

Evaluation of Bioactive Potential of Basidiocarp Extracts of *Ganoderma lucidum*

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Subject: Biology

Abstract

The present study was carried out to evaluate antimicrobial potential of *Ganoderma lucidum* using various concentrations of chloroform extract of basidiocarp against the pathogenic Bacteria (*Salmonella typhi*, *Baillus subtilis*, *Escheriachia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) and Fungi (*Aspergillus niger*, *Aspergillus flavus*, *Candida albicans*, *Curvularia species* and *Fusarium oxysporum*). The study revealed that the chloroform extract of basidiocarp of *G.lucidum* showed antibacterial activity against *S.typhi* (18mm at 100µl concentration) and antifungal activity against *C.albicans* (17mm at 100µl concentration). Preliminary phytochemical screening of *Ganoderma lucidum* was carried out for different extracts to detect the bioactive principles which can help in drug discovery and development. FT-IR analysis of the sample *G.lucidum* was also carried out to study the distribution of functional group within organic fractions. TLC and HPLC study confirmed the presence of triterpenoids and polysaccharides. Antioxidant activity of *Ganoderma lucidum* was also assayed.

Key words: *G.lucidum*, antimicrobial, phytochemical, triterpenoids, polysaccharides, antioxidant.

Introduction

The current world is frantically in search of new antibiotics because of alarmingly increased in bacterial and fungal resistance to existing antibiotics due to their inappropriate and indiscriminate use. Mushroom (wood decay fungi) is considered to have the antimicrobial and immense medicinal properties (5). The response of microorganisms to mushroom extracts might vary depending upon the nature of environment in which it has been grown. (6).

Ganoderma species are regarded as higher fungi because the carpophores are visible enough to be seen with naked eyes. *Ganoderma lucidum* is commonly known as “The King of Mushroom” or “The Mushroom of Immortality” has been used for thousands of year’s by far Eastern countries for its medicinal uses. It belongs to the family Polyporaceae, class Basidiomycetes. They are regarded as polypores because they possessed tiny pores underside their cap which contained reproductive spores. The caps are spongy when fresh, hardening to a shiny, smooth woody structure when matured. The colour of the caps ranges from brown to yellowish with reddish-brown being typical of the

polypore. The pore surface is cream in colour and the spores are brown. *Ganoderma lucidum* is widely distributed all over in tropical and temperate regions. In natural forests, it occurs on old declining trees and decayed wood stumps causing white rots removing lignin as well as cellulose and related polysaccharides (13). This annual mushroom grows on a wide variety of dead or dying trees, e.g., deciduous trees especially oak, maple, elm, willow, sweet gum, magnolia, and locust (16&20). *Ganoderma* occurs in Coconut palm causing Basal Stem Rot diseases, referred as Thanjavur wilt. (10). *Ganoderama lucidum* is less frequently found on coniferous trees (e.g., Larix, Picea, Pinus) in Europe, Asia, and North and South America (in temperate rather than subtropical regions (17).

The fruiting bodies contains a variety of chemical substances, major components are terpenoids and polysaccharides currently 130 triterpenoids (11) and more than 100 types of polysaccharides are reported from *Ganoderma lucidum*. It has been reported that the major secondary metabolites of *Ganoderma lucidum* are ganoderic acid, triterpenes and

carcinostatic polysaccharides (7&8). These bioactive compounds have been implicated for their high antioxidant, immune regulatory and hypo glycaemic activities. *Ganoderma lucidum* has gained wide popularity as a health food in both China and Japan and it has many medicinal effects. (16). Although, *Ganoderma* species could not be consumed directly, they have been known all over the world as highly medicinal mushrooms (9). The growth of this mushroom is greatly influenced by the environmental conditions, specific host species and the nature of habitat. A study of Biodiversity was carried out in our college campus (Ethiraj College For Women, Chennai, Tamil Nadu), and interestingly, the basidiocarp of *Ganoderma Lucidum* was found on the dried wood logs of *Guazuma tomentosa* (Sterculiaceae), host plant not to be reported earlier. As an approach towards a measure of conservation, and also to evaluate its bioactive potentials, the following studies were carried out namely - antimicrobial assay, phytochemical screening for different extracts of basidiocarp of *Ganoderma*, FT-IR study, the presence of triterpenoids by TLC and HPLC, and anti oxidant assay.

Materials and Methods

Sample Collection and Identification

The mushroom species white rot fungi used in present study was collected from dried wood log of *Guazuma tomentosa* (family-sterculiaceae) at Ethiraj College for Women Campus, Chennai, Tamil Nadu and identified and authenticated by Dr.Kaviyarasan, Centre for Advanced Studies in Botany, University Of Madras, Chennai-25. (plate1a &1b).



Fig. Aa: Plate 1a- *G. lucidum* at young stage



Fig. Ab: Plate 1b- *G.lucidum* at mature state

Place of collection: Ethiraj College For Women, Chennai, Tamil Nadu.

Host plant: *Guazuma tomentosa* (sterculiaceae family).

Crude Extract Preparation

The fresh fruit bodies of *Ganoderma lucidum* were dried and powdered. The powder was dissolved in different organic solvents namely (chloroform, ethanol, methanol, ethyl acetate and water) and filtered after 48 hrs. These filtrates were concentrated at 40 to 50 degree centigrade using a flash evaporator (3). The paste that was formed was freeze dried, and stored in a refrigerator in air tight containers for further study.

Antimicrobial Assay

Preliminary antimicrobial study was done with different extracts of *Ganoderma lucidum* and only chloroform extract was taken for further study. Different concentrations chloroform extract of the *Ganoderma lucidum* (25 μ l, 50 μ l, 100 μ l) was assayed for its antibacterial and antifungal potential. Bacterial pathogens such as *Bacillus subtilis*, *Salmonella typhi*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and fungal pathogens such as *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans*, plant pathogens-*Curvularia sps*, was used. Antimicrobial assay was carried out using well diffusion method. Muller Hindon Media (bacterial media) & PDA (Fungal media) were used. For anti bacterial assay, 24hrs old cultures were swabbed in the respective plates and wells were made on agar surface with sterile cork borer (7mm). For anti fungal assay, an aliquot (0.02ml) of spore suspension was used as inoculum for pour plate

technique, after solidification, the appropriate wells were made on agar plates by using sterile cork borer. The different concentration of chloroform extract of Basidiocarp was poured into well using sterile syringe. Gentamycin discs and Carbendazim were used as positive control respectively. The plates were incubated at 37°C for bacteria & at room temperature for fungi. Zone of inhibition around the well was observed after 24hrs for antibacterial assay and 48hrs for anti fungal assay. Triplicates were maintained for all the samples.

Phytochemical Screening

Phytochemical screening for secondary metabolites for all the 5 extracts (chloroform, ethanol, methanol, ethyl acetate, water) of Basidiocarp was carried out by Mayer's test, Wagner's test & Hager's test (alkaloids), Fehling's test, Barfoed's test, Benedict's test (carbohydrates, glycosides), Million's test, Biuret test, Ninhydrin test (protein and amino acid), spot test (fixed oil and fats), ferric chloride test, lead acetate test, alkaline reagent test (phenolic compounds and tannins), detection of gum and mucilage (Whistler and BeMiller, 1993), detection of terpenoids: (Salkowski test). (4)

Fourier Transform Infrared Spectrophotometer (FT-IR)

For the FT-IR study, Spectrum FT-IR system (Shimadzu, IR Affinity 1, Japan), equipped with a DLATGS detector with a mirror speed of 2.8mm/sec. scan range: from 400-4000cm⁻¹ with a resolution of 4cm⁻¹ was used. The methanolic, ethanolic, chloroform extract of *Ganoderma lucidum* was prepared. The extract was evaporated by flash evaporator and it was mixed with a KBr salt, using a mortar and pestle and compressed into a thin pellet. Infrared spectra were recorded on KBr pellet on a Shimadzu FTIR spectrometer 4000 - 500cm⁻¹. (22)

Thin Layer Chromatography (TLC)

The solvent system used to identify triterpenoids was chloroform: methanol: water (30:4:1 v/v). The silica gel coated TLC plates were spotted with all 5 extracts of *Ganoderma lucidum* at equal distance. The plate was allowed to run until it reaches ¾th positions, 10% sulphuric acid in alcohol was used as spraying agent and plates were dried. The spots were noted under UV lamp. (15). The R_f value was calculated and recorded.

High Performance Liquid Chromatography (HPLC)

2gms of powdered *Ganoderma lucidum* was mixed with 100ml of alcohol and kept for 24hrs in rotating shaker. Filtrate was collected and evaporated to dryness (1) A final volume of 1.5ml of extract was prepared as a stock solution with addition of methanol. HPLC equipment consisted of a SHIMADZU (Kyoto, Japan). LC 20 AD pump, an SPD 20 A- UV detector (SHIMADZU) with wavelength set at 254 nm and then LC solution software recorder (Integrator, Spectra-physics, Mountain view, CA). Room temperature was maintained. Separation was carried out at C18 column (Phenomenax 250) (Merck, Darmstadt, Germany). 20 microlitres of the stock or sample solution was introduced with a Rheodyne valve equipped with 20µl external loop. The mobile phase rate was 1ml/min and consisted of Acetonitrile: Glacial acetic acid: HPLC grade distilled water (69.3: 0.01: 30.69) for triterpenoids, Distilled water (HPLC grade) for polysaccharides. Low pressure was maintained.

Antioxidant Assay (DPPH) Radical Scavenging Activity)

25mg of DPPH (150µm) was prepared in 100 ml of ethanol. To 1.9ml of DPPH, 0.1ml of extract of different concentration (100,300,500,700&1000µg/ml) was added. Ethanol was used as blank solution. The absorbance of test mixture was read at 517nm. (21) The percent inhibition was calculated and expressed as percent scavenging of DPPH radical. The percent DPPH inhibition was calculated from the following formula:

Percent DPPH activity

$$= \frac{\text{O.D of control} - \text{O.D of test} \times 10}{\text{O.D of control}}$$

O.D of control

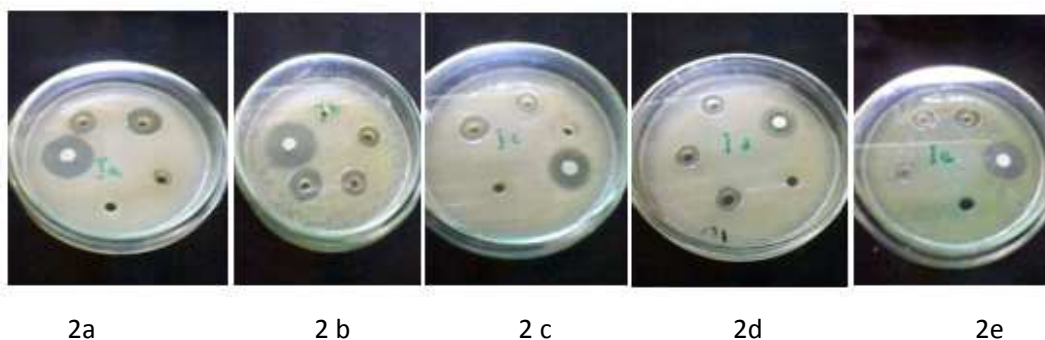
Results and Discussion

Antimicrobial Assay

Chloroform extract of Basidiocarp of *Ganoderma lucidum* showed maximum result in the preliminary antimicrobial assay. Further the antimicrobial potential of *Ganoderma lucidum* done with chloroform extract of different concentrations (25µg, 50µg, 100µg) revealed that chloroform extract of

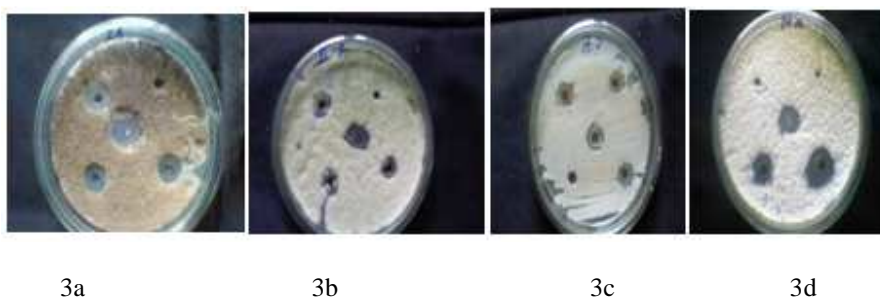
Basidiocarp of *Ganoderma lucidum* (at 100 μ g) showed maximum antibacterial activity against *Salmonella typhi* (18mm) and anti fungal activity against *Candida albicans* (17mm). (Figure1), (plate2&3), (Table 1&2) *Ganoderma lucidum* could be used for the invitro control of bacteria and fungi causing human diseases. Depending on the solvent used for extraction, it was observed that *Ganoderma lucidum* extracts behaved differently which might be the reason for the differences in their antimicrobial effectiveness (3). The bioactive secondary metabolites of mushroom extracted may be different

depending on the extractive solvents used (17&18). Variation in antimicrobial activity may also be due to its molecular configuration. The high level of effectiveness of different solvent of extraction could be linked with higher concentration of metabolites extracted. The most active components are generally water insoluble, hence it is expected that low polarity organic solvents would yield more active extracts (2).The antibacterial activity of chloroform extract of *G.lucidum* from Iran, showed the growth inhibitory effects on *B.subtilis* & *S.aureus*. (12 &14).



- 2a- Antibacterial activity of chloroform extract of *G.lucidum* against *Bacillus subtilis*
- 2b- Antibacterial activity of chloroform extract of *G.lucidum* against *Salmonella typhi*
- 2c- Antibacterial activity of chloroform extract of *G.lucidum* against *Escherichia coli*
- 2d- Antibacterial activity of chloroform extract of *G.lucidum* against *Klebsiella pneumonia*
- 2e- Antibacterial activity of chloroform extract of *G.lucidum* against *Pseudomonas aeruginosa*.

Fig B a-e: Plate2 -Antibacterial activity of chloroform extract of *Ganoderma lucidum*



- 3a- Antifungal activity of chloroform extract of *G.lucidum* against *Aspergillus niger*
- 3b- Antifungal activity of chloroform extract of *G.lucidum* against *Aspergillus flavus*
- 3c- Antifungal activity of chloroform extract of *G.lucidum* against *Candida albicans*
- 3d- Antifungal activity of chloroform extract of *G.lucidum* against *Curvularia Sp.*

Fig C a-d: Plate 3 Antifungal activity of chloroform extract of *Ganoderma lucidum*.

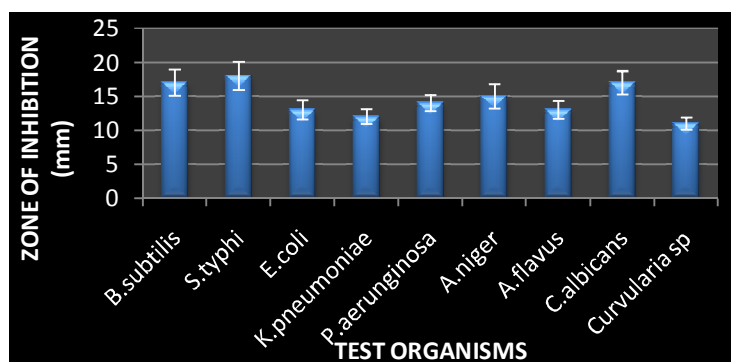


Fig.1: Maximum inhibition zone showed by chloroform extract for both bacteria and fungi

Table 1: Antibacterial activity of chloroform extract of *Ganoderma lucidum*

S.NO	ORGANISM	ZONE OF INHIBITION(mm)			
		Control	25µl	50 µl	100 µl
1	<i>Bacillus subtilis</i>	20±1.2	8±0.4	14±1.8	17±1.9
2	<i>Salmonella typhi</i>	22±1.8	12±0.6	16±1.5	18±2.1
3	<i>Escherichia coli</i>	14±1.5	8±0.9	11±0.8	13±1.4
4	<i>Klebsiella pneumonia</i>	13±0.8	9±1.0	11±0.6	12±1.1
5	<i>Pseudomonas aeruginosa</i>	17±0.9	9±0.4	12±1.1	14±1.2

Control: (Gentamicin disc)

Table 2: Antifungal activity of chloroform extract of *Ganoderma lucidum*

S.NO	ORGANISM	ZONE OF INHIBITION(mm)			
		Control	25µl	50 µl	100 µl
1	<i>Aspergillus niger</i>	17±0.8	13±0.7	14±1.5	15±1.8
2	<i>Aspergillus flavus</i>	14±1.1	10±0.3	12±1.3	13±1.3
3	<i>Candida albicans</i>	18±0.5	12±0.9	13±1.6	17±1.7
4	<i>Curvularia sp</i>	13±0.4	7±0.2	9±0.4	11±0.9

Control: Carbendazim disc

Phytochemical Analysis

(Table 3) The phytochemical screening of *Ganoderma lucidum* done for different extracts revealed the presence of alkaloids, carbohydrates, saponins, proteins, aminoacids, phytosterols, fixed oils and fats, triterpenoids, flavonoids, phenolic compounds and tannins in different tests. The polyphenols, flavonoids found in this study are known to be the source of plant based antioxidants which can protect nerves, heart, liver and other

organs and tissues. The presence of alkaloids in the mushroom powder explains its antibacterial activity, since this phytochemical is reported to have antibacterial activity (6). The presence of tannins which can complex with the metal ions and macro molecules such as proteins and carbohydrates obtained in powdered sample can be utilized in weight reduction management. Saponins have the anti-inflammatory, expectorant and immune stimulating effects. (21)

Table: 3 Phytochemical screening of different extracts of *Ganoderma lucidum*

S.No	Phytochemical tests	Chloroform extract	Ethanol extract	Methanol Extract	Ethyl acetate extract	Aqueous extract
<u>Alkaloid test</u>						
1	Mayer's test	+	+	+	+	+
	Wagner's test	+	-	-	+	-
	Hager's test	+	-	+	-	-
<u>Carbohydrates & Glycosides test</u>						
2	Fehling's test	+	+	+	+	+
	Barfoed's test	+	+	+	+	+
	Benedict's test	-	-	+	+	+
3	<u>Saponin test</u>	+	+	+	+	+
4	<u>Protein and Amino acid tests</u>					
	Million's test	+	+	+	-	+
	Biuret test	-	+	-	+	-
	Ninhydrin test	-	-	-	-	-
5	<u>Phytosterols</u>	+	+	+	+	+
6	<u>Fixed oils and Fats</u>					
	Spot test	+	+	+	+	+
7	<u>Phenolic compounds and Tannins</u>					
	Ferric chloride test					
	Lead acetate test	-	-	-	-	-
	Alkaline reagent test	+	-	+	+	-
		+	+	+	+	+
8	<u>Triterpenoids</u>	+	+	+	++	+
9	<u>Phlobatanins</u>	-	-	-	-	-
10	<u>Flavonoids</u>					
	Vanillin test	+	+	+	-	+

Fourier Transform Infrared Spectrophotometer (Ft-Ir)

FT-IR analysis of the sample *Ganoderma lucidum* informs about the distribution of functional group within the organic fractions. The IR spectra for all the 3 extracts (chloroform, methanol, ethanol) of

Ganoderma lucidum showed the presence of alkyl halides, alcohols, esters, carboxylic acids, ethers, alkenes, phosphines, monomeric alcohols and phenols (Fig2,3&4). The presence of various functional groups is attributed to the existence of variety of potential phytochemicals in this medicinal mushroom *Ganoderma lucidum*. (22)

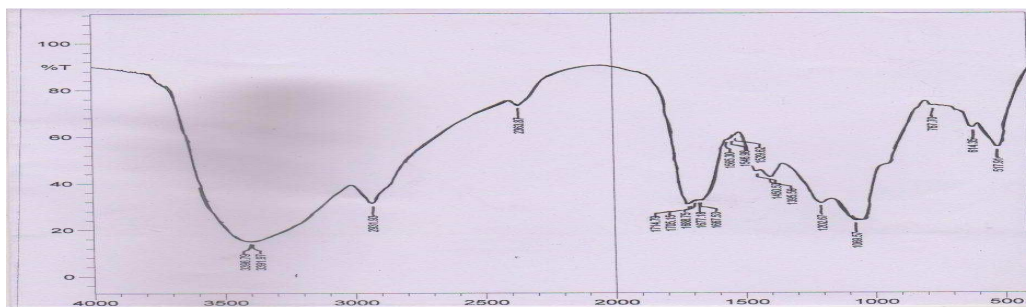


Fig. 2: FT-IR for *Ganoderma lucidum* ethanol extract.

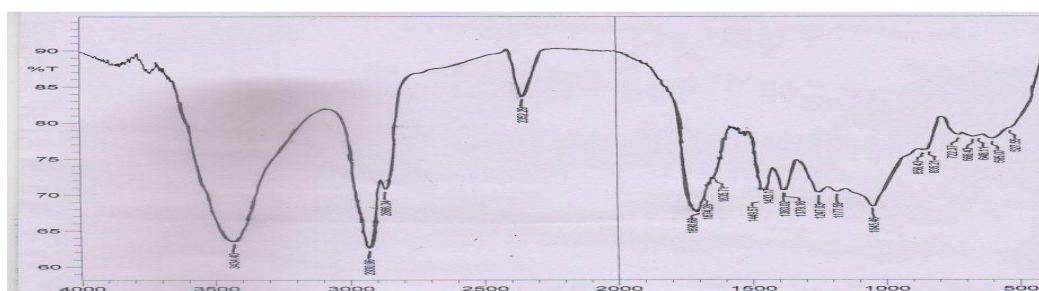


Fig. 3: FT-IR for *Ganoderma lucidum* chloroform extract.

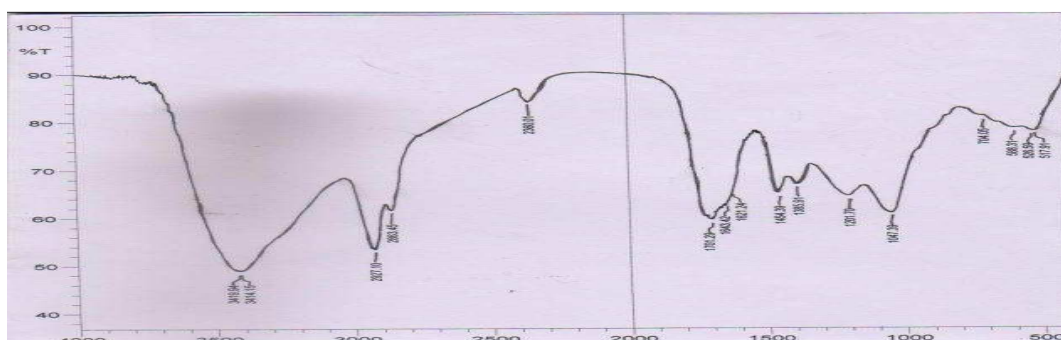


Fig. 4: FT-IR for *Ganoderma lucidum* methanol extract.

Thin Layer Chromatography

The TLC study for triterpenoids was done for different extracts of *Ganoderma lucidum*. Plate with yellow to green fluorescent bands was observed under UV. It indicates the presence of triterpenoids. Strong spots were obtained for the chloroform and ethanol extracts of *Ganoderma lucidum* which has the R_f value 0.271 and 0.312 respectively.(figure:5) Our result is in accordance with the result of (15), which showed the R_f value ranging from 0.51 to 0.901 in benzene extract with carrprice reagent spray under UV light. Variation in R_f value of triterpenoids may be due to the strains of *Ganoderma lucidum*. It may also be due to the host plant from where the mushroom is collected, soil and environmental factors, geographical location, extraction solvents and reagents used in this study.

High Performance Liquid Chromatography:

HPLC study is followed to identify and confirm the presence of triterpenoids and polysaccharides. Fig6 shows the chromatographic peaks 12.075 and 19.889 and confirms the presence of triterpenoids. The presence of polysaccharides in the samples of *Ganoderma lucidum* was also confirmed by HPLC study (Fig 7). Individual peak with the retention time of 5.056 (13). Apart from this chromatographic peaks additional peaks are also present before, in between and after the chromatographic peaks of ganoderic acid C2 (15.95) and B (21.93) which indicates the existence of additional triterpenoids, (Fig 8) (1). Triterpenoids of chloroform extract might be responsible for the antioxidant and inflammatory activities. These findings suggest that the therapeutic potentials of the chloroform extract of this mushroom for the prevention and the control of inflammation and diseases mediated through oxidative stress (19)

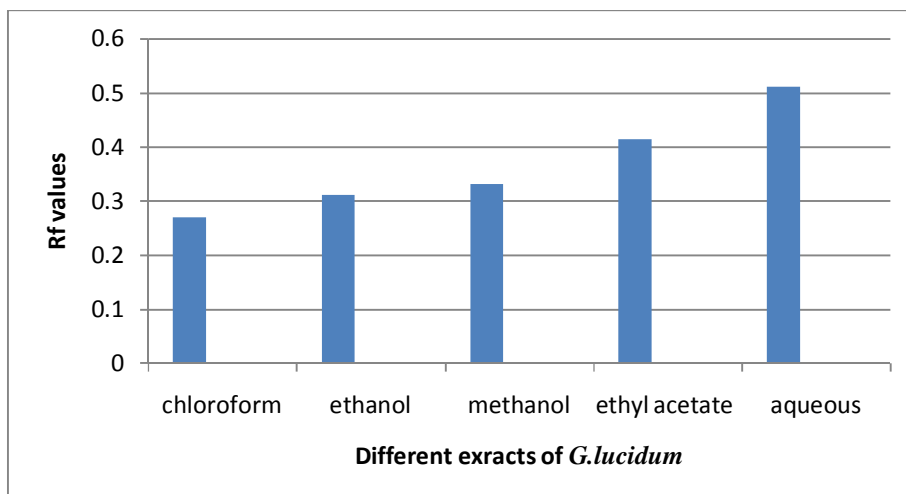


Fig. 5: TLC study for triterpenoids

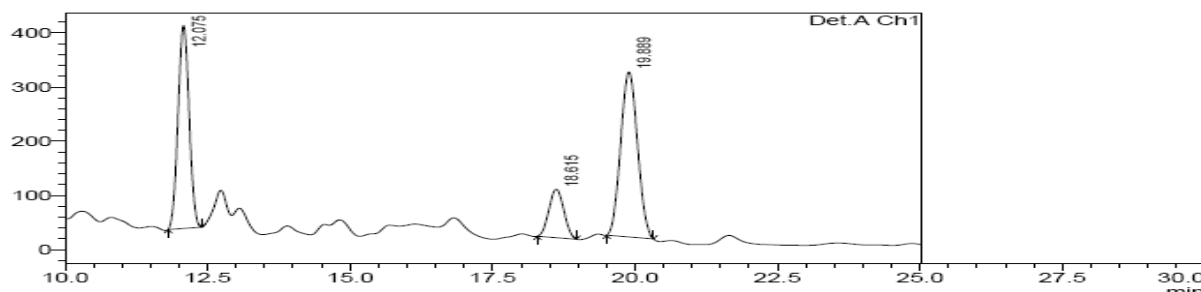


Fig.6: HPLC study in *Ganoderma lucidum* for Triterpenoids

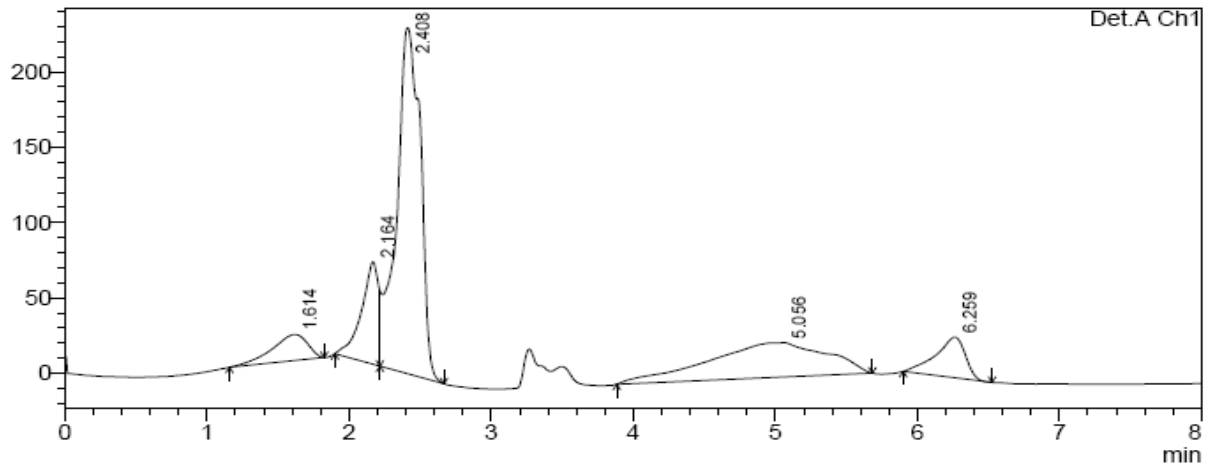


Fig. 7: HPLC study in *Ganoderma lucidum* for polysaccharides.

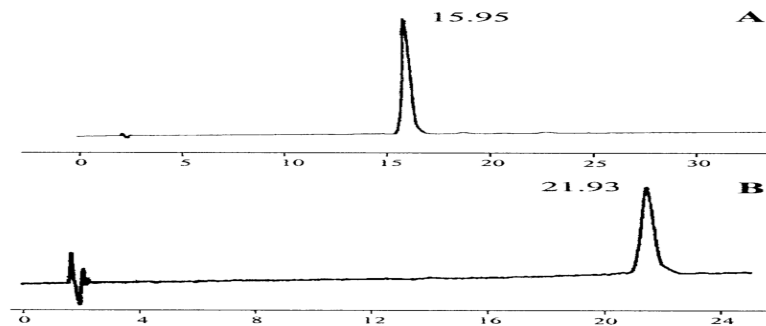


Fig. 8: Standard HPLC chromatograms of the ganoderic acids C₂ (A) and B(B). (1)

Antioxidant Activity by Dpph Radical Scavenging

Activity

Figure 9 shows the DPPH radical scavenging activity of different organic extracts (chloroform, ethanol and methanol) of *Ganoderma lucidum*. The results indicate that the chloroform extract at 1000 µg/ml of *Ganoderma lucidum* has potent radical scavenging activity (89.71% of inhibition) compared to that of the other two extracts, ethanol (64.72% of inhibition) and methanol (74.05% of inhibition). In the DPPH assay, the ability of antioxidant to scavenge stable purple coloured primary radical DPPH is tested by its depolarization at 515nm. This shows the ability of the extract to scavenge stable free radicals. The antioxidant property may be responsible for reduction of hepatic damage. Human diet containing medicinal mushrooms possessing anti oxidative properties would be potentially useful to help human body to reduce oxidative damage. The broad-spectrum medicinal property of *Ganoderma lucidum* might be due its significant anti oxidative activity (21).

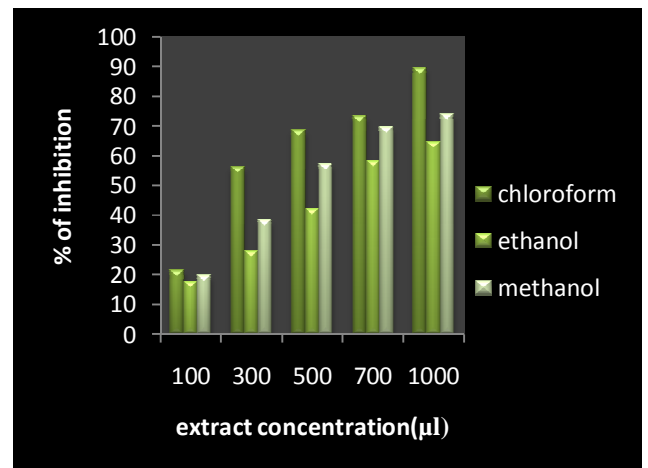


Figure 9: Free radical scavenging by DPPH assay of different extracts of *Ganoderma lucidum*

Conclusion

The whole world is in search of new antibiotics because of alarmingly increased bacterial and fungal resistance to existing antibiotics due to their inappropriate and indiscriminate use. *Ganoderma lucidum* is the macro fungi which have high antimicrobial potential against various pathogens. And interestingly this potent medicinal mushroom was found to occur in our college campus Ethiraj College for Women, Chennai, Tamil Nadu in the host plant *Guazuma tomentosa*. This Research work on *Ganoderma lucidum* demonstrates that mushrooms, similar to plants have a great potential for the production of useful bioactive metabolites and those they are considered to be prolific resource for drugs. The responsible bioactive compounds belong to several chemical groups very often they are polysaccharides and triterpenoids. The *Ganoderma lucidum* contains more of triterpenoids and also contains proteins and other bioactive compounds. The variation in the bioactive potentials of *Ganoderma lucidum* may be attributed to type of Host plant, soil, environmental condition existing in that area and geographical location. Resistance to antibiotics is emerging in a wide variety of organism and multiple drug resistant organisms pose a serious threat to the treatment of infectious diseases. Hence, mushroom derived antimicrobial substances have received considerable attention in recent years. It is apparent from the present study that mushroom extracts from *G. lucidum* could be employed to combat several diseases caused by pathogenic microorganisms (16). Following are some measures of conservation strategies taken for this indigenous mushroom: (I) Habitat is protected by planting more seedlings of host plants (II) Soil remains undisturbed (III) Periodical analysis of soil microbial load. It has also been proposed to cultivate *Ganoderma lucidum* in wood chips/ saw dust of *G.tomentosato* promote invitro cultivation. Isolation and characterization of *Ganoderma lucidum* using appropriate media will be carried out and the culture will be sent to MTCC for further identification and characterization to confirm the strain.

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