

Antibacterial Activity Determination of Polar and Non-Polar Solvent Extracts of *Ganoderma lucidum* Against Human Pathogenic Bacteria

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Abstract: Mushrooms have been used as valuable food for a very long time because they are highly nutritious, with the necessary tremendous amount of quality proteins, vitamins, minerals, etc. Mushrooms are rich, diverse sources of various bioactive compounds extracted quickly by different extraction procedures. In the present study, *Ganoderma lucidum* was possessed for extract preparation of mixed polar and non-polar solvents. The extracts solvents were analyzed for potential antimicrobial property determination of crude extracts by disk diffusion and minimum inhibitory concentration (MIC) methods and successfully investigated the antimicrobial potency. The results indicated that polar and non-polar extracts from *Ganoderma* possessed potential antimicrobial activities against the tested organisms, i.e., *Bacillus tequilensis* and *Bacillus subtilis*. The present study suggests that the *Ganoderma* mushroom containing bioactive compounds has potent antibacterial capacity; future complete profiling of bioactive compounds will be a new dimension for research and drug discovery.

Index Terms - Mushroom, bioactive compound, extraction procedure, minimum inhibitory concentration, antimicrobial.

I. INTRODUCTION

Infectious diseases still remain one of the major threats to Human health. Many natural and synthetic antimicrobial agents have been discovered-developed to kill the harmful microorganisms present in the body. Antibiotics have saved millions of lives but also lead to an increase in antibiotic resistance. Bacterial and fungal pathogens have evolved numerous defense mechanisms against antimicrobial agents. Global antimicrobial resistance is creating a problem worldwide. There can be several reasons behind the increasing antimicrobial resistance (Mehmat *et al.*, 2010). There are some possible reasons such as overprescription of antibiotics, patients not completing the entire antibiotic course, overuse of antibiotics in fish farming and livestock, poor hygiene and sanitation, and absence of new antibiotics being discovered, etc. This is the reason why drug resistance among human pathogenic microorganisms has necessitated a continuous search for new antimicrobial agents (Ibiam-Udu *et al.*, 2014). Feeding practices of different ethnic groups across the globe have encouraged the development of research on natural products. This has led to the discovery of the relationship between chemical structures found in natural products and their biological properties as well as their significance to human health (Viegas and Bolzani, 2004). Currently, natural compounds are the focus as many biotechnological companies are looking for new antimicrobial drugs. The natural products derived are of great use in the production of the new antibacterial substance. Macro fungi or Mushrooms have been used as a portion of valuable food for a very long time because they are highly nutritive, low-calorie food with good quality proteins, vitamins, and minerals. They produce a wide range of secondary metabolites with high therapeutic value. Some of them are even used as medicines worldwide (Nedelkoska *et al.*, 2013; AKYUZ *et al.*, 2010). *G. lucidum* (Fr.) Krast, a basidiomycete belonging to the Ganodermataceae family is one of the most famous traditional medicinal herbs used for healthy food and medicine (Fang and Zhong, 2002). *Ganoderma* has been reported to have a variety of biologically active components such as phenols, terpenoids, steroids, glycoprotein, polysaccharides, nucleotides, and their derivatives. Polysaccharides have been reported to contribute to immune enhancing and tumor retarding effects. It is seen that anti-tumor and anti-cancer effects are based on the enhancement of the body's immune system (Mizuno *et al.*, 1995; Liu *et al.*, 1996; Chandrawanshi *et al.*, 2017). Therefore, mushroom attracts attention because of their activities such as anti-tumor, immunomodulatory, cardiovascular, and respiratory effect (Ha *et al.*, 2000; Chang and Mshigeni, 2001).

Antibacterial Assay Methods

Antibacterial activity is the most used antimicrobial activity reported (Joseph P. Michael, 2017). Antibiotics are produced by microorganisms or they are fully prepared by chemical synthesis. They inhibit the growth of microorganisms by minimal concentrations. A variety of methods can be used to determine the *in vitro* antibacterial activity of a compound or an extract. The most common are disk diffusion and agar dilution methods. According to Balouiri *et al.* (2016). The agar disc diffusion method is the official method used in most laboratories for determining antibacterial susceptibility. Antimicrobial agent diffuses into the agar and inhibits the growth of the microbes. A clear zone of inhibition is measured (Balouiri *et al.* 2016). The broth dilution method involves creating two-fold dilutions of the antimicrobial agent using 96-well microtitre plates. The extracts with different concentrations are added by diluting them with distilled water. Then each well is inoculated by microbial inoculums. This is incubated under desired conditions and used with 2, 3, 5 triphenyl tetrazolium chlorides (TTC, tetrazolium red) to analyze the growth of treated bacteria (Tiwari *et al.* 2010; Balouiri *et al.* 2016). Furthermore, determined the minimum inhibitory concentration (MIC). MICs are used in laboratories to confirm resistance but most often as a research tool to determine the activity of new antimicrobials (Quereshi *et al.* 2010). The present study targeted to extract preparation of *Ganoderma* by using polar and non-polar various solvents, finally determining their antimicrobial capacity by two different assay methods.

II. MATERIALS AND METHOD

2.1 Samples Collection and Identification

Fruiting bodies of *G. lucidum* mushroom germplasm were collected from the Charama forest (Kanker District) Raipur Chhattisgarh, in July 2017. This collected mushroom sample was kept in air-tight 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). For the antibacterial activity *Bacillus subtilis* and *Bacillus tequilensis* (human pathogen) were used, which were collected from the Microbial Laboratory, School of Studies in Biotechnology, Pt. RSU Raipur.

2.2 Preparation of Mushroom Extracts

The extraction method of mushrooms was determined by the protocol of Nithya et al. (2016). Initially, mushrooms samples were sun-dried for 1-2 days and each mushroom sample was subjected to grinding in a mixer (Bajaj GX7) to make a fine powder and further packed in airtight zipper bags for extraction and this was applied by using different solvents. The antibacterial compounds were extracted from *G.lucidum* samples with aqueous and organic solvents, in order to separate the chemical constituents into groups of different polarities. The solvents used in extraction are chloroform, ethanol, methanol, and aqueous extraction as hot water and normal water extract. 20g powdered were submerged into 200ml of different solvents (chloroform, ethanol, and methanol) in an air-tight flat bottom container incubates at an orbital shaker at 100 rpm for 24 hrs at 30°C respectively (Chandrawanshi et al., 2018). Similarly, performed for inorganic solvents (normal and hot water (60 °C), 20g powder was submerged in solvents and normal water was incubated overnight at room temperature and hot water extract was processed after one hour. The normal water was further incubated in an orbital shaker at 100 rpm for 24 hrs at 30°C respectively. 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, either 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.

2.3 Yield of extract

After the extraction process of mushroom samples by using different solvents, the initial weight of mushroom samples and the final weight of the extracted sample was 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.

$$\text{Yield of extract (\%)} = \frac{\text{Amount of obtained weight (gm)}}{\text{Amount of obtained used weight (gm)}} \times 100$$

2.4 Antibacterial studies

2.4a Determination of *in vitro* antibacterial assay through Disk diffusion method

The antibacterial activity of the mushroom extract was analyzed, following the procedure of Ibiyam *et al.* (2014). Firstly prepared nutrient agar basal medium (NAM), after the prepared medium, without contamination, the sterile disk treated with varying concentrations of extracts, then placed on the basal medium. Then after broth medium with *B. subtilis* and *B. tequilensis*. Then after being treated placed and incubated in an incubator for 24 hrs. The next days the treated and control plates were measured by radial zone scale (HiMedia).

2.4b Determination of *in vitro* antibacterial assay through MIC

The *in vitro* antibacterial activity of the mushroom extract was analyzed, according to the modified protocol of Heleno *et al.* (2013). *B. subtilis* and *B. tequilensis* (human pathogen) were used as a test organism. Minimum inhibitory concentration (MIC) was performed by dilution techniques using 96-well microtiter plates. The extracted samples with different concentrations (20, 40, 60, 80, and 100µl) containing (v/v) (1 mg/mL) and added bacterial broth medium with bacterial inoculums of the microdilution test were performed. Initially, 5µl of bacterial suspension in a suitable growth media was added to wells of a sterile 96-well microtitre plate, already containing 100 µl of the mushroom extract with various concentrations (20, 40, 60, 80, and 100µl), with (v/v) (1 mg/mL). The final volume is maintained in each well up to 250 µl. The control well was poured only with broth medium and bacterial suspension (5 µl) respectively. The contents of each well were mixed on the microplate through a micropipette, finally covered with aluminum foil to protect them from other microbial contamination, and incubated for 24hrs at 37 °C. After 24 hrs 10 µl of tetrazolium salt was added to each well and it was further incubated for 30 minutes. The lowest concentration without visible growth was defined as the concentration that completely inhibited bacterial growth (MICs). This was calculated based on color reduction compared with positive control of each bacterial strain.

III. RESULTS AND DISCUSSION

Spices and herbal plants are among food's most commonly used antibacterial agents. They have been used traditionally for thousands of years to control various health complications, including infectious diseases (Udu-Ibiyam et al., 2014). Many antibacterial compounds, such as lectins, terpenes, polysaccharides, etc., act on the bacterial cytoplasmic membrane (Quereshi *et al.*, 2010). The disk diffusion method and broth dilution minimum inhibitory concentration determined the antibacterial activities of different extracts with different concentrations. The present study revealed that the extracts of *G.lucidum* as presented by the various extracts solvents used for antibacterial effects. The study revealed that the ethanol extracts showed the highest percentage yield of 19.05%, and the minuscule yield of 1.40 % was observed in chloroform. Similarly, normal water showed a 6.05% yield compared to hot water, where the percentage yield was 4.85%. A yield percentage of 3.35 % was observed in methanol-derived extracts. Table 1, given below, shows the percentage yields of the various extract from *G.lucidum*.

Table 1: Percent yield of the extract

S.N.	Solvents used	Yield of extract (%)
1	Normal water	6.05
2	Hot water (60°C)	4.85
3	Ethanol	19.05
4	Methanol	3.35
5	Chloroform	1.40

3.1 Antimicrobial activity of polar and non-polar solvent extracts

3.1a Disk Diffusion Method

According to the results obtained by the disk diffusion method, increasing the concentration of extracts observed that the bacterial growth gradually decreased. The present study revealed that the non-polar solvent exhibited potent antimicrobial capacity against *B. tequilensis*. The methanol extracts showed potent inhibitory actions against *B. tequilensis* bacteria with a concentration of 100 µl had shown the highest zone of inhibition recorded (11.5 mm). Similarly, the hot water extracts showed antibacterial activity with a concentration of 100µl of maximum zone inhibition with 11mm. The ethanol extracts showed a zone of inhibition of diameter at 11mm for 100µl extracts. The hot water extracts showed inhibitory actions against *B. subtilis* bacteria with a concentration of 100 µl, with the highest zone of inhibition being 10 mm; likely methanolic extracts also showed antibacterial activity with a concentration of 100µl shown at 10.2 mm. The zone inhibition data has presented in table 2. The maximum inhibition zone was obtained against *B. tequilensis* in methanolic extract. The minimum zone of inhibition was obtained against the test organisms in different hot water and methanolic extract concentrations. In this study, the antibacterial activities of *G. lucidum* were examined using the disk diffusion method and Minimum Inhibitory concentration (MIC). The results indicated that aqueous and organic extracts from *Ganoderma* possessed activities against the tested organisms.

Table 2: Antimicrobial activity of *G.lucidum* polar and non polar solvent extract against microbes by disk diffusion assay method

Concentration of extracts (µl)	<i>B. tequilensis</i>			<i>B. subtilis</i>		
	Methanol (mm)	Ethanol (mm)	Hot water (mm)	Methanol (mm)	Ethanol (mm)	Hot water (mm)
20	nd	nd	10.00	6.00	5.00	7.00
40	nd	nd	nd	6.5 mm	6.90	7.50
60	10.00	8.50	6.00	9.00	7.50	8.00
80	11.00	10.00	8.00	10.00	9.00	8.50
100	11.50	11.00	11.00	10.20	11.00	10.00
DMSO (control)	nd*	nd	nd	nd	nd	nd

*nd =not detected

Some other researchers reported the antimicrobial potency for *Ganoderma* species. Djide *et al.* (2014) extracted fruiting bodies of *Ganoderma* by maceration method using 50% acetone, 50% ethanol, 50% methanol, and boiling water respectively and they found that the extracts compounds had antibacterial activity. Hoque *et al.* (2015) observed the antimicrobial various extracts of *G.lucidum* and found that methanol had the highest antioxidant activity as compared to petroleum ether and ethanol whereas antibacterial activity extracts showed mild to moderate zone. Ibiam *et al.* (2014) examined the antibacterial activities of mushrooms, ginger, and garlic by agar well diffusion method and found that extracts from mushrooms have antibacterial properties. Kamble *et al.* (2011) studied the antimicrobial activity of *G. lucidum* mycelia by agar well diffusion method where methanol, chloroform, and acetone extracts were used and found that all four extracts showed maximum inhibition at a concentration of 100 mg/ml. Quereshi *et al.* (2010) evaluated the antibacterial activity of *G. lucidum* extracts and found that acetone extract of the strain possessed strong antibacterial activity. Shikongo *et al.* (2013) did antimicrobial screening of extracts of *G. lucidum* mushrooms in Namibia and reported the presence of more than one biochemical class of compounds having antimicrobial effects on microbial strains tested.

3.1b Minimum Inhibitory Concentration of *G.lucidum* extracts

The broth dilution method is a very good alternative method for assaying to antimicrobial potency of isolated or extracted bioactive compounds. The methodology is involved in developing two-fold dilutions of an antimicrobial agent with 96 microtitre well plates. In the present study, 96 wells in microtitre plates were poured with different concentrations of solvent like 20 μ l, 40 μ l, 60 μ l, 80 μ l, and 100 μ l, which was then diluted with distilled water. Tetrazolium salts are used to indicate biological activity because the colorless compound present acts as an electron acceptor and is then reduced to a colored compound. The tetrazolium used in this activity gave good results against the two test organisms. The bacterial suspension changed to red or pink within 30 minutes where bacterial growth occurred. *B. tequilensis* MIC has shown against methanolic extract was 40 μ l whereas it was 10 μ l against hot water extracts. The value of MIC with the extract of normal water turned out to be 80 μ l, against ethanolic extracts the MIC was 10 μ l and with chloroform, extract MIC came out to be 10 μ l. Similarly, *B. subtilis* the MIC with methanolic extract was found to be 8 μ l, in ethanolic extract it was 60 μ l. The minimum inhibitory concentration of various extracts has presented in table 3 and Fig 1.

Table 3: MIC of different extracts of *Ganoderma* against tested bacteria

Concentration of extracts (μ l)	<i>B. tequilensis</i>	<i>B. subtilis</i>
Methanol	40 μ l	8 μ l
Ethanol	10 μ l	60 μ l
Chloroform	10 μ l	6 μ l
Normal water	80 μ l	10 μ l
Hot water	10 μ l	20 μ l
DMSO (control)	nd	nd

Figure 1: Broth dilution assay method for MIC estimation of different extracts against *B. tequilensis* and *B. subtilis*



Furthermore, MIC was recorded for chloroform (6 μ l) and in normal water it was 10 μ l. The highest MIC of 80 μ l was obtained in normal water extract against *B. tequilensis* and 60 μ l was obtained in ethanolic extract against *B. subtilis* and the lowest MIC of 6 μ l was obtained in chloroform extract against *B. subtilis*. Correspondingly, other worldwide researchers reported the potency of the antimicrobial capacity of *Ganoderma*. Gahlaut and Chhillar (2013) evaluated the antibacterial potential of plant extracts using resazurin as an indicator in microtitre plates where five different solvents were used and percentage yield was calculated. Heleno *et al.* (2013) reported that p-hydroxybenzoic and cinnamic acid were the two compounds identified in *G. lucidum* and methanolic extracts were active against all the tested bacteria with a minimum inhibitory concentration of 0.0125-0.75 mg/ml and bacterial concentration of 0.035-1.5 mg/ml. Wadt *et al.* (2015) evaluated the antimicrobial activity of *G. lucidum* extracts and demonstrated that the hydroethanolic extracts showed tannins, flavonoids, terpenes, and steroids. It showed good anti-inflammatory and antimicrobial activity. The recent experiment revealed that the *Ganoderma* has the potential bioactive compounds series, successfully extracted and analyzed by rapid antimicrobial screening methods.

IV. Conclusion

The present situation of resistance to antibiotics is emerging in a wide variety of different organisms and this poses a serious threat to the treatment of diseases. The various bioactive compounds have been isolated from plants and fungi. The macro fungus has big resources for a variety of bioactive compounds. *G.lucidum* have a good potential for the production of many different useful bioactive metabolites and they serve as a good source of antimicrobial drugs. The bioproducts of *Ganoderma* have multi-beneficial effects on human welfare. The antibacterial activity of various polar and non-polar solvent extracts i.e. methanol, ethanol, chloroform, normal water, and hot water of *G. lucidum* was tested against bacteria. The antimicrobial activity was executed by both the disk diffusion method and serial broth dilution method and the antibacterial activity was expressed by minimum inhibitory concentration (MIC) where both the zone of inhibition as well as MIC was observed. The present study justifies conducting further research to characterize the antibacterial activity. Antibacterial substances derived from *G.lucidum* have received considerable attention in recent years. It is quite clear from the present study that the mushroom extracts of *G. lucidum* could be used to combat several infectious diseases caused by pathogenic organisms.

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