nucleosides) in *Ganoderma* spp. Chen *et al*. (2012) described zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) method for the simultaneous determination of 16 nucleosides and nucleobases. With this method, the nucleosides and nucleobases in *Ganoderma* species and origins were quantified.

Germanium and Selenium:

For several years organo-germanium containing medicine has been used for cancer and AIDS. The active substance "Ge-132" as Bis (β)-carboxyethylgermanium sesquioxide ($(GeCH_2CH_2COOH)_2.3O$, spiro-germanium and germanium-lactate-citrate organic germanium has never been seen toxic in human whereas germanium dioxide is toxic. CEGS is a unique organic germanium compound first made by Mironov and coworkers in Russia with strong anticancer effect. CEGS induces interferon-gamma (IFN-γ), enhances natural killer cell activity and inhibits tumor and metastatic growth-effects often detectable after a single oral dose. In addition, oral consumption is readily assimilated and rapidly cleared from the body without prolonged toxicity. Selenium as cofactor and

selenocysteine is also found in *Ganoderma* spp. Selenium and Ge-132 in rats liver microsomes treated with Fe_2SO_4-NADPH system was studied with electron spin resonance (ESR) technique on hydroxyl free radical production. Hydroxyl free radical was decreased significantly on adding selenium, Ge-132 and combined selenium and Ge-132 indicating a direct scavenging effect on hydroxyl free radical even at low concentration.

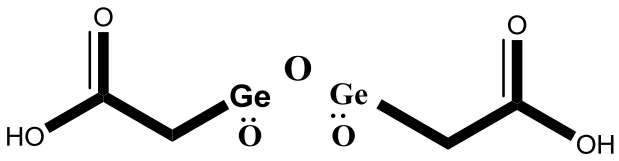


Figure 2.4: Germanium sesquioxide (Ge-132)

Immune modulating proteins

Fungal immune modulating proteins are glycoproteins. Four such immune modulating proteins as LZ8, FIP-gts, FIP-vvo and FIP-fve have been discovered and purified from *Ganoderma lucidum* and other mushrooms. Amongst other LZ8 shows considerable similarity to immunoglobulin heavy chain with respect to amino acid sequence and predicted secondary structure. LZ8 acts as mitogen for antitumor activity for human peripheral blood lymphocytes activating macrophages and T lymphocytes inducing TNF-α and cytokine.

2.4. Pharmaco-medicinal activities

Health foods, neutraceauticals, supplements**,** medicine, medicinal surplus should have immediate or long lasting antimicrobial, antioxidant, immune enhancing, anti aging, anticancer and non toxic to embryo, stem cells, cells and whole body sooner or on long consumption. Several biochemical and medicinal *in vitro, ex vivo* and *in vivo* assays are done in research laboratories to determine their medicinal efficacy as well as toxicity. Below are given some effects that *Ganoderma lucidum* impose on body. Adulteration, inappropriate formulation, misunderstanding body and drug interactions have led to adverse reactions sometimes life threatening or lethal (Elvin-Lewis, 2001).

2.4.1. Tyrosinase inhibiting effect

Guesnet (2003) showed that Ganoderic acids are used as cosmetic agents to lighten skin and as medicines to treat or prevent skin disorder. Tyrosinase is a copper-containing

enzyme present in animal epithelial tissues that catalyzes the melanin produced from tyrosine. ‘‘Lingzhi’’ is a common ingredient in various cosmetic lines that contribute to the skin whitening as it contains tyrosinase inhibitors like arbutin or kojic acid. Tyrosinase inhibitors are effective components in skin lightening compounds and other cosmetics. Inhibitory effects on tyrosinase activity among the tested mushrooms (*Ganoderma lucidum*, *Antrodia, camphorata*, *Agaricus brasiliensis*, and *Cordyceps militaris*), *G. lucidum* exhibited significant inhibition (IC_{50} value 0.32 mg/ml), compared to those prepared from other Basidiomycetes. One hundred-microliter tyrosinase (350 units/ml) was mixed with 0.9 ml various extracts (0.1 to 1 mg/ml) in phosphate buffer (pH 6.8) and 1 $ml.L^{-1}$ tyrosine (1 mmol/l). The reaction mixture was incubated at 37°C for 25 min. Absorbance at 280 nm was measured to determine the tyrosinase activity. Experimental controls were performed with the same reaction mixture in no extracts with or without tyrosinase. The tyrosinase activity inhibition rate was calculated as the inhibition (%) = $[1 - (B - B_0)/(A - A_0)]x100$, where A and A_0 represent the absorbance of control with and without tyrosinase, respectively; B and B_0 represent the absorbance of the experimental sample with and without tyrosinase, respectively. About 80% tyrosinase activity was inhibited when the reaction mixture contained 1 mg/ml extract and still about 40% inhibition effect was observed when the reaction mixture contained 0.1 mg/ml extract. No difference in inhibitory effects on tyrosinase activity was observed on three different extraction methods (75%, 50% ethanol, and distilled water extraction). Results from this study suggested another possible mechanism about this magic traditional Chinese herbal on preventing aging and can also be useful as additives in skin care cosmetics (Chien *et al*., 2008)

2.4.2. Anti - HIV effect

El-Mekkaway *et al.* (1998) reported that GA-C1-α and -H inhibited HIV-1 protease activity at concentration 0.17–0.23 mM. Min *et al*. (1998) described that GA-β and triterpenes from spores showed strong anti-HIV-1 protease activity with an IC_{50}-20 µM. Recently Sato *et al*. (2009) also demonstrated that GA-GS-2 isolated from *Ganoderma sinense* was significantly inhibitory against HIV-1 protease, with an IC_{50}-30 µM. About 42 million people living with HIV or AIDS worldwide and more than 3 million die from AIDS-related illnesses. Nucleoside analogues, such as 30’-azidothymidine are the major effective approaches to inhibit HIV reverse transcriptase (RT) and protease. However, due to their toxicities and drug-resistant new variants severely limit their long-term effectiveness (Menendez, 2002). Recent studies have indicated that some natural products belonging to different structural classes, *viz.* coumarins, flavonoids, tannins, alkaloids, terpenes, naphtho- and anthraquinones and

polysaccharides (Vermani & Garg, 2002) are active against HIV. Different *in vitro* studies indicated that various triterpenoids from *G. lucidum* had potent inhibitory activity against HIV. Lucidenic acid O and lucidenic lactone not only inhibited calf DNA polymerase-α and rat DNA polymerase-β but HIV-1 RT also (Gao *et al*., 2003). Ganoderiol F and ganodermanontriol isolated from the sporophore are active against HIV-1 growth with an IC_{100}-7.8 µg/ml (McKenna *et al.*, 2002 and Sahar *et al*., 1997). Ganoderic acid B and ganoderiol B showed potent inhibitory effect on HIV protease with an IC_{50}-0.17 mM. Other triterpenoids including ganoderic acid C-1, 3β-5α-dihydroxy-6β-methoxyergosta-7, 22-diene, ganoderic acid-α, ganoderic acid H and ganoderiol A had moderate activity against HIV-1 protease with IC_{50}: 0.17–0.23 mM. In addition, ganoderic acid-β, lucidumol B, ganodermanondiol, ganodermanontriol and ganolucidic acid A showed significant anti-HIV-1 protease activity with IC_{50} value 20, 59, 90, 70 and 70 mM, respectively (Min *et al*., 1999). Ganoderic acid A, B and C-1 had minor inhibitory activity against HIV protease with IC_{50} values 140–430 mM. C-3, C-24, or C-25 atoms in Ganoderic acids are vital for the anti-HIV activity. The aqueous low molecular-weight fraction extracted from *G. lucidum* also exhibited anti-HIV activity using the XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)- 5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide] antiviral assay, which quantitatively measures its cytopathic effects on CEM cells, a human T lympho-blastoid cell line (Kim *et al*.,1997). The IC_{50} and EC_{50} values were 125 and 11 µg/ml respectively. This aqueous low-molecular-weight extract was further fractionated to eight sub-fractions by methanol: GL-A (methanolic extract), GL-B (hexane soluble), GL-C (acetic ether soluble), GL-D (water soluble), GL-E (neutral), GL-F (acidic), GL-G (alkaline) and GL-H (amphoteric). All sub-fractions except GL-D, GL-F and GL-H exhibited anti-HIV activity with IC_{50} and EC_{50} values 22–44 µg/ml and 14–44 µg/ml, respectively. GL-C and GL-G inhibited HIV RT showing consistency. Incubating Jurkat T cells with GL-C (50 µg/ml) or GL-G (100 µg/ml) gave 75% and 66% inhibition on HIV growth, respectively. Both low-molecular-weight and high molecular-weight fractions from *G. lucidum* had negligible toxicities to CEM cells. Sato *et al*. (2009) reported that ganoderic acid GS-2, 20-hydroxylucidenic acid N, 20 (21)-dehydrolucidenic acid N and ganoderiol F inhibited HIV-1 protease with IC_{50} values 20-40 mM compared to Acetyl pepstatin as positive control.

2.4.3. Immune modulation

Ganoderma lucidum has been reported to contain several major substances with potent immuno-modulating action which include polysaccharides as β-D-glucan, proteins as Ling Zhi-8 and triterpenoids. Major immuno-modulating effects include mitogenicity and

immune effector cells' activation such as T cells, macrophages and natural killer cells resulting in the cytokines production including interleukins, tumor necrosis factor-α (TNF- α) and interferons.

2.4.3.1. Mitogenic activity

Polysaccharide fractions, methanolic extracts and protein LZ-8 have mitogenic effect on mouse splenocytes and human peripheral blood mononuclear cells (PBMC) incubated with various immuno-stimulating or immuno-suppressive agents such as phytohemagglutinin (PHA) and 12-O-tetradecanoylphorbol 13-acetate (Mao *et al*., 1999). Methanolic fraction restored the CsA inhibitted cell proliferation in PBMC, which might be through inhibiting protein kinase C signal pathway that accelerates the CsA signal pathway (Kim *et al*., 1997). Recombinant Fan-jets had the same blast stimulatory activity in human peripheral blood lymphocytes as native Fip-gts (Lin *et al*., 1997). Using deletion analysis, about 10 amino acids sequence from the N-terminal amphipathic alpha-helix domain in Fip-gts has been identified to be responsible for the immuno-modulatory activity.

2.4.3.2. Effect on T-cells

G. lucidum extracts are potent T cells activator and cytokines inducer particularly IL-2 (Wang *et al*., 1997). In human PBMC (T cells), the crude *G. lucidum* water-extract induced cytokines' expression such as IL-10, TNF-α, IL-1β, IL-6 and IL-2 *in vitro* (Mao *et al.,* 1999). Crude polysaccharide fractions isolated from fresh fruiting bodies made human T-cells release interferon (IFN-γ). Polysaccharide fraction (GL-P) promoted IL-2 release in a dose-dependent manner and markedly capacitated cytotoxic T lymphocytes. Protein LZ-8 is also a potent T-cell activator mediating its effects via integrin expression. Human peripheral blood lymphocytes stimulated with LZ-8 resulted in IL-2 expression and correspondingly up-regulating IL-2 receptor. In addition to T-cell proliferation, microscopic examination revealed that it induced cellular aggregate formation. The aggregate formation correlated with a dramatic rise in ICAM-1 expression and cytokines (IFN- γ, TNF-α and IL-1 β) production associated with ICAM-1 regulation. Both the aggregate formation and the proliferative effects were blocked on adding monoclonal antibody to either CD-18 or CD-11a, the counter receptor complex components for ICAM-1. Furthermore, adding neutralizing antibodies to both IL-2 receptor and TNF-α blocked aggregate formation, cellular proliferation and ICAM-1 expression.

2.4.3.3. Effects on Natural Killer (NK) Cells

Water-extracted polysaccharide fraction from *G. lucidum* enhanced splenic NK cells' cytotoxicity in tumor-bearing mice (Lee *et al*., 1995). The water-extracted fractions from *G. tsugae* mycelium have been shown to activate NK cells in mice. The water soluble extract and alcohol insoluble sub fraction, but not the alcohol soluble sub fraction, increased the splenic NK cytotoxic activity in a dose-dependent manner in mice. The alcohol soluble sub fraction also increased the serum IFN level in mice which was reduced by either IFN-(α, β) antiserum or IFN-γ monoclonal antibody *in vitro*.

Macrophages

Macrophages constitute first defence in the body. Activating them with substances from *G. lucidum* released cytokines, nitric oxide (NO) and other mediators. Polysaccharide from *G. lucidum* particularly β-D-glucans stimulates murine and human macrophages *in vitro* and *in vivo* (Li *et al*., 2000). CR-3 receptors on macrophages are bound with β-D glucans and internalized, priming down molecular events. Water-extracted crude polysaccharides isolated from fresh fruiting bodies potentiated the cytokines production including IL-1β, IL-6, IFN-γ and TNF-α from human macrophages, which were anti-proliferative and apoptosis inducing to the HL-60 and the U-937 leukaemic cells. IFN-γ and TNF-α released from macrophages acted synergistically and inhibited leukaemic cells. GLB7, a *G. lucidum* polysaccharide, decreased free radicals (ROS) and antagonized PMA induced respiratory burst in murine peritoneal macrophages (Li *et al*., 2000). These observations suggest that GLB7-decreased oxygen free radicals production in murine peritoneal macrophages further suggesting its antiaging effect. Ganoderan (GAN), a β-D-glucan isolated from *G. lucidum*, enhanced NO's production in the RAW 264.7 macrophages (Han *et al*., 1998). The cell proliferation in GAN-treated Raw 264.7 cell lines was inhibited. These results indicate that the β-glucan-related polysaccharides from the higher fungus activate macrophage releasing NO, which is an important chemical messenger for inducing many biological responses. A protein-polysaccharide fraction (GLB) extracted from its growing tips is a strong stimulator. When analyzed using a flow cytometer, GLB (100 μg/ml) increased the phagocytic activity in mouse peritoneal macrophages as well as chicken macrophage BM2CL cells against FITC-labeled *Candida albicans* by 55.2% and 21.2%, respectively. GLB also spread and expressed MHC class II molecules in BM2CL cells as well as in mouse peritoneal macrophages.

Mast Cells

Water extract from fruiting body inhibits histamine secretion from rat peritoneal mast cells induced by compound 48/80 or antigen-antibody reaction and on passive cutaneous anaphylaxis reaction in guinea-pigs and rats (Nogami *et al*., 1986). Two Ganoderic acids (C and D) isolated in methanol from fruit body inhibited the histamine release from rat mast cells induced by compound 48/80 and concanavalin-A. Chloroform extract from *G. lucidum* broth also significantly inhibited histamine release from rat peritoneal mast cells induced by A-23187 and compound 48/80. Further studies on the mechanism(s) about the inhibitory activity on histamine release from mast cells revealed that oleic acid present in the active fraction induced membrane stabilization in model membrane systems. Cyclo-octasulfur extracted from the culture medium decreased calcium uptake from the extracellular medium by a disulfide exchange reaction in the cell membrane leading to inhibition in histamine release from mast cells. LZ-8 prevented systemic anaphylaxis reaction in mice *in vivo* when it was administered repeatedly which is thought to be due to reduced antibody formation (Kino *et al*., 1991)

2.4.3.4. Effects on complement system

An alkali extract isolated from cultured mycelium activated both classical and alternative complement pathways (Lee *et al*., 1990). This fraction also activated murine reticulo endothelial system in the carbon clearance test and increased hemolytic plaque forming cells. The alkali extract contained 10% carbohydrate and 49% proteins respectively. Recently, a clinical study in old patients with insomnia and palpitation has shown that taking *G. lucidum* essence for 4-6 weeks increased C-3 levels in their serum.

2.4.4. Anti oxidant activity

Some metabolic perturbations and stresses and environmental stresses like pollution, radiation and pathogen invasion result in oxidative damage to cellular components and macromolecules. Normal oxygen with two paired electrons having opposite spin is the triplet one that cannot react with divalent reductant. Absorbing adequate energy enough to reverse its paired oppositely spinning electrons, it forms singlet oxygen. Singlet Oxygen which is the excited state having two electrons with parallel spin, can combine with organic compounds with paired electrons (triplet state) in cascade and damage cellular components.

Oxidative stress might have natural causes such as extreme exercise, inflammation and biotic stress or non-natural causes such as xenobiotics or chronic situations related to several diseases. In fact, their non-controlled production has been correlated to more than one hundred diseases including several cancers, diabetes, cirrhoses, cardiovascular diseases (Shah & Channon, 2004), neurological disorders (Moreira *et al*., 2008) and aging processes. Considering that 70% chronic diseases and related costs controllable, the knowledge about ROS and checking their overproduction is crucial. Maintaining antioxidants levels and free radicals scavengers in diets and increasing their quality (vegetables, legumes and fruits) or avoiding behaviours that lead to higher ROS production such as tobacco addiction and excessive exposure to environmental pollutants and xenobiotics are good control methods.

Free radical is defined as a generally unstable and very reactive atom or molecule possessing unpaired electrons in the outer orbit. Free radicals derived from molecular Oxygen (O_2) are usually known as reactive Oxygen species (ROS) and represent the most important radical species generated in living systems. In fact, despite O_2 is important to aerobic life in some conditions it can be toxic. This phenomenon is called "oxygen paradox" (Gilbert, 2000). One electron added to molecular Oxygen forms reactive superoxide ($^{\bullet}O_2$) which is "primary ROS". Superoxide is mostly produced in mitochondria due to a small but continuous electrons leaking (1–3%) from the mitochondrial electron transport system (ETS) without reducing oxygen to water. Superoxide can also be produced by different endogenous enzymatic systems present in the cell like NADPH oxidases and xanthine oxidase. Excess $^{\bullet}O_2$ causes several diseases (Kovacic *et al*., 2005). Even though $^{\bullet}O_2$ is not a very active radical, it can interact with other molecules generating what are considered as "secondary ROS" such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$). Hydroxyl radical has a very short life time but is the most toxic among all ROS which attacks DNA and damages purines, pyrimidines and deoxyribose. Hydroxyl radical is the chargeless hydroxide ion and it is formed through an electron transfer from transition metals to H_2O_2 and interacts with bio-molecules immediately after generation. Eventual permanent damages in the genetic material caused by oxidative stress might represent the first step to mutagenesis, carcinogenesis and aging (Valko *et al*., 2007). Mitochondria generate ROS and also become their first targets because ROS have an easier access to its membrane lipids which are susceptible to free radicals attack. This attack is called lipid per-oxidation and it promotes different ROS production subsequently. In the lipid per-oxidation some reactive species like $HO^{\bullet}$ abstract hydrogen atom from polyunsaturated lipid (LH) chain. This generates a highly reactive lipid radical ($L^{\bullet}$) that can react with O_2 to form a peroxyl radical ($LOO^{\bullet}$). If not neutralized, the peroxyl radical will react with other adjacent lipids producing

hydro-peroxides lipids (LOOH) that can be decomposed to form new $L^{\bullet}$ radicals initiating a process that is known as chain propagation reactions. This process when not stopped can lead to much superior damage than the ROS that started the reaction.

Radicals with Nitrogen are called reactive Nitrogen species (RNS). The principal RNS is nitric oxide ($NO^{\bullet}$) and it is generated in biological tissues by specific nitric oxide synthase (NOS) which metabolises arginine to citrulline (Ghafourifar & Cadenas, 2005). $NO^{\bullet}$ is an abundant reactive radical that acts as an important oxidative biological signaling molecule in numerous physiological processes including neurotransmission, blood flow into sex organs, penile erection, defence mechanisms and immune regulation. Their over expression is called nitrosative stress can lead to proteins' nitrosylation and so affect their normal function (Ridnour *et al*., 2005). Some immune cells produce both superoxide and nitric oxide during the oxidative burst triggered in inflammatory processes. Under these conditions $NO^{\bullet}$ combines with $^{\bullet}O_2$ to produce significant peroxynitrite anions ($ONOO^-$) which are potent oxidizing agents that can cause DNA fragmentation and lipid oxidation (Goetz & Luch, 2008).

Exposure to free radicals has led organisms to develop defence mechanisms. These were the evolution response to unavoidable oxygen radicals in aerobic life conditions and can be classified into enzymatic and non-enzymatic defences. There are many different endogenous enzymatic antioxidant defences in an organism either in intracellular or extracellular medium. Examples include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), and glutathione reductase (Gred) among others. The endogenous non-enzymatic antioxidant defences include glutathione (GSH), α-tocopherol (vitamin E), ascorbic acid (vitamin C), lipoic acid, phenolics, carotenoids and other antioxidants (Lee *et al*., 2004). SOD converts $^{\bullet}O_2$ into H_2O_2, which is then detoxified into water either by CAT in the peroxysomes or by GPx in the mitochondria, cytosol or nucleus. GPx requires Selenium on their biosynthesis. Foods or dietary supplements enriched with Selenium are crucial for strong antioxidant activity. Mushrooms have been found to contain Selenium in good quantity especially wild edible mushrooms (Falandysz, 2008). Another important enzyme is Gred which regenerates GSH that is used as a hydrogen donor by GPx. GPx can also transform hydroperoxide lipids into corresponding alcohols (LOH). Glutathione (GSH) is a low molecular weight tripeptide consisting glutamate, cysteine and glycine being the main intracellular redox buffer and its capacity to regenerate the most important antioxidant molecules is linked with glutathione disulphide/glutathione (GSSG/GSH) redox couple (Pastore *et al*., 2003). GSH effectively scavenges ROS ($HO^{\bullet}$, H_2O_2, $LOO^{\bullet}$ and $ONOO^-$)

either directly or indirectly as a cofactor of several detoxifying enzymes, eg GPx, GST, among others. In the neutralization process GSH is oxidized to glutathione disulphide (GS-SG), which can be further reduced to two GSH by the enzyme Gred. GSH is also able to regenerate other antioxidant molecules such as vitamins C and E. GSH can also react with electrophilic xenobiotics in reactions catalysed by glutathione-S transferases (GST) generating products with higher solubility and thus easier to eliminate. GSH can also neutralize $NO^{\bullet}$ resulting in S-nitrosoglutathione (GSNO). Vitamin E is a liposoluble vitamin present in the membranes thus playing an important role to prevent lipid per oxidation. Among the eight vitamin E forms α- tocopherol is the most active form in humans. ROS (hydroxyl and peroxyl radicals etc) react with vitamin E generating a poorly reactive radical vit. $E^{\bullet}$. Vitamin C then reacts with vit. $E^{\bullet}$ producing vitamin C radical (vit. $C^{\bullet}$) and regenerating vitamin E. Both vit. $E^{\bullet}$ and vit. $C^{\bullet}$ radicals are poorly reactive species owing to their unpaired electron (Hensley *et al*., 2004).

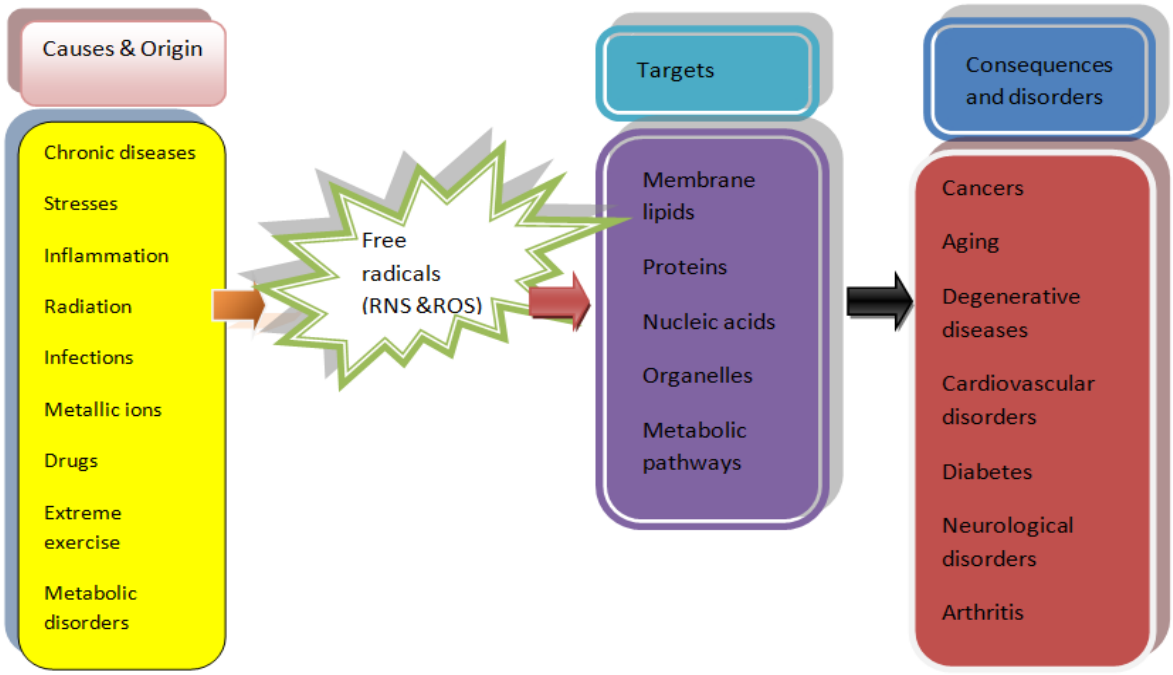


Fig 2.5: Free reactive radicals: causes and consequences

Besides all mentioned endogenous defences, antioxidant supplements or antioxidant containing foods shall be used to help the organism to reduce oxidative damage or to protect food quality. In recent years restricted synthetic antioxidants such as BHA (2-*tert*-butyl-4-methoxyphenol) and BHT (2, 6-di-*tert*-butyl-4-methylphenol) have caused an increased interest towards natural antioxidant substances. Natural antioxidants are being extensively studied for their capacity to protect organisms and cells from oxidative stresses.

Natural antioxidants have already been isolated from different plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs. Epidemiological studies have consistently shown that fruits and vegetables intake is strongly associated with reduced chronic diseases, such as cancer and cardiovascular disease (Soobrattee *et al*., 2005). In fact, oxidative and nitrosative stresses in the etiology for several acute and chronic clinical disorders have led to the suggestion that antioxidants can have health benefits as prophylactic agents. This suggests that changes in dietary behavior and consuming plant-based foods, containing significant bioactive phytochemicals, shall provide desirable health benefits beyond basic nutrition to reduce the risk on chronic diseases. Phytochemicals already identified in fruits and vegetables is estimated to be more than 5,000 but their large percentage still remains unknown thus need to be identified to fully understand their health benefits in whole foods (Liu, 2004).

In another mechanism oxygen is reduced in stepwise enzymatic reactions to form reactive O_2 species (ROS): superoxide ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$). Superoxide is strong reductant or oxidant can oxidize sulphur, ascorbic acid, NADPH and reduce cytochrome c and metal ions. Superoxide anion combines with hydrogen ions to form hydrogen peroxide.

$$O_2 \rightarrow {}^1O_2 + e \rightarrow {}^{\cdot}O_2^- + H^+ \rightarrow H_2O_2 + {}^{\cdot}O_2^- \rightarrow {}^{\cdot}OH.$$

Naturally anti-oxidants are present in plants abundantly. Animals consume plants and plant derived products and meet antioxidant demands. Ascorbic acid, α tocopherol, β carotene, glutathione reductase, glutathione synthase, superoxide dismutase etc are natural anti oxidants that scavenge radicals or reduce them. *G. lucidum* possesses marked free radical scavenging and anti lipid per oxidation properties. Aqueous, alcoholic and chloroform extracts exhibited anti oxidative activities. The chloroform extract showed significant superoxide scavenging activity (IC_{50}: 144.6 ± 1.5 µg/ml). *Ganoderma* were available in mature and baby Ling chih, mycelia and fermentation filtrate. From these four forms, hot water extracts were prepared and their antioxidant properties were studied. Hot water extracts from mature and baby Ling chih showed high antioxidant activities (78.5% and 78.2%) at 20 mg/ml, and had EC_{50} values 7.25 and 5.89 mg/ml, respectively. EC_{50} values in reducing power were 1.12, 1.37, 2.48 and 1.41 mg/ml, whereas those in scavenging abilities using DPPH radicals were 0.30, 0.40 and 0.72 mg/ml for Ling chih, immature Ling chih and mycelia respectively. Total phenols were the major naturally occurring antioxidant components found in hot water extracts and in the range of 40.86–42.34 mg/g. From EC_{50}

values obtained, fruit bodies (Ling chih and immature Ling chih) were good in antioxidant properties except for the chelating ability on ferrous ions (Mau *et al*., 2005). In 2006, Tao and others synthesised sulfated mushroom polysaccharides and compared their physico-chemical and antitumor properties with the original polysaccharides and their solubility in water was the most important factor for their antitumor activity.

2.4.5. Anti-tumour activity

Polysaccharides (β-D-glucans, heteropolysaccharides and glycoproteins) isolated from *G. lucidum* demonstrated antitumour activity against sarcoma-180 in mice (Wasser & Weis., 1999). Lanostane terpenoids such as ganoderic acids T-Z showed cytotoxic activity *in vitro* on hepatoma cells. A lanostanoid: 3β-hydroxyl-26-oxo-5α- lanosta-8, 24-dien-11-one and a steroid: ergosta 7, 22 -diene-3β, 3α, 9α-triol demonstrated potent inhibitory effects on KB cells and human PLC/PRF/5 cells *in vitro* (Lin *et al*., 1991). The polysaccharide mediated immune potentiation is thought to be the major anti-tumor action mechanism. Among the multiple polysaccharides active β D-glucans are responsible for anti-tumor effect. Beta D glucan binds to leucocyte surfaces or serum specific proteins resulting macrophages, T helper cells, NK cells and other effector cells activation (Mueller *et al*., 2000). All these increase the cytokines production such as TNF-α, interleukins and interferons often nitric oxide and antibodies by the activated effector cells. In addition to host defence potentiation, other mechanisms are also involved in the anti-tumour effect. Compound karst from *G. lucidum* suppressed K-562 leukemic cells in a dose and time-dependent manner and induced their differentiation into more mature erythrocytic cells. The conditioned medium from *G. lucidum* PS-stimulated human blood mononuclear cells (PSG-MNCCM) significantly inhibited U-937 cells and stimulated them to differentiate into mature monocytes macrophages which functioned as phagocytosis and produced cytoplasmic superoxide (Wang *et al*., 1997). DNA polymerase Inhibition and post-translational modification in oncoproteins are reported to contribute to the anti-tumour activity (Mizushina *et al*., 1998). The organic Germanium in *G. lucidum* also contributes to its anti-tumour activity (Mirabelli *et al*., 1989)

2.4.6. Anti cancer activity

Zhoua *et al*. (2011) explained Ganoderic acid Me induceing apoptosis through mitochondrial dysfunctions in human colon carcinoma cells. Johnson *et al*. (2010) described Ganoderic Acid DM as an alternative agent treat advanced prostate cancer. Jiang *et al*. (2011) predicted Ganoderic acid Me inhibits multidrug resistance and induces apoptosis in

multidrug resistant colon cancer cells. Jiang *et al*. (2008) reported that Ganoderic acids suppresses growth and invasive behavior through AP-1 and NF-κb signaling in breast cancer cells. Li *et al*. (2005) showed that Ganoderic acid X, a lanostanoid triterpene inhibits topoisomerase and induces apoptosis in cancer cells. Tang *et al*. (2006) showed that Ganoderic acid-T from *Ganoderma lucidum* mycelia induces mitochondria mediated apoptosis in lung cancer cells. *Ganoderma lucidum* inhibits constitutively active transcription factors like nuclear factor kappa B (NF-κB) and AP-1 which in turn inhibit urokinase type plasminogen activator (uPA) and its receptor uPAR. And it also suppressed cell adhesion and cell migration in highly invasive breast and prostate cancer cells, suggesting its potency to reduce tumor invasiveness (Sliva, 2006). Anti cancer activity was suggested to be mediated through the complement receptor: CR3 which binds β-glucan. In addition to β-D glucans isolated from *Ganoderma lucidum*, other polysaccharides were isolated from *Ganoderma applanatum* (Schaeff.) With *Ganoderma japonicum* (Fr.) Sewada and *Ganoderma tsugae* Murr**.** (Zhang *et al*., 1994). Some antitumor activity was demonstrated with glucuronoglucan, manno-galactoglucan, arabinoglucan, and glucogalactan from *Ganoderma* species. Polysaccharides were demonstrated to prevent oncogenesis and tumor metastasis indirectly enhancing natural killer cells, T cells, B cells and macrophage dependent immune responses (Wasser, 2005). PS-G-induced cytokines suppressed the human leukaemic proliferation and clonogenicity. Ganoderic acids like U, V, W, X and Y demonstrated cytotoxicity in hepatoma cells *in vitro*. Ganoderic acid (A and C) inhibited farnesyl protein transferase and induced cytotoxicity against hepatoma cells *in vitro*. More recently, ganoderic acids A and C inhibited farnesyl protein transferase crucial for activating Ras oncoprotein responsible for cell transformation. The ganoderic alcohols lucidimols A and B, ganodermanondiol, ganoderiol F and ganodermanontriol demonstrated cytotoxic effects on both mouse sarcoma (Meth-A) and mouse Lewis lung carcinoma (LLC) cells tumor cell lines (Min *et al*., 2000). The triterpenoid fraction inhibited primary solid-tumor growth in the spleen, metastasis in liver and secondary metastatic tumor growth in the liver (Kimura *et al*., 2002). In addition, the triterpenoid fraction inhibited Matrigel-induced neo-vascularization and the biologically active compound responsible for antiangiogenesis was identified as Ganoderic acid F. Lipids extracted from the germinating spores remarkably inhibited the mouse hepatoma, sarcoma S-180 and reticulocyte sarcoma L-II cells in mouse. Highly metastatic cancer cells activate transcription factors AP-1 and NF-κB constitutively. Furthermore, both GS and GFB down regulated receptor uPAR as well as uPA secretion thus inhibited metastasis in breast and prostate cancer cells (Sliva *et al*., 2002). *Ganoderma lucidum* also suppressed cell adhesion to fibronectin (FN) which binds to the α3-β1 integrin receptor and to vitronectin (VN) which binds to the αV-β3 integrin

receptor and finally inhibits the uPA-uPAR, FN-α3β1 and uPA-uPAR-VN-αVβ3 complexes resulting in inhibited cell adhesion and highly invasive breast and prostate cancer cells' metastasis (Sliva, 2006). Alcohol extract inhibited breast cancer cells proliferation arresting cell-cycle at the G1 phase, upregulating the cell-cycle inhibitor p21/Waf-1 and downregulating cyclin D1. Furthermore, the alcohol extract also induced apoptosis in breast cancer cells which was mediated through pro-apoptotic Bax protein up-regulation (Hu *et al*., 2002). Extract rich in triterpene inhibited hepatoma cells but not a normal human liver cell line (Lin *et al*., 2003). The inhibitory effect was caused through deactivation in the PKC activity and activation in c-Jun N-terminal kinase (JNK) and p38 MAPK resulting in G2 cell cycle arrest (Lin *et al*., 2003). Water extract from *Ganoderma lucidum* induced the neuronal differentiation and prevented apoptosis in rat pheochromocytoma PC-12 cells derived from adrenal medulla tumor, suggesting neuroactive compounds are present in *Ganoderma lucidum*. These effects were probably mediated through the ras/extracellular signal-regulated kinase (Erk) and cAMP-response element binding protein (CREB) signaling pathways because *Ganoderma lucidum* activates Erk1, Erk2, and CREB (Cheung *et al*., 2000). Although there are different compounds with various pharmacological activities extracted from mycelia, fruiting bodies or spores, the anti-cancer and anti-metastatic activities are almost completely due to its polysaccharide and triterpenoid components (Yang & Zhang, 2002). As mentioned above, some effects on cancer cells are indirect through polysaccharides induced immune stimulation and cytokines release from activated macrophages and T lymphocytes. Other effects are targeted directly to the cancer cells modulating their intracellular signaling and behavior (Cheung *et al*., 2000 and Sliva *et al*., 2002). Liu & Zhong (2011) described Ganoderic acid Mf and S induce mitochondria mediated apoptosis in human cervical carcinoma HeLa cells

2.4.7. Anti bacterial activity

Aqueous extract showed synergistic antimicrobial activity in combination with cefazolin against *Klebsiella oxytoca* ATCC 8724, *Bacillus subtilis* ATCC 6603, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25933 and *Salmonella Typhi* ATCC 650950. Several studies demonstrated that *G. lucidum* contained antibacterial constituents that are able to inhibit gram positive and/or gram-negative bacteria (Suay *et al*., 2000). The aqueous extract from carpophores inhibited 15 gram-positive and gram-negative bacteria. The studies further indicated that combining *G. lucidum* extract with four antibiotics (ampicillin, cefazolin, oxytetracycline and chloramphenicol) resulted in additive effects in most instances, synergism in two instances when combined with cefazolin against *Bacillus subtilis*

and *Klebsiella oxytoca* and antagonism in two instances (Yoon *et al*., 1994). Klaus & Miomir (2007) have studied various extracts isolated from *G. lucidum* on *E. coli*, *Bacillus* species, *S. aureus* and *Salmonella* species. The aqueous carpophoric extract showed maximum inhibition zone against *Bacillus* species while least inhibition zone was reported for *E. coli* and *Salmonella* species. Extracts from *G. applanatum* (Smania *et al*., 2007) and *G. cupreolaceatum* (Kalch.) Torr. (Mothana *et al.,* 2000) have shown significant antibacterial activity against *E. coli*. Sheena *et al*. (2003) reported that its methanol extract showed remarkable antibacterial activity against *E. coli*, *Salmonella* species and *B. subtilis*. Keypour *et al*. (2008) investigated the antibacterial activity on its chloroform extract from Iran. The results from disc diffusion tests showed that the chloroform extract had growth inhibitory effects on *B. subtilis* and *S. aureus*. Smania *et al*. (2007) observed MIC value 2 mg/ml for *E. coli* and *P. aeruginosa* while 1 mg/ml for *S. aureus* and 0.25 mg/ml for *Bacillus* species with *G. australate* extract.

2.4.8. Anti diabetic activity

Animal studies have demonstrated that reishi polysaccharide fractions have potential hypoglycemic and hypolipidemic activities. The aqueous extract (1000 mg/Kg) normalized blood glucose level in alloxan induced diabetes in Wistar rats (Mohammed *et al*., 2007). Water reishi extract reduced the increase in blood glucose level in rats following oral glucose test. Following adrenaline (i.v.) or oral glucose in rats, the mushroom inhibited increase in blood glucose without raising blood insulin levels. Glycans and ganoderans B and D have shown significant hypoglycemic activity in mice. A clinical study aimed at evaluating the antidiabetic efficacy and safety of polysaccharide fractions extracted from *G. lucidum* (Ganopoly) in a patented technique in 71 patients with confirmed type II diabetes mellitus (DM) was carried out (Gao *et al*., 2004). Treatment with Ganopoly significantly decreased the mean HbA1c from 8.4% at baseline to 7.6% at 12 weeks. Significant changes in mean FPG and PPG levels at the last visit paralleled the changes in mean HbA1c levels. Changes in fasting insulin 2-hr postprandial insulin, fasting C-peptide and 2-hr postprandial C-peptide were consistent with the between-group differences in these end points being significant at the last visit. Overall, Ganopoly was well tolerated. This study demonstrated that Ganopoly is efficacious and safe in lowering blood glucose concentrations. A 2- month open label comparative clinical study on a reishi powder extract (1 g t.i.d.) for eight diabetic patients (four with NIDD and four with IDDM) found hypoglycemic effects comparable to those found in controls who were administered insulin (100 IU=ml for 60 days) or oral hypoglycemic agents (250 mg/day for 60 days) (Gao *et al*., 2004).

2.4.9. Hypotensive activity

Komoda *et al*. (1989) showed Ganoderic acid and their derivatives as cholesterol synthesis inhibitors. Hajjaj *et al*. (2005) showed 26-oxygenosterols from *Ganoderma lucidum* were active as cholesterol biosynthesis inhibitors via acetate or mevalonate. And also demonstrated that lanosterol 14α-demethylase which converts 24, 25-dihydrolanosterol to cholesterol can be inhibited using 26-oxygenosterols from *G. lucidum.* Mycelia's water extract administered to rats and rabbits (3–30 mg/kg i.v.) produced significant hypotensive effects (Lee & Rhee, 1990). The powdered mycelium at 5% fed in spontaneously hypertensive rats for 4 weeks, caused systolic blood pressure to be significantly lower (approximately 10 mmHg) without causing a significant difference in the heart rate (Kabir *et al*., 1988). Jin *et al*. (1996) conducted a double blind, placebo-controlled clinical study in 54 patients with primary stage-II hypertension who had not responded to previous drug treatment (captopril 25mg t.i.d. or nomodipine 20mg t.i.d.). In the group which was administrated *G. lucidum* extract tablets (2 tablets b.i.d. or 220mg/day), systemic blood pressure significantly improved in 82.5% patients, with capillary and arterial blood pressure showing significant improvements in as little as 14 days. No significant changes were found in the placebo group. According to Soo (1996) in treating hypertension, *G. lucidum* was highly effective in numerous treated cases. In the more successful cases, blood pressure was back to normal within 2 months and in some cases, within 2 weeks.

2.5. Dosage and toxicity

Ganoderma lucidum is usually prescribed in various forms. May it be injected as a solution containing powered spore. Mushroom mycelia can be ingested in diverse forms as soup, syrup, tea, tablets, capsules, tincture or bolus (Wasser, 2005.). The dose as tincture (20%) is 10 mL three times daily, tablet as 1 g tablets three times daily and syrup as 4-6 mL/day (Stamets, 2000).

2.5.1. Toxicity

Conventional drugs and chemotherapeutics pose hematological and non-hematological toxicities. *Ganoderma lucidum* does not present toxicity and has demonstrated to be safe due to its long historic oral administration not associated with toxicity. In animal experiments, *Ganoderma lucidum* extracts showed a very low toxicity. There are few reported data on the possibility about adverse effects on long-term consumption. In a clinical trial 88 men over 49 years who had slight-to-moderate lower urinary tract

symptoms, were randomly assigned to 12 weeks' treatment with *G. lucidum* extract (6 mg once a day) or placebo. Evaluation on the changes in the International Prostate Symptom Score (IPSS) and variables in uroflowmetry was done. *G. lucidum* was effective and significantly superior to placebo for improving total IPSS. Overall treatment was well tolerated with no severe adverse effects. There were no observed hematological, hepatic or renal toxicity (Noguchi *et al*., 2008). The aqueous extract administered to mice (5 g/kg during 30 days) produced no changes in body weight, organ weight or hematological parameters (Wasser, 2005). The mushroom produced no changes in the estrus cycles in ovariectomized mice from a dosage 10 g/kg and no increase in the weight on levator cavernosa and testicles in male mice from the same dosage. Li *et al.* (2007) in a study about acute and genetic toxicity due to spore powder capsule found that LD_{50} was higher than 10 g/kg. Ames test, Micronucleus test in bone marrow cell in mice and sperm shape abnormality test in mice had negative reaction and lacked toxicity, hence indicating that *Ganoderma lucidum* spore powder capsule has non-toxicity.

No toxicity was observed in the rabbits' organs who took a reishi syrup preparation in progressive doses 4-140 mL/kg daily during 10 days or in dogs 2 mL/kg and 4 mL/kg during 10 days. When an alcoholic extract was administered to young rats (1.2 and 12 g/kg daily during 30 days), no toxic signs were produced in major organs, hepatic function, growth or development. In dogs administered with an alcoholic extract (12 g/kg daily during 15 days and 24 g/kg daily during 13 days), there were no toxic reactions but they displayed lethargy (McKenna *et al*., 2002). In rural area in Hong Kong, the toxicity due to wild Reishi was evaluated using harvested fruit bodies as a freeze-dried powder extract (1 g/20 g freeze-dried fruit bodies and 50 mL extract solution/100 g freeze-dried fruit bodies). Acute toxicity was tested administering the extract solution to male mice at a dosage equivalent to that one commonly recommended in commercial concentrated extracts. Neither was acute toxicity found nor was abnormal serum urea GOT or GPT compared to controls. No abnormalities were found in livers and kidneys histology, organ weights (liver, kidney, heart, lung and spleen) or organ/body weight ratios compared to controls (Chiu *et al*., 2000). In other work, toxicity was evaluated feeding 70 rats with *G. lucidum.* No significant toxicity was detected in the rats (Liang *et al*., 2008). In a double-blinded, placebo-controlled, cross-over intervention study, Reishi supplementation for 4 weeks biomarkers for antioxidant status, coronary human disease risk, DNA damage, immune status, inflammation as well as markers for liver and renal toxicity were investigated. In this study, blood and urine from healthy consenting 18 adults (aged 22–52 years) were collected before and after 4 weeks supplementation with a commercially available encapsulated Reishi preparation (1·44 g

Reishi/day: equivalent to 13·2 g fresh mushroom/day) or placebo. No significant change in any studied variables was found although a slight trend toward lower lipids was observed, while antioxidant capacity in urine increased. The results showed no liver, renal or DNA toxicity with Reishi intake (Wachtel-Galor *et al*., 2004). The previous study was performed as a follow-up to a study which showed that antioxidant power in plasma increased after Reishi ingestion and that 10 days supplementation was associated with a trend towards an improved CHD biomarker profile.

As regards long term toxicity, rats in three experimental groups were given *Ganoderma lucidum* capsule at doses 0.47, 0.94 and 1.87 g/kg·day during twenty six weeks. There was found no abnormality induced in all results and the pathological figures were normal. There is no toxicity given to rats for long term indicating that it is safe to administrate *Ganoderma lucidum* capsule continuously (Gao & Han, 2008). To observe the long term toxic reactions on rats, 80 rats were randomly divided into 4 groups -20 in each i.e. one blank group and 3 treating groups. The low, middle and high dosages were perfused respectively to the three treating groups for 30 days continuously and the body weight was weekly measured. The hematological and biochemical indexes, organ coefficient and patho-histology changes were tested after stopping administration. No evident abnormal change in every index was observed in every group, indicating that administrating *Ganoderma* spores to rats during 30 days is safe (Wu, 2005).

2.5.2. Alerginicity

Human sensitization due to *Ganoderma lucidum* antigen was first reported in the *Journal of Allergy and Clinical Immunology* in 1979 in a work performed in Ontario. The researchers found that 8.2% allergic patients positively reacted to *Ganoderma lucidum* antigen. In similar work in Auckland, this allergic reaction occurred in the 16% patients studied. In India, sensitization was also reported in 1995 and found that 28 % and 17% atopic patients showed marked skin reactivity to spores extract and whole fruit bodies respectively. On the other hand concerning food immuno-modulation, it was seen that diet and nutrition can affect the various immune parameters functioning. This concept can be utilized in attempts to prevent or mitigate allergic reactions through development on targeted food products or ingredients. In this sense, there are food products and ingredients that show potential with special emphasis on pro- and prebiotics i.e. β-glucans and fungal immunomodulatory proteins (Wichers, 2009). Beta-glucans bind to immune cells such as macrophages and NK cells these appear to exert their immunomodulatory effects by activating innate pathways as in macrophages (Volman *et al*., 2008) and were found to stimulate the TNF-α, IFN-γ and

IL-12 production. Mitigant effects to peanut allergy were observed in a rat model after using preparation containing β-glucans from *Ganoderma lucidum* (Srivastava *et al.*, 2005) even providing long term protection from anaphylaxis by inducing a beneficial shift in allergen-specific immune responses mediated largely by elevated CD_{81}, T-cell and IFN- γ production (Srivastava *et al*., 2009). The biological relevance about fungal immunomodulatory proteins (FIPs) for allergy mitigation lies in the observation that they were able to inhibit food allergic and respiratory-allergic reactions in mouse models when applied orally or nasally (Wichers, 2009). When *G. lucidum* LZ-8 FIP preparations were orally supplied to 50 male rats it could be observed that they were effective immunotherapeautic against inflammation caused in respiratory allergy by *Dermatophagoides pteronyssinus* (Liu *et al*., 2003). In a double-blind trial in 91 subjects with moderate-severe, persistent asthma with Prednisone therapy was studied to compare the efficacy, safety and immunomodulatory effects with ASHMI treatment (formula which contains *Ganoderma lucidum*) in comparison with prednisone therapy during 4 weeks. From this study the authors concluded that antiasthma herbal medicine intervention appeared to be a safe and effective alternative medicine for treating asthma. In contrast to prednisone, ASHMI had no adverse effects on adrenal function and had a beneficial effect on TH_1 and TH_2 lymphocyte balance. Recently it was completed a study examining the safety, tolerability and immunological effects using complementary ASHMI administration (which includes *Ganoderma lucidum*) to standard therapy using cortico-steroid (Budesonide–Pulmicort Turbohaler) in 5-14 years old children with persistent asthma with or without allergic rhinitis in China (Li, 2009). The results showed that ASHMI was safe and well tolerated in children. As expected both standard and ASHMI plus standard groups significantly the improved clinical symptoms. However, symptom scores improvement was greater in the ASHMI plus group than in standard therapy alone group, particularly in the nasal symptoms. Furthermore, ASHMI plus standard group showed significantly greater reductions in serum total IgE ($p<0.05$) and serum eosinophil cationic protein ($p<0.05$) but higher serum IFN-γ levels ($p<0.001$) after 3 months' treatment as compared to the standard therapy. Numerous treatments commonly used in Western medicine are linked with allergies, penicillin is one such example. It does not seem surprising therefore that *G. lucidum* have been also related to some allergies, too. However, it is important to bear this downside in mind when considering the various healing claims consisting *Ganoderma,* it occurs with the penicillin's case (Dunham, 2009).

2.5.3. Side effects

In oral dosages 1.5-9 g/day, some patients when initially took a powder have experienced temporary symptoms like sleepiness, thirst, rashes, bloating, frequent urination, abnormal sweating and loose stools, reactions which were considered to be a response to its detoxifying effect (Wasser, 2005). Large oral doses vitamin C (6-12 g/day) taken at the same time as Reishi powder extract (2-10g/day) counteracted loose stools. Because Reishi potentiates the immune system, it may be advised precaution in people who receive immunosuppressive therapies. The platelet aggregation inhibition activity demonstrated in Reishi (Hobbs, 1995) may present an additive effect in those taking blood thinning medications such as daily aspirin or warfarin (Wen *et al*., 2005) .

2.6. Product economization

During GLPP preparation water-insoluble dark-colored *G. lucidum* polysaccharide residues are generated and treated as low-value wastes. Developing value-added neutraceutical products from these water-insoluble polysaccharide residues (GLP) is a timely demand to improve the profitability for producing and processing industries and enhance the local agricultural economy. Recent study showed that carboxy-methylation improved its water solubility and antioxidant capacity (Xu *et al*., 2009) suggesting that improving water solubility is a possible approach to develop novel bioactive polysaccharide derivatives from GLP. Structural modification has shown to improve water solubility of polysaccharides effectively as well as their biological activities. Compared to other modification methods, hydroxy-propylation has several advantages: simple, low cost, non-toxic and effective in improving the water solubility and other functional properties. Hydroxy-propylation was effective in modifying the structure and properties such as chitosan, starch and cellulose.

Second, reishi cultivation generates spent substraes or post harvest byproducts that can be pasteurized and used as high value animal feed. Synthetic logs covered with mycelia are reused for mushroom cultivation or dumped in soil with agricultural wastes, plant leaves and scrap woods to compost them and used later as fertilizer or soil conditioner for bioremediation.

2.7. Common culture methods

The macro fungus is rare in nature rather not sufficient for commercial exploitation and vital therapeutic emergencies, so its cultivation on solid substrates, stationary liquid

medium or submerged cultivation has become an essential aspect to meet the increasing demands in the international market (Sanodiya *et al*., 2009). Currently, the methods most widely adopted for commercial production are the wood log, short wood segment, tree stump, sawdust bag and submerged culture (Stamets, 2000). Diverse chemical compounds with pharmacological activity have been isolated from the mycelium, fruiting bodies and sclerotia: triterpenoids, polysaccharides, proteins, nucleotides, alkaloids, steroids, lactones, and metal complexes. Till now polysaccharides, Ganoderic acid and other metabolites are mainly extracted from solid state cultivated fruit body, submerged cultured biomass and its broth.

Hsieh & Yang (2004) reported mycelial growth rate 6 mm/day on soy residue substrate having C: N ratio 80 in test tubes. However, 7.5 mm/day growth rate was observed in C: N ratio 70-80 in 500 ml flasks. Habijanic and Berovic (2000) have reported a unique work which was carried out in the horizontal stirred tank bioreactor (30 L) maintaining 80 rpm, 30 °C, air flow 2 L/min, consisting beech sawdust, olive oil, $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$ and distilled water to produce animal feed supplements.

2.8. Culture advancements

2.8.1. Immobilized Culture

Yang *et al*., (2000) introduced a polyurethane foam sheet into the submerged fermentation medium in an Erlenmeyer flask. The mycelium adhered to the surface on the foam matrix with almost no mycelia remaining free in the bulk liquid. The biomass density and EPS obtained were both markedly higher in this culture method than in freely suspended cultures. The polysaccharide secretion occurred at a slow rate and after 2–3 weeks, a large polysaccharide portion was adsorbed on the support. This might enable an alternative strategy for product and biomass recovery in which the support can simply be removed and pressed. There is no report and scientific data available on Ganoderic acid production in immobilized cultures.

2.8.2. Elicitation in submerged culture

Traditional methods include microbial strain selection, culture improvement, media development, and, bioreactor and process design. These methods, however, suffer from severe drawbacks such as the long time required for successful outcomes, high expenses and, in many cases, low success rate. Two novel strategies have been introduced for overproduction of industrially desirable microbial products. These strategies are based on

microbial response to the microbes in their vicinity (quorum sensing) and, to their surrounding environment (elicitation). Elicitors are added to a biological system as stress factors that induce or enhance the biosynthesis of secondary metabolites. For example, jasmonic acid supplemented *Catharanthus roseus* cultures had an increase in the specific yields of serpentine, ajmalicine, tabersonine and lochnericine. *Sophora flavescens* water extract of 11 g/l promoted *Ganoderma lucidum* biomass and extracellular polysaccharides production to increase at 14.5 and 2.4 g/l from 10.5 and 1.2 g/l respectively. The extract increased the pH buffer capacity and decreased the broth viscosity (Li & Zhi, 2012). Rutin, methyl jasmonate (Ren *et al*., 2010), jasmonic acid, fungal polysaccharides and extracts have been used as elicitor to bring enhancement on usual biomass growth and secondary metabolite accumulation. Their exact role is not known but it is believed that elicitors provide essential precursors for secondary metabolism helping to over produce either biomass or natural metabolites important medicinally. Elicitation mycelia exogenously with methyl jasmonate stimulated them to accumulate *GlFPS* gene. Subsequently promoter analysis indicated that its 50 upstream region possessed various potential regulatory elements associated with physiological and environmental factors.

Quorum sensing is the inter-cell communication between cells through the release of chemical signals when cell density reaches a threshold concentration (critical mass). The quorum sensing signals differ in different microbial systems; examples are acyl-homoserine lactones, modified or unmodified peptides, complex γ -butyrolactone molecules and their derivatives. Numerous physiological activities in microbes such as symbiosis, competence, conjugation, sporulation, biofilm formation, virulence, motility and the production of various secondary metabolites are regulated through the quorum-sensing.

2.9. Biotechnical advancements

2.9.1. Molecular breeding

Protoplast-fusion techniques are widely applied on mushroom breeding have made greater progress. Transgenic engineering techniques applied on the medicinal mushroom breeding are technological innovation on the molecular level. Tissue separation and spore separation methods are used for obtaining pure strain (Zhou *et al*., 2012). Mutation breeding is also done as: spore (or protoplast) suspension liquid → viable count and mutagenizing → spreading plate for cultivation → picking up strain and inoculation → initial screening → slope culture → rescreening → superior strain selection. The proto-plast is usually chosen for mutation breeding and increased bioactive components such as polysaccharides (Gao *et*

al., 2008), triterpenoids and organic germanium are regarded as the breeding objectives. DXN claims to have used bred and patented *Ganoderma lucidum* in its different products.

2.9.2. Cloning

LZ-8 gene was cloned into pPIC9K to construct a yeast expression vector pPIC9K-LZ8 and then was transformed to *Pichia pastoris* strain GS115. Recombinant *LZ*8 with molecular weight 17 kDa was hemagglutinating *in vitro* on mouse red blood corpuscles but not in human RBC and its yield reached 270 mg/l at optimum culture conditions. rFIPs expressed in insects showed more activity than those expressed in *E. coli* due to poor glycosyltion and folding. So the most efficient *P. pastoris* system was chosen to enhance production and bioactivity (Xue *et al*., 2008). Ding *et al*. (2008) cloned farnesyl diphosphate synthase (*GlFPS*) gene producing enzyme GlFPS having 360 amino acids with a calculated molecular mass 41.27 kDa involved in iso-prenoid biosynthetic pathways. Homology based PCR method was used to clone a cDNA encoding Lanosterol synthase gene (*LS*) from *Ganoderma lucidum* which produces triterpenes. Functional complementation in an *erg7* yeast strain lacking *LS* activity demonstrated that cloned cDNA contains functional *LS* gene. *Gl-LS* transcript profiles analysis revealed a positive correlation between *LS* gene expression pattern and triterpenes production. *LS* gene over expression due to methyl jasmonate in the mycelia was also seen with RT-PCR (Shang *et al*., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1. Strain collection and culture

Ganoderma lucidum strain Philippine (*Ganoderma multipileum*) was purchased from Centre for Agricultural and Technological Training, Imadol, Lalitpur and cultured in PDA plates and slants. The cultured plates were kept at 4°C for further use.

3.2. Mass culture

3.2.1. Culture in Synthetic logs

Following constituents were mixed properly wearing hand gloves. Water was added in calculated amount just to make it 65% available in media with formula

Water = [65x/ 35 – {90 x (a-p)/100a + 4x (b – q)/100b + 4x (c – r)/100c}] kg

Where x= total dry media substrate (kg), a=raw saw dust wt (kg), b= raw wheat bran wt (kg), c=raw rice bran wt (kg), p= dried saw dust wt (kg), q= dried wheat bran wt (kg), r= dried rice bran wt (kg)

(20 x12) cm^2 Polypropylene bags were filled up to 200 g with substrates (Appendix I A) and their mouths were inserted into 1'' diameter PVC ring and their necks were tied with rubber to let their mouth open. Cotton plugs used to close their neck tightly and bags were autoclaved at 15 *lb.* psi at 121°C for an hour, synthetic logs were removed from autoclave and cooled down to room temperature in Laminar Air Flow. The prepared synthetic logs were inoculated with fresh spawn. Packs were tightly closed with cotton plugs and kept on the sterile place for 25 days.

3.2.2 Growth environment management for fruiting

Synthetic logs were run with spawn at 28 °C in diffused light. Relative humidity 90-95 %, was maintained with sprinkling sterile water twice daily with calcium salt solution (0.1%) into cotton plugs. As soon as spawn run completed, carbon dioxide concentration was reduced making some holes in sacks to ensure ventilation so that fresh air diffuses into plastic bags.

After logs became fully colonized with mycelia and browning began to appear, PVC rings and rubber bands were removed to open the packs and the optimum moisture and relative humidity was maintained by sprinkling sterile water.

3.2.3. Stationary submerged culture in potato broth

Potatoes were peeled, weighed 300 g and chopped into small and thin pieces. Then it was boiled in 1000 ml distilled water to get its infusion slightly thick, transparent and gravid. Then it was strained and its filtrate was taken, 25 g Glucose was added, pH was adjusted to 5.0 and it was poured in sterile bottles so as to fill only 25 % volume. All bottles with broth media were autoclaved at 15 *lb* psi at 121 °C for 30 minutes, cooled to room temperature and then prepared media were inoculated with fresh mycelia equally cut with 6 mm cork borer from homogenously growing region. Bottles were kept at 28 °C for 20 days for maximum growth and secondary metabolite accumulation.

3.2.4. Plate culture

39.5 g potato dextrose agar (PDA) was weighed and dissolved in 1000 ml distilled water, sterilized in autoclave under 15 *lb* psi (103 kPa) at 121°C. After cooling it down to 50°C for an hour it was poured into previously sterilized and dried plates. After gel set, properly fresh and actively growing mycelia were inoculated in the middle with sterile forceps under laminar air hood. Plates were kept at 28 ˚C for 1 week for fresh inoculum and for 1 month to excise the upper mat for extraction.

3.2.5. Shaking submerged culture

The fungus was maintained in PDA medium at 4 °C. Inoculum contained mycelia in 5 mm sized agar disc from a 10-day-old culture grown on PDA. Cultures were performed in 125 mL Erlenmeyer flasks containing 25 mL liquid medium. The natural media contained variable glucose concentrations along with the natural component PD and sometimes peptone. Non-inoculated Erlenmeyer flasks served as controls for possible pre-existing polysaccharides contained in the natural media. These plates were shaken at 100 rpm at room temperature for 20 days.

3.3. Growth parameters optimization in plate culture

3.3.1. pH Optimization

300 g potatoes were peeled, cut into thin and small pieces and boiled in 1000 ml distilled water. Boiling for an hour, it was strained with muslin cloth and volume was adjusted to one liter. In each sterile conical flask, 100 ml PD broth was poured and its pH was varied from 3.5 to 6.75 with 0.1 N HCl and 0.1 N NaOH. Then 1.5 g agar powder was added to each bottle, shaken and autoclaved before pouring into sterile Petri plates in triplicates. Very minute mat with agar was plugged by borer and inoculated into plates, incubated at 30 °C for four days and were marked at their margin. On the following day at the same

time, increase in mycelial length was reported which indicated lengthwise growth per day. Lengthwise growth rate was measured.

3.3.2. Temperature optimization

PD broth was prepared as in 3.3.1. pH was maintained 5.0 in whole media and poured into plates labeled temperature in triplicates and corresponding control. Plates were sealed with paraffin and incubated for 4 days at different temperature. On the following day at the same time, increase in mycelia length was measured which indicated lengthwise growth per day.

3.3.3. Carbon source optimization

Liquid media composition as mentioned in 3.2.5 was prepared with no carbohydrate at all. pH was adjusted to 5.0 and 100 ml medium was poured into sterile conical flask. Then 15 g different carbohydrate sources (sucrose, trehalose, glucose, lactose, cellulose, maltose, sorbose and xylose) as labeled in flask were added into respective flask and autoclaved to dissolve them. Each medium was poured into plates in triplicates and the latter were inoculated with small mycelial spawn. Plates were sealed with paraffin and incubated for 4 days at 30°C and their margin (apex) was marked. On the following day at the same time, increase in mycelia length was measured which indicates lengthwise growth per day.

3.4. Sample preparation for extraction

For extraction, carpophores and mycelia were taken from variously cultivated *Ganoderma lucidum*.

3.4.1. From solid cultivation

Fruiting bodies were harvested, crushed and the powder was dried in Petri plates in hot air oven at 60°C until it was totally dry.

3.4.2. From lawn culture

Upper mycelial mat was carefully excised with forceps from plates then it was pulled whole over and cut into small pieces.

3.4.3. From submerged culture

Broth was filtered with whatmann's no 1 filter paper. The biomass was dried in petri plates in hot air oven at 60°C until it was totally dry. Filtrate was saved and allowed to air dry at room temperature.

3.4.4. Powdering

The biomass was again separately dried in liquid nitrogen (-196°C) and ground in mortar and pestle until it was totally powdered**.** Powder was kept in hot air oven at 60°C.

3.5. Soxhletion

Powder was weighed; 10 g was taken in thimble and kept inside Soxhlet's apparatus. Nearly 400 ml ethanol was kept in round bottomed flask to be fitted in the apparatus with cooling jacket and it was run for 48 hrs until ethanol came down clear**.** The solution containing crude bioactive compounds was dried in rotary vacuum evaporator and the remnant in hot air oven at 60°C.

3.6. Working solution preparation

Dried extract was weighed in pre weighed eppendorf tubes and 1 ml ethanol was added per 100 mg crude extract i.e. 100 mg/ml solution as working solution.

3.7. Antibacterial activity

Required materials: MHA plates, bacteria, penicillin discs (Hi-Media), filter paper discs,

Procedure:

Six opportunistic bacteria (*Staphylococcus aureus, Pseudomonas aeroginosa, Bacillus subtilis, Salmonella typhii, Klebsiella pneumonae and Klebsiella oxytoca)* were brought from Man Mohan Memorial Hospital, stored in NA plates in deep freeze. For antibacterial test, bacteria were inoculated into MHA plates and each pure colony was transferred to nutrient broth which was incubated for 24 hrs at room temperature and on the very day their opalescence (turbidity) was equalized with 0.5 Mc-Farland's standard. MHA plates were swabbed with separate bacteria using cotton swab inside Laminar Air Flow. In each plate 6 paper-discs (4 mm) were placed equally separated and different extract solutions (20 µl) were aliquot. Penicillin and erythromycin discs (Hi-Media, India) were used as standard antimicrobial drug and ethanol was aliquot in one disc as negative control. Plates were sealed with paraffin tape and incubated at 37°C for 24 hrs and inhibition zones were measured on the following day.

3.8. In vitro antioxidant assay

3.8.1. DPPH free radical scavenging assay

Required materials: DPPH (0.2 mM) (Sigma), ascorbic acid (Hi-Media), test tubes, spectrophotometer

Procedure:

Free radical scavenging activity was determined as described by Singh *et al*. (2002). Ethanol solution (1 ml) containing *Ganoderma lucidum* extract at various concentrations was added to 0.4 ml DPPH (0.2 mM) solution. And ascorbic acid (3.125 µg/ml - 100 µg/ml) as standard was taken in different test tubes. The sample volume was adjusted to 1 mL adding ethanol. Then, 0.4 ml DPPH was added to these tubes and shaken gently and allowed to stand for 45 min at 27°C in dark. The control was prepared as above without sample or ascorbic acid. Ethanol was used for the baseline correction. Their absorbance was measured at 517 nm in Genesys UV Vis spectrophotometer. Radical scavenging activity was expressed as the inhibition percentage and calculated using the following formula,

% Radical scavenging activity (pi) = [(Control abs-sample abs)/Control abs)] x100%

IC_{50} was calculated using formula IC_{50} = EXP (LN (conc>50%)-((pi>50%-50)/ (pi>50%-pi<50%)*LN (conc>50%/conc<50%))) in Microsoft office excel 2007 (Maes and Cos, 2010).

3.8.2. Reducing power ability

Required materials: phosphate buffer (0.2 M, pH 6.6), potassium ferricyanide (1%), trichloro-acetic acid (10%), ferric chloride, UV-spectrophotometer (Genesys).

Procedure: Reducing power ability was measured mixing 1.0 ml extract (prepared in distilled water) to 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1 %) and incubated for 30 min at 50°C. After that 2.5 ml trichloroacetic acid (10%) was added to the mixture and centrifuged for 10 min at 3000 g. 2.5 ml supernatant was diluted with 2.5 ml distilled water and shaken with 0.5 ml freshly prepared ferric chloride (0.1%). The absorbance was measured at 700 nm using Genesys UV Vis spectrophotometer. The reference solution was prepared by following the same method as described above, but water was added instead of the sample. Increased absorbance of the reaction mixture indicated increased reducing power. All experiments were done in triplicate using butylated hydroxy toluene as positive control. (Subhashini *et al*., 2011)

3.8.3. Total antioxidant potential estimation

Required materials: Conc. sulphuric acid, Sodium phosphate, Ammonium molybdate, Spectrophotometer, test tubes etc.

Procedure:

To 0.1 ml extract (100 µg/ml) solution in water was mixed in 1 ml reagent (0.6 M Sulphuric acid 28 mM Sodium phosphate, and 4 mM ammonium molybdate). Capped tubes were

incubated in boiling water bath for 90 min and cooled to room temperature. Absorbance against blank was measured at 695 nm in Genesys UV Vis spectrophotometer. Blank contained 1 ml reagent solution plus water. Total antioxidant activity is expressed as ascorbic acid equivalent (Subhashini *et al*., 2011).

3.9. Phytochemicals estimation

3.9.1. Total phenolic content estimation

Required materials: Gallic acid (HiMedia), ethanol, Folin–Ciocalteu reagent ((Hi-Media), sodium carbonate

Procedure:

Total phenolics was determined with the Folin–Ciocalteu reagent using Panovska *et al.* (2005) method. Each 100 µl prepared sample in different concentration was dissolved in 500 µl (1:10 v/v dilution) Folin–Ciocalteu reagent and 1000 µl distilled water was added. The mixtures were gently shaked and incubated at room temperature for 10 min. After incubation, 1500 µl sodium carbonate (20%) solution was added. The final mixture was shaken and then incubated for 2 hrs in the dark at room temperature. Standard Gallic acid solution was prepared in ethanol (90%) simultaneously, and their absorbance was recorded at 765 nm in Genesys UV Vis spectrophotometer and standard curve was plotted using Microsoft office excel 2007 with absorbance versus concentration and the results were expressed as mg Gallic acid equivalent (GAE) per g dried *Ganoderma* sample biomass comparing with its standard graph.

3.9.2. Total flavonoid content estimation

Required materials: Aluminium chloride, quercetin (Sigma), absolute ethanol, potassium acetate

Procedure:

Total flavonoid in the extract was determined using Govindarajan *et al*. (2004) method with aluminium chloride using quercetin as a standard. Each 0.5 ml plant extract (20 mg/ml) mixed in 1 ml ethanol (90 %) was added to test tubes containing 0.1 ml aluminium chloride (10 %), 0.1 ml potassium acetate (1 M) and 1.3 ml ethanol (90%). Their absorbance was measured at 415 nm in Genesys 6 UV Vis spectrophotometer after incubating for 40 min at room temperature. Total flavonoid concentration was calculated using quercetin as mg flavonoid equivalent to quercetin (FEQ) per gram biomass.

3.10. Toxicity analysis

Herbal medicines contain mixed compounds. Some compounds and contaminants can pose silent poisoning after medication. Empirical toxicity can be determined using lethality test and heavy metal quantification.

3.10.1. Heavy metal quantification

Required materials: Conc. HNO_3, Ashless whatmann's 40 Filter paper, quartz tubes, fume hood, AAS, Sand furnace

3.10.1.1. Sample collection and preparation

For cadmium (Cd) and lead (Pb) quantification, four supplement samples were collected in Nepal: Lab grown *Ganoderma lucidum*, gano coffee (DXN), gano capsules (DXN) and wild *Ganoderma* from Chitlang, Makwanpur. Each sample was dried and ground into fine powder in grinder. The ground samples were again dried to remove the remaining moisture and 1 g each sample was digested with conc. nitric acid. Heavy metals were quantified by Acid digestion method (Zheljazkov and Nielson, 1996).

3.10.1.2. Sample digestion

The samples were dissolved with 5 ml conc nitric acid separately overnight. On the very day again 5 ml nitric acid was added to each tube kept in sand furnace over stove just heating at 70°C for 2 hours. Then their temperature was raised up to 180°C and then kept constant for four hours until all fumes escaped. At last the clear tubes, with no fume, were cooled and the solution was filtered with ashless whatmann no. 40 filter paper. The tubes were rinsed with deionized water poured to unused falcon tubes each not exceeding 25 ml. Samples were analyzed for lead (Pb) and cadmium (Cd) in Flame Atomic absorption spectrometry (FAAS) with Perkin Elmer VMAA 240 FS instrument using APHA 21st edition 3111B test method (APHA, 2005) at Nepal standards and metrology Department, Balazu.

3.10.2. Brine shrimp bioassay

Required chemicals: shrimp eggs, hatching beaker, test tubes, artificial sea water, Disposable pipettes, micropipettes,

Procedure:

Brine shrimp bioassay was followed according to Meyer *et al*. (1982). All the apparatus were properly sterilized initially.

Artificial sea water preparation: Freshly prepared artificial sea water needed for the bioassay was prepared dissolving the chemicals in distilled water.

Shrimp hatchling: For the hatching, brine shrimps eggs (50 mg) were sprinkled on the beaker filled with artificial sea water and illuminated with table lamp (60 watt) for 48 hrs with temperature adjusted at 30°C.

Sample preparation: 20 mg sample to be tested was dissolved in chloroform or methanol depending upon its solubility. The solution thus prepared was used as stock solution. From the each stock solution 500 µl (eqv. 1000 ppm), 50 µl (eqv. 100 ppm) and 5 µl (eqv. 10 ppm) were transferred to total nine test tubes for each doses level. Then the solvent was evaporated standing overnight.

Lethality bioassay: After complete evaporation, 5 ml artificial sea water and ten matured shrimps were transferred into all test tubes containing samples. Similarly, three controlled vials were taken and ten matured naupalii were introduced to each vial. After 24 hrs, survivors' number was counted using disposable pipettes.

Samples taken:

Four samples were assessed for brine shrimp lethality bioassay. Samples were labeled as GL= wild *Ganoderma* collected in Chitlang, Makwanpur, LG= lab grown *Ganoderma lucidum*, CA= *Ganoderma* capsule mix (DXN), CF= *Ganoderma* coffee (DXN).

LC_{50} Calculation:

LC_{50} is the lethal concentration dose required to kill 50% shrimps. LC_{50} is calculated as follows. Here "n" is the replicates, x is logarithm of constituent concentration taken for lethality test in µg/ml and y is the probit for average survivors in all replicates.

We have, $\boldsymbol{\beta = \frac{\sum xy - \frac{\sum x . \sum y}{n}}{\sum x2 - (\sum x)\ 2/n}}$ and $\boldsymbol{\alpha = [\sum y - \beta \sum x]/n}$

From **probit regression**, $Y = \alpha + \beta.X$ **=> $X = (Y - \alpha)/\beta$**, where Y is a constant having value 5 for calculating LC_{50} value. Thus, **LC_{50} = Antilog X**

CHAPTER 4

RESULT AND DISCUSSION

This research was designed to evaluate biological, medicinal activities and empirical toxicity on potent medicinal mushroom *Ganoderma lucidum.* As it is well known that *Ganoderma lucidum* has a long medicinal applications, its growth trial, parameters optimization, medicinal activities and toxicity evaluation are contextual with current global consciousness regarding herbal medicine consumption and obstacles in their trade.

4.1. Growth pattern

4.1.1. Growth pattern in semisolid media

Semisolid media or lawn culture is used for subculture, transport, mother culture storage and sometimes for spawning. Mycelias growth characteristics can easily be studied using semisolid media. Amongst four media tested for growth trial for *Ganoderma lucidum*, the best growth was observed in PDA media. The mycelia on PDA media had colony characteristics like straightforward spreading, velvety and whitish cottony intermingled fabric mat on earlier incubation turning to yellowish brown patches on long incubation (45 days) depositing secondary metabolites as in **photo 4.1**. Fresh growing apex had straight silken micro-fibres heading ahead with inter-fiber distance nearly 0.5 mm. *Ganoderma lucidum* on MEA shows slow growth and smooth mat. Similar patterns were seen on SDA. So PDA was used as medium for culture, subculture and storage throughout the thesis work.

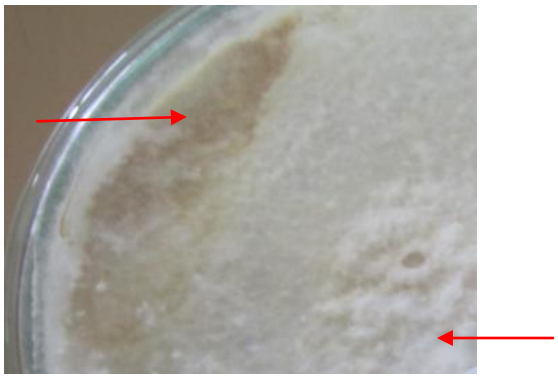


Fig 4.1: Ganocelia in PDA plate culture on long incubation with red arrows showing whitish mass in the middle and brownish patches on periphery

4.1.2. Growth variation in liquid media

Liquid potato dextrose broth media (100 ml) in marmalade bottles (500 ml capacity) were shaken continuously on shaker at 120 rpm at room temperature keeping 3 bottles (control) still for 18 days. Obviously higher growth was seen in control (dried 1.2 g/100 ml) culture. In shaking culture the biomass remained submerged and round wet cotton plug like bulk whereas in stationary culture it remained floating and spread fully forming

a disc 8 mm in thickness outwardly and 4 mm inwardly with hydrophobic upper surface and jellied underneath. Biomass was filtered, dried and weighed about 1.08 g/100 ml from shaking culture. On long incubation (45 days) upper surface in stationary culture changed to yellowish brown. Mycelia from the disc started to grow upward adhering to the bottle glass wall as substratum with almost no mycelia remaining free in the bulk liquid similar to the immobilized culture with foam sheet (Yang *et al*., 2000). The biomass density was higher than in shaking culture. Higher the biomass growth higher would be Ganoderic acids and polysaccharides accumulation. The reason behind higher biomass in stationary culture than in shaking culture is biomass and bioactive compounds accumulation favor less Oxygen in the medium. Moreover, the medium which was shaken for four days and then kept still had the most biomass and Ganoderic acid accumulation (Sanodiya *et al*., 2009).

In submerged culture environment, growth and nutrient parameters can be manipulated as we will. In modern culture method, elicitors like rutin, jasmonic acid and fungal polysaccharides are used to increase the bioactive components in submerged culture medium. Submerged culture is efficient with respect to productivity, control, cost and downstream processing. Carpophores maturation takes about 90 days whereas reasonable biomass and specific metabolites can be enriched in less than 20 days (Sanodiya *et al*., 2009).

4.1.3. Growth pattern in solid substrate culture

In our lab, spawn covered the 200 g substrate packed in polypropylene bags in 22-25 days. However, bags were opened prior to browning. Yellowish brown color appeared at the top and sides in still closed bags which indicates more oxygen is necessary for sporophore formation as lignin degradation takes place only in aerobic condition. After six days, crown changed into thick cap with white margin. With its thickening, concentric striations were seen on flattened cap and deep brown coloration at the centre. Once harvested, second or third fruiting also occurred from the synthetic log having enough substrates going on decomposition. Somewhere logs are also buried in humid soil rich in humus prior to primordia formation to facilitate irrigation and nutrient absorption from soil for further fruiting.

4.2. Growth parameters optimization in plate culture

For easiness to manipulate the growth variables, semisolid medium was chosen to optimize variables for the best growth. Mycelia spreading behavior and growth rate varies in different conditions. PDA plates were incubated at room temperature in

diffused or no light and relative humidity 90-95%. Ventilation was facilitated to increase fresh air to exchange carbon dioxide gas.

4.2.1. pH optimization

pH indicates acidity or alkalinity. Many fungi can grow in acidic to slightly alkaline media adjusting to various pH adversities within pH 2.0 to 9.0 (Jay *et al*., 2005). However, in this research for *Ganoderma lucidum*, growth was tested varying pH between 3.5 and 6.75 in triplicate gapping 0.25 in PDA medium prepared in lab. Significantly higher growth was seen between pH 4.5 to 5. 5 (**figure 4.2)**. Nearly up to 1.08 cm increase in length per day was seen at these pH however, average showed a little less. Sharp rise in growth was seen in increasing pH towards optimum. Higher and sustained growth was obtained in alkaline medium than in acidic medium whereas growth tended to stop nearly below pH 3.0. According to Jayasinghe *et al*. (2008) this Mushroom has a broad pH range (5~9) for its mycelial growth and mostly favorable growth was found at pH 5.

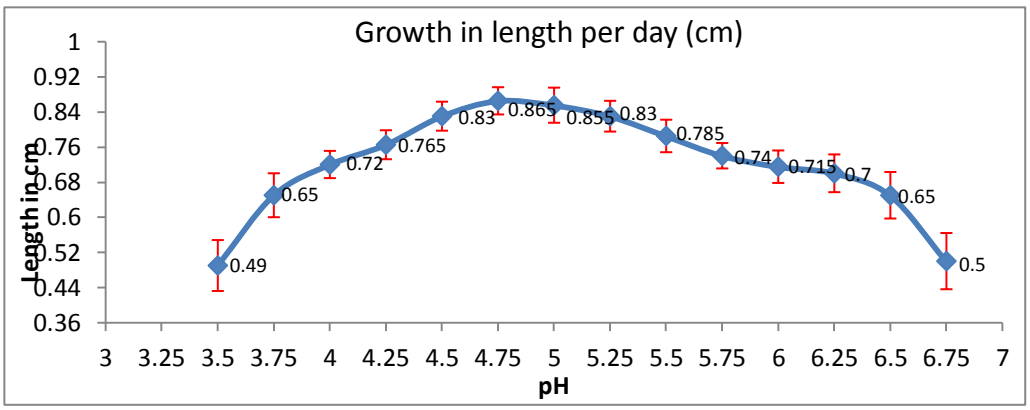


Fig 4.2: pH optimization for *Ganoderma lucidum* growth in PDA plates

4.2.2. Temperature optimization

Fungi naturally can adjust various temperatures inducing sporulation on adversities as their spores can live long armoring their germplasm alive inside. So *Ganoderma lucidium* is not exception to this. Since it grows better in temperate regions, temperature was varied in between 20 and 36 °C in PDA to observe its growth pattern in this thesis. Magnificently, highest growth was seen at temperature between 28 and 32 °C (**figure 4.3)** and at pH 5.0 as optimized previously. *Ganoderma lucidum* seemed to tolerate and sustain its growth at lower temperature than higher temperature because at higher temperature enzymes for growth get inhibited and sometimes get denatured. So lengthwise growth increased slowly and reached its highest peak at 30 °C and then plunged harshly onwards. Jayasinghe *et al*. (2008) reported the most suitable temperature for the mycelial growth was obtained at 30℃ whilist

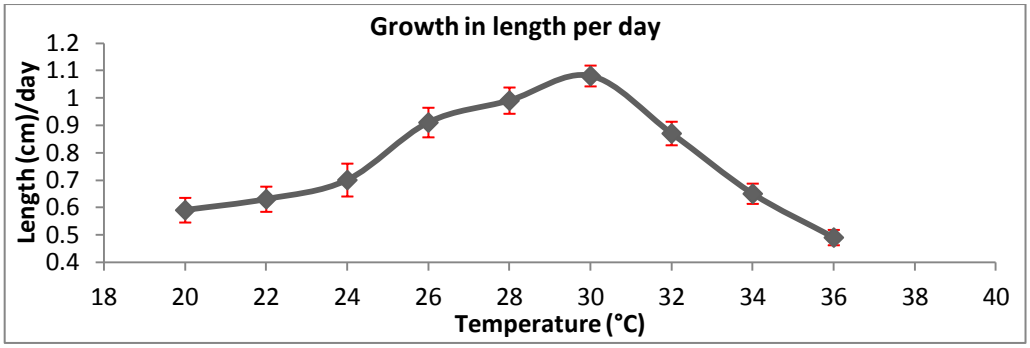


Fig 4.3: Temperature optimization for *Ganoderma lucidum* growth in PDA

4.2.3. Carbon source optimization

Various carbohydrates were supplied in basal minimum (10 g/L) into sugar free nutrient media so that their limiting effects could be studied. Temperature and pH were used 30 °C and 5.0 as optimized previously. Fortunately, *Ganoderma lucidum* was able to utilize them all but some little nevertheless. Lengthwise growth per day showed sorbose and trehalose to be the most effective carbohydrate where maximum lengthwise growth per day was 1.08 cm. On the contrary, *Ganoderma lucidum* was almost reluctant to utilize cellulose and lactose as carbon source (**figure 4.4)** because it seemed to utilize more popular biochemical precursors than unusual precursors. Most efficient carbon sources were in the descending order: sorbose> trehalose> glucose> maltose> starch.

In the first instance, Carbon is required for structural buildup and energy which comes from initially supplied monosaccharides or disaccharides in the media. Then, initially supplied carbohydrate is used for fueling spawn run, substrates colonization and their degradation.

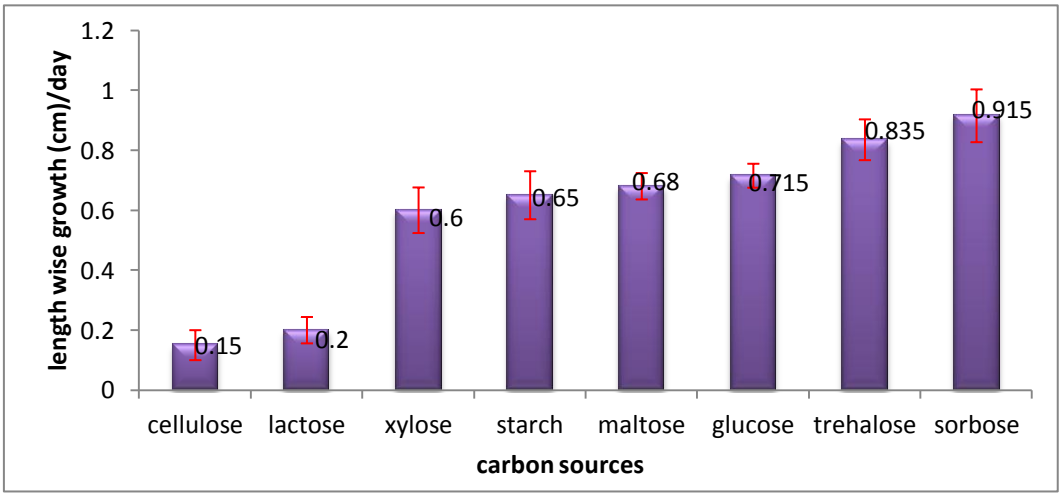


Fig 4.4: Carbon source optimization for *Ganoderma lucidum* growth in PDA

4.3. Extract yield calculation

From percolation method, antioxidant assay and antibacterial activity were not satisfactory. Again soxhletion was used to recover the crude extract in ethanol. Since ethanol is polar and organic solvent, it could extract both polar and some non polar organic compounds. Ethanol could easily be evaporated *in vacuuo* in Rotatory vacuum evaporator.

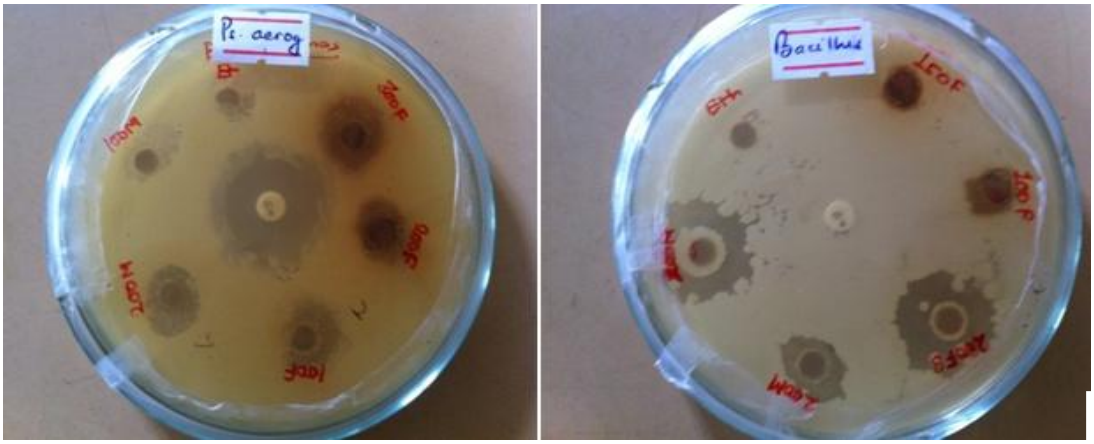
SN	Raw material	Input crude weight	Extract Yield	Yield %
1	Mature fruit	8g	0.85g	10.625%
2	Immature fruit	8g	0.98g	12.25%
3	Lawn mycelia	5g	0.55g	11%

As presented in above table, immature fruits gave the highest crude extract and mature fruits the lowest. So, immature fruits were possibly rich in metabolites due to incomplete polymerization which were readily soluble in ethanol. But mature fruits contain tough and insoluble matrix and thus lower yield than immature fruits.

4.4. Antibacterial activity

In the present research, the soxhletted ethanol extract exhibited better results. All results are average amongst three. However, inhibition zone was smaller in extract from percolation method for it being inappropriate method for extraction. Inhibition zone was nearly independent to extract's concentration applied on discs. Immature fruit extracts showed better inhibition than mature fruit extracts. Since temperature during extraction was around 70 °C, higher temperature possibly denatured some antimicrobial proteins, so results obtained might be all below the real capacity.

Raw *Ganoderma lucidum* obviously should exhibit higher activity than soxhletted extracts owing to higher temperature during extraction and incomplete solubility on particular solvent. That mushrooms possess normal antimicrobial substances for primary defense like other plants is not an exception. Moreover, Mushrooms are more prone to microbial attack since these themselves grow on debris, dead and degrading substrates, so nature has made these recyclers more potent anti-microbial than other organisms through 'Natural selection'. Resultantly, mycelia's extracts were more effective than fruit's extracts. Even highly pathogenic *Salmonella typhii* was amazingly inhibited with *Ganoderma lucidum* extracts. Clear zones were seen in *Bacillus*, *Staphylococcus* and *Pseudomonas* whilst blurred inhibition zones were seen with other probably due to their bacterio-static effect during incubation.



A B

Fig 4.5: Antibacterial effects on MHA plate incubated at 37 °C for 24 hrs *Pseudomonas aerogenosa* (A) and *Bacillus subtillis* (B) with various extracts (20 µl) from mature fruit (Fr), and ganocelium (M) at concentration 300 µg/ml, 200 and 100 µg/ml with standard drug erythromycin (ERM) and penicillin (PNC) respectively.

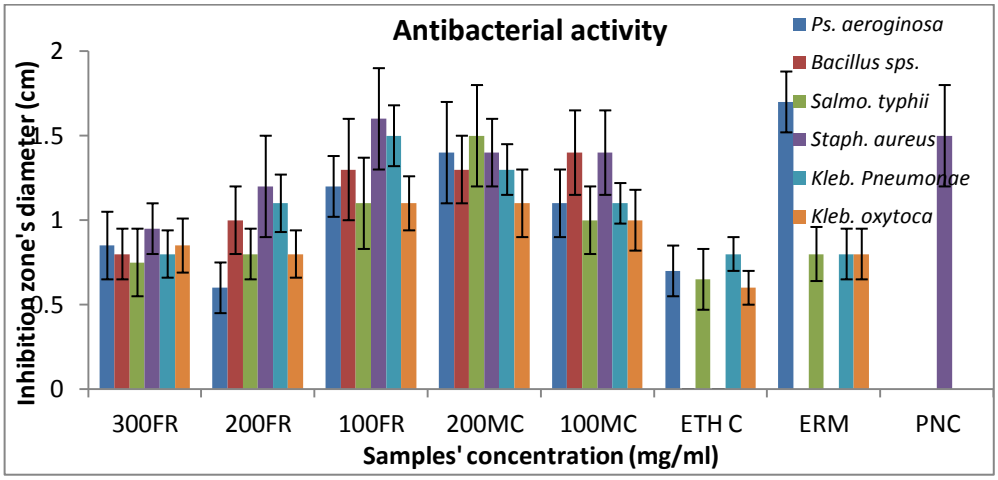


Fig 4.6: Bar graph showing inhibition zones with *Ganoderma lucidum*'s extracts on various opportunistic bacteria compared to standard antibiotics and control ethanol.

From the above bar graph, it is seen that inhibiting effect was independent to extract's concentration. 100 mg/ml fruit extract was the most effective amongst fruit extracts. But 200 mg/ml and 100 mg/ml both were effective to all bacteria. Several substances exhibit antimicrobial action like lectins, defensins, PR protein (chitinase, glucanase), lysozyme, thionins, attacin E, cercopin A, siderophore, depolymerase, temporins, esculentins, Ace-AMPs and gallerimycin. Such compounds degrade pathogen's cell wall and bio-membrane selectively (Slater *et al*., 2008). Antimicrobial compounds in mushrooms such as terpenes, lectins, polysaccharides etc act on the bacterial cytoplasmic membrane (Lin & Chou, 1984). For *Ganoderma lucidum* various extracts have been found to be equally effective when compared with Gentamycin sulphate. Cowan (1999) reported that the most active antimicrobial components are generally

water insoluble, it is expected that low polarity organic solvents would yield more active antimicrobial extracts and so ethanol is suitable to some extent. According to Gao *et al*. (2003) *Ganoderma lucidum* and other species more often in combination with chemotherapeutic agents have been used to treat various bacterial diseases. Polysaccharide components were found to be the bioactive principle which plays an important role in antibacterial activity. However, terpenoids, isoflavonoids and tannins have stronger antimicrobial effects than other compounds singly. Smania *et al.* (2007) observed methyl australate, a derivative from *Ganoderma lucidum* showing maximum antibacterial activity against *Escherischia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* while least inhibition zone was recorded for *Bacillus* species. Moreover, it appears that some constituents such as ganomycin, triterpenoids and aqueous extracts from *Ganoderma lucidum* species have a broad spectrum *in vitro* antibacterial activity against gram-positive, gram-negative bacteria and *Helicobacter pylori.* Thus, it is possible that its antibacterial activity might be beneficial for those patients with chronic infections (eg chronic bronchitis) and those with *H. pylori*-positive peptic ulcer diseases, though clinical studies are required to confirm this. Resistance to antibiotics is emerging in almost all pathogens and multiple drug resistant pathogens pose serious threats to their treatment. Hence, multidrug mushroom derived antimicrobial substances have received considerable attention in recent years for chronic diseases and against *Helicobacter pylori*.

4.5. *In vitro* antioxidant assay

Free radicals are produced in the normal natural metabolism inside aerobic cells through Fenton's reaction, mostly as reactive oxygen species (ROS) and reactive nitrogen species (RNS). Cellular antioxidants (enzymes and non-enzymatic molecules) neutralize most radicals. Maintaining equilibrium between free radicals production and cellular antioxidants is an essential condition for organism's normal functioning (Valko *et al*., 2007). Nevertheless, the equilibrium between ROS production and antioxidant defence might be displaced either due to their overproduction or due to masked neutralization process. This disequilibrium is known as oxidative stress. In this case, the excess ROS can oxidize and damage cellular lipids, proteins and DNA leading to their modification and inhibiting their normal function (Ridnour *et al*., 2005). Their beneficial effects occur at low or moderate concentrations and involve cellular physiological roles on signalization and regulation (Fang *et al*., 2002).

4.5.1. DPPH Scavenging Assay

DPPH has been used in evaluating natural antioxidants for their free radical scavenging capacity. DPPH or 1, 1- Diphenyl-2-picrylhydrazyl radical is stable radical with purple

color in alcoholic solution within visible range which has maximum absorbance at 517nm. DPPH reacts with natural antioxidants, receives Hydrogen radical gets reduced and turns into yellow colored diphenylpicryl hydrazine. Antioxidant capacity is expressed as percent scavenging capacity and their IC_{50} is calculated and compared with standard such as ascorbic acid. Lower the IC_{50} higher will be its antioxidant potential and vice versa (Jain and Jain, 2011). When DPPH reacts with an antioxidant compound it is bleached, the resulting decrease in absorbance at 517 nm is recorded at 10 min intervals up to 30 min using a UV–Vis Spectrophotometer. As it gets scavenged, electrons become paired through electron (unpaired) delocalization all over the molecule and resulting discoloration stoichiometrically coincides with electrons seized. DPPH bleaching (speed and extent) within 90 minutes is equivalent to test drug's capacity to scavenge free radicals independently.

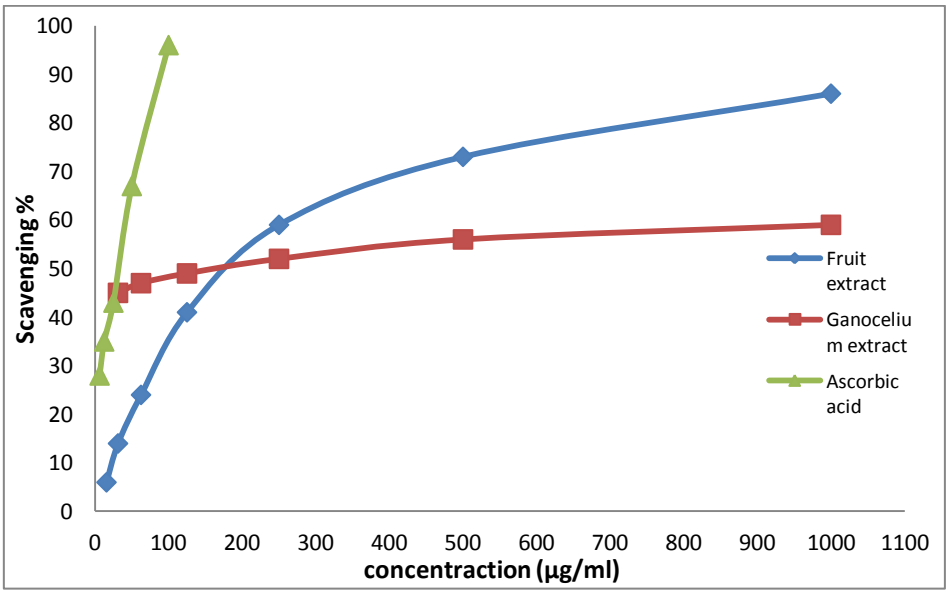


Fig 4.7: DPPH scavenging activity with *Ganoderma lucidum* extracts taking ascorbic acid as standard.

IC_{50} calculation

Using formula IC_{50} = EXP (LN (conc>50%)-((pi>50%-50)/ (pi>50%-pi<50%)*LN (conc>50%/conc<50%))) (Maes and Cos., 2010)

For ascorbic acid, IC_{50} =30.60134 µg /ml,
For *Ganoderma* Fruit IC_{50} =176.7767 µg/ml and
For *Ganoderma* Mycelia IC_{50} = 157.4901 µg/ml
From the above calculation, it is seen that ascorbic acid is far more potent antioxidant than *Ganoderma lucidum* extract having far more IC_{50} values. IC_{50} value should be lower than 250 µg/ml. *Ganoderma lucidum* carpophore showed a little higher IC_{50} value than

mycelia but it was good antioxidant value as stated above. However, *in vitro* antioxidant assay shows obviously less values than using whole plant because during extraction many antioxidant proteins, enzymes and compounds might get obviously denatured and deactivated at high temperature during soxhlet's operation. Ascorbic acid being pure compound gave high antioxidant activity. Despite its less value, *Ganoderma lucidum* extracts showing positive trend with ascorbic acid signified potent antioxidant ability as cited in research papers.

However, DPPH is not considered effective radical for antioxidant assay. First, with increase in extract concentration, absorbance decreases initially and then amazingly increases. So a parabolic curve for scavenging activity is obtained puzzling where to locate and which trend to take for antioxidant activity. Second, reduced DPPH (2, 2-diphenyl-1-picrylhydrazine i.e. DPPH-H) is major product and its nitro derivative (NO2-DPPH-H) formed with its own nitric oxide is unavoidable side product.

Barros *et al*. (2007) explained that heat used in the cooking procedure could destroy polyphenols and decrease in their antioxidant activity. Nevertheless, at low heating temperatures, an increase in phenolics concentration shall occur. Choi *et al*. (2006) observed in the dried mushrooms that heat increased the overall free polyphenolic and flavonoid compounds in *Lentinus edodes*. The authors suggested that heat treatment might produce changes in their extractability due to the cell wall disruption thus bound polyphenolic and flavonoid compounds get released more easily relative to raw materials. Another reason for the improved antioxidant activity could be novel compounds formation during heat treatment or thermal processing having antioxidant activities. Some advantages using mushrooms are that often the fruiting body can be produced in much less time even mycelium can be rapidly produced in liquid culture and the culture medium can be manipulated to overproduce desired active products.

4.5.2. Total antioxidant assay

Phospho-molybdenum method is used to evaluate total antioxidant potential. If test extract posses strong antioxidants, phospho-molybdenum (VI) in the reagent is reduced to phospho-molybdenum (V) complex which is green and has maximum absorbance at 695 nm. The graph fig 4.8 shows that ascorbic acid (positive control) was strong antioxidant among all. The antioxidant capacity has been expressed as ascorbic acid equivalent.

Absorbance records against various extract concentrations plotted on this graph showed that Ascorbic acid has higher anti-oxidant potential than *Ganoderma lucidum*'s extracts. Since ascorbic acid used was pure, its graph line was almost straight with trend-line

equation y = 0.005x – 0.058 whilst those extracts contained other dissolved constituents so their graphs were not so linear.

Due to precipitation in acidic conditions turbidity developed in higher extract concentration so particular phospho-molybdenum (V) complexes were undetectable to spectrophotometer (at 695 nm) which gave lower values and disturbed their graphs.

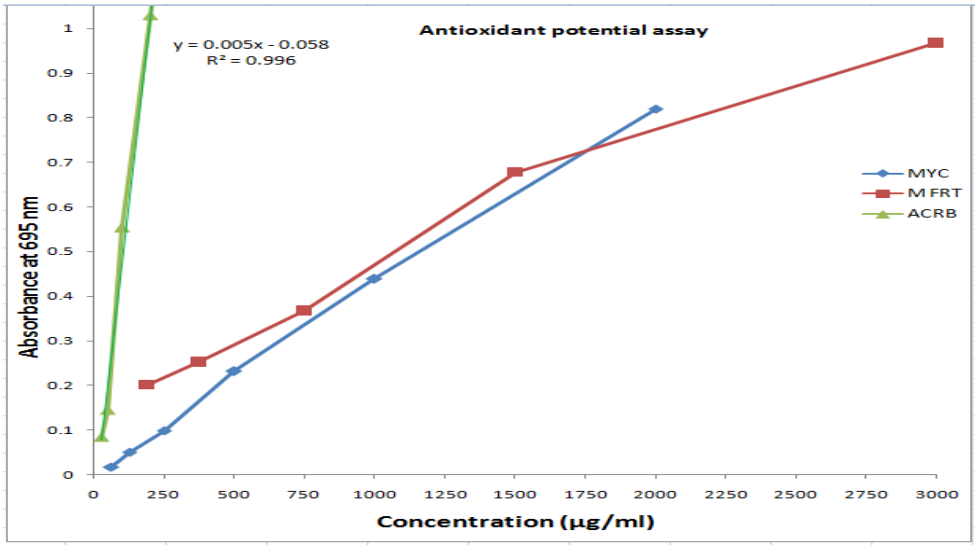


Fig 4.8: Graph for total antioxidant potential assay by Phospho-molybdenum method Myc = mycelia, MFRT = Mature Fruit and ACRB = Ascorbic acid.

Their graphs were more correlated at lower concentration with ascorbic acid's trend line than at higher concentration which gave the lowest calculated values. Both extracts showed similar trends however, fruit extract showed higher antioxidant potential (276.2667 mg ascorbic acid equivalent/g extract) than mycelial extract (240 mg ascorbic acid equivalent/g extract).

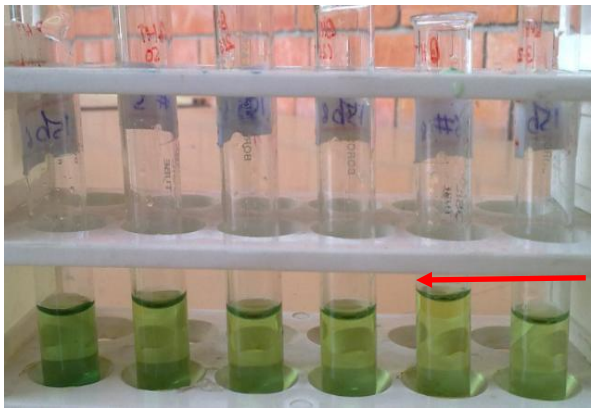


Fig 4.9: Green complexes seen in test-solution in phospho-molybdenum assay with arrow indicating increase in green color with respect to sample concentration.

Average antioxidant potential taken from five observations showed that fruit extract had 144.28 ± 81.72 mg ascorbic acid equivalent/g extract whereas mycelial extract had 150.6 ± 56.92 mg ascorbic acid equivalent/g extract.

4.5.3. Reducing power assay (FRAP)

Reducing ability transforms Fe^{3+} state to Fe^{2+} state developing green blue color with *Ganoderma lucidum* extracts at different concentration. Reducing capacity is also an antioxidant capacity since antioxidant activities arise due to various mechanisms among which are preventing chain initiation and progression, binding to transition metal ion catalysis, decomposing peroxides, preventing continued hydrogen abstraction, reducing

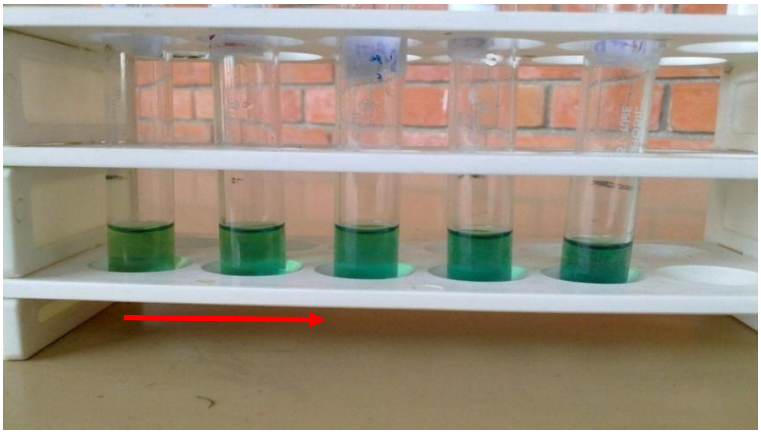


Fig 4.10: Blue green complexes seen in test-solution (Fruit) in reducing power assay with an arrow indicating increase in blue color with respect to increase in sample concentration.

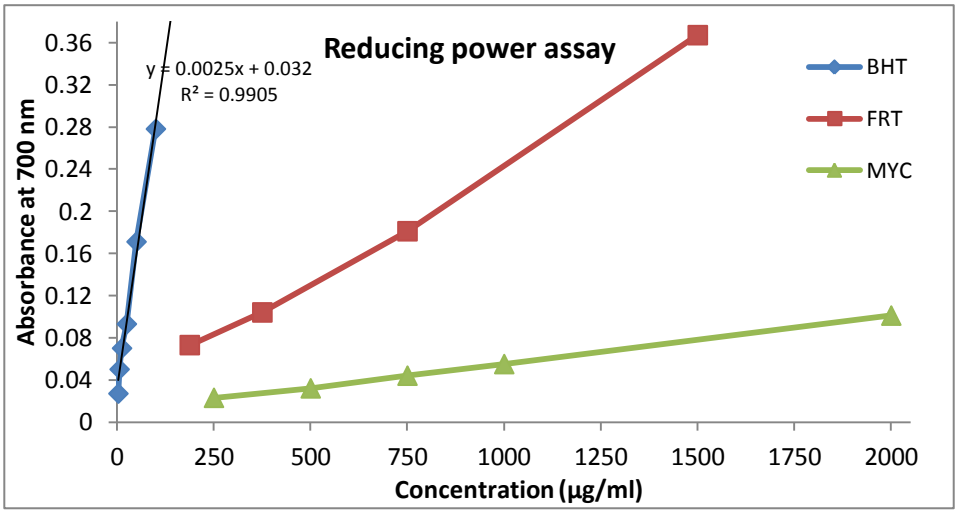


Fig 4.11: Graph illustrating reducing power of *Ganoderma lucidum* extracts compared to BHT as standard, BHT = Butylated Hydroxy Toluene, Myc = Mycelia and FRT = Fruit.

capacity and radical scavenging activity (Gulcin *et al.,* 2003). The reductants (antioxidants) in the GLEEX reduces Fe^{3+} (ferric cyanide complex) to Fe^{2+} (ferrous form) (Amarowicz *et al.,* 2004). In this research, yellow color in the test solution changed to various green and blue shades depending upon its reducing power. Reducing ability was compared with BHT as standard. Antioxidants or reductants show antioxidant potential by breaking the free radical chain and donating hydrogen atoms. Moreover, antioxidants in extracts reduced ferricyanide complex (Fe^{+3}) to Fe^{+2} Perl's Prussian blue complex. This state was measured spectrophotometrically at 700 nm (Genesys UV Vis spectrophotometer).

From standard linear trend line equation y = 0.002x + 0.032, reducing power of *Ganoderma lucidum* extracts at different concentration was calculated. As seen in graph mycelia's extract had far lower reducing power value than its fruit's extract did. Moreover, values such as -4 and 0 mg eqv BHT/g extract were even seen for mycelia's extract at 250 µg/ml and 500 µg/ml respectively. Taking them as technical error of standard trend line's equation, only appropriate values were considered that gave average reducing power value 13.58333 ± 3.185252 mg eqv BHT/g and maximum only 17.25 mg eqv BHT/g at 2 mg/ml mycelial extract. Similarly, fruit extract had higher values as seen in graph. Average reducing power value was 104.0833 ± 7.5932 mg eqv BHT/g with maximum value 111.67 mg eqv BHT/g at 1.5 mg/ml fruit extract.

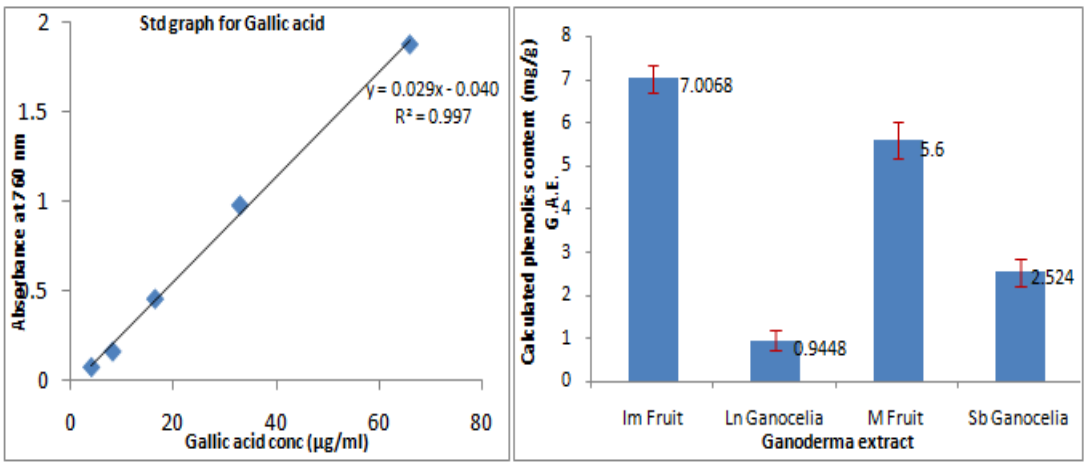
Besides all mentioned endogenous defences, antioxidant supplements or antioxidant containing foods shall be used to help the organism to reduce oxidative damage or to protect food quality. In recent years restricted synthetic antioxidants such as BHA (2-*tert*-butyl 4-methoxyphenol) and BHT (2, 6-di-*tert* butyl 4-methylphenol) have caused an increased interest towards natural antioxidant substances. Natural antioxidants are being extensively studied for their capacity to protect organisms and cells from oxidative stresses. Natural antioxidants have already been isolated from different plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs. Epidemiological studies have consistently shown that fruits and vegetables intake is strongly associated with reduced chronic diseases, such as cancer and cardiovascular disease (Soobrattee *et al*., 2005). In fact, oxidative and nitrosative stresses in the etiology for several acute and chronic clinical disorders have led to the suggestion that antioxidants can have health benefits as prophylactic agents. This suggests that changes in dietary behavior and consuming plant-based foods, containing significant bioactive phytochemicals, shall provide desirable health benefits beyond basic nutrition to reduce the risk on chronic diseases (Liu, 2003 and 2004).

4.6. Antioxidant quantification

4.6.1. Phenolics quantification

Using equation y = 0.029x – 0.040 that represented the standard graph for Gallic acid, phenolics content was quantified. Immature fruit was found to contain 7.0068 mg phenolics equivalent to Gallic acid per gram dry biomass which was much higher than mature fruit 5.6 mg per gram biomass. Similarly liquid cultured mycelia contained 2.524 mg phenolics equivalent to Gallic acid per gram biomass which was higher than lawn mycelia 0.9448 mg per gram dry biomass.

A



B

Fig 4.12: A: Standard trend line graph for Gallic acid absorbance at 765 nm vs concentrations and B: bar graph showing Phenolics content in extracts calculated compared with standard trend line equation.

Helvella crispa from India revealed the highest phenolics content expressed as per g extract (34.65 mg/g), while *Sparassis crispa* from Korea revealed the highest value expressed in a dry weight basis (0.76 mg/g extract). In fact, fruiting bodies in a mature stage with mature spores revealed low antioxidant values such as phenolics content, ascorbic acid and β-carotene. This was explained with involving those compounds in defense mechanisms inherent to the aging process resulting in the lowering their contents in the most advanced stage.

Owing to structural diversity, isomerism, cumbersome chromatographic separation techniques and inadequate commercial standards, phenolics quantification has been done according to Folin- Ciocalteau's method despite some interference in this assay. Phosphotungstic acid and phosphomolybdic acid in Folin-Ciocalteau's reagent also react with other non-phenolic reducing compounds leading to their overvaluation. For instance, ascorbic acid is a widespread reducing agent that could interfere in the Folin-

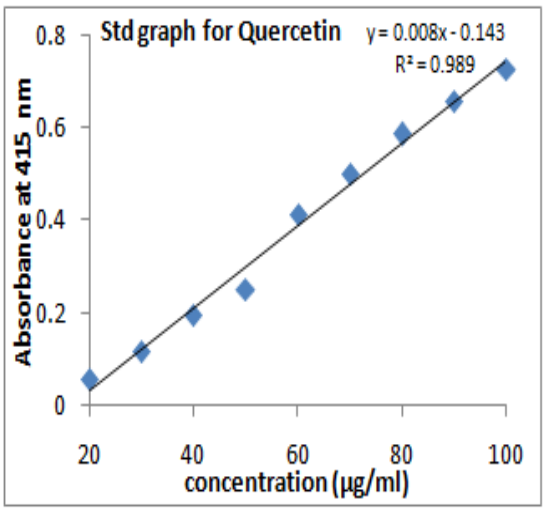
Ciocalteu's reaction. Other reducing substances such as some sugars and amino acids could also interfere.

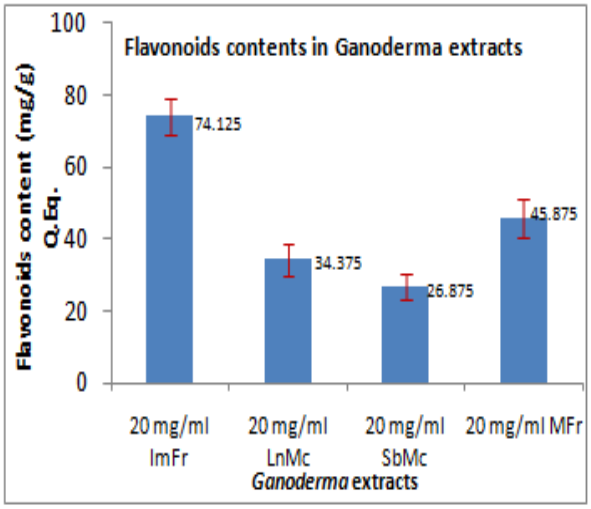
Phenolic compounds, most potent and therapeutically useful bioactive substances are aromatic hydroxylated compounds possessing one or more aromatic rings with one or more hydroxyl groups. Natural phenolic compounds accumulate as end-products from the shikimate and acetate pathways and can range from relatively simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins), with flavonoids representing the most common and widely distributed sub-group including different subclasses: flavonoids, phenolic acids, stilbenes, lignans, tannins and oxidized polyphenols. *Ganoderma lucidum* has been reported to contain protocatechic acid, gallic acid, 5-sulphosalicylic acid, quercetin and kaempferol (Kim *et al.*, 2008). Their overall effectiveness depends on the phenolic hydrogen involved in radical reactions, the radical's stability formed during radical chain reactions and the chemical substituent present on the structure. The substituents on the structure are probably the most significant with respect to the natural antioxidant's ability to control radical reactions and to form resonance-stabilized natural antioxidant radicals. Phenolic antioxidants (ArOH) interrupt the chain reaction according to: $RO^{\bullet}_2$ + ArOH $\longrightarrow$ ROOH + $ArO^{\bullet}$. To be effective $ArO^{\bullet}$ must be a relatively stable free radical, so that it reacts slowly with substrate RH but rapidly with $RO^{\bullet}_2$, hence the term "chain-breaking antioxidant. Like other phenolic compounds, phenolic acid's antioxidant activity is due to the phenolic hydrogens. Hydroxyl substitutions at ortho and para positions will enhance antioxidant activity. Intramolecular hydrogen bonds are formed in ortho substituted phenols during radical reactions, which impart stability to the phenoxy radical. Tri-hydroxybenzoic acid (gallic acid) has higher antioxidant activity than 3, 4-dihydroxybenzoic acid (protocatechuic acid) due to three hydroxyl groups in trihydroxybenzoic acid. And more stable phenoxy radical is formed through intramolecular hydrogen bond so it is used as standard in Folin-Ciocalteau's method.

4.6.2. Flavonoids quantification

Using the equation y = 0.008x – 0.143 that represented the standard trend line for Quercetin, flavonoids content in each percolated sample extract was calculated. Using this standard graph, lawn mycelia extract solution contained 34.375 mg flavonoids equivalent to Quercetin per gram biomass. Similarly, mature fruit extract gave 45.875 mg, immature fruit extract contained 74.125 mg and submerged mycelia extract contained 26.875 mg flavonoids equivalent to Quercetin per gram biomass.

A





B

Fig 4.13: A: Standard trend line graph for Quercetin absorbance vs concentration and B: Bargraph showing flavonoids conc^{n} in extracts calculated with trend line equation.

But in general, it was assumed that only plants possess the biosynthetic ability to produce flavonoids and not animals and fungi. Recently, Barros *et al*. (2007) reported that no flavonoids were detected in sixteen Portuguese wild mushrooms. However, Kamra & Bhatt (2012) and Rajasekaran & Kalaimagal (2011) have reported and quantified flavonoids in *Ganoderma lucidum*.

Flavonoids are most frequently found in nature as conjugates in glycosylated or esterified forms but can also occur in food as aglycones. Flavonols are the most abundant flavonoids in foods. Multiple mechanisms have been identified as involved in the health-promoting effects including anti-oxidant, anti-inflammatory and anti-proliferative activities. Similarly, inhibiting bio-activating enzymes or inducing detoxifying enzymes regards to their protective effect against cardiovascular diseases and scavenge oxidizing molecules singlet oxygen and various free radicals. Phenolics and flavonoids are non-nutritive compounds but have roles in donating hydrogen and quenching singlet oxygen thus vital role in the food products stability. Flavonoids have been shown to exhibit their actions through effects on membrane permeability, and membrane-bound enzymes inhibition such as the ATPase and phospholipase A2 (Li *et al*., 2003) and this properties explain the anti-oxidative mechanisms consuming *Ganoderma lucidum*. Flavonoids serve as health promoting compound as a results of its anion radicals (Wanda *et al.,* 2010).

4.7. Toxicity analysis

4.7.1. Heavy metal analysis

Heavy metals might have accumulated in *Ganoderma lucidum* mushroom through soil, organic substrates, water used in irrigation and atmosphere over storage. Mushroom products might have contaminated with heavy metals also due to negligence in processing, leaded petrol used in industrial area and polluted urban atmosphere. Heavy metals were screened in Reishi products sold in Nepal to detect their level and to aware public about their safety for consumption. Lead and cadmium were measured in conc. Nitric Acid digested each gram samples through Atomic Absorption Spectrometry in Nepal Standards and Metrology Bureau, Balaju, Kathmandu. In AAS the sample is atomized at very high temperature 3000 °C and line spectrum is emitted. Light intensity that free electrons absorb to be excited from ground state to higher energy level is measured (Varian, 2004). Lead as Pb and Cadmium as Cd were found inversely i.e. higher lead (Pb) concentration was associated with lower cadmium (Cd) concentration.

For instance, if a person consumes various Reishi products A/C physician's prescription,
2 LG capsules b.i.d. + 2 GL capsules b.i.d. + 2 cups Gano-coffee per day
= 2*2*270 mg +2*2*450 mg + 1 g =1.08 g + 1.8 g + 1 g ≈ 4 g per day i.e. 28 g at most per week.
Considering he consumes products with maximum lead concentration 9 µg/g (9 ppm), total lead consumed per week = 28*9 = 252 µg = 0.252 mg per week which is 1.75 mg/0.252 mg = 7 times less than Codex Alimentarius Commission (1993). Similarly, considering he consumes products with maximum cadmium concentration 0.65 µg/g (ppm), total cadmium consumed per week = 28*0.65 = 18.2 µg per week which is 490 µg/18.2 µg = 27 times less than Codex Alimentarius Commission (1993).

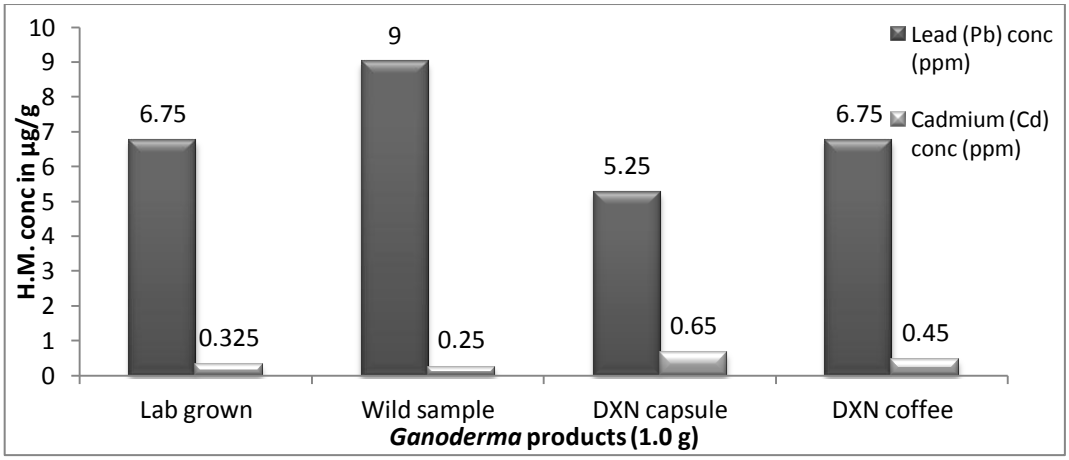


Fig 4.14: AAS quantified lead (Pb) and cadmium (Cd) levels in *Ganoderma lucidum* samples.

Most heavy metals pose threats to consumers in two ways. On one hand, their sudden and large single dose causes immediate metabolic anomalies and poisoning. On the other hand, their chronic intake is major concern for toxicologists being cumulative poison. So their measurement in popular reishi products is contextual to declare their safety or awareness about their toxicity.

According to Codex Alimentarius Commission (2010), Lead has comparatively high affinity for proteins. Lead ions consumed bond with the hemoglobin and the plasma proteins. This leads to inhibited red blood cell physiology and thus vital oxygen transport. If the bonding capacity to them is exceeded, lead passes into the bone-marrow, liver and kidneys. Particularly dangerous to all are the organic lead compounds that cause injuries to mental development such as reduction in intelligence, growth disturbances and spasticity. Children are particularly at risk from lead consumption before and after birth as they absorb lead more rapidly than adults. Such intoxication leads to- i: Encephalopathies in the central nervous system, (CNS), ii: Disturbances in kidney and liver functions resulting in necrosis, iii: Damage to the reproductive organs and iv: Anaemias and many metabolic deficiency symptoms. According to Codex Alimentarius Commission (1993), Lead uptake limit is 1.75 mg per week per 70 kg person.

Codex Alimentarius Commission (2010) stated that Cadmium is concentrated particularly in the kidneys, the liver, the blood forming organs and lungs and results in kidney damage (due to necrotic protein precipitation) and metabolic anomalies through enzyme inhibitions. *Itai-itai* sickness in Japan (with bone damage) was due to the cadmium contaminated rice. Cadmium unlike lead is a cumulative poison i.e. the danger lies primarily on consuming foodstuffs with low contamination regularly. However, in contrast to lead, exact toxicity limit is not possible for cadmium. The decisive point is whether the existing cadmium absorption actually takes place. This is, firstly, dependent upon the diet composition as a whole and secondly, on its compound's bio-availability. According to Codex Alimentarius Commission (1993) Cadmium uptake limit is 490 µg per week per 70 kg person. No connection with cancerous disorders has been found.

From AAS values it is seen that all products are below the maximum guideline limit. From above calculation, it can be recommended that all Reishi edible products sold in Nepal were safe for consumption because toxic heavy metals: lead and cadmium were 7 times and 27 times below Codex Alimentarius Commission declared in 1993. Standard sampling, frequent monitoring their presence and their radioactivity level is required to declare them safe. Radioactive metals are carcinogenic, deleterious for human reproductive physiology and responsible for defective births. Thus, this thesis should

draw their attention towards SOP, GMP, ISO 9001- 2000 and HACCP about using the best substrates, best methods, best location and best downstream processing.

4.7.2. Brine shrimp lethality bioassay

Brine shrimp lethality test is performed before advancing towards other tests like drug function tests in pharmacology. This assay strongly correlates with cytotoxicity and anti-tumor activity in cancer cell lines despite it being a systemic toxicity or lethality test. The brine shrimp lethality test (Meyer *et al.*, 1982) can be used as a simple tool to guide screening and fractionating physiologically active plant extracts, where the simplest biological response to monitor bioactivity is lethality with only one criterion: either dead or alive. One basic premise here is that toxicology is equivalent to pharmacology at a higher dose, thus as we find toxic compounds, a lower, non-toxic dose might elicit a pharmacological perturbation on a physiologic system. Many reports are there on using this animal model for environmental studies, screening for natural toxins and as a general screening for bioactive substances in plant extracts.

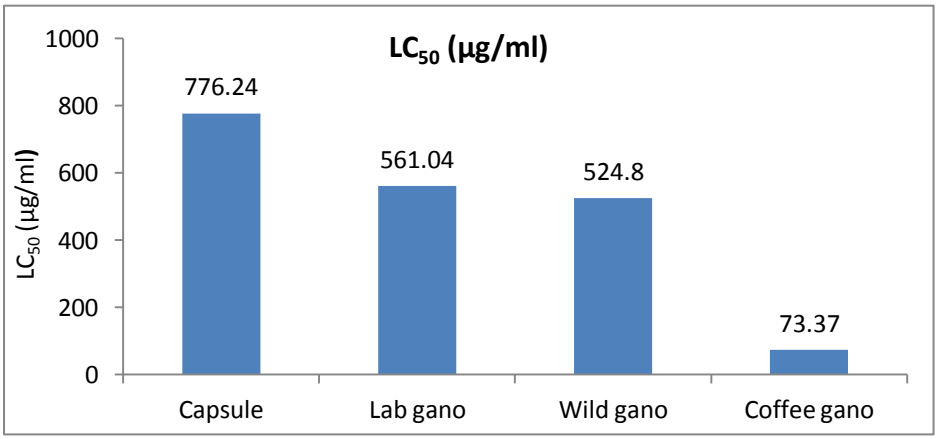


Fig 4.15: Bar Graph showing LC_{50} calculation

Those tested shrimps that escaped death were also obviously at risk i.e. oscillating between death and life at the moment. Crude alcoholic percolated extract were used in this research. Seeing comparatively higher LC_{50} values, one might conclude that these extracts were less bioactive and pharmacologically less potent. However, this is not the case here. That pure, isolated and mixed bioactive compounds gives lower LC_{50} value is universal. However, ethanol dissolves polar organic and some inorganic constituents, so crude alcoholic extract contained bioactive as well as non bioactive compounds. Thus crude extracts giving LC_{50} value something above 1000 ppm shall also be acceptable bioactive mixture pharmacologically. Coffee gano seemed most pharmaco-potent amongst 4 samples. This might be due to mixed bioactive compounds from *Ganoderma lucidum* and strong bioactive compounds from coffee seeds. Lab grown *Ganoderma*

lucidum and wild sample showed nearly equivalent LC_{50} values. DXN capsules gave lowest values. This might be due to industrial cleverness in adulterating edible substances in capsules, mixing hard residual biomass powder, chemically altering insoluble matrix to soluble substances and mixing them in capsules or extracting in multiple solvents.

Moreover, the extract produced concentration dependent increment in percent mortality. Lethality rate was found to be directly proportional to the concentration of the extract. In addition, positive result in cytotoxic activity test led us to the inference that the plant extract may contain bioactive compounds which may aid ongoing anticancer drug discovery from floristic resources.

Cumulative toxicity in crude extracts arises due to heavy metals, xenobiotics and potent bioactive constituents. So it cannot be declared any pronounced relationship between heavy metal content and brine shrimp toxicity assay i.e. heavy metal content is not the sole cause for brine shrimp's lethality even though unexpected inverse parallelism was seen in respective heavy metal contents and LC_{50} values.

LC_{50} is minimum drug or toxin's dose that kills 50% tested population. LC_{50} is the boundary line red signal for possible lethality. For a compound (formulation) to be biologically active, LC_{50} value should be below 1000 ppm. For example, considering paracetamol's LC_{50} value 100 ppm, this means if 100 animals each weighing 1 kg consumes 100 mg paracetamol, 50% will possibly die. So its daily dose should be far below the LC_{50} value. This explanation applies to human as well. LC_{50} value gives next significance too. A substance with high LC_{50} value is required to formulate in unusually high amount in its case or bottle which might be economically expensive and pose a nuisance handling for consumers or patients.

Summary

Exploring the scientific basis among known cures used in folk medicines has resulted in purifying potential natural products that could combat many diseases, particularly cancer. So the natural and organic products are being increasingly demanded worldwide. *Ganoderma lucidum* being the third potent mushroom supplement leaving *Ophiocordyceps sinensis* and *Antrodia camphorata* on the top, its cultivation feasibility, medicinal values and toxicity were evaluated as much possible as a dissertation in Master in Biotechnology within some financial and time constraint. This research made a strong background to establish a baseline on *Ganoderma lucidum* based biotech industry in Nepal because its environment, situation and neighbouring and international industrial context are favourable here for its production and export. Since *Ophiocordyceps sinensis* and *Antrodia camphorata* are hard to culture as compared to *Ganoderma lucidum*, this research will be contextual and judicious enough to build industrial foundation.

Ganoderma lucidum strain Philippinnes or *G. multipileum* was brought from 'Centre for Agricultural Technology and Training' (CATT), Lalitpur and cultured in PDA slants and plates. *Ganoderma lucidum* was cropped in solid substrates, cultured in liquid medium and PDA semisolid media to harvest its biomass. Growth parameters were optimized in laboratory in semisolid media subsequently with respect to carbon source, pH and temperature. The harvested biomass was dried, weighed, ground and extracted in ethanol using Soxhlet's extraction system at 65˚C. Suspension was filtered and filtrate solution was dried *in vacuuo* in Rotary Vacuum Evaporator in Chemistry Department. Almost 11% extract yield was dissolved in ethanol to use in Antimicrobial activity by disc diffusion method, Antioxidant activity by DPPH method, Reducing power (FRAP) assays, Total antioxidant potential by phospho-molybdenum method and Phenolics estimation by Folin- ciocalteaue's method and Flavonoids estimation. Worldwide popular reishi products were sampled from market in order to evaluate their toxicity or edibility with regard to heavy metal contamination and brine shrimp lethality assay. For Heavy metal quantification the samples were dried and weighed 1 g and digested in concentrated nitric acid overnight and boiled to evaporate its fumes in sand's furnace on the following day. Filtered samples were assessed for lead and cadmium concentration in national bureau of standards and metrology. For brine shrimp lethality bioassay crude extracts were dissolved in DMSO and shrimp's eggs were made to hatch in artificial sea water overnight and larvae were pippetted to crude extract containing solution and incubated for 24 hours and live shrimps were counted.

Ganoderma lucidum was cultured successfully in solid substrates, liquid and PDA media. As per optimization, maximum growth rate as lengthwise mycelial spread was almost 9.8 mm per day in

PDA. Maximum growth was seen at temperature 30°C and pH 5 ±0.25 with sorbose and trehalose. *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* were most susceptible with all extracts almost in all concentration and *Salmonella typhii* showed striking record with prominent inhibition zone. Ganocelia was potent in antioxidant activity with IC_{50} 157.49 µg/ml and Fruit 176.77 µg/ml compared to standard ascorbic acid (30.60 µg/ml). Moreover, total antioxidant potential for carpophore extract calculated by Phosphomolybdenum method showed 144.28 ± 81.72 mg ascorbic acid equivalent/g extract whereas mycelial extract had 150.6 ± 56.92 mg ascorbic acid equivalent/g extract. Reducing power activity by FRAP method showed that average reducing power value was 104.08 ± 7.59 mg eqv BHT/g carpophore extract and average reducing power value 13.58 ± 3.18 mg eqv BHT/g mycelial extract. Immature fruit was found to contain 70.068 mg phenolics equivalent to Gallic acid per gram dry biomass which was higher than mature fruit 56 mg eqv GA per gram biomass. Similarly liquid cultured mycelia contained 25.24 mg phenolics equivalent to Gallic acid per gram biomass compared to lawn mycelia only 9.448 mg per gram dry biomass. Lawn mycelia extract contained 34.375 mg flavonoids equivalent to Quercetin per gram biomass. Similarly, mature fruit extract gave 45.875 mg, immature fruit extract contained 74.125 mg and submerged mycelia extract contained 26.875 mg flavonoids equivalent to Quercetin per gram biomass. Heavy metal quantification by AAS (Atomic Absorption Spectroscopy) on four crude products viz; capsules, coffee, lab grown chips and wild Reishi powder commonly sold in Nepal revealed toxic heavy metals: lead and cadmium 7 times and 27 times far below Codex Alimentarius Commission (1993). Brine shrimp bioassay performed on above four crude items revealed them pharmaco-potent (cytotoxic) with LC_{50} around 500 mg/L highly cytotoxic being coffee with LC_{50} 73.37 µg/ml and pharmacologically low potent being capsules with LC_{50} 776.24 µg/ml (ppm).

Due to economic and time constraints, neither molecular profiling could be done nor could organic germanium be estimated on wild and cultivated native *Ganoderma lucidum*. Nevertheless, bioactivities and safety study performed are of immense value. However, total medicinal analysis was not possible due to scarcity in laboratorial sophistication, economic and time constraints. This research has opened a door to establish a bio-industry to process and produce *Ganoderma lucidum*, world's highly demanded medicinal mushroom in Nepal with versatile economic and health opportunities. Secondly, this thesis possibly guides producers and consumers towards safe and potent herbal and medicinal supplements rather than towards brand.

Assurance

Worldwide accepted principles like HACCP, GMP, ISO, SOP and WHO standards for foods, supplements and medicines are mandatory. Since *Ganoderma lucidum* has been found medicinally active and nutritionally safe for human consumption, all people are advised to use its products as prescribed without hesitation.

Conclusion

This thesis was honestly conducted with scientific methods and worldwide accepted principles. Data presented hereunder are also similar to the researches done worldwide in *Ganoderma lucidum.* Wild and bred *Ganoderma lucidum* could be successfully cultured in laboratory in suitable parameters such as temperature: 30 $^{\circ}$C, pH: 5 ± 0.25 and carbon sources: sorbose, trehalose and glucose. *Ganoderma lucidum* showed various medicinal bioactivities clearly *in vitro* and antioxidants potential and their quantification could be measured effectively. Though a trustable validity on its products has not been approved in Nepal, this thesis provided a glimpse about real potency and toxicity regarding its commercial advertisements and suspects. Products and crude samples collected in Nepal were safe with respect to Heavy metal content and effective pharmacologically.

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Appendix I

A. Solid substrate formulation

SN	Ingredients	Amount
1	Rice bran	800g (4%)
2	Wheat bran	800g (4%)
3	Sugar	200g (1%)
4	$CaCO_3$	200g (1%)
5	Saw dust	18000g (90%)
6	Moisture	~65%

B. Moisture content management

Suppose a kg saw dust, b kg wheat bran and c kg rice bran were kept in hot air chamber at 60°C for 24 hrs and weighed p, q and r kg respectively. Total water required was 65% of the final wet media. Suppose total x kg dry media was to be formulated excluding water.

Now,

Calculated required saw dust = 90% of x = 90x/100

Calculated required wheat bran = 4% of x = 4x/100

Calculated required rice bran = 4% of x = 4x/100

Water percent in saw dust = (a – p)*100%/a

Water content in saw dust = 90 x (a-p)/100a kg

Dry saw dust weight = 90x/100 - 90 x (a-p)/100a kg =90xp/100a

Required saw dust weight in media = 90ax/100p kg

Similarly,

Water percent in wheat bran = (b – q)*100%/ b

Water content in wheat bran = 4x (b – q)/100b kg

Dry wheat bran weight = 4x/100 - 4x (b – q)/100b kg = 4qx/100b

Required wheat bran weight in media = 4bx/100q kg

Similarly,

Water percent in rice bran = (c – r)*100%/c

Water content in rice bran = 4x (c – r)/100c kg

Dry rice bran weight = 4x/100 - 4x (c – r)/100c kg = 4rx/100c

Required rice bran weight in media = 4cx/100r kg

Finally, required water was 65 % in the media i.e. x kg dry media + 65% of total wet media = z kg

Water content = 65% of z kg

But 65 % of z kg = water content in (saw dust + wheat bran + rice bran) + some water to be added

Water to be added = 65 % of z kg – water content in (saw dust + wheat bran + rice bran)

= **[65x/ 35 – {90 x (a-p)/100a + 4x (b – q)/100b + 4x (c – r)/100c}] kg**

NB: 1 litre water at 25 °C = 997 g at 25°C and 998 g at 20°C

C. Mueller Hilnton Agar's composition (HIMEDIA)

Ingredients	**amount (g/l)**
Beef Extract.	2.0 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g
Agar	17.0 g

D. Luria Bertani's Broth composition (HIMEDIA)

Ingredients	**Gms / Litre**
Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	10.000
Agar	15.000
Final pH (at 25°C)	7.5±0.2

E. MEA media composition (HIMEDIA)

Malt extract = 1.2 %
Glucose = 1 %
Agar = 2 %

F. Mc Farland's standard preparation

0.5 McFarland standard is prepared by mixing 0.05 mL barium chloride dihydrate (1.175%) ($BaCl_2 \bullet 2H_2O$), with 9.95 mL sulfuric acid (1%) (H_2SO_4). Sometimes, there are McFarland standards prepared from latex particles, which stabilizes suspensions, their shelf life and stability. The standard can be compared visually to a suspension of bacteria in sterile saline or nutrient broth.

McFarland Nephelometer Standards:

McFarland Standard No.	0.5	1	2	3	4
1.0% Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1.0% Sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1X10^8 CFU/mL)	1.5	3.0	6.0	9.0	12.0
% Transmittance*	74.3	55.6	35.6	26.4	21.5
Absorbance*	0.132	0.257	0.451	0.582	0.669

*at wavelength 600 nm

G. DPPH standard solution preparation

Molecular weight = 394.3 amu

DPPH to be used =0.2 millimolar = 0.0002 M

393.3 g in 1000 ml ethanol makes it 1M

393.3/10 g in 1000/10 = 100 ml ethanol makes it 1 M

39.33 g * 0.0002 in 100 ml ethanol makes it 0.0002 M i.e. 0.007886 g = 7.886 mg

So nearly 8 mg DPPH was weighed and completely dissolved in 100 ml ethanol to use it freshly for that or the following day only.

H. Media formulation for submerged culture

SN	Ingredients	Amount (g/l)
1	$MgSO_4.7H_2O$	0.5 g
2	Thiamine hydrochloride	0.1 mg
3	KH_2PO_4	0.5 g
4	K_2HPO_4	0.6 g
5	$CuSO_4.5H_2O$	0.4 mg
6	$FeCl_3$	1 mg
7	Asparagine monohydrate	Variable
8	Lactose	10g
9	$MnCl_2.4H2O$	0.09 mg
10	Sucrose	10g
11	Glucose,	10g
12	Crystalline cellulose	10g
13	H_3BO_3	0.07 mg
14	$Na_2MoO_4.H_2O$	0.02 mg
15	$ZnCl_2$	3.5 mg

I. Phosphate buffer (0.2 M, pH 6.6) preparation

Molecular wt of $Na_2HPO_4.7H_2O$ =268.05 a.m.u. Molecular wt of $NaH_2PO_4.2H_2O$ =156 a.m.u. So, 1000 ml 1 M $NaH_2PO_4.2H_2O$, requires its 268.05 g

100 0.2 M Na_2HPO_4 requires its 268.05 / (5*10) = 5.361 g

Similarly, 1000 ml 1 M $NaH_2PO_4.2H_2O$ requires its 156 g

100 ml 0.2 M $NaH_2PO_4.2H_2O$ requires its 156/ (10*5) g = 3.12 g

Now, 62.5 ml $NaH_2PO_4.2H_2O$ sol^n and 37.5 ml $Na_2HPO_4.7H_2O$ sol^n were mixed well in another 0.2 M, P^H 6.6 labeled reagent bottle, P^H was maintained 6.6 with Ortho phosphoric acid and used in reducing power assay.

J. Phosphomolybdenum reagent preparation

Reagent composition = 0.6 M Sulphuric Acid, 28 mM Sodium Phosphate and 4 mM Ammonium Molybdate

For conc. Sulphuric Acid, density = 1.84 g/ml

Assay = 0.98

Density 1.84 g/ml = 1.84 g Sulphuric Acid in 1 ml = 1840 g in 1000 ml = 1840*0.98 g in 1000 ml = 1803.2 g/98.08 M = 18.3849918 M {Molarity = weight of compound/ molecular weight}

Now, for 100 ml phospho-molybdenum reagent,

x = (100 x 0.6) /18.3849918 = 5.4377379 ml = 5437.7379 µl

For Sodium Phosphate,

Molarity = 380.13 g/l, So

1 M = 1000 millimolar contains 380.13 g

100 millimolar contains mg 38.013 x 100/98 = 38.7887755 mg Sodium Phosphate in 100 ml.

x = 2.8/0.1 = 28 ml

Similarly, for 4 millimolar Ammonium molybdate

Molarity = 1235.9 g/ l,

1000 millimolar contains 1235.9 g in 1000 ml = 123.59 g in 100 ml

50 millimolars contains 0.12359*50 g in 100 ml = 6.1795 g in 100 ml

x = 8 ml

Finally, 5437.7379 µl conc. Sulphuric Acid, 28 ml Sodium Phosphate (100 mM) and 8 ml Ammonium molybdate (50 mM) were mixed together to make up 100 ml with distilled water.

K. Calculation for Heavy metal estimation

Suppose Lead conc^{n} on *Ganoderma* wild sample = 0.36 mg/l

Since its 1 g was dissolved to 25 ml Nitric acid sol^{n},

0.36 mg/l = 0.36 mg dissolved in 1000 ml

= 0.36 x 25/1000 in 25 ml sol^{n} = 0.009 mg in 25 ml sol^{n}

= 0.009 mg in 1 g dried *Ganoderma* wild sample

So, 1 g dried *Ganoderma* wild sample contains 0.009 mg Lead (Pb)

1000 g " " " " 0.009 x 1000 mg Lead (Pb)

= 9 mg Lead (Pb)

i.e. 9 mg per 1000 g = 9 ppm Lead (Pb)

L. Media formulation for Brine shrimp Bioassay

SN	Composition	Amount(g/l)
1	NaCl	23.50
2	Na_2SO_4	4.00
3	KCl	0.68
4	H_3BO_3	0.027
5	$MgCl_2.2H_2O$	10.68
6	$CaCl_2.2H_2O$	1.48
7	$NaHCO_3$	0.197
8	Na_2EDTA	0.0003

M. FC reagent composition

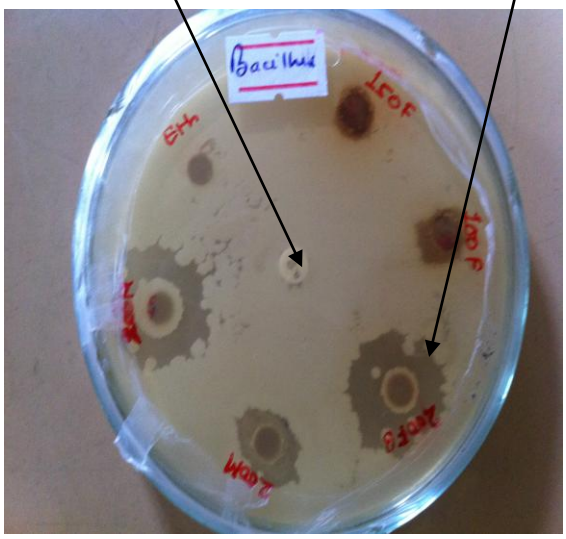
First sodium tungstate ($Na_2WO_4.2H_2O$, 100 g), sodium molybdate ($Na_2MoO_4.2H_2O$, 25 g), concentrated hydrochloric acid (100 mL), 85% phosphoric acid (50 mL) and water (700 mL) was boiled (for 10 h). After boiling, lithium sulfate ($Li_2SO_4.4H_2O$, 150 g) is added to the mixture to give an intense yellow solution, the FC reagent

.

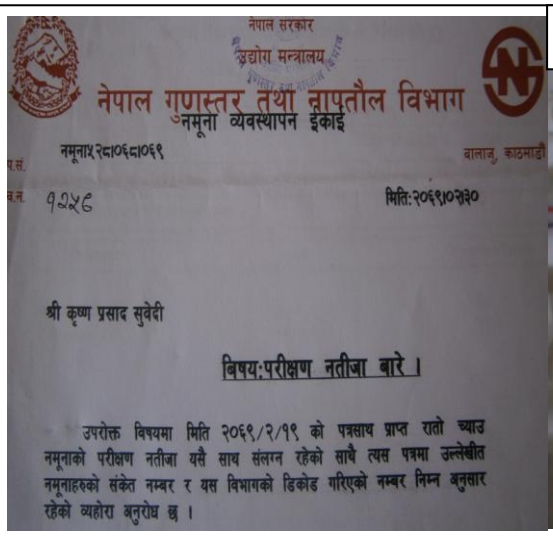
Appendix II

Photographs

Ganoderma lucidum spreading on PDA plate

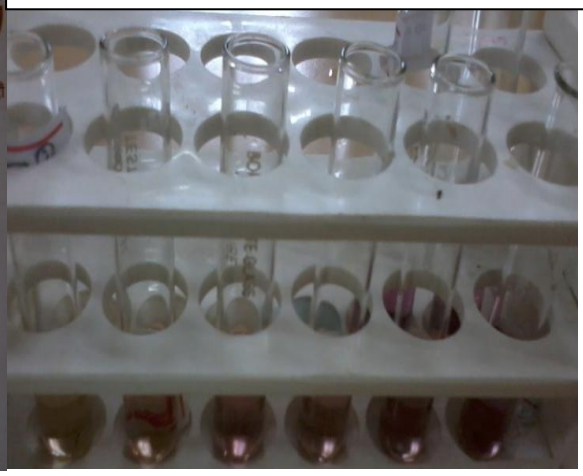


Antibacterial test on *B. subtiliss*: Disc diffusion method

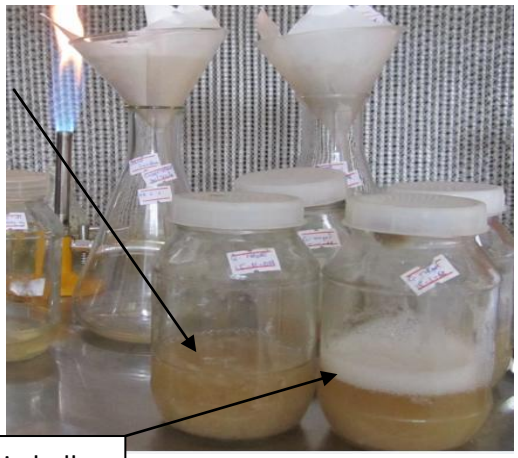


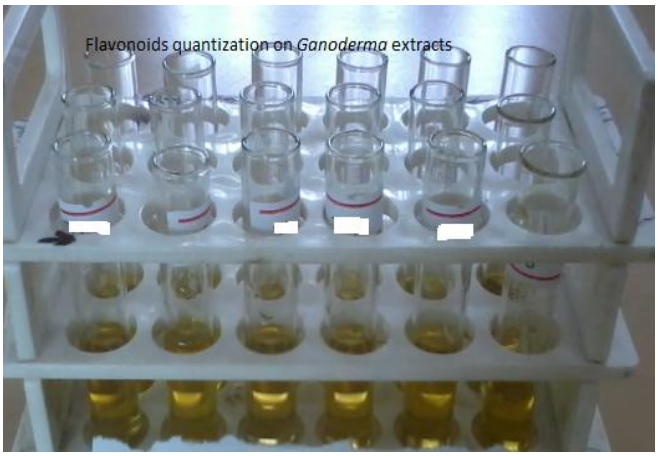
नेपाल सरकार
उद्योग मन्त्रालय
नेपाल गुणस्तर तथा नापतौल विभाग
नमूना व्यवस्थापन इकाई
नमूना२८०६८०६९
बालाजु, काठमाडौं
९२५६
मिति:२०६९।०२।३०
श्री कृष्ण प्रसाद सुवेदी
विषय:परीक्षण नतीजा बारे ।
उपरोक्त विषयमा मिति २०६९/२/१९ को पत्रसाथ प्राप्त रातो च्याउ नमूनाको परीक्षण नतीजा यसै साथ संलग्न रहेको साथै त्यस पत्रमा उल्लेखीत नमूनाहरुको संकेत नम्बर र यस विभागको डिकोड गरिएको नम्बर निम्न अनुसार रहेको व्यहोरा अनुरोध छ ।

DPPH scavenging assay on extracts



control



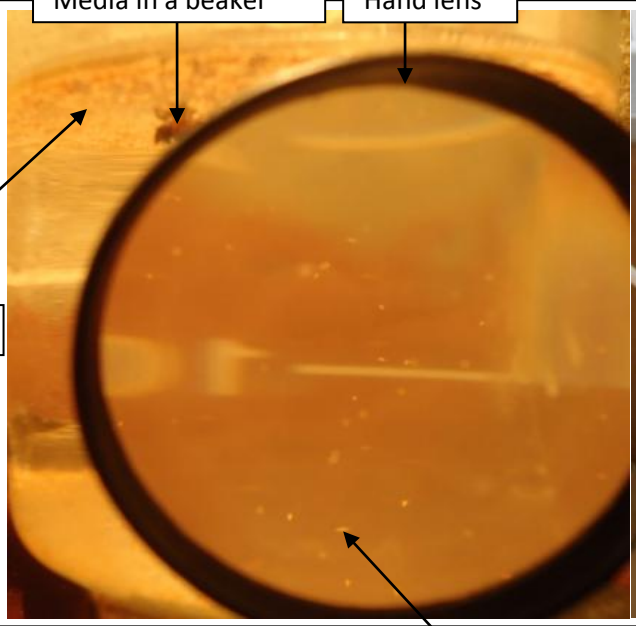


Mycelia in bulk

A. Filtering mycelia biomass cultured in liquid medium B. Positive tests for flavonoids

Media in a beaker

Hand lens



Hatching Eggs

A. *Artemia salina* hatchling on sea water medium B. Soxhlet's extraction system

Observing Live Shrimp with hand lens