

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

PHYTOCHEMICAL AND PHARMACOLOGICAL INVESTIGATIONS OF
GENODERMA LUCIDUM



A DISSERTATION SUBMITTED TO THE DEPARTMENT OF PHARMACY, EAST WEST UNIVERSITY IN PARTIAL FULFILLMENT OF REQUIREMENT FOR THE DEGREE OF MASTERS OF PHARMACY IN CLINICAL PHARMACY & MOLECULAR PHARMACOLOGY.

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DECLARATION

I, do hereby declare that the thesis, entitled “**PHYTOCHEMICAL AND PHARMACOLOGICAL INVESTIGATIONS OF *Ganoderma lucidum***.” submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the degree of Masters of Pharmacy in Clinical Pharmacy & Molecular Pharmacology, is the outcome of the investigations performed by Md. Rafi-Uz-Zaman Akanda, ID. 2012-3-79-004, under the supervision of Ms. Nazia Hoque, Senior Lecturer, Department of Pharmacy, East West University. I also declare that no part of this dissertation has been or is being submitted elsewhere for the award of any Degree/ Diploma.

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DECLARATION

I, do hereby declare that the thesis, entitled “**PHYTOCHEMICAL AND PHARMACOLOGICAL INVESTIGATIONS OF *Ganoderma lucidum***.”submitted to the Department of Pharmacy, East West University, in partial fulfillment of the requirements for the degree of Masters of Pharmacy in Clinical Pharmacy & Molecular Pharmacology, is the outcome of the investigations performed by Md. Rafi-Uz-Zaman Akanda, ID. 2012-3-79-004, under my supervision . I also declare that no part of this dissertation has been or is being submitted elsewhere for the award of any Degree/ Diploma.

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ABSTRACT

The popular edible mushroom *Ganoderma lucidum*(strain GL-4) has been widely used for the general promotion of health and longevity in Asian countries. The present study was carried out to evaluate the antibacterial and antioxidant activity of the methanolic extract of *Ganoderma lucidum*. The phytochemicals evaluation reveals the presence of Flavonoids, Steroids, Terpinoids, Alkaloids, Gums carbohydrates, Saponins & Tannins.

The antibacterial and antifungal activity of methanolic extract of *Ganoderma lucidum* was evaluated with selected twelve bacterial pathogenic such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* etc and one fungi was *Candida albican*. For antimicrobial test, disc diffusion technique was used and the zone of inhibition of microorganisms was measured in mm. The extract of *G. lucidum* showed moderate antimicrobial activities against the selected strains and maximum inhibition zone 15 mm was recorded from 1500ug/disc of aqueous extract of *G. lucidum* against *Escherichia coli* and minimum (8mm) by the *Salmonella paratyphi*, *Betahemolytic streptococcus*, *Beta megaterinium*, and *Vibrio mimicus* at 1000ug/disc of extract. The methanolic extract showed the maximum antifungal activity 11.5mm inhibition zone was recorded from 1500ug/disc of extract against *Candida albican*.

These extracts were also checked for their cytotoxicity using a sensitive *in vitro* Brine shrimp lethality bioassay. The results of brine shrimp lethality bioassay are showed different mortality rate at different concentration. The mortality rate of brine shrimp *napulii* was found to be increased with the increase with the concentration of the sample.

The antioxidant effect of methanolic extract of *G. lucidum* was significant in the DPPH method used. Free radical scavenging abilities of the test samples were determined by measuring the change in absorbance of DPPH (1,1-Diphenyl-2-picrylhydrazyl radical) at 517 nm by the UV-Spectrophotometer. Concentration providing 50% inhibition (IC₅₀) was calculated from the graph plotted inhibition percentage versus concentration of the extract. IC₅₀ value of the standard and methanol extract of *G. lucidum* were 12.6 ug/ml and 581.52 µg/ml respectively. So, comparison with the ascorbic acid, it is clear that plant extracts possess moderate antiradical activity. In conclusion, Further investigations are needed to identify the active constituents and the exact mechanism(s) of action responsible for the reported antimicrobial, cytotoxicity and antioxidant properties of *G.lucidum*.

Key Words: *Ganoderma lucidum*, Antimicrobial, Cytotoxicity, Antioxidant.

CHAPTER 1 INTRODUCTION

1.1 General Introduction

1.1.1 Introduction

Nature always stands as a golden mark to exemplify the outstanding phenomenon of symbiosis. The biotic and abiotic elements of nature are all interdependent. The plants are indispensable to man for his life. The three important necessities of life – food, clothing and shelter- and a host of other useful products are supplied to him by the plant kingdom. Nature has provided a complete store-house of remedies to cure all ailments of mankind. The knowledge of drugs has accumulated over thousands of years as a result of man's inquisitive nature so that today we possess many effective means of ensuring health-care.

The human being appears to be afflicted with more diseases than any other animal species. There can be little doubt then he, very early, sought to alleviate his sufferings from injury and disease by taking advantage of plants growing around him. In the past, almost all the medicines used were from the plants, the plant being man's only chemist for ages. Today, a vast store of knowledge concerning therapeutic properties of different plants has accumulated. All phyla of plants viz. Thallophyta, Bryophyta, Pteridophyta and Spermatophyta contain species that yield official and unofficial products of medicinal importance. (Balick J.M. and P.A. Cox, 1996.)

1.1.2 Medicinal plants

Many familiar medications of the twentieth century were developed from ancient healing traditions that treated health problems with specific plants. Today, science has isolated the medicinal properties of a large number of botanicals, and their healing components have been extracted and analyzed. Many plant components are now synthesized in large laboratories for use in pharmaceutical preparations.

“A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs.” This

definition of Medicinal Plant has been formulated by WHO (World Health Organization). The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as “Medicinal Plants”. Although there are no apparent morphological characteristics in the medicinal plants growing with them, yet they possess some special qualities or virtues that make them medicinally important. It has now been established that the plants which naturally synthesis and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatile oils and contain minerals and vitamins, possess medicinal properties. (Srivastava J.J. et al, 1996)

1.1.3 Significances of medicinal plants to human being

1. Many of the modern medicines are produced indirectly from medicinal plants, for example aspirin.
2. Plants are directly used as medicines by a majority of cultures around the world, for example Chinese medicine and Indian medicine.
3. Many food crops have medicinal effects, for example garlic.
4. Medicinal plants are resources of new drugs. It is estimated there are more than 250, 000 flower plant species.
5. Studying medicinal plants helps to understand plant toxicity and protect human and animals from natural poisons.
6. Cultivation and preservation of medicinal plants protect biological diversity, for example metabolic engineering of plants.

1.1.4 Herbal medicine

Herbal medicine is the oldest form of healthcare known to mankind. Herbs had been used by all cultures throughout history. It was an integral part of the development of modern civilization.

Primitive man observed and appreciated the great diversity of plants available to him. The plants provided food, clothing, shelter, and medicine. Much of the medicinal use of plants seems to have been developed through observations of wild animals, and by trial and error. As time went on, each tribe added the medicinal power of herbs in their area to its knowledgebase. They methodically collected information on herbs and developed well-defined herbal pharmacopoeias. Indeed, well into the 20th century much of the pharmacopoeia of scientific medicine was derived from the herbal lore of native peoples. Many drugs commonly used today are of herbal origin. Indeed, about 25% of the prescription drugs dispensed in the United States contain at least one active ingredient derived from plant material. Some are made from plant extracts; others are synthesized to mimic a natural plant compound.

The therapeutic use of herbal medicines is gaining considerable momentum in the world during the past decade. The World Health Organization (WHO) estimates that herbal medicine is still the mainstay of about 75-80% of the world population, mainly in the developing countries, for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side-effects.

1.1.5 Global status of medicinal plants

Medicinal plants have played a key role in world health. It is estimated that about 25 – 30% of all modern medicines are directly or indirectly derived from higher plants. The herbal products industry comprises a number of inter related sub sectors including as Herbal teas; Functional foods; Nutraceuticals; Phytochemicals; Ethical OTC medicines; Flavours and fragrances; Aroma therapy; Culinary herbs and Spices. As per World Bank reports trade in medicinal plants, botanical drug products and raw material is growing at an annual growth rate between 5 to 15%. The Global pharmaceutical market has risen from US \$550 billion in 2004 worth to a close to US \$900 billion in the year 2008. The herbal industry shares about US \$62 billion with good growth potential. In India the value of botanicals related trade is about US \$10 billion per annum with annual export of US \$1.1 billion while China annual herbal drugs production is worth US \$48 billion with export of US \$3.6 billion. Presently the United States is the largest market for Indian

botanical products accounting for about 50% of the total exports. Japan, Hong Kong, Korea and Singapore are the major importer of the herbal drugs making 66% share of China botanical drug export. Within the European community botanical medicine represents an important share of the pharmaceutical market.

1.1.6 Medicinal plants in Bangladesh

In an estimate, the international market of medicinal plants related to trade stood at 60 billion US Dollar per year. The demand for medicinal plants based raw materials are growing at an approximate rate of 10-15% per year internationally. Medicinal plant sector has traditionally occupied an important position in the socio-cultural, spiritual and medicinal arena of rural and tribal lives of Bangladesh. In recent years, the growing demand for herbal product has led to a quantum jumping in volume of plants materials trade within and across the country. In Bangladesh there are no systematic cultivation process or conservation strategies about medicinal plants. The local people conserve traditional knowledge through their experience and practice, which is handed down orally without any documentation. This knowledge is now under threat to extinction. This is a very alarming situation with regard to natural growth of medicinal plants in the wilderness in this country. In a survey on “Traditional and industrial use and market Scenario of Medicinal plants in Bangladesh.” conducted by the DEBTEC researchers at Chakbazar, Dhaka, Bangladesh, found that there is worth of 11 million US dollars medicinal plant market in Bangladesh, which have been imported but not in the name of medicinal plants rather in the name of spices and other products. Another research aimed at documenting the ‘Present Status and Market Scenario of Medicinal Plants’ in Bangladesh shows that 84.1% of the respondent use medicinal plants in health care. 18.3% of the villagers use Kabirazi in the disease in medium category. 55.0% of respondent’s source of knowledge of using medicinal plant is family where 34.7% gained knowledge from neighbor. Only 14.3% of the respondents are involved with trading of medicinal plant. About 10.4% of the villagers are involved in cultivation, collection or business of medicinal plant. From the survey report it has been found that 46.6% industries are using above 60% of imported medicinal plants as their raw materials and 53.3% of the industries are using below 40%. The study revealed that 86.7% industries are

importing Indian raw materials, 53.3% are importing the Pakistani one and very few of them are importing the raw materials from Nepal, Iran and Korea. According to the response of shop owners, the local raw materials of their products are mostly coming from 5 different areas of the country. Among those 90% are coming from Chittagong and again 76.6% from Tangail, 30% from Gazipur and another 30% from Khulna. In this scenario, appropriate steps must therefore be taken immediately in order to save this situation with regard to growth, conservation and supply of medicinal plants in the country.

1.1List of Medicinal Plants in Bangladesh and their uses					
SL No	Local name	Scientific Name	Family	Using part	Control Disease Name
1	Apang	<i>Achyranthes Paniculata</i>	Amaranthaceae	whole plant	Dysentery, Constipation, piles, Arthritis, Skin disease.
2	কালোমেঘ	<i>Andrographis pariculata</i>	Acanthaceae	whole plant	Metabolic problem, Gastric, Fever, worm killer, Dysentery, Liver Disease, Strengthen.
3	আকন্দ	<i>Calotropis procera</i>	Asclepiadaceae	Root, leaf. Bark flower extract of leaf.	Ulcer, Tooth pain chronic dysentery, cold, Asthma
4	অনন্ডুল	<i>Hemidesmus indicus</i>	Asclepia- daceae	Root and whole plant	Strength increaser, apetiser. Arthritis, Diabetes.
5	অর্জুন	<i>Terminalia arjuna</i>	Combretaceae	Bark	Heart disease, Diarrhea, piles, Tuberculosis.
6	উলটকম্বল	<i>Abroma augusta</i>	Sterculiaceae	Root, Bark &	Vaginal pain sexual disease.

				Leaf.	
7	ঘতকুমারী	<i>Aloe indica</i>	Liliaceae	Extract of leaf	Headache, sexual disease, metabolic problem. Fever.
8	পুদিনা	<i>Mentha viridis</i>	Labiatae	Whole plant	Metabolic disorder, Gastric.
9	বাসক	<i>Adhatoda vasica</i>	Acanthaceae	Leaf, root of plant	Cough, asthma, tuberculosis, cold, blood refine.
10	জৈষ্ঠিমধু	<i>Hydrangea arborescens</i>	Saxifraga zaceae	Leaf flower fruit	Liver disease, adrenal peptic Ulcer, hormonal disease, cold, throat pain.
11	বান্ধীশাক	<i>Becopa moniera</i>	Scrophulariaceae	Leaf	Heart disease, nural pressure, Asthma.
12	শর্পগন্ধা	<i>Rauwolfia Serpentina</i>	Apocynaceae	Leaf and root.	Blood Pressure, brain abnormal, dysentery diarrhea pain killer.
13	কুরচি	<i>Holarrhena antidysenterica</i>	Apocynaceae	Bark & Seed	Diarrhea, dysentery, worm killer constipation, intestinal weakness.
14	নিম	<i>Azadirachta indica</i>	Meliaceae	Root, leaf Bark	Skin disease, worm killer Arthritis, Insecticide, Anti vomiting, Tooth disease, Jaundice etc. Antiviral.
15	উলটচাঁপাল	<i>Gloriosa superba</i>	Liliaceae	Leaf and Steam	Arthritis Adrenals peptic, ulcer.

16	শতমূলী	<i>Asparagus racemosus</i>	Liliaceae	Leaf and root	Fever dysentery Diabetics.
17	বেল	<i>Aegle marmelos</i>	Rutaceae	Fruit	Dysentery Diarrhoea.
18	আনারস	<i>Ananas comosus</i>	Bromeliaceae	Fruit & leaf	Jaundice
19	জাম	<i>Syzygium cumini</i>	Myrtaceae	Fruit & seed	Diabetics, Dysentery.
20	আসামলতা	<i>Mikania scandens</i>	Compositae	Leaf	Bleeding control, Dysentery, Daud etc.
21	নিশিন্দা	<i>Vitex negundo</i>	Verbenaceae	root. leaf	Asthma, Arthritis, fever etc.
22	দুর্বা	<i>Cynodon dactylon</i>	Grominae	Leaf	Blood bleeding control. Skin disease.

1.1.7 Natural products as a resource for established new drugs

Natural products have served as a major source of drugs for centuries, and about half of the pharmaceuticals in use today are derived from natural products. Quinine, Theophylline, Penicillin G, Morphine, Digitoxine, Vincristine, Doxorubicin, Cyclosporin and vitamin A, all share two important characteristics: they are the cornerstones of modern pharmaceutical care and they are all natural products. The use of natural substance, particularly plants, to control diseases is centuries old practice that has led to the discovery of more than half of all “Modern” pharmaceuticals. (Rahman A.M. et al 2007)

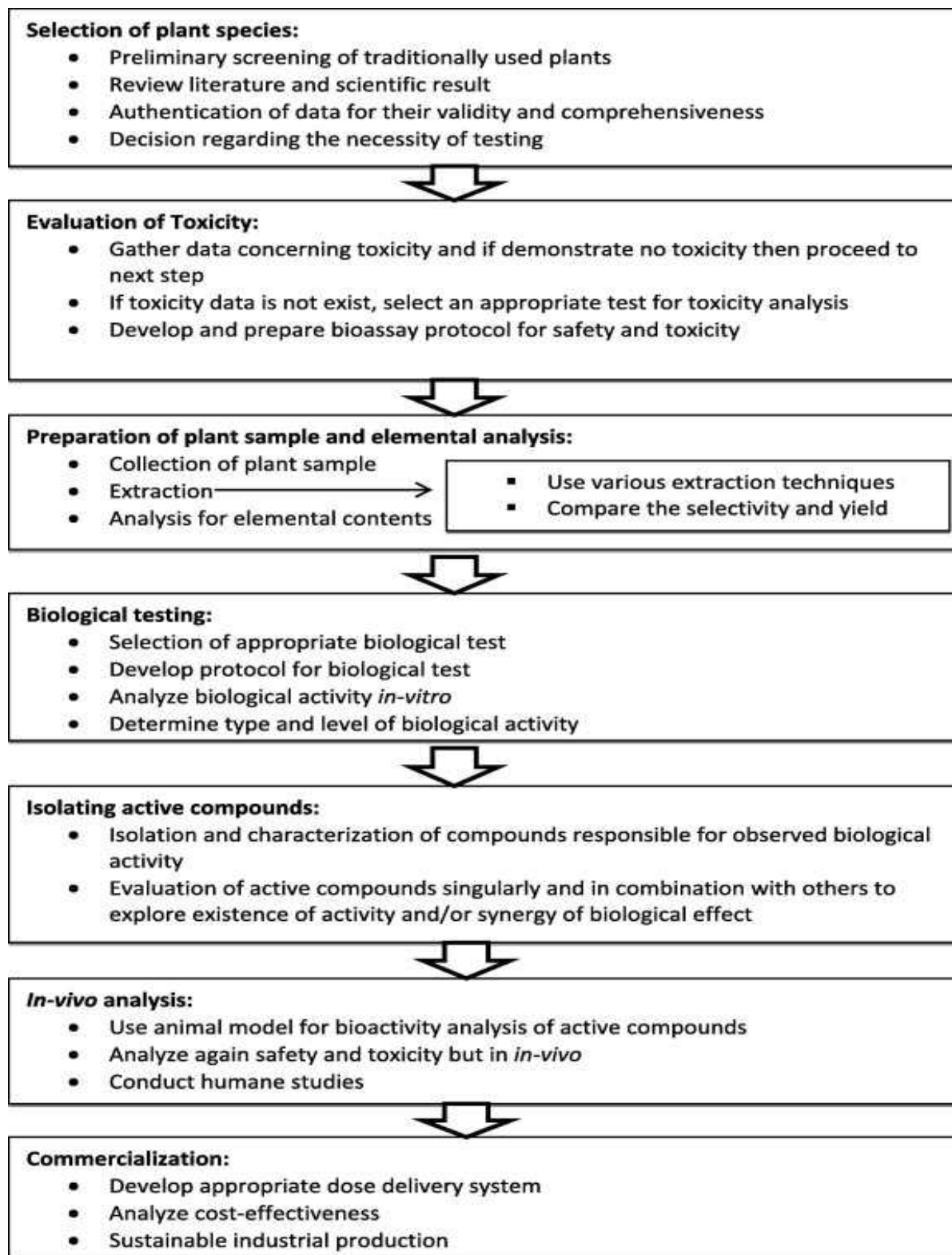
1.1.7.1 Plant resources for new medicine

1. **Bryophytes** (nonvascular plants, e.g. liverwort and moss) have about 15,350 species.
2. **Seedless vascular plants** (commonly called fern) are estimated about 12,157 species.

3. *Gymnosperm* has about 760 species.
4. *Angiosperm* is estimated to have more than 250,000 species.

1.1.7.2 Approaches to Drug Development from Plant Sources

The major portion of the present day knowledge of the medicinal properties of plants is the sum total of some observations and experiences. According to some generous estimates, almost 80 percent of the present day medicines are directly or indirectly obtained from plants (Ghani, 1998).



Flow-chart 1.2: Steps of drug development from plant sources

1.1.7.3 Some established drugs of natural origin

1.1.7.3.1 Antimalarial agents

Quinine is considered to be the drug of choice for severe chloroquine-resistant malaria due to *P. falciparum*. In the United States, the related alkaloid quinidine is recommended for this purpose because of its wide availability, there in its use as an antiarrhythmic agent.

1.1.7.3.2 Cardiovascular drugs

The cardiac glycosides, which include Digoxin, Digitoxin and Deslanoside, exert a powerful and selective positive inotropic action on the cardiac muscles.

1.1.7.3.3 CNS – drugs

One of the most cited examples of important natural product-derived drugs is the neuromuscular blocker, d-tubocurarine, which recently helped recognition of the possibility that a number of vastly different CNS and peripheral nervous system diseases may be therapeutically controlled by selective nicotinic acetylcholine receptor (nAChR) agonists and has opened a new area of drugs design based on the Nicotine molecule.

1.1.7.3.4 Antibiotics

Today, new important anti-infectives are being discovered from microbial, plant and animal sources. For example, the antimalarial agent, Artemisinin, was isolated from the Chinese medicinal plant *Artemisia annua*.

1.1.7.3.5 Antineoplastic agents

The major anticancer drugs are natural products from plants or microorganisms. Examples of such important anticancer drugs are Bleomycin, Doxorubicin, Daunorubicin, Vincristine, Vinblastine, Mitomycin, Streptozocin and now the recent additions of Paclitaxel (Taxol™), Irinotecan, Etoposide and Tenoposide.

1.1.7.3.6 Cholesterol-lowering agents(hypolipidimics)

These drug acts by inhibition of 3-hydroxy-3- methylglutaryl coenzyme- A reductase (HMG-CoA reductase), an enzyme critical in the biosynthesis of cholesterol. The first of the HMG-CoA reductase inhibitors were isolated from *Penicillium sp.*

1.1.7.3.7 Immunomodulators

The immunomodulator Cyclosporin was originally isolated from a soil fungus, *Trichoderma polysporum*. This compound was a major breakthrough for organ transplantation.

1.1.7.3.8 Antihyperglycemics

The major antihyperglycemic drugs are natural products derived from popular plant such as *Momordica charantia* (Karela); *Tinospora cordifolia* (Guduchi); *Gymnema sylevestre* (Gurmar); *Azadirachta indica* (Neem); *Ficus benghalensis* (Indian banyan tree); *Aegle marmelos* (bel or bilva); *Aloe vera*; *Allium sativum* – Garlic etc.

1.1.7.3.9 Hepatoprotectives

Numbers of plants are used for the effective treatment of liver disorder. Some of these plants are – *Tinospora cordifolia* (Guduchi or Gulvel), *Astracaula longifolia*, *Cleoma viscosa*, *Bauhania variaata*, *Alstoma scholaris* etc.

1.1.8 Purpose for the following study

The potential of higher plants as source for new drugs is still largely unexplored. Among the estimated 250,000-500,000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller. Historically pharmacological screening of compounds of natural or synthetic origin has been the source of innumerable therapeutic agents. Plants are used in different countries and are a source of many potent and powerful drugs. A wide range of medicinal plant parts is used for extract as raw drugs and they possess varied medicinal properties. The different parts used include seeds, root, stem, flower, fruit, twigs exudates and modified plant organs.

Considering the vast area of potentiality of plants as sources for drugs and taking into account the local traditional uses, a systematic investigation was undertaken to screen the phytochemical and pharmacological activity of *Ganoderma Lucidum* . belonging to the family of Ganodermatacaea. It is used as medicinal mushroom in traditional Chinese medicine for more than 2,000 years, making it one of the oldest mushrooms known to have been used medicinally.

1.2Plant review

1.2.1.Scientific name: *Ganoderma Lucidum* .



Picture : *Ganoderma Lucidum*

1.2.2Family: Ganodermataceae

1.2.3 Bengali/Vernacular Name:

Mushroom

1.2.4 Tribal Name:

Lingzhi (Chinese);

Reishi , (Japanese);

Yeong Ji or *Yung Gee* ,(Korean);

linh chi (Vietnamese).

1.2.5 Description of the plant

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

Ganoderma lucidum 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. The three main factors that are greatly influencing the shape it takes are light, temperature, and humidity (although less significant water and air quality can have an impact).(David Arora, 1986)

1.2.6 Using information:

The plant possesses anti-tumor, anti-cancer, immunomodulatory and immunotherapeutic qualities, supported by studies on polysaccharides, terpenes, and other bioactive compounds isolated from fruiting bodies and mycelia of this fungus. It has also been found to inhibit platelet aggregation, and to lower blood pressure (via inhibition of angiotensin-converting enzyme, cholesterol, and blood sugar.

1.2.7 Accepted scientific name:

Ganoderma lucidum (accepted name)

1.2.7.1 Synonyms:

Ganoderma mongolicum (synonym)

Ganoderma pseudoboletus (synonym)

Ganoderma nitens (synonym)

1.2.8 Taxonomy:

Kingdom: Fungi

Division: Basidiomycota

Class: Agaricomycetes

Order: Polyporales

Family: Ganodermataceae

Genus: *Ganoderma*

Species: *Ganoderma lucidum*

1.2.9 Pharmacological effects of this plant:

GANODERMA LUCIDUM POLYSACCHARIDE

Enhancing the immune system, suppressing and inhibiting growth and metastasis of a tumor, regulating blood pressure, preventing cardiovascular and cerebral-vascular diseases, activating insulin secretion, adjusting blood sugar; as well as producing anti-oxidation and anti-aging effects, among others.

TRITERPENE GANODERMA LUCIDUM ACID

Showing effects in degrading alcohol, protecting the liver, killing cancer cells, relieving pain, alleviating inflammation, improving irritability, reducing cholesterol, preventing vascular stiffening, strengthening liver and digestive functions

ORGANIC GERMANIUM

Boosting the blood's oxygen-carrying capacity, scavenging free radicals, suppressing tumor pervasiveness

MICROELEMENT SELENIUM

Preventing cancer and relieving pain for cancer patients. (www.superganoderma.com)

1.3. Aims and objectives

1.3.1 Aims:

There is a continuous and urgent need to discover new medicinal compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases. Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against the harmful diseases. The increasing failure of chemotherapeutics, severe adverse effects with increase doses and repeated use of drugs, problems with multiple dosage regimens and antibiotic resistance exhibited by pathogenic microbial infectious agents and emergence of new diseases has led to the screening of medicinal plants throughout the world for their potential activity.

Bangladesh imports a large quantity of pharmaceutical raw materials including medicinal plants and semi-processed plant produce drugs and medicines. Thus huge foreign exchanges can be saved if the manufacturers, to satisfy their needs, utilize the indigenous medicinal plants or their semi processed products.

Ganoderma Lucidum is a medicinal plant used traditionally in Bangladesh. Upon significant literature survey it was found only a little research work has been performed on this plant to evaluate its medicinal value and active constituents those are responsible for its pharmacological activities.

As a student of Pharmacy, I had a fascination to investigate the secret behind the medicinal uses of the plant, to find out other medicinal uses which could be present in the plant and to establish a scientific data on its medicinal value. Therefore, taking into consideration the traditional uses of the plant and facilities available for conducting the study, this research work was performed on the plant *Ganoderma Lucidum*.

1.3.1.1 Principal aim

The principal aim of the present study was to investigate the scientific basis of the traditional uses of the plant *Ganoderma Lucidum*. to study other medicinal uses possible and in the same time to find out the chemical groups present in the active plant parts to get preliminary idea about the active constituents.

1.3.1.2 Secondary aims

The primary goal of the project will be complemented by a series of further secondary aims.

1. Separation of the plant material by using different solvent sequentially based on their ascending order of polarity as because different compounds of varying polarity are soluble in different solvents.
2. Qualitative analysis of different chemical groups present in the plant extracts to get preliminary idea about the compounds present in the extracts.
3. Screening of various pharmacological activities.

1.3.2 Objectives

In order to achieve these aims, the following research objectives have been identified:

Table 1.3: Plant : <i>Ganoderma lucidum</i> (Methanol Extract of mushroom)	
SI No	Experiment
1	Chemical Analysis
a.	Phytochemical Analysis (test for Alkaloids, Flavonoids, Saponins, Tannins, Terpinoids, Gum & carbohydrates, steroids)
	Pharmacological Activity Test
2	Antimicrobial Activity Study
3	Brine Shrimp lethality bioassay of cytotoxic activity
4	Antioxidant Activity Study
a.	Total Anti-oxidant Capacity by DPPH method
b.	Total Phenol Content

The overall purpose and objective of the study is to analyze phytochemical substances present in the plant and evaluate the biological activities of *Ganoderma Lucidum*.

1.3.3 Study area

The research was carried out in the Pharmacognosy Lab, Microbiology Lab, Chemistry Lab and Pharmacology Lab of Department of Pharmacy, East West University, Dhaka.

1.3.4 Data collection

All the relevant data has been collected from two types of sources:

1. Primary sources: direct personal contact and observations of the experiments carried out in the laboratory.
2. Secondary sources: various publications like journals, papers, documents and websites.

1.3.5 Research protocol:

1. Selection, identification, collection, drying and grinding of plants.
2. Extraction of the powders with methanol and collection of extract.
3. Phytochemical analysis of the plant extract.
4. Antioxidant activity determination.
5. Anti Microbial activity determination.
6. Studying and comparing the results obtained.

1.3.6 Information processing and analysis

The data and the results collected were reviewed, compared, processed and organized. Some tests were repeated to be sure of the results. Some data were analyzed into flow charts and statistical tables where possible.

1.4 Phytochemical Screening

“Phyto” is the Greek word for plant. There are many “families” of phytochemicals and they help the human body in a variety of ways. Phytochemicals may protect human from a host of diseases. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Plant produces these chemicals to protect itself but recent research demonstrates that many phytochemicals can protect humans against diseases.

Plant synthesizes a wide variety of chemical compounds which can be sorted by their chemical class, biosynthetic origin and functional groups into primary and secondary metabolites.

1.4.2 Phytochemical investigation

1.4.2.1 Phytochemistry

Phytochemistry is in the strict sense of the word the study of phytochemicals. These are chemicals derived from plants. In a narrower sense the terms are often used to describe the large number of secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant diseases. They also exhibit a number of protective functions for human consumers.

The preliminary phytochemical studies were conducted for the extracts of *Ganoderma Lucidum* find out the presence of various phytoconstituents. (Mojab F. et al, 2003)

1.4.2.2 Important Phytochemicals Present in Plant

a) Alkaloids: Alkaloids are a group of naturally occurring chemical compounds which mostly contain basic nitrogen atoms. This group also includes some related compounds with neutral and even weakly acidic properties. Also some synthetic compounds of similar structure are attributed

to alkaloids. Beside carbon, hydrogen and nitrogen, molecules of alkaloids may contain sulfur and rarely chlorine, bromine or phosphorus.

Distribution of Alkaloids in Plants:

Alkaloids are generated by various living organisms (bacteria, fungi, plants, and animals), especially by higher plants – about 10 to 25% of those contain alkaloids. The alkaloids content in plants is usually within a few percent and is inhomogeneous over the plant tissues. Depending on the type of plants, the maximum concentration is observed in the leaves (black henbane), fruits or seeds (Strychnine tree), root (*Rauwolfia serpentina*) or bark (cinchona). Furthermore, different tissues of the same plants may contain different alkaloids.

Biological role of Alkaloids:

The role of alkaloids for living organisms which produce them is still unclear.

1. Initially it was assumed that the alkaloids are the final products of nitrogen metabolism in plants, and urea in mammals. Later it was shown that alkaloid concentrations vary over time and this hypothesis was refuted.
2. Most of the known functions of alkaloids are related to protection. For example, aporphine alkaloid liriodenine produced by the tulip tree protects it from parasitic mushrooms. In addition, presence of alkaloids in the plant prevents insects and chordate animals from eating it.
3. Some animals adapted to alkaloids and even use them in their own metabolism.
4. Besides, such alkaloid-related substances as serotonin, dopamine and histamine are important neurotransmitters in animals.
5. Alkaloids are known to regulate plant growth.
- 6.

Applications of Alkaloids

1. **In medicine:** Medical use of alkaloid plants has a long history, and thus when the first alkaloids were synthesized in the 19th century, they immediately found application in clinical practice. Many alkaloids are still used in medicine, usually in the form of salts, including the following:

Table 1.4: Alkaloids that are used in medicine

Alkaloid	Action
Ajmaline	Antiarrhythmic
Atropine, scopolamine, hyoscyamine	Anticholinergic
Vinblastine, vincristine	Antitumor
Vincamine	Vasodilating, antihypertensive
Codeine	Cough medicine
Cocaine	Anesthetic
Colchicine	Remedy for gout
Morphine	Analgesic
Reserpine	Antihypertensive
Tubocurarine	Muscle relaxant
Physostigmine	Inhibitor of acetylcholinesterase
Quinidine	Antiarrhythmic
Quinine	Antipyretics, antimalarial
Emetine	Antiprotozoal agent
Ergot alkaloids	Sympathomimetic, vasodilator, antihypertensive

Many synthetic and semisynthetic drugs are structural modifications of the alkaloids, which were designed to enhance or change the primary effect of the drug and reduce unwanted side effects. For example, naloxone, an opioid receptor antagonist, is a derivative of thebaine which is present in opium.

1. **In agriculture:**

Prior to the development of a wide range of relatively low-toxic synthetic pesticides, some alkaloids, such as salts of nicotine and anabasine, were used as insecticides. Their use was limited by their high toxicity to humans.

2. **Use as psychoactive drugs:**

Preparations of plants containing alkaloids and their extracts, and later pure alkaloids have long been used as psychoactive substances. Cocaine and cathinone are stimulants of the central nervous system. Mescaline and many of indole alkaloids (such as psilocybin, dimethyltryptamine and ibogaine) have hallucinogenic effect. Morphine and codeine are strong narcotic pain killers. There are alkaloids that do not have strong psychoactive effect themselves, but are precursors for semi-synthetic psychoactive drugs. For example, ephedrine and pseudoephedrine are used to produce methcathinone (ephedrine) and methamphetamine.

b) Flavonoids:

Flavonoids (or bioflavonoids), also collectively known as Vitamin P and citrin, are a class of plant secondary metabolites.

Classification:

According to the IUPAC nomenclature, they can be classified into:

1. Flavonoids, derived from 2-phenylchromen-4-one (2-phenyl-1,4-benzopyrone) structure (examples: quercetin, rutin)

2. Isoflavonoids, derived from 3-phenylchromen-4-one (3-phenyl-1,4-benzopyrone) structure
3. Neoflavonoids, derived from 4-phenylcoumarine (4-phenyl-1,2-benzopyrone) structure

Distribution of Flavonoids in Plants:

Flavonoids are widely distributed in plants fulfilling many functions.

Biological roles of flavonoids:

1. Flavonoids are the most important plant pigments for flower coloration producing yellow or red/blue pigmentation in petals designed to attract pollinator animals.
2. Flavonoids secreted by the root of their host plant help Rhizobia in the infection stage of their symbiotic relationship with legumes like peas, beans, clover, and soy. Rhizobia living in soil are able to sense the flavonoids and this triggers the secretion of Nod factors, which in turn are recognized by the host plant and can lead to root hair deformation and several cellular responses such as ion fluxes and the formation of a root nodule
3. They protect plants from attacks by microbes, fungi and insects

Potential for Biological Activity of Flavonoids:

1. In vitro studies of flavonoids have displayed anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities. Flavonoids (both flavonols and flavanols) are most commonly known for their antioxidant activity in vitro.
2. Consumers and food manufacturers have become interested in flavonoids for their possible medicinal properties, especially their putative role in prevention of cancers and cardiovascular diseases. Although physiological evidence is not yet established, the beneficial effects of fruits, vegetables, tea, and red wine have sometimes been attributed to flavonoid compounds rather than to known micronutrients, such as vitamins and dietary minerals.

3. Cancer: Physiological processing of unwanted flavonoid compounds induces so-called Phase II enzymes that also help to eliminate mutagens and carcinogens, and therefore may be of value in cancer prevention. Flavonoids could also induce mechanisms that may kill cancer cells and inhibit tumor invasion. UCLA cancer researchers have found that study participants who ate foods containing certain flavonoids, such as catechins found in strawberries and green and black teas; kaempferol from brussel sprouts and apples; and quercetin from beans, onions and apples, may have reduced risk of obtaining lung cancer.
4. Diarrhea: A study done at Children's Hospital & Research Center Oakland, in collaboration with scientists at Heinrich Heine University in Germany, has shown that epicatechin, quercetin and luteolin can inhibit the development of fluids that result in diarrhea by targeting the intestinal cystic fibrosis transmembrane conductance regulator Cl⁻ transport inhibiting cAMP-stimulated Cl⁻ secretion in the intestine.
5. Capillary stabilizing agents: Bioflavonoids like rutin, monoxerutin, diosmin, troxerutin and hidrosmin have potential vasoprotective proprieties still under experimental evaluation

c) Saponins:

Saponins are a class of chemical compounds, one of many secondary metabolites found in natural sources, with saponins found in particular abundance in various plant species. Specifically, they are amphipathic glycosides grouped phenomenologically by the soap-like foaming they produce when shaken in aqueous solutions, and structurally by their composition of one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative. A ready and therapeutically relevant example is the cardio-active agent digoxin, from common foxglove.

Distribution of Saponins in Plants:

Saponins have historically been understood to be plant-derived, but they have also been isolated from marine organisms. Saponins are indeed found in many plants, and derive their name from the soapwort plant (Genus *Saponaria*, Family Caryophyllaceae), the root of which was used historically as a soap. Saponins are also found in the botanical family Sapindaceae, with its defining genus *Sapindus* (soapberry or soapnut), and in the closely related families Aceraceae (maples) and Hippocastanaceae (horse chestnuts; ref. needed). It is also found heavily in *Gynostemma pentaphyllum* (Genus *Gynostemma*, Family Cucurbitaceae) in a form called gypenosides, and ginseng (Genus *Panax*, Family Araliaceae) in a form called ginsenosides. Within these families, this class of chemical compounds is found in various parts of the plant: leaves, stems, roots, bulbs, blossom and fruit. Commercial formulations of plant-derived saponins – e.g., from the soap bark (or soapbark) tree, *Quillaja saponaria*, and from other sources—are available via controlled manufacturing processes, which make them of use as chemical and biomedical reagents.

Medical uses of Saponin:

There is tremendous, commercially driven promotion of saponins as dietary supplements and nutraceuticals.

1. There is evidence of the presence of saponins in traditional medicine preparations, where oral administrations might be expected to lead to hydrolysis of glycoside from terpenoid (and obviation of any toxicity associated with the intact molecule). But as is often the case with wide-ranging commercial therapeutic claims for natural products: the claims for organismal/human benefit are often based on very preliminary biochemical or cell biological studies; and mention is generally omitted of the possibilities of individual chemical sensitivity, or to the general toxicity of specific agents, and high toxicity of selected cases. While such statements require constant review (and despite the myriad web claims to the contrary), it appears that there are very limited US, EU, etc. agency-approved roles for saponins in human therapy. In their use as adjuvants in the production of vaccines, toxicity

associated with sterol complexation remains a major issue for attention. Even in the case of digoxin, therapeutic benefit from the cardiotoxin is a result of careful administration of an appropriate dose. Very great care needs to be exercised in evaluating or acting on specific claims of therapeutic benefit from ingesting saponin-type and other natural products.

d) Tannins:

Tannins are astringent, bitter plant polyphenols that either bind and precipitate or shrink proteins and various other organic compounds including amino acids and alkaloids. Tannins have molecular weights ranging from 500 to over 3,000. The astringency from the tannins is what causes the dry and puckery feeling in the mouth following the consumption of unripened fruit or red wine. Likewise, the destruction or modification of tannins with time plays an important role in the ripening of fruit and the aging of wine.

Distribution of Tannins in Plants:

The compounds are widely distributed in many species of plants, where they play a role in protection from predation and perhaps also in growth regulation.

Medicinal Uses of Tannins:

Tannins may be employed medicinally in antidiarrheal, hemostatic, and antihemorrhoidal compounds.

2. The anti-inflammatory effect of tannins help control all indications of gastritis, esophagitis, enteritis, and irritating bowel disorders. Diarrhea is also treated with an effective astringent medicine that does not stop the flow of the disturbing substance in the stomach; rather, it controls the irritation in the small intestine.
3. Tannins not only heal burns and stop bleeding, but they also stop infection while they continue to heal the wound internally. The ability of tannins to form a protective layer over the exposed tissue keeps the wound from being infected even more.

4. Tannins are beneficial when applied to the mucosal lining of the mouth. Tannins can also be effective in protecting the kidneys.
5. Tannins have been used for immediate relief of sore throats, diarrhea, dysentery, hemorrhaging, fatigue, skin ulcers and as a cicatrizant on gangrenous wounds.
6. Tannins can cause regression of tumors that are already present in tissue, but if used excessively over time, they can cause tumors in healthy tissue.
7. Tannins are used indirectly as molluscicides to interrupt the transmission cycle of schistosomiasis.
8. Tannins have shown potential antiviral, antibacterial and antiparasitic effects. It is believed that tannins isolated from the stem bark of *Myracrodruon urundeuva* are of neuroprotective functions capable of reversing 6-hydroxydopamine induced toxicity. The plant has shown promising futures for therapeutic use, which may be of benefit to neuro disease patients . Souza et al. discovered that the tannins isolated from the stem bark also has the antiinflammatory and antiulcer potency on rodents, showing a strong anioxidant property for possible therapeutic applications
9. Foods rich in tannins can be used in the treatment of HFE hereditary hemochromatosis, a hereditary disease characterized by excessive absorption of dietary iron resulting in a pathological increase in total body iron stores

e) Terpenoids:

The terpenoids, sometimes called isoprenoids, are a large and diverse class of naturally-occurring organic chemicals similar to terpenes, derived from five-carbon isoprene units assembled and modified in thousands of ways. Most are multicyclic structures that differ from one another not only in functional groups but also in their basic carbon skeletons.

Distribution of Terpenoids:

These lipids can be found in all classes of living things, and are the largest group of natural products.

Biological Activity of Terpenoids:

1. Plant terpenoids are used extensively for their aromatic qualities.
2. They play a role in traditional herbal remedies and are under investigation for antibacterial, antineoplastic, and other pharmaceutical functions.
3. Terpenoids contribute to the scent of eucalyptus, the flavors of cinnamon, cloves, and ginger, and the color of yellow flowers. Well-known terpenoids include citral, menthol, camphor, Salvinorin A in the plant *Salvia divinorum*, and the cannabinoids found in *Cannabis*.
4. The steroids and sterols in animals are biologically produced from terpenoid precursors.
5. Sometimes terpenoids are added to proteins, e.g., to enhance their attachment to the cell membrane; this is known as isoprenylation. Many of these are substrates for plant Cytochrome P450.

1.5 Antimicrobial Screening of *Genoderma Lucidum*

The main objective of performing the antibacterial screening is to determine the susceptibility of the pathogenic microorganisms to test compound which, in turn is used to selection of the compound as a therapeutic agent. In general, antimicrobial screening *in-vitro* is undertaken in following two steps:

i) Primary assay

It is essentially a qualitative or semi qualitative test that indicates the sensitivity or resistance of microorganisms to the compound. However this technique cannot be used to distinguish between bacteriostatic and bactericidal agents (**Reiner R. et al 1982**). The primary assay can be performed in vitro by disk diffusion assay method , which includes

- a) Plate diffusion test
- b) Streak test

The plate diffusion test utilizes different concentrations of a test compound absorbed on sterile filter paper disks on the same plate whereas the streak test permits the determination of the antibacterial effect of a test compound on several microorganisms simultaneously and is suitable for the estimation of the spectrum of the activity. However, the plate diffusion test is commonly used.

ii) Secondary assay

It quantifies the relative potency such as minimum inhibitory concentration (MIC). The lowest concentration of an antimicrobial agent required to inhibit the growth of the microorganisms in vitro is referred to as minimum inhibitory concentration (MIC). It is done by serial dilution technique. (**Reiner R. et al, 1982**).

1.5.2 Antimicrobial drug

Antimicrobial drug/Antibiotics are the greatest contribution at the present century at therapeutic. Antibiotics are special kind of chemotherapeutic agent usually obtained from living organism. The term chemotherapeutic agent mean “All chemical substance that destroy all kind of cell wall

such as bacterial cell wall, viral cell wall even human cell wall. “Antibiotics one kind of chemotherapeutic agent, but it does not destroy the human cell wall, it destroy the bacterial & viral cell wall. So all antibiotics are chemotherapeutic agent but all chemotherapeutic agents are not antibiotic. The word antibiotic come to refer to a metabolic of one microorganism that is very small amount is detrimental or inhibitory to their microorganism. The term antibiosis was first defined by vuillemin in 1889. The first systematic search for & study of antibiotics made by “Gratia & both about 1924. In 1929 Alexander Fleming discovers one kind of Antibiotics named by penicillin from the penicillium tree. Characteristics of Antibiotic: To be useful as chemotherapeutic agent antibiotics must have the following qualities:

- (1) They should have the ability to destroy or inhibit many different species of pathogenic microorganism.
- (2) They should prevent the ready development of resistant forms of the parasites.
- (3) They should not produced undesirable side effects in the host, such as sensibility or allergic reaction, never damage or irritation of the kidneys & gastrointestinal tract(G.I.T).
- (4) They should not eliminate the normal microbial flora of the host.(**Cui J.L. et al, 2011**)

1.5.3 Classification of Antibiotics:-

Antibiotic drug are classified in several way: For example, some are bactericidal & other are bacteriostatic. Here the term Bactericidal mean “ Stop the Bacterial growth & it also kill the bacteria”, and bacteriostat mean “ stop the bacterial growth but can not kill the bacteria.”

Antibiotic may be grouped on the basis of chemical structure.

- 1) Sulfonamide & relative drugs
- 2) Diaminopyrimidines.
- 3) Quinolones
- 4) β - lactam antibiotics
- 5) Nitromemzene derivatives
- 6) Amino glycosides.
- 7) Polypeptide Antibiotics.
- 8) Nitrofurans derivatives etc.

Antibiotics & their mode of action:-The major points of attack of antibiotics on microorganism include:

- 1) Inhibition of cell wall synthesis Drug- penicillin, Bacitracin.
- 2) Damage to the cytoplasmic membran. Drug- ploymxins, Hamycin.
- 3) Inhibition of nucleic acid & protein synthesis, Drug-Tetracyclines, Clidamycin.
- 4) Inhibition of specific enzyme systems. Drug-Pyridine, Pyrimidine.
- 5) Interfere with DNA synthesis. Drug-Acyclovir.
- 6) Interfere with intermediary metabolism. Drug-Sulfonamides, PAS(Para amino salicylic acid).

1.5.4 Uses of Antibiotics Drug that promote Resistance:-

- 1) Inappropriate treatment of cold and other viral infection.
- 2) Indiscriminate prophylaxis.
- 3) Overuse of potent, broad-spectrums antibiotics.
- 4) Administering doses that are too small, or not continuing therapy long enough to eliminate the most resistant microbes.

Antibiotics resistance can be prevent by following:-

- 1) Limiting the use of newer antibiotics so long as the currently used are effective.
- 2) Avoidance of indiscriminate use of antibiotics.
- 3) Using Antibiotics combinations in selected cireums-tances e.g- tuberculosis, leprosy
- 4) Constant monitoring of resistance patterns in a hospital or community.

Causes of failure of antibiotics therompyhos: The possible causes of failure of antibiotics therapy are:

- 1) Antibiotics resistance to microorganism.
- 2) Failure of selection of the best drugs, which in specific for specific micro organism.
- 3) Sub optimal use of antibiotics.

- a) Inadequate dose b) interval between doses too long. c) Duration of course's too short d) Unsuitable route.
- 4) Treatment begun too late to save patient
- 5) Super infection by other pathogens.
- 6) Undrained pus, retained infection foreign body, dead tissue.

1.5.5 Misuses of antibiotics:-

- 1) Treatment of untreatable infection: The majority of the diseases caused by viruses will not respond to anti-infective agent. Thus antimicrobial therapy of measles, chickenpox, mumps & upper respiratory infection-90% is totally ineffective.
- 2) Therapy of fever of undetermined origin.
- 3) Improper doses.
- 4) Reliance on chemotherapy with omission of surgical drainage.
- 5) Lack of adequate bacteriological information.

Factors to be considered in selecting antibiotics drug:-

- 1) Clinical symptoms & site of infection.
- 2) Identity of the pathogen-samples for laboratory analysis must be collected before any chemotherapy begins.
- 3) Drug toxicity.
- 4) Cost
- 5) Prior history drug allergy.

1.5.6 Rational use of antibiotics:-

- 1) Use appropriate antibiotics with adequate bacteriological information.
- 2) Antibiotics should be used in proper dose & for appropriate duration.
- 3) Combination of antibiotic should be used where single drug is ineffective or to overcome the chance of microbial resistance to antibiotics.
- 4) A bactericidal antibiotic should not be used with a bacteriostatic antibiotic at the same time (antibiotic antagonism).

- 5) Broad spectrum antibiotics should not be used indiscriminately.
- 6) Consider the patient condition during selection of antibiotics (e.g-in renal failure ciprofloxacin is contraindicated).
- 7) Should not use the newer member of group of antibiotics so long as the currently used drug is effective.

1.6 Cytotoxicity Study

1.6.1 Cytotoxicity:

Cytotoxicity is the quality of being toxic to cells. Examples of toxic agents are a chemical substance, an immune cell or some types of venom (e.g. from the puff adder or brown recluse spider).

1.6.2 Measuring cytotoxicity:

Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries. Researchers can either look for cytotoxic compounds, if they are interested in developing a therapeutic that targets rapidly dividing cancer cells, for instance; or they can screen "hits" from initial high-throughput drug screens for unwanted cytotoxic effects before investing in their development as a pharmaceutical.

Assessing cell membrane integrity is one of the most common ways to measure cell viability and cytotoxic effects. Compounds that have cytotoxic effects often compromise cell membrane integrity. Vital dyes, such as trypan blue or propidium iodide are normally excluded from the inside of healthy cells; however, if the cell membrane has been compromised, they freely cross the membrane and stain intracellular components. Alternatively, membrane integrity can be assessed by monitoring the passage of substances that are normally sequestered inside cells to the outside. One commonly measured molecule is lactate dehydrogenase (LDH). Protease biomarkers have been identified that allow researchers to measure relative numbers of live and dead cells within the same cell population.

The live-cell protease is only active in cells that have a healthy cell membrane, and loses activity once the cell is compromised and the protease is exposed to the external environment. The dead-cell protease cannot cross the cell membrane, and can only be measured in culture media after cells have lost their membrane integrity.

Cytotoxicity can also be monitored using the MTT or MTS assay. This assay measures the reducing potential of the cell using a colorimetric reaction. Viable cells will reduce the MTS

reagent to a colored formazan product. A similar redox-based assay has also been developed using the fluorescent dye, resazurin. In addition to using dyes to indicate the redox potential of cells in order to monitor their viability, researchers have developed assays that use ATP content as a marker of viability. Such ATP-based assays include bioluminescent assays in which ATP is the limiting reagent for the luciferase reaction.

Cytotoxicity can also be measured by the sulforhodamine B (SRB) assay, WST assay and clonogenic assay.

A label-free approach to follow the cytotoxic response of adherent animal cells in real-time is based on electric impedance measurements when the cells are grown on gold-film electrodes. This technology is referred to as electric cell-substrate impedance sensing (ECIS). Label-free real-time techniques provide the kinetics of the cytotoxic response rather than just a snapshot like many colorimetric endpoint assays. (Meyer B.N et al, 1982)

1.7 ANTI-OXIDANTS ACTIVITY

1.7.1 ANTIOXIDANTS:

Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Plant sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the mono-phenols are weak antioxidants. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydro peroxide or lipid peroxy and thus inhibit the oxidative mechanisms that lead to degenerative diseases. There are a number of clinical studies suggesting that the antioxidants in fruits, vegetables, tea and red wine are the main factors for the observed efficacy of these foods in reducing the incidence of chronic diseases including heart disease and some cancers.

An **antioxidant** is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols.

Free radicals are highly charged and active particles which are made of unstable molecules or atoms due to their single and unbalanced electrons. The common free radicals are oxygen

reactive species (ROS), namely, super oxide radical, hydroxyl radical, and peroxy radical which can be internally produced by cellular metabolism, inflammation by immune cells and externally by radiation, pharmaceuticals, hydrogen peroxide, toxic chemicals, smoke, alcohol, oxidized polyunsaturated fats and cooked food. They are unstable and through chain reaction can attack vital biomolecules (DNA, lipids, proteins) in cells and body fluids. They also weaken the cells in our bodies leaving us vulnerable to disorders and diseases such as arteriosclerosis, coronary heart disease, stroke, hypertension, emphysema, diabetes, cataracts, rheumatoid arthritis, nephritis, Alzheimer disease, cancer, AIDS, etc. Aging process is also a result of the oxidation by free radicals in the body. They are formed naturally, both internally by metabolism and externally by chemicals. These include alcohol consumption, drugs, toxic metals, emotional stress, smoking, pesticides, herbicides and air pollutants.

Fortunately, nature provides us with plenty of "protecting molecules" or the so called "antioxidants" which can trap or destroy free radicals and subsequently protect us from damage due to the oxidative stress.

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions.

Antioxidants are substances or nutrients in our foods which can prevent or slow the oxidative damage to our body. When our body cells use oxygen, they naturally produce free radicals (by-products) which can cause damage. Antioxidants act as "free radical scavengers" and hence prevent and repair damage done by these free radicals. Health problems such as heart disease, macular degeneration, diabetes, cancer etc are all contributed by oxidative damage.

1.7.2 Natural Antioxidants

There are two groups of natural antioxidants.

- The first group is our body enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase. Wheat and barley grain products are rich in SOD.
- The other group is nutrient antioxidants which are vitamin E, vitamin C and beta-carotene (the pre-form of vitamin A).

In addition, there are still numerous other antioxidants such as bioflavonoids, carotenoids (such as lutein and lycopene) and phenolic compounds. Selenium is also an important mineral antioxidant. Selenium is commonly found in onions, garlic, mushrooms, whole grain cereals, particularly in the wheat germ and rice bran.

1.7.3 The Antioxidant Process

Antioxidants block the process of oxidation by neutralizing free radicals. In doing so, the antioxidants themselves become oxidized. That is why there is a constant need to replenish our antioxidant resources. How they work can be classified in one of two ways:

1. Chain-breaking –

When a free radical releases or steals an electron, a second radical is formed. This molecule then turns around and does the same thing to a third molecule, continuing to generate more unstable products. The process continues until termination occurs -- either the radical is stabilized by a chain-breaking antioxidant such as beta-carotene and vitamins C and E, or it simply decays into a harmless product.

2. Preventive –

Antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase prevent oxidation by reducing the rate of chain initiation. That is, by scavenging initiating radicals, such antioxidants can thwart an oxidation chain from ever setting in motion. They can also prevent oxidation by stabilizing transition metal radicals such as copper and iron.

The effectiveness of any given antioxidant in the body depends on which free radical is involved, how and where it is generated, and where the target of damage is. Thus, while in one particular system an antioxidant may protect against free radicals, in other systems it could have no effect at all. Or, in certain circumstances, an antioxidant may even act as a "pro-oxidant" that generates toxic oxygen species.

1.7.4 Free Radicals, Antioxidants and Health

Prevention is better than cure. The more toxic chemicals which produce free radicals we obtain, the more antioxidants in our diet we have to consume for the detoxification. We should be healthy and free from disease by preventing our body from any oxidative damage. In order to protect our better health or slow our aging, we should carefully avoid those factors which increase the production of free radicals, and daily eat more fresh vegetables and fruits, at least one pound or 400 grams, as well as sufficient amounts of nuts, seeds and pulses.

Plant food contains a wide range of phytochemicals and antioxidants, which are protecting agents against free radicals. Beta-carotene is common in dark green, yellow, orange and red vegetables and fruits such as carrots, pumpkin, tomatoes, spinach, peppers, watercress, broccoli, cherries, peaches, papaya, watermelon and apricots. Several tropical fruits and vegetables are good sources of antioxidants.

- Vitamin C: Found in all fresh fruits and vegetables, particularly citrus fruits, melon, strawberries, leafy green vegetables and tomatoes.

- Vitamin E: Found in all whole-grain cereals including brown rice, oats, whole meal bread, wheat germ, soya beans, cold-pressed vegetable oils, nuts and seeds, parsley, broccoli and asparagus. In conclusion, if we can control free radicals, then we can reduce the degenerative diseases. Vegetarians have an advantage over non-vegetarians, because they eat a large variety of fresh vegetables, fruit and whole grains.

1.7.5 Types of Antioxidants

Examples of Antioxidants

Natural antioxidants	Synthetic antioxidants
Ascorbic acid, Gallic acid, Vitamin C& E, Coenzyme Q 10, Lipoic acid, Gluthatione	Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), Tert-butylhydroquinone Propyl gallate

1.7.6 Assay Methods to Evaluate Anti-oxidant Activity

1.7.6.1 Free Radical Scavenging Activity (DPPH Assay Method)

A rapid, simple and inexpensive method to measure antioxidant capacity involves the use of the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity.

DPPH(1,1-Diphenyl-2-picrylhydrazyl) is a dark-colored crystalline powder composed of stable free-radical with red color(absorbed at 517nm. DPPH is a well-known radical and a trap ("scavenger") for other radicals. Therefore, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. Because of a strong absorption band centered at about 520 nm, the DPPH radical has a deep violet color in solution, and it becomes colorless or pale yellow when neutralized. This property allows visual monitoring of the reaction, and the number of initial radicals can be counted from the change in the optical absorption at 520 nm or in the EPR signal of the DPPH.

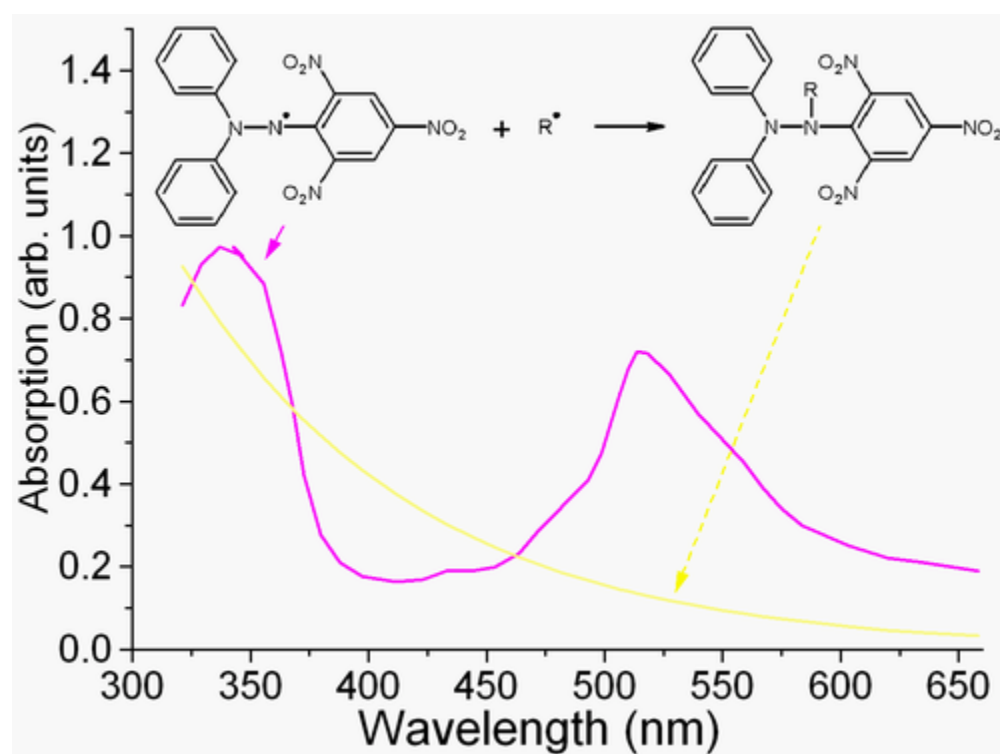


Fig 1.1: DPPH state change

1.7.6.2 Total Phenol Content

Phenolic compounds are important to human health because of their antioxidant activity. A sample if high in phenolics this could potentially be a source of phenolic compounds for use as natural antioxidants.

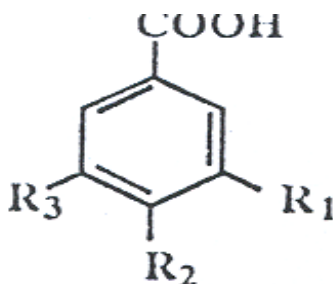
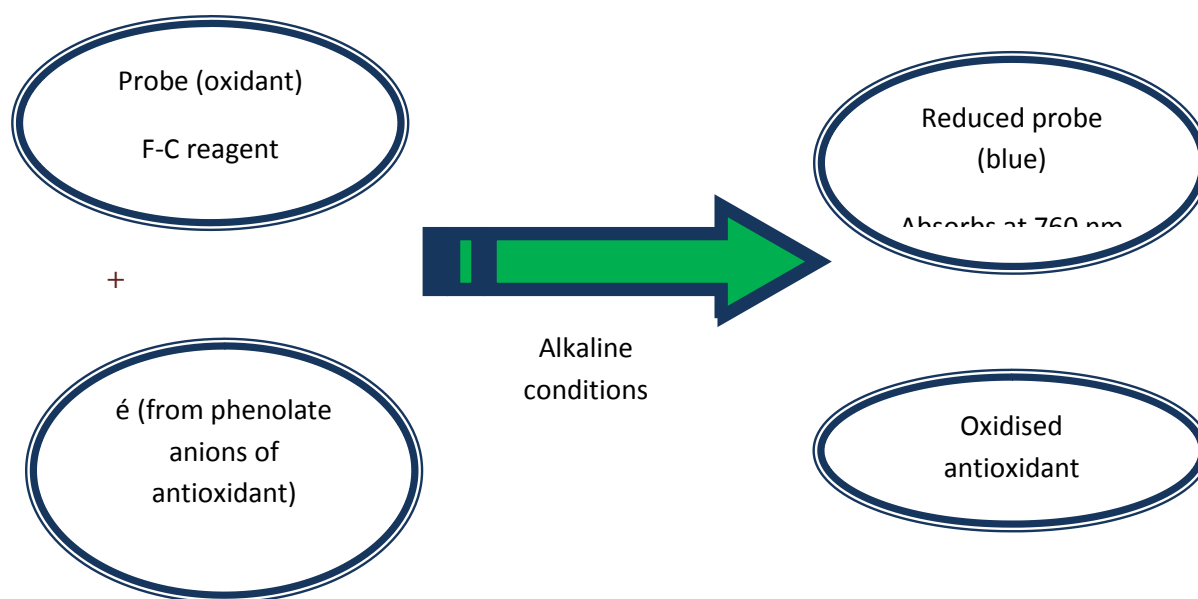
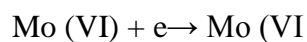


Figure 1.2: Phenolic antioxidants

Total Phenol Content can be measured by Folin-Ciocalteu method (Singleton & Rossi, 1965) where Folin – Ciocalteu reagent (FCR) is used to estimate the amount of phenolic hydroxyl groups based on the principle of electron transfer.



The FCR actually measures a sample's reducing capacity. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates molybdates. Sequences of reversible one or two electron reduction reactions lead to blue species, possibly $(\text{PMoW}_{11}\text{O}_{40})^{4-}$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI).



2.1 Antimicrobial activity of *Ganoderma lucidum* extract alone and in combination with some antibiotics.

Antimicrobial activity of GL (the aqueous extract from the carpophores of *Ganoderma lucidum* (F_R)K_{ARST}) was tested *in vitro* against Gram positive and Gram negative bacteria by serial broth dilution method, and the antimicrobial activity was expressed by minimal inhibitory concentration (MIC). Among fifteen species of bacteria tested, the antimicrobial activity of GL was the most potent against *Micrococcus luteus* (MIC, 0.75 mg/ml). To investigate the effects of antimicrobial combinations of GL with four kinds of antibiotics (ampicillin, cefazolin, oxytetracycline and chloramphenicol), the fractional inhibitory concentration index (FICI) was determined by checkerboard assay for each strain. The antimicrobial combinations of GL with four antibiotics resulted in additive effect in most instances, synergism in two instances, and antagonism in two instances. Synergism was observed when GL was combined with cefazolin against *Bacillus subtilis* and *Klebsiella oxytoca*. (Sang, Y.Y et al, 1994)

2.2 Anticancer Properties of *Ganoderma Lucidum* Methanol Extracts In Vitro and In Vivo.

Anticancer activities of various extracts of the medicinal mushroom, *Ganoderma lucidum*, have been widely demonstrated and are mainly associated with the presence of different bioactive polysaccharides and triterpenoids. We have evaluated and compared *in vitro* and *in vivo* the antitumor effects of two preparations from *Ganoderma lucidum*: a methanol extract containing total terpenoids (GLme) and a purified methanol extract containing mainly acidic terpenoids (GLpme). Both extracts inhibited tumor growth of B16 mouse melanoma cells inoculated subcutaneously into syngeneic C57BL/6 mice and reduced viability of B16 cells *in vitro*, whereby GLme exhibited stronger effect. Furthermore, anticancer activity of GLme was demonstrated for the first time against two other rodent tumor cell lines, L929-mouse fibrosarcoma and C6-rat astrocytoma. The mechanism of antitumor activity of GLme comprised inhibition of cell proliferation and induction of caspase-dependent apoptotic cell death mediated by upregulated p53 and inhibited Bcl-2 expression. Moreover, the antitumor effect of the GLme was associated with intensified production of reactive oxygen species, whereas their neutralization by the antioxidant, N-acetyl cysteine, resulted in partial recovery of cell viability. Thus, our results suggest that GLme might be a good candidate for treatment of diverse forms of cancers. (Ljubica, M. et al, 2009)

2.3 Cellular and Physiological Effects of *Ganoderma lucidum* (Reishi).

In Asia, a variety of dietary products have been used for centuries as popular remedies to prevent or treat different diseases. A large number of herbs and extracts from medicinal mushrooms are used for the treatment of diseases. Mushrooms such as *Ganoderma lucidum* (Reishi), *Lentinus*

edodes (Shiitake), *Grifola frondosa* (Maitake), *Hericium erinaceum* (Yamabushitake), and *Inonotus obliquus* (Chaga) have been collected and consumed in China, Korea, and Japan for centuries. Until recently, these mushrooms were largely unknown in the West and were considered “fungi” without any nutritional value. However, most mushrooms are rich in vitamins, fiber, and amino acids and low in fat, cholesterol, and calories. These mushrooms contain a large variety of biologically active polysaccharides with immunostimulatory properties, which contribute to their anticancer effects. Furthermore, other bioactive substances, including triterpenes, proteins, lipids, cerebrosides, and phenols, have been identified and characterized in medicinal mushrooms. This review summarizes the biological effects of *Ganoderma lucidum* upon specific signaling molecules and pathways, which are responsible for its therapeutic effects. (Sliva & Daniel, 2004)

2.4 Enhanced transformation of malachite green by laccase of *Ganoderma lucidum* in the presence of natural phenolic compounds.

In this study, investigated the efficacy of phenolic extract of wheat bran and lignin-related phenolic compounds as natural redox mediators on laccase-mediated transformation of malachite green (MG) using purified laccase from the white-rot fungus *Ganoderma lucidum*. *G. lucidum* laccase was able to decolorize 40.7% MG dye (at 25 mg l⁻¹) after 24 h of incubation. Whereas, the addition of phenolic extract of wheat bran enhanced the decolorization significantly ($p < 0.001$) by two- to threefold than that of purified laccase alone. Among various natural phenolic compounds, acetovanillone, *p*-coumaric acid, ferulic acid, syringaldehyde, and vanillin were the most efficient mediators, as effective as the synthetic mediator 1-hydroxybenzotriazole. Characterization of MG transformation products by HPLC, UV–Vis, and liquid chromatography-mass spectrometry-electrospray ionization analysis revealed that *N*-demethylation was the key mechanism of decolorization of MG by laccase. Growth inhibition test based on mycelial growth inhibition of white rot fungus *Phanerochaete chrysosporium* revealed that treatment with laccase plus natural mediators effectively reduced the growth inhibitory levels of MG than that of untreated one. Among all the tested compounds, syringaldehyde showed the highest enhanced decolorization, as a consequence reduced growth inhibition was observed in syringaldehyde-treated samples. The results of the present study revealed that the natural phenolic compounds could alternatively be used as potential redox mediators for effective laccase-mediated decolorization of MG. (Kumarasamy, M. et al,2009)

2.5 *Ganoderma lucidum*: A Potent Pharmacological Macrofungus.

Ganoderma lucidum (Ling Zhi) is a basidiomycete white rot macrofungus which has been used extensively as “the mushroom of immortality” in China, Japan, Korea and other Asian countries for 2000 years. A great deal of work has been carried out on therapeutic potential of *Ganoderma lucidum*. The basidiocarp, mycelia and spores of *Ganoderma lucidum* contain approximately 400

different bioactive compounds, which mainly include triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins/peptides and trace elements which has been reported to have a number of pharmacological effects including immunomodulation, anti-atherosclerotic, anti-inflammatory, analgesic, chemopreventive, antitumor, chemo and radio protective, sleep promoting, antibacterial, antiviral (including anti-HIV), hypolipidemic, anti-fibrotic, hepatoprotective, anti-diabetic, anti-androgenic, anti-angiogenic, anti-herpetic, antioxidative and radical-scavenging, anti-aging, hypoglycemic, estrogenic activity and anti-ulcer properties. *Ganoderma lucidum* has now become recognized as an alternative adjuvant in the treatment of leukemia, carcinoma, hepatitis and diabetes. The macrofungus is very rare in nature rather not sufficient for commercial exploitation for vital therapeutic emergencies, therefore, the cultivation on solid substrates, stationary liquid medium or by submerged cultivation has become an essential aspect to meet the driving force towards the increasing demands in the international market. Present review focuses on the pharmacological aspects, cultivation methods and bioactive metabolites playing a significant role in various therapeutic applications.(Sanodiya et al,2009)

2.6 Hepatoprotective Activity and the Mechanisms of Action of *Ganoderma lucidum* (Curt.:Fr.) P. Karst. (Ling Zhi, Reishi Mushroom) (Aphyllophoromycetidae) (Review)

Herbal medicines are always considered to be a safe and useful approach for the treatment of chronic hepatopathy. *Ganoderma lucidum* (Curt.:Fr.) P. Karst. [(Ling Zhi, Reishi mushroom) (Aphyllophoromycetidae)], a highly ranked medicinal mushroom in Oriental traditional medicine, has been widely used for the treatment of chronic hepatopathy of various etiologies. Data from *in vitro* and animal studies indicate that *G. lucidum* extracts (mainly polysaccharides or triterpenoids) exhibit protective activities against liver injury induced by toxic chemicals (e.g., CCl₄) and Bacillus Calmette-Guerin (BCG) plus lipopolysaccharide (LPS). *G. lucidum* also showed anti-hepatitis B virus (HBV) activity in a duckling study. Recently, a randomized placebo-controlled clinical study showed that treatment with *G. lucidum* polysaccharides for 12 weeks reduced hepatitis B e antigen (HBeAg) and HBV DNA in 25% (13/52) patients with HBV infection. The mechanisms of the hepatoprotective effects of *G. lucidum* have been largely undefined. However, accumulating evidence suggests several possible mechanisms. These include antioxidant and radical-scavenging activity, modulation of hepatic Phase I and II enzymes, inhibition of b-glucuronidase, antifibrotic and antiviral activity, modulation of nitric oxide production, maintenance of hepatocellular calcium homeostasis, and immunomodulating effects. *G. lucidum* could represent a promising approach for the management of various chronic hepatopathies. Further studies are needed to explore the kinetics and mechanisms of action of *G. lucidum* constituents with hepatoprotective activities.(Yihuai G. et al, 2003)

2.7 Polysaccharide purified from *Ganoderma lucidum* inhibits spontaneous and Fas-mediated apoptosis in human neutrophils through activation of the phosphatidylinositol 3 kinase/Akt signaling pathway.

Ganoderma lucidum has been widely used as a remedy to promote health and longevity in China. The polysaccharide component with a branched (1→3)-β-D-glucan moiety from *G. lucidum* (PS-G) has shown evidence of enhancement of immune responses and of eliciting anti-tumor effects. In this study, we investigated the effect of PS-G on neutrophil viability, which is manifested by spontaneous apoptosis. Annexin V staining and MTT assays reveal that PS-G is able to inhibit spontaneous and Fas-induced neutrophil apoptosis, and this effect of PS-G is enhanced by the presence of zVAD (a caspase inhibitor) and GM-CSF. The antiapoptotic effect of PS-G is diminished by the presence of wortmannin and LY294002 (two PI-3K inhibitors), but is not altered by PD98059 (a MEK inhibitor). Western blotting indicates the stimulating effect of PS-G on Akt phosphorylation and its inhibition of procaspase 3 degradation, which occurs in neutrophils undergoing spontaneous apoptosis or triggered death by Fas. Taken together, PS-G elicitation of antiapoptotic effects on neutrophils primarily relies on activation of Akt-regulated signaling pathways. (Ming-Jen H. et al, 2002)

2.8 Antifungal activity of a toothpaste containing Ganodermalucidum against *Candida albicans* -an in vitro study

Candida albicans is the most common oral fungus associated with oral candidial infections. Various antifungal agents are in use and the search is on for more agents showing anti candidial properties. Ganodermalucidum has been in use in Traditional Chinese Medicine for years. Literature supports the use of this *Ganoderma lucidum* as a medicinal mushroom for its antimicrobial, antiviral properties. Objectives: Varying concentrations of a toothpaste containing Ganodermalucidum was tested in vitro for its antifungal properties against *Candida albicans*. Method: The activity of a Ganoderma containing toothpaste against *Candida albicans* was tested by serial broth dilution method and was expressed by minimum inhibitory concentration (MIC). Results: The toothpaste exhibited antifungal properties against the tested organism. The MIC value of *Candida albicans* was found to be less than 02 mgm /ml. (Arati N. et al, 2010)

2.9 The effect of an extract from *Ganoderma lucidum* (reishi) on the labeling of blood constituents with technetium-99m and on the survival of *Escherichia coli*

This study evaluated effects of an aqueous extract of *Ganoderma lucidum* (reishi) on the labeling of blood constituents with technetium-99m (^{99m}Tc) and on the survival of cultures of *Escherichia coli* treated with stannous chloride. Blood samples from Wistar rats were treated with reishi extract, radiolabeling procedure was performed, plasma (P), blood cells (BC) and insoluble (IF) and soluble (SF) fractions of P and BC were separated. The radioactivity was counted for the determination of the percentages of radioactivity (%ATI). Cultures of *Escherichia coli* AB1157 were treated with stannous chloride in the presence and absence of reishi extract. Blood samples

and bacterial cultures treated with NaCl 0.9% were used as controls. Data indicated that reishi extract altered significantly ($p < 0.05$) the %ATI of P, BC, IF-P, SF-P, IF-BC and SF-BC, as well as increased the survival of bacterial cultures treated with stannous chloride. Our results suggest that reishi extract could present a redox/chelating action, altering the labeling of blood constituents with ^{99m}Tc and protecting bacterial cultures against oxidative damage induced by stannous chloride. (Qi-zhen C. et al, 2004)

2.10 Cellular and Molecular Mechanisms of Immuno-modulation by *Ganoderma lucidum*

Ganoderma lucidum (Leyss. ex Fr.) Karst. (*Lingzhi* or *Reishi*) has been used for a long time in China to prevent and treat various human diseases. *G. lucidum* polysaccharides extracted from *G. lucidum* are one of efficacious ingredient groups of *G. lucidum*. A number of reports have demonstrated that *G. lucidum* polysaccharides modulate immune function both in vivo and in vitro. The immuno-modulating effects of *G. lucidum* polysaccharides were extensive, including promoting the function of antigen-presenting cells, mononuclear phagocyte system, humoral immunity, and cellular immunity. Cellular and molecular mechanisms, possible receptors involved, and triggered signaling cascades have also been studied in vitro. However, whole animal experiments are still needed to further establish the mechanism of the immuno-modulating effects by *G. lucidum*. Evidence-based clinical trials are also needed. (Zhi-Bin L. 2005)

2.11 Hypoglycemic effect of *Ganoderma lucidum* polysaccharides

AIM: To investigate the hypoglycemic effect of *Ganoderma lucidum* polysaccharides (*Gl-PS*) in the normal fasted mice and its possible mechanism. **METHODS:** Normal fasted mice were given a single dose of *Gl-PS* 25, 50, and 100 mg/kg by ip and the serum glucose was measured at 0, 3, and 6 h after administration. *Gl-PS* 100 mg/kg were also given by ip and the serum glucose and insulin levels were measured at 0 min, 30 min, 1 h, 3 h, 6 h, and 12 h. Pancreatic islets were isolated and incubated with glucose 5.6 mmol/L and different concentration of *Gl-PS*, the insulin content of islets and insulin release were examined. The islets fluorescent intensity of $[\text{Ca}^{2+}]_i$ was also studied with a confocal microscope. Verapamil and egtazic acid were used to testify whether the insulin-releasing effect of *Gl-PS* was mediated by its ability to raise the Ca^{2+} influx. **RESULTS:** *Gl-PS* dose-dependently lowered the serum glucose levels at 3 h and 6 h after administration. *Gl-PS* 100 mg/kg raised the circulating insulin levels at 1 h after administration. *In vitro*, *Gl-PS* had no effect on islets insulin content, but it stimulated the insulin release after incubation with glucose 5.6 mmol/L. Confocal microscope showed that *Gl-PS* 100 mg/L had the capacity to raise the $[\text{Ca}^{2+}]_i$. The insulin-releasing effect of *Gl-PS* was inhibited by verapamil/egtazic acid. **CONCLUSION:** *Gl-PS* possesses the hypoglycemic effect on normal mice; one mechanism is through its insulin-releasing activity due to a facilitation of Ca^{2+} in flow to the pancreatic cells. (Hui-na Z. et al, 2004)

2.12 Effects of *ganoderma lucidum* on apoptotic and anti-inflammatory function in HT-29 human colonic carcinoma cells

Ling Zhi extract (LZE) is a herbal mushroom preparation which been used world wide for the prevention and treatment of various cancers. The current study was designed to evaluate these claims in human colon cancer cells in terms of cancer preventive mechanisms. Results have demonstrated induction of apoptosis, anti-inflammatory action and differential cytokine expression during induced inflammation in the human colonic carcinoma cell line, HT -29. LZE caused no cytotoxicity in HT-29 cells at doses less than 10 000 µg/ml. Increasing concentrations of LZE reduced prostaglandin E₂ production, but increased nitric oxide production. LZE treatment induced apoptosis by increasing the activity of caspase-3. RT-PCR showed that LZE at a concentration of 5000 µg/ml decreased the expression of cyclooxygenase-2 mRNA. Among 42 cytokines tested by protein array in this study, supplementation of LZE at doses of 500 and 5000 µg/ml to HT-29 cells reduced the expression of interleukin-8, macrophage inflammatory protein 1-delta, vascular epithelial growth factor, and platelet-derived growth factor. These results suggest that LZE has pro-apoptotic and anti-inflammatory functions, as well as inhibitory effects on cytokine expression during early iflammation in colonic carcinoma cells, which may be of significance in the use of Chinese herbal alternative medicines for cancer prevention. Copyright © 2004 John Wiley & Sons, Ltd.(Kee-jong H. et al, 2004)

2.13 Anti-Inflammatory and Anti-Tumor-Promoting Effects of Triterpene Acids and Sterols from the Fungus *Ganoderma lucidum*

A series of lanostane-type triterpene acids, including eleven lucidenic acids (3, 4, 9, 10, 13–19) and six ganoderic acids (20–22, 24, 26, 27), as well as six sterols (28–33), all isolated from the fruiting bodies of the fungus *Ganoderma lucidum*, were examined for their inhibitory effects on the induction of *Epstein–Barr* virus early antigen (EBV-EA) by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in *Raji* cells, a known primary screening test for anti-tumor promoters. All of the compounds tested, except for ganolactone (27) and three sterols (29–31), showed potent inhibitory effects on EBV-EA induction, with *IC*₅₀ values of 235–370 mol ratio/32 pmol TPA. In addition, nine lucidenic acids (1, 2, 5–8, 11, 12, 18) and four ganoderic acids (20, 23–25) were found to inhibit TPA-induced inflammation (1 µg/ear) in mice, with *ID*₅₀ values of 0.07–0.39 mg per ear. Further, 20-hydroxylucidenic acid N (18) exhibited inhibitory effects on skin-tumor promotion in an *in vivo* two-stage mouse-skin carcinogenesis test based on 7,12-dimethylbenz[*a*]anthracene (DMBA) as initiator, and with TPA as promoter.(Toshihiro A. et al, 2007)

In this study cold extraction method was followed:

450gm of powdered material was taken in 3 clean, conical flask and soaked in 1.5 liter of Methanol. The container with its contents was sealed with aluminum foil and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a series of filtrations and it was done in four steps:

1. **Step 1:** First it was filtered with a piece of clean, white, pure cotton material. After the filtrate was collected, the residue on the cloth was squeezed to collect the remaining extract.
2. **Step 2:** Then the filtrate was filtered using absorbent cotton.
3. **Step 3:** The same step as repeated using fresh cotton
4. **Step 4:** Finally the filtrate was filtered through Whatman filter paper.



Fig 3.1: Sample dissolved in Methanol.



Fig 3.2: Filtration of the sample

After filtration evaporation was followed.

The filtrate (Methanolic extract) obtained was evaporated by Rotary evaporator (Bibby RE-200, Sterilin Ltd., UK) at 4 rpm and at 65°C temperature. It rendered a gummy concentrate of chocolate black color. The gummy concentrate was designated as crude extract or methanolic extract. Then the crude methanolic extract was dried by heat in water bath and preserved it.

Imp. Note: - Moisture content was measured after the filtrate obtained.

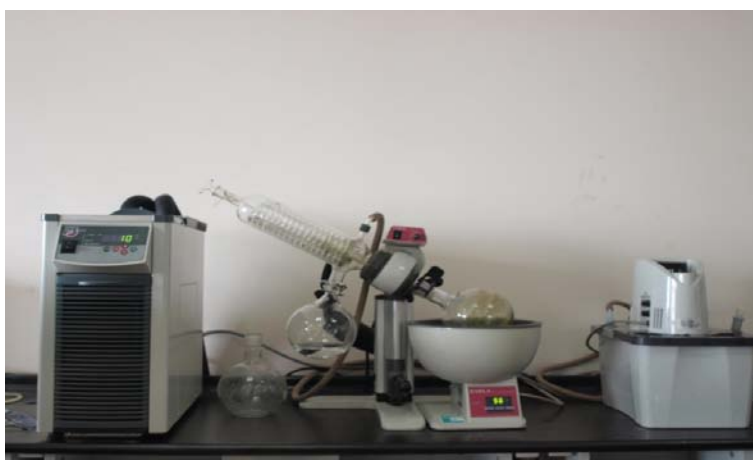


Fig 3.3: Ethanol evaporated using Rotary Evaporator



Fig 3.4: Extract obtained after evaporation

3.2 PHYTOCHEMICAL INVESTIGATION

3.2 Phytochemical Investigation

Presence of different chemical groups present in the extract represents the preliminary phytochemical screening. For phytochemical screening, 20 ml of the methanolic extracts of *Ganoderma Lucidum* were used before it was evaporated. The extracts were transferred in 50 ml beakers respectively using pipettes for the extracts with proper labeling. Then 10 clean test tubes were taken in two test tube racks and labeled with the name of the extracts & test names to prevent confusion. Phytochemical screening was performed using standard procedures and specific reagents were used for the different chemical group test. (Mojab F. et al, 2003)

3.2.1 Apparatus and Instruments

Glass rod	Dropper
Electronic Balance	Vortex
Water bath	Test tube
Filter paper	Micropipette
Funnel	Pipette
Conical flask	Beaker

3.2.2 Methods of Different Phytochemical Test

1. Test for Alkaloids

Preparation of Wagner's Reagent

1.27 gm of iodine and 2 gm of potassium iodide (KI) were weighed accurately in an electronic balance and dissolved in 5ml of water, and finally made it 100 ml with distilled water.

Procedure

1. 200mg of methanolic extracts of *Ganoderma Lucidum* were weighed accurately and dissolved in 10 ml of methanol and then filtered using filter paper
2. One thousand microlitres (1ml) of the filtrate was then mixed with 6 drops of Wagner's reagent

Identification

1. Creamish, brownish-red or orange precipitate indicated the presence of alkaloids
2. A sign (+) denotes low concentration if addition of the reagent produce a faint turbidity
3. A sign (++) denotes moderate concentration if the addition if reagent produce light opalescence precipitate
4. A sign (++++) denotes high concentration if the addition of reagent produce yellowish-white precipitate

2. Test for Tannins

Procedure

1. About 0.5 g of the extracts were boiled in 10 ml of water in a test tube and then filtered
2. A few drops of 0.1% ferric chloride was added and observed for nongreenish black coloration and precipitation.

Identification

1. A sign (+) denotes lower level of tannins when a slight precipitate was observed
2. A sign (++) denotes moderate level of tannins when a medium precipitate was observed
3. A sign (+++) denotes higher level of tannins when a heavy precipitate was observed

3. Test for Gum & Carbohydrates

Procedure

1. Several tests are present to determine carbohydrates, Barford's test, Molisch's test, Seliwanoff's test, Bial's test, Benedict's test and Iodine/Potassium iodide test
2. In the present study Molisch's test was performed to determine the presence of carbohydrates. Molisch's test (named after Austrian botanist Hans Molisch) is a sensitive chemical test for the presence of carbohydrates, based on the dehydration of the carbohydrate by sulfuric acid to produce an aldehyde, which condenses with two molecules of phenol resulting in a red-or-purple-colored compound

Identification

1. All carbohydrates should give a positive reaction. All carbohydrates such as monosaccharide's gives a rapid positive test(60%-70%) where as disaccharides(40%) and polysaccharides(15%) react slowly with Molisch's test
2. Either of the aldehydes if present will condense with two molecules of naphthol to form a purple colored product

4. Test for Steroids

Procedure

1. 0.12 to 1 ml concentrated H₂SO₄ was added at the side of the test tube containing the methanol extract

Identification

1. Reddish brown colored ring forms in some samples indicating the presence of steroids

5. Test for Flavonoids

Procedure

1. 0.1-1ml of conc. HCl was added with 2.5ml of the filtrate of the plant extracts.

Identification

1. An immediate red coloration indicates the presence of flavonoids
2. The yellow coloration disappeared on standing.
3. A sign (+) denotes low conc. of flavonoids if pale yellow color was observed
4. A sign (++) denotes moderate conc. of flavonoids if moderate color was observed
5. A sign (+++) denotes high conc. of flavonoids if high color was observed

6. Test for Saponins

Procedure

1. To 1ml of the extracts were added 10 ml of distilled water in a test tube
2. The solutions were shaken vigorously and observed for a stable persistent froth

3. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which Foam was formed which was unstable. So, saponins were absent

Identification

1. A sign (+) denotes low concentration of saponins when the froth reached a height of 0.5cm
2. A sign (++) denotes moderate concentration of saponins when the froth reached a height of 0.6- 1 cm
3. A sign (+++) denotes high concentration of saponins when the froth reached a height of more than 1 cm

7. Test for Terpenoids

Procedure (Salkowski Test)

1. In 0.5 gm of extracts, 2ml of dichloride methen was added
2. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer

Identification

1. Reddish- brown coloration at the interface indicates the presence of terpenoids
2. A sign (+) denotes when a faint reddish brown coloration was observed
3. A sign (++) denotes when a medium reddish brown coloration was observed
4. A sign (+++) denotes when a deep reddish brown coloration was observed.

3.3 Antimicrobial Screening Study

3.3 Antibacterial Screening:

The antibacterial assay was performed by disc diffusion technique (Bauer AW et al, 1988) ^[21]. Disc diffusion technique is highly effective for rapidly growing microorganisms.. 0.50g or 500 mg and 0.75g or 750mg of sample (methanol extracts of *Ganoderma Lucidum*) was dissolved in 10ml of methanol solvents to prepare stock solutions respectively. The concentration of sample solution 50 mg/ml and 75mg/ml (i.e. each μl contain 50 μg and 75ug of sample)(**Sang YY et al, 2003**). 20 μl of such solution was applied on sterile disc (5mm diameter, filter paper) and allowed to dry off the solvent in an aseptic hood. Thus, such discs contain 1000 μg and 1500ug of crude extracts. To compare the activity with standard antibiotics, Kanamycin (30 $\mu\text{g}/\text{disc}$) and vancomycin(30 $\mu\text{g}/\text{disc}$) was used. The extract of *Ganoderma Lucidum* was tested against six Gram-positive and six Gram-negative (*Bacillus megaterium*, *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, *Sarcina lutea*, *Betahemolytic streptococcus*, and *Salmonella typhi*, *Salmonella paratyphi*, *Escherichia coli*, *Pseudomonas areus* ,*Vibrio parahemolyticus*, *Vibrio mimicus*) bacteria and one fungi *Candida albican* .

Briefly, in this study the test discs and standard disc were placed in a Petri dish seeded with particular bacteria and then left in a suitable container for extraction. The Petri dishes were then incubated at 37°C for overnight to allow the bacterial growth. The antibacterial activities of the extracts were then determined by measuring the respective zone of inhibition in mm.

3.3.1 Test materials used for the study:

- i) The methanolic crude extracts of *Ganoderma Lucidum* for the investigation of antimicrobial activity.
- ii) Solvent (methanol) were used for dissolving the compounds.
- iii) Kanamycin, K-30 (30 $\mu\text{g}/\text{disc}$) and Vancomycin, VA-30 (30 $\mu\text{g}/\text{disc}$) as standard disc.

3.3.2 Reagents:

- i) Rectified spirit
- ii) Agar purified powder
- iii) Methanol

3.3.3 Apparatus:

- i) Filter paper discs (Sterilized)
- ii) Petridishes
- iii) Inoculating loop
- iv) Sterile cotton
- v) Test tubes
- vi) Sterile forceps
- vii) Micropipette
- viii) Electric balance(4 digits)
- ix) Nose mask and hand gloves
- x) Spirit burner and match box
- xi) Laminar air flow unit
- xii) Incubator
- xiii) Refrigerator
- xiv) Autoclave

3.3.4 Test organism used for the study:

12 Pathogenic bacteria were selected for the test, 6 of which were Gram-negative and the remaining 6 were Gram-positive and one fungi. The bacterial strains used for this investigation are listed in the following table:

Table 3.1: List of the test pathogenic Bacteria and Fungi:

Serial No.	Name of the test organism
Gram Positive	
1	<i>Staphylococcus aureus</i>
2	<i>Bacillus megaterium</i>
3	<i>Bacillus subtilis</i>
4	<i>Bacillus cereus</i>
5	<i>Betahemolytic streptococcus</i>
6	<i>Sarcina lutea</i>
Gram negative	
7	<i>Salmonella typhi</i>
8	<i>Salmonella paratyphi</i>
9	<i>Vibrio parahemolyticus</i>
10	<i>Vibrio mimicus</i>
11	<i>Eschericia coli</i>
12	<i>Pseudomonas areus</i>
Fungi	
13	<i>Candida albicun</i>

3.3.5 Sterilization procedures:

The antibacterial screening was carried out in a laminar air flow unit and all types of precautions were highly maintained to avoid any type of contamination during the test. UV light was switched on for half an hour before working in the laminar hood to avoid any accidental contamination. Petridishes and other glass wares were sterilized in the autoclave at

121⁰C temperature and a pressure of 15 lbs./sq. inch for 15 minutes. Micropipette tips, Culture media, cotton, forceps, blank disks etc. were also sterilized .

3.3.6 Culture media:

For demonstrating the antibacterial activity and subculture of the test organism's agar purified powder media was used.

3.3.7 Preparation of the nutrient agar medium:

The agar purified powdered was weighed and then reconstituted with distilled water in a conical flask according to specification (agar purified powdered 5.6 g for 200 ml of distilled water). It was then heated in a water bath to dissolve the agar until a transparent solution was obtained.

3.3.8 Preparation of fresh culture of the pathogenic organisms:

The nutrient agar medium was prepared and dispersed in a number of test tubes to prepare slants (5 ml in each test tube). The test tubes were plugged with cotton and sterilized in the autoclave at 121⁰C temperature and a pressure of 15 lbs./sq. inch for 15 minutes. After sterilization, the test tubes were kept in an inclined position for solidification. These were then incubated at 37⁰ C to ensure sterilization. The test organisms were transferred to the agar slants from the supplied pure cultures with the help of an inoculating loop in a aseptic condition. The loop was burned after each transfer of microorganism to avoid contamination very carefully. The inoculated slants were then incubated at 37⁰ C for 24 hours to assure the growth of test organisms. These fresh cultures were used for the sensitivity test.

3.3.9 Preparation of the test plates:

The test plates were prepared according to the following procedure.

- A. The nutrient agar medium prepared in the previous section was pored in 10 ml quantity in each in the clean test tubes and plugged with the cotton.

- B. The test tubes and a number of petridishes were sterilized in an autoclave at 121⁰C temperature and a pressure of 15 lbs. /sq. inch for 60 minutes and were transferred into a laminar air flow unit and then allowed to cool to about 45 to 50⁰C.
- C. The test organisms were transferred from the fresh subculture to the saline water containing 1 ml with the help of an inoculating loop in an aseptic condition. Then shaken by rotation to get a uniform suspension of the organism.
- D. The bacterial suspensions were immediately transferred to the sterile petridishes in an aseptic area. The petridishes were rotated several times, first clockwise and then anticlockwise to assure homogenous distribution of the test organisms. The media were poured into petridishes in such a way that to give a uniform depth of approximately 4 mm.
- E. Finally, after medium was cooled to room temperature in laminar air flow unit, it was in incubator (37⁰C).

3.3.10 Preparation of the discs containing samples:

For the Preparation of the discs containing samples, following procedures were used.

a) Sample discs

Sterilized filter paper discs (5 mm in diameter) were taken by the forceps in the plates. Sample solutions of desired concentrations (1000 µg/disc and 1500 µg/disc) were applied in the disc with the help of the micropipette in an aseptic condition. These discs were left for a few minutes in aseptic condition for complete removal of the solvent.

b) Standard discs

These were used to compare the antibacterial activity of the test material. In the present study, ready-made Kanamycin, K-30 discs and Vancomycin, VA-30 disc containing 30 µg/disc of antibiotic were used as a standard disc for comparison purpose.

3.3.11 Placement of the disc and incubation:

For the placement of the discs, the following procedures were utilized:

- i) By means of a pair of sterile forceps, the sample impregnated discs and standard disc were placed gently on the solidified agar plate seeded with the test organisms to insure contact with the medium.
- ii) Finally, the plates were incubated at 37⁰ C for 24 hours in an incubator.

3.3.12 Precaution:

The discs were placed in such a way that they were not closer than 15 mm to the edge of the plate and far enough apart to prevent overlapping the zones of inhibition.

3.4 Cytotoxicity Studies

3.4.1 : Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is a recent development in the bioassay for the bioactive compounds, which indicates cytotoxicity as well as a wide range of pharmacological activities (e.g. anticancer, antiviral, insecticidal, pesticidal, AIDS etc) of the compounds. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a higher dose. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, antimicrobial and pharmacological activities of natural products. Natural products extracts, fractions or pure compounds can be tested for their bioactivity by this method. Here in vitro lethality of a simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening an fractionation in the discovery of new bioactive natural products, Generally the median effective dose (ED₅₀) values for cytotoxicities are one tenth (1/10) of median lethal dose (LD₅₀) values in the brine shrimp test (Meyer B.N. et al, 1982)

3.4.2 Principle

Brine shrimp eggs are hatched in simulated sea water to get nauplii. Test samples are prepared by the addition of calculated amount of DMSO (dimethyl sulfoxide) for obtaining desired concentration of test sample. The nauplii are counted by visual inspection and are taken in vials containing 5 ml of sea water. The samples of different concentrations are added to pre-marked vials with a micropipette. The vials are left for 24 hours and then nauplii are counted again to find out the cytotoxicity of the test agents. These data are processed in a simple program for profit analysis to estimate LC₅₀ values with 95% confidence intervals for statistically significant comparisons of potencies.

3.4.3 Test materials

- i) *Artemia salina* Leach (brine shrimp eggs)
- ii) Sea salt non ionized NaCl)
- iii) Small tank with perforated deviding dam to hatch the shrimp
- iv) Lamp to attract the nauplii

- v) Pipette (1 ml and 5 ml)
- vi) Micropipette (5-50 μ l & 10 to 100 μ l)
- vii) Glass vials (5ml)
- viii) Magnifying glass
- ix) Test sample for experimental plants

3.4.4 Procedure

3.4.4.1 Preparation of the simulated sea water

37 grams sea salt (nonionized NaCl) was weighted accurately, dissolved in 1 liter of sterilized distilled water and then filtered to get clear solution. The p^H of the sea water was maintained between 8.5 using 1N NaOH solution.

3.4.4.2. Hatching of brine shrimp eggs

Artemia salina Leach (brine shrimp eggs) collected from the pet shop was used as the test organism. Simulated sea water was taken in the small tank and the shrimp eggs (1.5 gm /L) were added to one side of the tank and this side was covered. The shrimps were allowed to one side of tank and this side was covered. The shrimp were allowed for two days to hatch and mature as nauplii (larvae). Constant oxygen supply was carried out during the hatching time. The hatched shrimps were attracted to the lamp on the other side of the divided tank through the perforated dam. These nauplii were taken for this bioassay.

3.4.4.3 Preparation of the sample solution

Clean test tubes were taken. These test tubes were used for different concentration (one test tube for each concentration) of test samples.

5 mg of methanol extracts (*Ganoderma Lucidum*) were accurately weighed and dissolved in 1000 μ l or 1 ml DMSO (dimethyl sulfoxide) in different beaker. Thus a concentration of 5 mg/ml was obtained which used as a stock solution .

From this stock solution 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml and 25 µg/ml were taken in five test tubes respectively each containing 5ml sea water and 10 nauplii.

3.4.4.4 Preparation of control group:

Control groups are used in cytotoxicity study to validate the test method and ensure that the results obtained are only due to the activity of the test agent and the effects of the other possible factors are nullified. Usually two types of control groups are used.

- i) Positive control
- ii) Negative control

3.4.4.5 Preparation of the positive control group:

Positive control in cytotoxicity study is a widely accepted cytotoxic agent and the result of the test agent is compared with the result obtained for the positive control. In the present study, vincristine sulphate was used as the positive control. 3 mg of vincristine sulphate was dissolved in 1.8 ml of distilled water to get a concentration of 5 mg/ml. This was used as stock solution of vincristine sulphate. With the help of a micropipette 10, 50, 100, 150 and 200 µl of the stock solution were transferred in 5 different vials. NaCl solution (brine water) was added to each vial making the volume up to 5 ml. The final concentration of vincristine sulphate in the vials became 10, 50, 100, 150 and 200 µg/ml respectively.

3.4.4.6 Preparation of the negative control group:

100 µl of distilled water was added to each of the three remarked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimp nauplii in these vials show a rapid mortality rate, then the test is considered as in valid as the nauplii died due to some reason other than the cytotoxicity of the samples.

3.4.4.7 Application of brine shrimp nauplii

With the help of the Pasteur pipette 10 living nauplii were added to each of the vials containing 5 ml of simulated sea water. A magnifying glass was used for convenient count of nauplii. If the counting of the 10 nauplii was not be possible accurately, then a variation in counting from 9-11 table might be allowed.

3.4.4.8 Counting of the nauplii

After 24 hours, the vials are observed using a magnifying glass and the number of survival nauplii in each vial were counted and recorded. From this data, the percentage of mortality of nauplii was calculated for each concentration of the sample. The median lethal concentration (LC50) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration.

Cytotoxicity, expressed as LC50 was calculated using the formula:

$\text{Log LC50} = \frac{\text{Log Concentration A} + \text{Log Concentration B}}{2}$

$\text{Log Concentration A} = 50\% - \frac{M}{N} - M$

Where concentration A is the concentration before LC50,

concentration B is the concentration after LC50,

M is the % killed before LC50, and

N is the % killed after LC50.

3.5 ANTI-OXIDANT ACTIVITY STUDY

3.5.1 Free Radical Scavenging Activity Test by DPPH Method

3.5.1.1 Principle

DPPH is a reactive free radical that acts as an electron acceptor (oxidant/ oxidizing agent) and causes oxidation other substances. On the other hand, antioxidants act as electron donors (reductant/ reducing agent). Antioxidants neutralize DPPH by being oxidized themselves. DPPH is found as dark-colored crystalline powder composed of stable free-radical molecules and forms deep violet color in solution. The scavenging of DPPH free radical (neutralization) is indicated by the deep violet color being turned into pale yellow or colorless. (Brand W.W. et al, 1995)

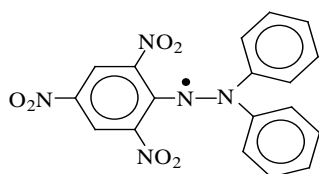


Fig3.16:1,1-diphenyl-2-

picrylhydrazyl

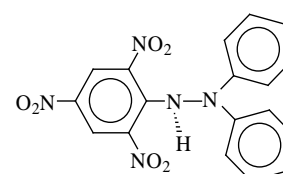
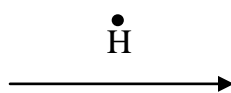


Fig 3.17:1,1-diphenyl-2

picrylhydrazine

1. Chemicals

1. 1,1-diphenyl-2-picrylhydrazyl (DPPH)
2. Ascorbic acid
3. Ethanol

4. Instruments and Apparatus

1. Electronic balance
2. Funnel
3. Test tube-5
4. Volumetric flask-3
5. Micropipette
6. Sonicator
7. Incubator
8. UV-Spectrophotometer

3.5.1.2 Procedure

1. Preparation of DPPH Solution

0.004gm of DPPH was taken into a 10ml Of volumetric flask and adjusted the volume with methanol.

2. Preparation of standard solutions

0.004 gm ascorbic acid was dissolved in 40 ml distilled water to prepare the standard solution for the experiments. The concentration of standard solution is 100 μ g/ml. From this solution, different required concentrations for different tests were prepared by serial dilution.

3. Preparation of Extract Solution

0.004gm of plant extract(*Ganoderma Lucidum*) was dissolved into 40ml of Methanol. The concentration is 100 μ g/ml. This is called the stock solution. From this solution, different required concentrations were prepared by serial dilution.

3.5.1.3 Different Concentration of Extract:

1. 1000 µg/100µl
2. 800 µg/100µl
3. 600 µg/100µl
4. 400 µg/100µl
5. 200 µg/100µl

Taken the Absorbance

1. Then the absorbance of the solution was measured at 517nm using a spectrophotometer (Shimadzu, UV – 1601 PC) against blank/ control.
2. A typical blank solution contains 3ml reagent solution and the appropriate volume 300µl of the same solvent used for the sample and it was incubated under the same conditions as the rest of the sample solution.

Formula of the calculation

The percentage scavenging activity of the extract was calculated using the formula:

$$\% \text{ scavenging activity} = \{(A_0 - A_1) / A_0\} * 100$$

Where,

A_0 is the absorbance of the control and

A_1 is the absorbance of the extract.

$$\% \text{ Inhibition} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of Control}}\right) \times 100$$

IC_{50} is the concentration at which 50% of the total DPPH free radical is scavenged/ neutralized and can be determined by linear regression method from plotting % inhibition against corresponding concentration

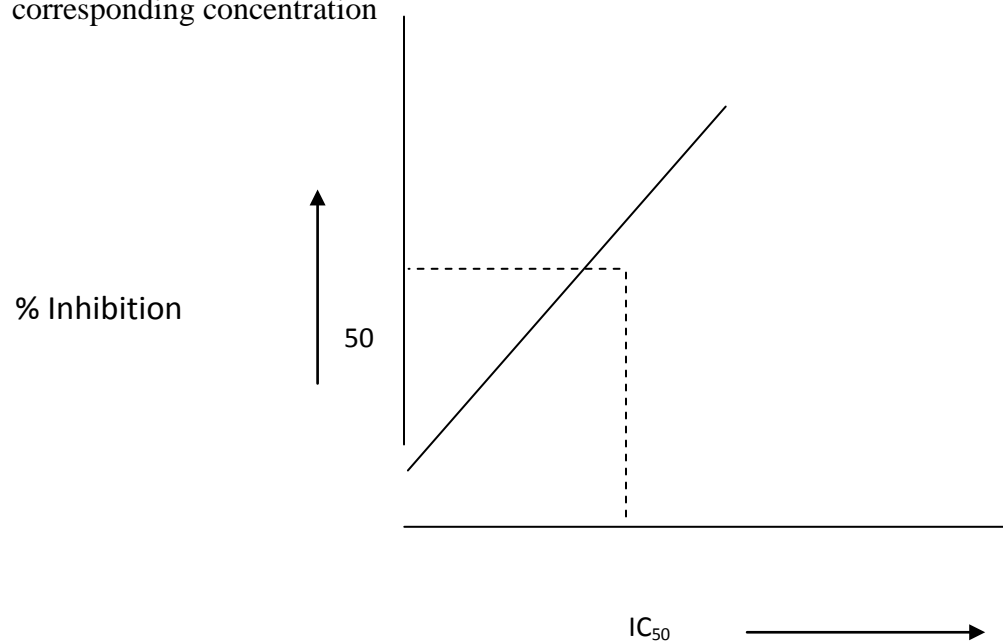


Fig 3.16: Diagram of IC_{50} value



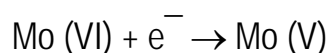
Fig 3.17: Anti-oxidant activity test by DPPH method

3.5.2 Determination of total phenolics content

Total phenolic content of *Genoderma lucidum* was determined employing the method as described by (Singleton *et al*,1965) involving Folin-Ciocalteu reagent as oxidizing agent and Gallic acid as standard.

Principle:

The content of total phenolic compounds of different fractions in the plant was determined by Folin–Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates - molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(\text{PMoW}_{11}\text{O}_{40})_4$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI):



Reagents:

- ✓ Folin – ciocalteu reagent
- ✓ Sodium carbonate (Na_2CO_3)
- ✓ Methanol
- ✓ Galic acid
- ✓ Ascorbic acid(Reagent grade)

Apparatus:

- ✓ Micropipette (10-100 μl)
- ✓ Pipette (1-10 ml)
- ✓ UV-spectrophotometer

Experimental procedure:

1. 1 ml of plant extract or standard of different concentration solution was taken in a test tube.

2. 5 ml of Folin – Ciocalteu (Diluted 10 times with water) reagent solution was added into the test tube.
3. 4 ml of Sodium carbonate (7.5%) solution was added into the test tube.
4. In case of standard, The test tube was incubated for 30 minutes at 20⁰C while test tube containing samples were incubated for 1hr at same temperature to complete the reaction.
5. Then the absorbance of the solution was measured at 765 nm using a spectrophotometer against blank.
6. A typical blank solution contained all reagents except plant extract or standard solution.
7. The total content of phenolic compounds in plant methanol extract and in different fractionates in gallic acid equivalents (GAE) was calculated by the following formula

$$C = (c \times V)/m$$

Where,

C = total content of phenolic compounds, mg/g plant extract, in GAE;

c = the concentration of galic acid established from the calibration curve, mg/ml;

V = the volume of extract,ml;

m = the weight of pure plant methanolic extracts, gm;

CHAPTER 4

RESULTS

4.1 Result of Phytochemical Analysis**Table4.1:** Qualitative analysis of the phytochemicals of *Ganoderma lucidum*

<i>Plant inextract</i>	<i>Tannins</i>	<i>Flavonoids</i>	<i>Saponins</i>	<i>Gums carbohydrates</i>	<i>Steroids</i>	<i>Alkaloids</i>	<i>Terpenoids</i>
Methanolic extract of <i>Ganoderma Lucidum</i>	++	+	+++	+++	++++	+++	+++

Symbol (+++) indicates presence in high concentration, Symbol (++) indicates presence in moderate concentration, and (+) indicates low concentration of the respective phytochemical.

4.2: Result of Antibacterial Screening :

4.2.1 The Results of Antibacterial Screening

The extract of methanol was active against all the test organisms except .The methanol extract (1000 µg/disc 1500ug/disc) of *Ganoderma Lucidum* showed antibacterial activity against **Gram positive** and **Gram negative bacteria**. and antifungal activity against *Candida albican*.

Table 4.2:The Antibacterial activity (in vitro) of Methanol extract of *Ganoderma Lucidum* and standard Kanamycin discs.

Serial No.	Name of the test organisms	Diameter of the zone of inhibition (in mm)	
		Methanol extract (1000 µg/disc)	Kanamycin disc (30 µg/disc)
Gram positive bacteria			
1	<i>Bacillus cereus</i>	10	30
2	<i>Bacillus megaterium</i>	8.0	26
3	<i>Betahemolytic streptococcus</i>	8.0	25
Gram negative bacteria			
6	<i>Salmonella paratyphi</i>	8.0	33
8	<i>Escherichia coli</i>	9.0	36

Table 4.3: The Antibacterial activity (*in vitro*) of Methanol extract of *Ganoderma Lucidum* and standard Vancomycin discs.

Serial No.	Name of the test organisms	Diameter of the zone of inhibition (in mm)		
		Methanol extract (1000 µg/disc)	Methanol extract (1500 µg/disc)	Vancomycin disc (30 µg/disc)
Gram positive bacteria				
1	<i>Sarcina lutea</i>	10	8.0	30
2	<i>Bacillus megaterium</i>	11	12.5	30
3	<i>Bacillus subtilis</i>	12	12	30
4	<i>Staphylococcus aureus</i>	9.5	9.5	34
Gram negative bacteria				
5	<i>Pseudomonas areus</i>	8	9.0	28
6	<i>Salmonella typhi</i>	6	7	17.5
7	<i>Salmonella paratyphi</i>	11	13	22
8	<i>Escherichia coli</i>	13	15	29
9	<i>Vibrio parahemolyticus</i>	10	10.5	20
10	<i>Vibrio mimicus</i>	8.0	10	13.5

Table 4.4: The Fungal activity (*in vitro*) of Methanol extract of *Ganoderma Lucidum* and standard Vancomycin discs.

1	<i>Candida albican</i>	9	11.5	21
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4.3: Result of Cytotoxic Activity:

4.3.1: Brine Shrimp Lethality Bioassay

The results of brine shrimp lethality bioassay are shown in the table 3.5. Test samples showed different mortality rate at different concentration. The mortality rate of brine shrimp nauplii was found to be increased with the increase with the concentration of the sample. The median lethal concentration (LC₅₀) was calculated. The LC₅₀ values of methanol extract of *Ganoderma lucidum* are 1.428 µg/ml.

So, it is evident that the methanol extract of *Ganoderma lucidum* was cytotoxic as well as biologically active.

Table 4.5: Effect of *Ganoderma lucidum* (methanol extract) on shrimp nauplii.

Conc. µg/ml	Log C	No. of nauplii taken	No. of nauplii dead	% mortality	LC50 µg/ml	Value of x
0	0	10	0	0	1.428	0.154
25	1.398	10	3	30		
50	1.699	10	4	50		
100	2.000	10	8	80		
200	2.301	10	10	100		
400	2.602	10	10	100		

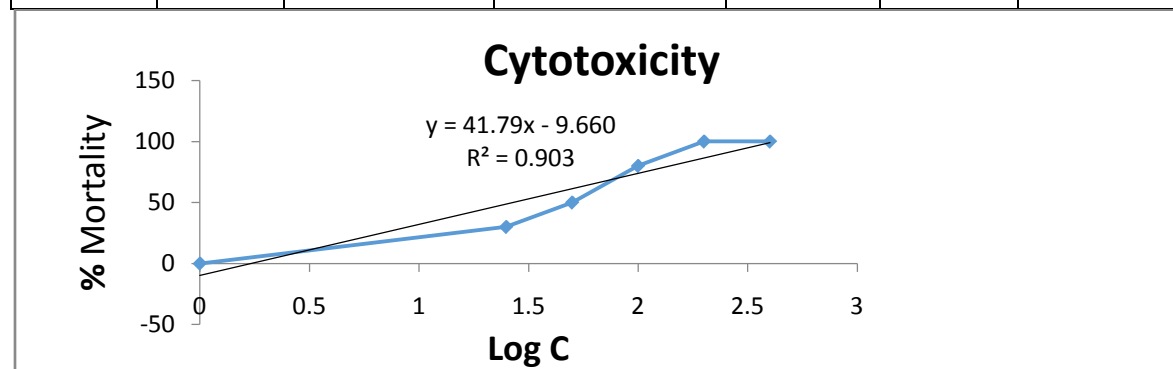


Fig-

4.1: Effects of various concentrations of Methanolic extract of *Ganoderma lucidum* on the viability of brine shrimp nauplii after 24 hrs of incubation.

4.4: Results of the Antioxidant activity by DPPH method:

Table 4.6: Free Radical Scavenging Activity of Standard Ascorbic Acid

Sample	Conc.($\mu\text{g/ml}$)	Mean % DPPH scavenging activity	IC50 Value($\mu\text{g/ml}$)
Standard(Ascorbic Acid)	200	95.65	12.6 $\mu\text{g/ml}$
	400	95.89	
	600	96.14	
	800	96.37	
	1000	96.59	

Table 4.7: Free Radical Scavenging Activity of Methanolic Extract of *Ganoderma Lucidum*

Sample	Conc.($\mu\text{g/ml}$)	Mean % DPPH scavenging activity	IC50 Value($\mu\text{g/ml}$)
Methanol Extract of <i>Ganoderma Lucidum</i>	200	28.50	581.525 $\mu\text{g/ml}$
	400	43.47	
	600	45.65	
	800	59.90	
	1000	79.95	

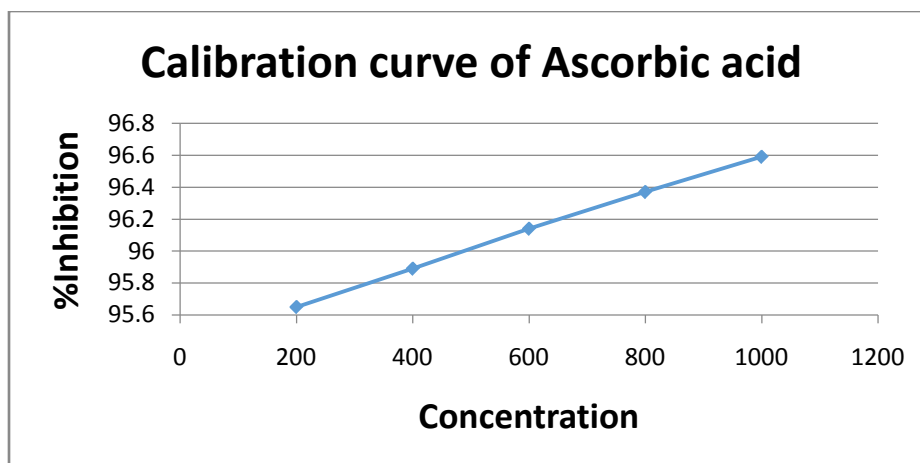


Fig 4.2: Calibration curve of Ascorbic Acid.

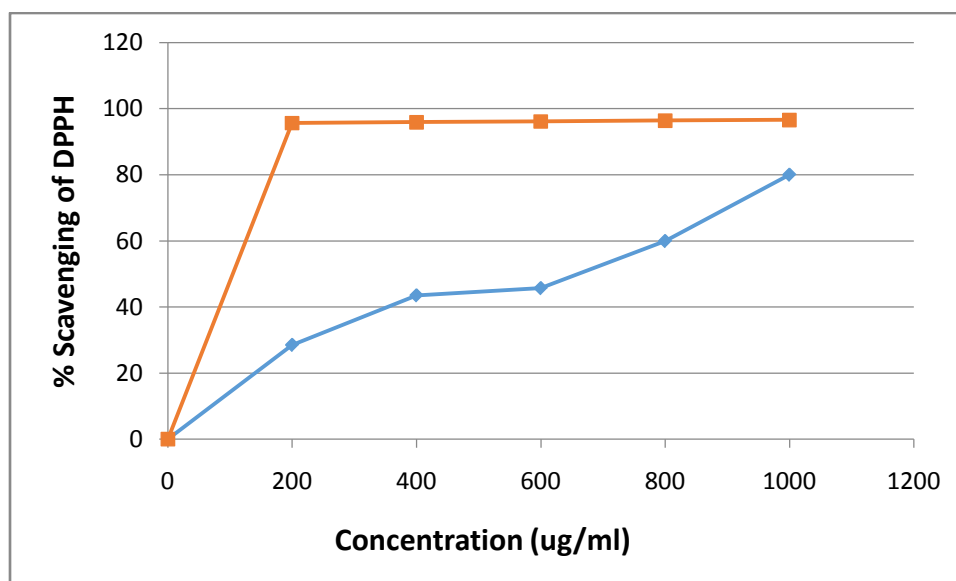


Fig 4.3 : Antioxidant Activity of *Genoderma lucidum* by DPPH Scavenging

4.2.2 Total Phenol Content

Total phenolic contents of the four fractions of the aerial parts of *G. lucidum* were determined by using the Folin-Ciocalteu reagent and were expressed as gallic acid equivalents (GAE) per gram of plant extract. The total phenolic contents of the test fractions were calculated using the standard curve of gallic acid ($y = 0.005x - 0.191$; $R^2 = 0.814$) Then the absorbance at 765 nm was determined. These data were used to estimate the phenolic contents using a standard curve obtained from various concentration of gallic acid. Total phenol content was expressed as mg of gallic acid equivalent.

The total phenol contents of methanol extract of *Ganoderma lucidum* , with total phenol contents of 107.2 mg/g had the highest amount among the samples in this study.

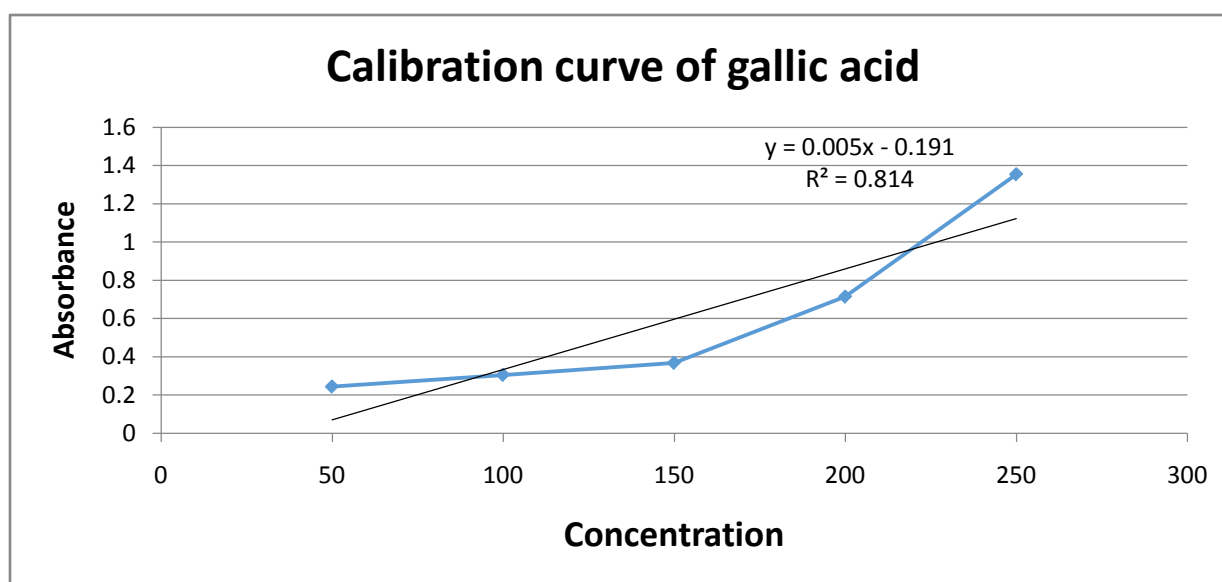
Table 4.7: Absorbance of Galic acid extract of *Ganoderma lucidum* at different concentration

Concentration ug/ml	Absorbance of Gallic Acid(Standard)	Absorbance of <i>Ganoderma lucidum</i> extract:
250	1.354	0.660
200	0.714	0.344
150	0.368	0.255
100	0.305	0.250
50	0.244	0.228

Blank: 0.222

Table: Total phenol content determination.

Concentration ug/ml	Absorbance	Best Fit Equation	R2 Value	X value(total phenolic content in mg/g)
250	1.354	Y=0.005x-0.191	0.814	170.2
200	0.714			107
150	0.368			89.2
100	0.305			88.2
50	0.244			83.8

**Fig. 4.3:** Calibration curve of **gallic acid** for total phenol content determination.

CHAPTER 5 Discussion

5.1 Discussion:

Phytochemical screening showed that the methanolic extract of *Ganoderma lucidum*(MEGL) was rich in chemical phyto-constituents. Such as Flavonoid, Gum & Carbohydrates, Steroids, Alkaloids and Terpinoids compounds. Thus further research is needed to work out the active medicinal compounds present in this extract; used for the treatment of various types of diseases.

The present study indicates that MEGL possesses significant antibacterial, cytotoxicity and antioxidant effects. The oral administration of MEGL did not present any mortality and abnormal behavior in the tested animals, so that MEGL can be considered to be safe and non toxic.

The antimicrobial screening using a sensitive *in-vitro* discs diffusion method & The extract of methanol was active against all the test organisms. The methanol extract (1000 µg/disc 1500ug/disc) of Ganoderma Lucidum showed antibacterial activity against **Gram positive** and **Gram negative** bacteria and antifungal activity against *Candida albican*.

Subsequently, these extracts were also checked for their cytotoxicity using a sensitive in vitro Brine shrimp lethality bioassay. The results of brine shrimp lethality bioassay are showed different mortality rate at different concentration. The mortality rate of brine shrimp *napulii* was found to be increased with the increase with the concentration of the sample. From result it can be well predicted that the crude extracts have considerable cytotoxic activity.

The antioxidant effect of MEGL was significant in the DPPH method used. Free radical scavenging abilities of the test samples were determined by measuring the change in absorbance of DPPH (1,1-Diphenyl-2-picrylhydrazyl radical) at 517 nm by UV-Spectrophotometer. Concentration providing 50% inhibition (IC₅₀) was calculated from the graph plotted inhibition percentage versus concentration of the extract. IC₅₀ value of the standard and methanol extract of *Ganoderma lucidum* were 12.6 and 581.52 µg/ml respectively. So, comparison with the ascorbic acid, it is clear that plant extracts possess moderate antiradical activity. The antioxidant power of MEGL was less than the antioxidant power of Ascorbic acid. The MEGL could have reduced the free radical production through the inhibition of NADPH oxidase during the

inflammation. Thus, the antioxidant property of MEGL would produce an additional therapeutic benefit enhancing its anti-inflammatory.

Total phenol content in the extract was determined with Folin-Ciocalteu reagent . Total phenol content was expressed as mg of Gallic acid . The total phenol contents of methanol extract of *Ganoderma lucidum* , was with total phenol contents of 170.2 mg/ml had the highest amount among the samples in this study. The present results suggest that all the tested plant extracts have mild to moderate antioxidant activity.

Since a variety of constituents is present in the extracts studied, it becomes difficult to describe the antioxidant properties selectively to any one group of constituents without further studies, which are beyond the scope of this paper.

Thus, further extensive investigations are necessary to find out the active antioxidative principles present in these plants.

CONCLUSION:

From the result of my study, it can be concluded that , using in vitro experiments established that methanol extract of *Ganoderma lucidum* inhibits the bacterial growth. In case of anticancer drug preparation this plant extracts may treated as a good candidate as it has notable cytotoxic effect. In case of antioxidant preparation this plant extracts may be treated as moderate antioxidant as it has moderate antioxidant effect.

The antimicrobial activity of the plant extracts were tested against twelve potentially bacterial pathogenic and one fungi by using disc diffusion method at different concentrations of the extracts of *Ganoderma lucidum* to understand the most effective activity. It has good antimicrobial and antifungal activity. The methanol extracts of *Ganoderma lucidum* possesses cytotoxic activity. It was observed that brime shrimp was died at the concentrations of 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml and, 400 µg/ml. The extracts of *Ganoderma lucidum* showed moderate 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and total phenol content. The antioxidant property depends upon concentration and increased with increasing amount of the extract in all the models.

This is only a preliminary study but the plant can be further screened against various diseases in order to find out its unexplored efficacy and can be a potential source of biologically important drug candidates.

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