Rapid *In Vitro* Antifungal Property Assessment of Organic and Aqueous Extracts of *Ganoderma lucidum* Against Human Pathogenic Fungus of *Aspergillus niger*

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Abstract: *G. lucidum* is commonly known as 'Reishi.' It has a variety of bioactive compounds. *Aspergillus niger* is common fungi, categorized with GRAS (generally recognized as safe) status by food and drug administration. However, due to its cosmopolitan nature, human beings get frequently exposed to spores and vegetative forms of *A.niger*. Mushroom has a wide diversity of bioactive components; the compound extraction process has an essential selection of suitable solvents for particular bioactive products for higher targetted efficiency activity. The present study took various organic and aqueous mediums for extract preparation. The rapid *in vitro* antifungal activity was analyzed. Among them, ethanolic extracts have shown potential, followed by other solvents in normal water, methanol, chloroform, and hot water. The current study result interpreted that owing to the new attraction to the properties of new antifungal products like fighting multidrug-resistant fungus. It is essential to develop a better understanding of the current methods available for screening and quantifying the antifungal effect of an extract or a pure compound for its applications in human health.

IndexTerms - **Reishi, bioactive compounds, good solvent, *in vitro,* antifungal activity.**

I. INTRODUCTION

The increasing rate of newly appearing fungal pathogens causes many diseases in humans and plants. Though there are a huge variety of drugs that are used for treatment. Pathogenic fungi produce an undesired product, cause unacceptable effects, or are very toxic to the host body, such as Amphotericin B; some, like Azoles. However, the entrance of these new pathogens gives rise to both primary and secondary drug resistance. So there is a huge need for the upcoming generation to be safer and more potent antifungal agents. One approach can be made to identify some photoactive bio-compound from medicinal plants, mushrooms, or some alternatives that can be directly used as antifungal agents or as templates for drug development (Suresh *et al.*, 2010). Traditional tribal communities, native peoples, and the eastern world have used plants, spices, and fungi for thousands of years as therapeutic agents. For the past few years, attention has turned to extracts of medicinal plants and biologically active compounds used in traditional herbal medicine to uncover the scientific basis of their remedial effects and to seek new lead compounds for development into therapeutic drugs. Many previous kinds of research have been carried out on fungi, determining their ability to mobilize the body's humoral immunity and prevent bacterial, viral, or fungal pathogens that are resistant to current therapeutic agents. Fungi are well known for producing important antibiotic compounds, potential antimicrobial sources in the fungi group class known as the Basidiomycetes family. Fungi are different from macrofungi from a diverse point. Macrofungi grow luxuriant and are found in many parts of the world (Smith, 1963). The most common group of macrofungi is mushrooms. Edible and medicinal mushrooms are used as food and also used to treat many human diseases against bacteria, yeast, fungi, parasites, and tumors (Chang *et al.*, 2004; Majeed *et al.*, 2017). The study of macrofungi, having edibility and medicinal property, still need to be studied (Jonathan & Fasidi, 2003). *G. lucidum* is a well-known mushroom species used in traditional medicine. It has prompted scientists worldwide to undertake mass cultivation and production of the bioactive metabolites of this fungus. It produces several functional metabolites with biological activity, such as polysaccharides, proteins, and trace elements. It might explain some of the observed medicinal properties. It is also known as lingzhi, reishi, and yeonghi (Shahid *et al.*, 2016). The extracts of *G.lucidium* contain bacteriolytic enzymes, lysozyme, and acid protease, which show antimicrobial effects (Hoque *et al.*, 2015). In the present study, taking potent diseases-causing fungi, *A.niger*, is commonly regarded as a pathogenic allergen. They are generally associated with a lung infection in individuals with a weak immune system because conidia and conidiophores are small, readily airborne, can easily breathe in, and cause deep or systemic mycosis. The ear is the location of this infection grows and multiplies. Various laboratory methods can evaluate or screen the *in vitro* antifungal activity of an extract or a pure compound. The most known and basic methods are the disk-diffusion and broth or agar dilution methods (Alves *et al.*, 2013). Numerous methods are appropriate for the *in vitro* antimicrobial property analysis, such as the disk diffusion method, agar well diffusion technique, micro dilution techniques, etc. Among all the above methods maintained particular period incubation periods, a determination needs to determine the growth pattern of the tested microorganism. Some drawback posse in both techniques (disk and well diffusion, agar plate) microbial agents diffuses into agar and growth is shown. It is measured by inhibition growth diameter since the fungal growth inhibition does not mean fungal death. This method cannot distinguish between fungicidal and fungi static effects. Further, it is not appropriate to determine the minimum inhibitory concentration (MIC) as it is impossible to quantify the amount of the antifungal agent diffused into the agar medium. Also, it is time-consuming as it takes around 7-9 days to complete the task (Balouiri *et al.*, 2016). While the micro dilution method comprises micro dilutions of the extract in a liquid

medium using micro plates to determine the minimal inhibitory concentration (MIC). In the disc diffusion method, the extract is incorporated into discs at different concentrations. The halo of growth inhibition is determined and represented by IZD (internal zone diameter (Hoffman *et al*., 2001). So therefore, the dilution method is the most appropriate for determining MIC values since they provide the possibility to estimate the concentration of the tested antifungal agent in the agar (agar dilution) or broth medium (macro dilution or micro dilution). Both broth and agar dilution methods may be used to measure the in vitro antifungal activity against fungi quantitatively. It is more useable in antifungal tests because it is less time-consuming and low-cost. MIC value recorded is defined as the lowest concentration of the test antimicrobial agent that inhibits the visible growth of the microorganism tested, and it is usually expressed in mg/mL or mg/L. Many approved guidelines are available for dilution antimicrobial susceptibility testing of critical or non-critical bacteria, yeast, and filamentous fungi (Balouiri *et al.*, 2016). The present study aims to contribute to selecting the suitable solvent for extract preparation and developing a rapid *in vitro* antifungal assay method of the *G. lucidum* mushroom extract against human pathogen fungus.

II. MATERIALS AND METHODOLOGY

2.1 Samples Collection and Identification

Fruiting bodies of *G. lucidum* mushroom samples were collected from the Charama forest (Kanker District), Raipur Chhattisgarh, in July 2018. This collected mushroom sample was kept in airtight polythene bags and preserved at the Pt. Ravishankar Shukla University Biotechnology Department, Raipur (C.G). The name designation of the collected mushroom and identification has been done based on critical observation of the specimens and examination of relevant literature (Kuo, 2004). *Aspergillus niger* (human pathogen) was used for the antifungal activity, which was collected from Microbial Laboratory, School of Studies in Biotechnology, Pt. RSU Raipur.

2.2 Reagents and chemicals

The following chemical was used as a solvent, and medium preparation as Ethanol, Methanol, Chloroform, and sterile water, Tween 80, Sodium Chloride, Dextrose, Agar, Dimethyl sulphoxide (DMSO) 20%, etc. are obtained from Sigma-Aldrich (Bangalore, India) and Himedia (Mumbai, India) respectively. The standard antifungal compound Fluconazole (Fluka® -150), manufactured in Cipla, India, and was purchased from the city medical store, Raipur, India.

2.3 Preparation of Mushroom Extracts

The extraction method of mushrooms was determined by following the modified protocol of Nithya *et al*. (2016) and Chandrawanshi *et al.* (2018). Initially, mushroom samples were sun-dried for 1-2 days. Each mushroom sample was grinding in a mixer (Bajaj GX7) to make a fine powder and further packed in airtight zipper bags for extraction using different solvents. The antifungal compounds were extracted from *G.lucidum* samples with aqueous and organic solvents to separate the chemical constituents into groups of different polarities. The solvents used in extraction are chloroform, ethanol, methanol, and aqueous as hot water and regular water extract. 20g powdered submerged into 200ml of different solvents (chloroform, ethanol, and methanol) in an airtight flat bottom container incubated at an orbital shaker at 100 rpm for 24 hrs at 30°C, respectively. Similarly, performed for inorganic solvents (normal and hot water (60 °C), taken 20g powdered, submerged in solvents and normal water incubated overnight at room temperature and hot water extracted processed after one hour. The average water continuing mushroom powder further incubates at an orbital shaker at 100 rpm for 24 hrs at 30°C. The crude extracts were gravity filtered through a Whatman No. 1 filter paper. Then the extracted materials were subjected to evaporation of solvents in an oven at 40°C. After the filtrations, all the solvents were evaporated at room temperature or on the hot plate at 40°C. All the dried extract was re-dissolved in DMSO (05%), stored, and used for further analysis.

2.4 Yield of extract

After the extraction process of mushroom samples using different solvents, the initial weight of mushroom samples and final weight of extracted sample were taken. According to Mujic *et al*. (2010), the bioactive substance was calculated and determined the total yield of extracts, as per the below-given formula.

$$\text{Yield of extract (\%)} = \frac{\text{Amount of obtained wieght (gm)}}{\text{Amount of obtained used wieght (gm)}} x100$$

2.5 Antifungal studies

2.5a Preparation of Fungal media (PDA)

Two Hundred grams (200g) of potato slices were boiled with 1000ml of distilled water. The potato infusion was used as a water source for media preparation. Then, 20g of dextrose was mixed with potato infusion, and 15g of agar was added as a solidifying agent. These constituents were mixed and autoclaved at 15 pounds per square inch (psi) above atmospheric pressure for 30 minutes (Hoffman *et al.* 2001).

2.5b Preparation of fungal inoculums

The fungal inoculums were prepared according to the modified method (Hoffman *et al.*, 2001; Tiwari *et al*., 2010). The micromycetes were maintained on PDA, and cultures were stored at 4°C and subcultured once a month. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80(v/v). The fungus spore suspension was adjusted with sterile saline to approximately a concentration. 1.0 X 10^5 in final volume 100 µl per well.

2.5c Determination of *in vitro* antifungal assay through MIC

The *in vitro* antifungal activity of the mushroom extract was analyzed according to Hoffman *et al.* (2001) and Chandrawanshi *et al.* (2017) modified protocol. *Aspergillus niger* (human pathogen) used for tested organisms. Minimum inhibitory concentration (MIC) determination was performed by dilution techniques using 96-well micro titer plates. The extracted samples with different

concentrations (20, 40, 60, 80, and 100µl) containing (v/v) (1 mg/mL) and added potato broth medium (PDB) with fungal inoculums of the micro dilution test were performed. Initially, 5µl of fungal suspension (fungal wash) in a suitable growth medium (PDB) was added to wells of sterile 96-well micro titer plate already containing 100 µl of mushroom extract containing various concentrations (20, 40, 60, 80, and 100µl), with (v/v) (1 mg/mL). The final volume was maintained in each well up to 250 µl. The control well was poured only with PDB medium and fungal suspension (5 µl). The extracted bioactive contents of each well were mixed on a micro plate through a micropipette. Finally covered with aluminum foil for protection from other microbial contamination, and then incubated for 72 hrs at 28 °C. The lowest concentrations without visible microbial biomass on the well (at the binocular microscope) were described as MICs. Fluconazole (Fluka® -150) used as positive control, dissolved in solvent with (v/v) (1 mg/mL) respectively.

III. RESULTS AND DISCUSSION

The current study applied various organic and aqueous solvent mediums for extract preparation from the medicinal mushroom of *G. lucidum*. The prepared extracts and their bioactivity were analyzed successfully.

3.1 Yield of extract

The amount of mushroom compound used for extraction of anti-fungal content was 20gm and dissolved in 200ml of solvent. For the extract preparation, various solvents were used. In the present study, ethanol showed the highest percentage recovery (19.05%), while normal water (6.05) and chloroform gave the minuscule recovery yield (1.40), respectively. The yield of extract gained has shown in table 1.

Table 1 Percent yield concentration of extracts

Solvents	Yield of Extract (%)
Ethanol	19.05
Normal water	6.05
Methanol	3.35
Chloroform	1.40
Hot Water (60ºC)	4.85

The extraction yield depends on the solvent with varying polarity, pH, temperature, extraction time, and sample composition (Do *et al.*, 2014). Extract in different solvents was treated at varying times. Ethanol took longest time for evaporation, around 48hrs, followed by methanol for 36hrs and chloroform for 6hrs at 60ºC. The aqueous solvent was kept in the hot plate at 100ºC for maximum evaporation. A viscous liquid was gained in the hot plate. In order to collect it in the dehydrated form they were further kept in hot air oven at 60ºC for 30 minutes for hot water and 24hrs for regular water. Hence, minimum inhibitory concentration of *A. niger* treated with various extracts was according to the concentration of the solvent extracted anti-microbial compound. The solubility of compounds of *G.lucidum* depends on the ecological and geological condition, herb age, and harvesting time.

3.2 Antifungal activity by Micro dilution technique

G. lucidum is used to study antifungal activity against fungal species i.e. *A. niger*. Antifungal activity was performed by micro dilution technique. There are 96 well in a titer plate which were poured with different extract (chloroform, ethanol, methanol, normal water and hot water) of having concentration 20µl, 40 µl, 60 µl, 80 µl, 100 µl. And nutrient broth was added along with fungal culture. The amount of mushroom compound used for extraction of anti-fungal content was 20gm and was dissolved in 200ml of solvent. The compound taken was low in amount due to which the % extract collected was in very low quantity. To study the anti fungal activity of the extracts, MIC was determined. The lowest concentrations without visible growth were defined as MICs. The minimum inhibitory concentration was shown by ethanol followed by normal water, methanol, chloroform and hot water. The percent growth of organic and aqueous extract from *A. niger* fruiting body shown in table 2.

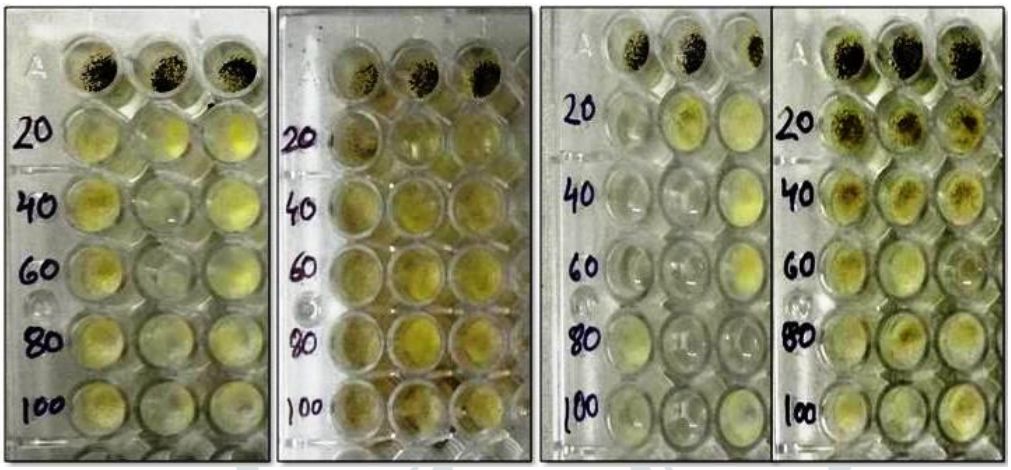
Table 2 Antifungal activity of *G. lucidium* extracts extracted by different solvents (10mg/ml)

S.N.	Concentration of extract	Organic extract (%)			Aqueous extract (%)	
		Methanol	Ethanol	Chloroform	Hot water	Normal water
1	Control	100	100	100	100	100
2	20ul	30	00	09	20	10
3	40ul	10	00	06	06	00
4	60ul	00	00	03	05	00
5	80ul	00	00	02	08	00
6	100ul	00	00	00	10	00

Experiment performed: n±2, 100% indicate full grown of microbial culture, 00 means no visual detection, or non microscopic view recorded on (40x X 10 xs)

In comparing organic and aqueous solvents, organic extracts were more potent in extracting the antifungal content from *G. lucidum*. Although extract prepared in cold water showed, antifungal activity ethanol was much more effective. The MIC of ethanol showed the most potent antifungal activity against *A. nige*r. The antifungal activity has shown in tables 2 &3, fig.1. The ethanol extract of *G. lucidum* showed activity against *A. niger* with MIC (0.002 mg/ml), which was higher than cold water with MIC (0.004 mg/ml). Similarly, the methanol with (0.006mg/ml), then chloroform with (10 mg/ml). Whereas hot water showed the lowest MIC value. All four extracts showed minimum inhibitory concentration at a specific range, but the hot water did not show complete inhibition; instead, the growth decreased.

Figure 1 Antifungal activity of *G. lucidium* extracts at micro dilution assay at micro titer plate method (10mg/ml)



(a) Chloroform (b) H_2O (Normal) (c) Ethanol (d) Methanol

Table 3 The ranges of minimum inhibitory concentrations (MIC) at mg/ml are shown below:

Solvents	**MIC (mg/ml)**
Ethanol	0.002
Normal water	0.004
Methanol	0.006
Chloroform	10
Hot water	No complete inhibition

Our results agree with the protocol of Rizi *et al*. (2015). They stated that compared to the disc-diffusion and broth micro-dilution methods, where a seven-day incubation period is recommended (Norris *et al*., 1999), SPOTi offers a significant advantage and ease of performance. The problem with the disc diffusion technique was that because of the lower extract concentration, no zone of inhibition was visible. One of the reasons may be due to the property of diffusion. According to Rizi *et al*. (2015), compared to the disc diffusion method, the main advantage of this assay is that the concentration of the drugs that fungal colonies are exposed to is uniform in each well as the drug is mixed thoroughly in the agar. The fact that mushroom extract likely has limited diffusion in agar-based assays relative to other antimicrobials might suggest that the proposed methodology could help provide quantitative data for similarly non-diffusible materials (Balouiri *et al.*, 2016). Similarly Jonathan and Fasid (2003**)** used different solvents (aqueous, methanol, and ethanol and prepared extract from *Lycoperdon pusilum* and *L. giganteum*, found the inhibition of growth of some disease-causing fungi in *in-vitro*, and ethanol was the best solvent for extracting antimicrobial substances. Balouiri *et al***.** (2016) stated antimicrobial testing methods such as disk-diffusion, healthy diffusion, and broth or dilution for the *in vitro* investigation of extracts and pure drugs as potential antimicrobial agents. Hoque *et al***.** (2015) investigated on antimicrobial potential of pet ether, chloroform, and methanol extracts of *G. lucidum.* They reported that the methanol extract had the highest antimicrobial activity compared to the pet ether and chloroform extract. The study is suggestive that *G. lucidum* can be used to develop new drugs as an antibacterial agent. Shahid *et al***.** (2016) focused to analyze the best extract of *G.lucidum* against plant pathogenic fungi for which infected marigold plant samples were collected, and both organic and aqueous extracts. Balakumar *et al***.** (2011) experimented on *Phellinus* polypore fungi to evaluate the antifungal activity of methanol and aqueous extract of fruit bodies on selected fungal and bacterial pathogens. They found antibacterial and antifungal activity in high levels, which was screened against the selected strain.

IV. CONCLUSION

This study showed that *Ganoderma lucidum,* the aqueous and organic solvents extract used against pathogenic fungal strains. They demonstrated a high level of antimicrobial activity in different proportions. These results guarantee the claims of traditional herbalists that *the Ganoderma* species could be used to treat some fungal infections in humans. *Ganoderma* species, primarily *G. lucidum* could be used as a feed supplement to resist microbial infections and boost immune system in human beings. Furthermore, it was observed that *G. lucidum* extracts behaved differently in their antimicrobial effectiveness depending on the

solvent used for extraction. The micro-dilution technique was rapid assay method and performed within 72 hours. Visible observations of MIC and ethanolic extract showed the most effective anti-fungal activity against *Aspergillus niger*. Thus, the efficiency of ethanolic extract and the standard drug over *A.niger* is identical. The present study output is direction for new potential bioactive screening for unexplored compounds.

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