Research Article

Phytochemical Analysis and *In Vitro* Antimicrobial Potential of *Ganoderma applanatum* (Pers.) Pat. of Shivamogga District-Karnataka, India

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ABSTRACT

Ganoderma applanatum, a wild macrofungi was screened for phytochemical composition and evaluate possible *in vitro* antimicrobial potential of various organic solvent extracts. The phytochemical screening was performed by Harborne method and antimicrobial activity against human pathogens was evaluated by agar well diffusion method. The phytochemical analysis reveals that the extracts were a rich source of phytoconstituents containing saponins, phenols, steroids, glycosides, terpenoids and flavonoids. Methanol extract was shown to be more effective against all the tested pathogens followed by aqueous, petroleum ether and chloroform extract. *S.auresus* was found to be more sensitive organism followed by *E.coli* and *B.subtilis*. Methanol extract have higher solubility for more active antimicrobial and phytochemical constituents, consequently displaying the highest antimicrobial activity. The extract could be potential source of new antimicrobial agents and scientifically validates the use of the macro fungi in traditional medicine.

Keywords: Agar well diffusion assay, Antimicrobial activity, *Ganoderma applanatum*, Phytochemicals.

INTRODUCTION

Ganoderma applanatum is a Basidomycete fungus belongs to the family of Polyporaceae. The Fungal genus *Ganoderma* contains about 400 species and is member of the Ganodermataceae, characterized by unique double walled Basidiospores[1]. The genus is distributed throughout the world, but is particularly diverse in the tropics [2]. The fruit body has been used as traditional medicine for anti-cancer in China and reported to have diverse physiological activities including anti-tumor[3], anti-virus[4], immuno-stimulation[5-6] and in treatment of chronic diseases such as hepatitis, bronchitis, asthma and haemorrhoids[7]. It is also noted that the majority of the studies concerning the Ganodermataceae family relate to the antitumour and antiviral effects, while the antioxidant properties associated with this fungus have only recently become apparent [8-10]. There appears to be limited information available that reports the antimicrobial properties of *Ganoderma* species.

Bacteremia is a scourge of modern medical care. In recent years, there have been a significant number of human pathogenic bacteria becoming resistant to antimicrobial drugs [11-14] and this is in part due to the misuse and overuse of current antibiotics [15]. In addition, bacteria and fungal pathogens have complicated the treatment of infectious diseases [16-17]. Given the increase in multiple drug resistance of human pathogenic microorganisms, it is imperative that new and effective therapeutic agents are developed.

Fungi are well known for the production of important antibiotic compounds such as penicillin, however, the occurrence of antibiotics in the class of fungi known as Basidiomycetes is less well documented[18] and there are only few reviews that summarize the antibacterial activity from these mushrooms [19-22]. Antifungal drugs available today are not always successful in treating immuno-compromised patients due to the ineffectiveness or toxicity that many of them have on the host [23] and hence, there is a need for the identification of novel antifungal agents.

MATERIALS AND METHODS

Sample collection and Extraction

The color, odour, ecology, distribution and other apparent properties of the macro fungi, were studied and noted in the study area Shivamogga, is one of the richest floristic area, located in between 13° 27' and 14° 39' N lat and 74° 38' and 76° 34' E long with a wide range of ecosystems and species diversity. The average rainfall is 140 cm, temp is 25°C and RH is 60 to 100%. The macro fungal diversity is very rich due to litter decomposition. Identification was done by comparing their morphological and anatomical characteristics[24] and also through the electronic data on identification keys of mushrooms

The fresh samples after removing the external material such as mud, bush, soil etc. by washing with demineralized water were shade dried, powdered and packed into Soxhlet column and extracted using the polarity difference of various solvents at 60^0 C, in order by petroleum ether, chloroform, methanol and distilled water.

Phytochemical analysis

The freshly prepared extracts were subjected to standard phytochemical analysis to ensure the presence of following phytoconstituents such as alkaloids, steroids,

saponins, tannins, phenols, glycosides, terpenoids, flavonoids and anthraquinones by standard procedures [25-27]. The tests were based on the visual observation of color change or formation of a precipitate after the addition of specific reagents.

Test for Alkaloids: Alkaloids are basic nitrogenous compounds with definite physiological and pharmacological activity. Alkaloid solution produces white yellowish precipitate when a few drops of Mayer's reagent were added to the extract solution. Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent [28].

Test for Terpenoids and Steroids : Four milligrams of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulphuric acid was added slowly and red violet color was observed for terpenoid and green bluish color for steroids.

Test for Saponins: Three milligrams of extract was treated with 5 ml of water and shaken well. Froth formation was observed for the presence of saponins, which was found to be stable for 15 minutes.

Test for Tannins: To 0.5 ml of extract solution 1 ml of water and 1 - 2 drops of ferric chloride solution was added. Blue color was observed for gallic tannins and green black for catecholic tannins.

Test for Phenols: Four milligrams of extract was dissolved in respective solvent, few drops of 5% ferric chloride solution was added. The presence of phenols was determined by obtaining the deep blue color.

Test for Glycosides: Glycosides are compounds which upon hydrolysis give rise to one or more sugars (glycones) and a compound which is not a sugar (aglycone or genine). To the solution of the extract in glacial acetic acid, few drops of ferric chloride and concentrated sulphuric acid were added and observed for a reddish brown coloration at the junction of two layers and the bluish green color in the upper layer.

Test for Flavonoids: Four milliliters of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated hydrochloric acid was added and red color was observed for flavonoids and orange color for flavones.

Test for Anthraquinone: To 0.5 g of the extract, 5 ml of chloroform was added and shaken thoroughly for 5 minutes. This was filtered and the filtrate shaken with an equal volume of 100% ammonia solution. No pink violet or red color in lower layer was seen, indicating the absence of anthraquinones.

Antimicrobial activity

Test microorganisms

The extracts were individually tested against a set of human pathogenic microorganisms, including two Gram-positive bacteria: *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 1134); four Gram-negative bacteria: *Escherichia coli* (MTCC 1559), *Pseudomonas aeruginosa* (MTCC 1034), *Klebsiella pneumonia* (MTCC 2113) and *Salmonella typhi* (MTCC 734), the four dermatophytic fungi were *Candida albicans* (MTCC 1637), *Chrysosporium indicum* (MTCC 2831), *Trichophyton rubrum* (MTCC 3272) and *Microsporum gypseum* (MTCC 2829). All these microorganisms were procured from IMTECH, Chandigarh, India.

Preparation of inoculums

The bacterial inoculums were revivified by transferring loop full of organisms from mother culture into a 250ml conical flasks containing sterilized Nutrient broth (Hi Media) .The flasks were incubated on a rotary shaker for 24 h at 37° C. The broth cultures were subjected to standard plate count method to enumerate the population and the dilution having 1 X 10^6cfu/ml were selected for antimicrobial test, for fungal inoculums sterilized Potato dextrose broth (Hi Media) was used and incubated for 24 h at 28^0 C. Using sterile saline solution, the cultures were harvested and diluted to bring the count to about 1 X 10^8cfu/ml. Commercially available Streptomycin and Chloramphenicol at the concentration 1mg/ml was used as positive (standard) control and 10% DMSO as negative control. Standard drug solutions were prepared in sterile water for injection.

Antimicrobial screening by agar well diffusion method

The antimicrobial activities of the different extract preparations from *G.applanatum* were determined by standard agar well diffusion method [29-30] with slight modifications. Mueller Hinton Agar (Hi Media) and Sabourd dextrose agar (Hi Media) plates were swabbed (sterile cotton swabs) with 24 h old - broth culture of respective bacteria and fungi. Wells of 6 mm diameter and about 2 cm apart were made in each of these plates using sterile cork borer. Stock solution of each extract was prepared at a concentration of 2 mg/ml. About 100 µl of different concentrations *viz.,* 25%, 50% and 75% of solvent extracts were added with the help of sterile syringe into the wells and allowed to diffuse at room temperature for 2hrs. Control experiments comprising inoculums without extract were set up. The plates were incubated at 37° C for 18-24 h for bacteria and 28° C for 72 h for fungi. The diameter of the inhibition zone (mm) was measured. Triplicates were maintained and the experiment was repeated thrice, for each replicates the readings were taken in three different fixed directions and the average values were recorded.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC is defined as the lowest concentration of the compound which will inhibit the growth of microorganism. MIC was determined by micro-dilution method [31-32] using serially diluted (2 folds) crude extracts according to the National Committee for Clinical

Laboratory Standards. We found that methanolic extract posses high inhibition zone, so we set out MIC and MBC only for methanolic extract. MIC of the extract was determined by dilution of methanolic extract with 20 mg/ml concentrations. Specifically, 0.1ml of standardized inoculums (1×10^7cfu/ml) was added in each tube. The tubes were incubated aerobically at 37° C for 18-24 h. The lowest concentration of the extract that produced no visible bacterial growth (no turbidity) when compared with the control were regarded as MIC. The highest dilution that yielded no bacterial was taken as MBC.

Statistical analysis

The experimental results were expressed as mean ± standard deviation of mean of three replicates. The results were processed using Microsoft Excel 2007 and ez Annova version 0.98. P values of less than 0.05 were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Phytochemical analysis

The test sample was successively extracted using the polarity difference of various solvents at 60° C. The corresponding extraction fraction for each solvent was obtained by concentrating under reduced pressure by using rotary evaporator. Table 1 provides some characteristics of either the solid extracts obtained after evaporation to dryness as the hygroscopic paste obtained for the chloroform extract. As described in Table 2, the phytochemical analysis of *Ganoderma applanatum* reveals the presence of saponins, steroids, phenols, glycosides, terpenoids and flavonoids. Last, alkaloids, tannins and anthraquinones were not detected in any extract.

Table 1: The appearance, consistency and yield of the organic extracts from *Ganoderma applanatum*.

Solvents	Appearance; Consistency	Yield in gms
Petroleum ether	Light yellow; Thick paste	2.1
Chloroform	Dark yellow; Gelly paste	1.8
Methanol	Brownish red; Solid powder	4.3
Aqueous	Dark brown; powder	2.5

Table 2: Showing analysis of Phytochemical constituents of *Ganoderma applanatum* sample

Phytochemical constituents	Petroleum ether Extract	Chloroform extract	Methanol extract	Aqueous Extract
Alkaloids	-	-	+	-
Steroids	+	-	+	-
Saponins	-	+	+	+
Tannins	-	-	-	+
Phenols	-	+	+	+
Glycosides	+	+	+	-
Terpenoids	-	-	+	+
Flavonoids	+	+	+	-
Anthraquinone	-	-	-	-

'+' Present ; **'-'** Absent

Antimicrobial screening by agar well diffusion method

The *in vitro* antibacterial potential of different solvent extracts of *G.applanatum* was carried out by agar well diffusion method and results were tabulated in Table 3. The methanol extract at 75 mg/ml, 50 mg/ml and 25 mg/ml of concentration were found to be having significant antibacterial activity against *S.aureus* (15mm) and *E.coli* (13mm) and moderate effect on *B.subtili* (12mm) *and P.aeruginosa* (12mm) and mild effect on growth of *K.pneumonia* (8mm) and *S.typhi* (8mm). Both petroleum ether and aqueous extract was found to be having moderate antagonistic effect on *S.aureus* (12mm) and mild effect on *E.coli* (8mm) and negligible effect on *K.pneumonia* (6mm), *S.typhi* (00 mm) and *P.aeruginosa* (7mm) at 75 mg/ml concentration and no activity was observed at 50 mg/ml and 25 mg/ml concentration. The chloroform extract showed moderate effect on *S.aureus* (8mm) followed by *B.subtilis* (6mm) and *E.coli* (5mm) at a concentration of 75 mg/ml and not at all showed any effect on *K.pneumonia*, *S.typhi* and *P.aeruginosa*.

The antifungal activity results of different solvent extracts were recorded in Table 4. The antifungal activity results reveals that, the methanol extract at 75 mg/ml and 50 mg/ml of concentration was found to be having significant antifungal activity against *C.albicans* (10mm) and *C.indicum* (7mm) compared to petroleum ether extract, where as the aqueous and chloroform extract showed very mild and less potent activity against all tested fungi.

The results suggested that only methanol extract possessed a broad spectrum antimicrobial effect including potent antibacterial and antifungal activities. The chloroform extracts exhibited little or no growth inhibiting activities.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of the methanolic extract were within the range of 6.8 to 45 mg/ml and 12 to 60 mg/ml respectively (Fig.1). Methanolic extract showing lowest MIC *i.e.,* 6.8 mg/ml against *S.aureus* followed by *E.coli* and *B.subtilis* and the highest MIC to *S.typhi*. The lowest MBC is 12mg/ml against *S.aureus* followed by *E.coli* and *B.subtilis*, whereas *K.pneumonia* and *P.aeruginosa* were showed MBC at 45mg/ml and the highest MBC was found to be 60 mg/ml against *S.typhi*.

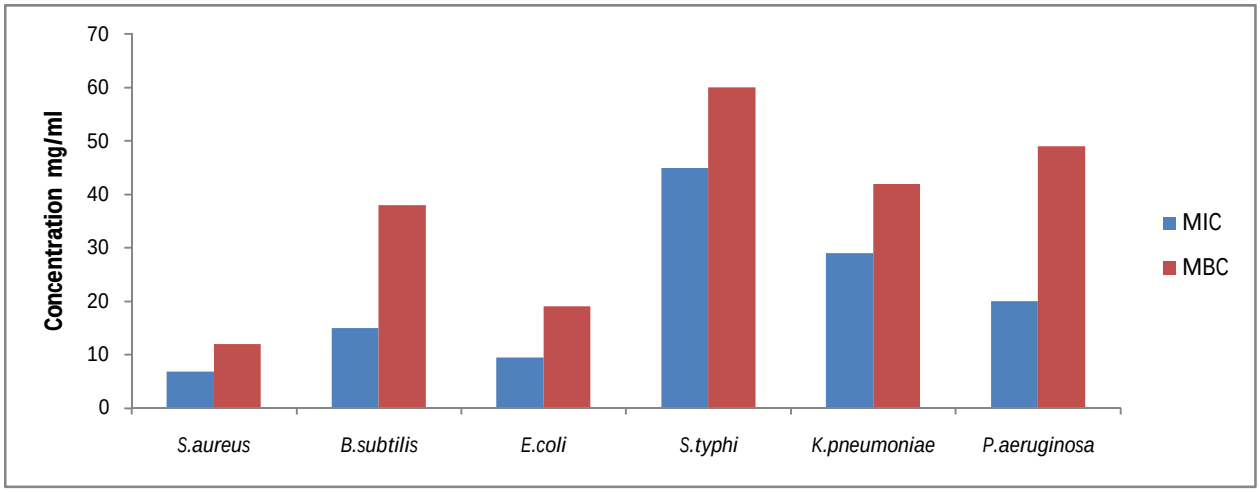

Figure 1: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of methanolic extract of *G.applanatum*. Values are mean ± SD of 3 experiments in each group.

Table 3: Showing antibacterial activity of *Ganoderma applanatum* against human pathogenic bacteria

Test microorganisms	Zone of Inhibition (mm)													
	Pet. Ether Extract			Chloroform Extract			Methanol Extract			Aqueous Extract			10% DMSO (Control)	Streptomycin 1mg/ml (Standard)
	75%	50%	25%	75%	50%	25%	75%	50%	25%	75%	50%	25%		
S.aureus	11±0.23[b]	8± 0.18[b]	0	8± 0.21[b]	0	0	15±0.21[b]	12±0.17[b]	8± 0.23[b]	12±0.23[b]	10±0.26[b]	0	0	19± 0.21[b]
B. subtilis	8± 0.23[b]	6 ±0.21[b]	0	6±0 .18[b]	0	0	12± 0.2[b]	9 ±0.2[b]	6± 0.2[b]	9± 0.14[b]	8± 0.17[b]	0	0	20± 0.21[b]
E.coli	8±0.22[b]	5±0.23[b]	0	5± 0.23[b]	0	0	13± 0.26[b]	8± 0.20[b]	6± 0.17[b]	10± 0.2[b]	8± 0.24[b]	0	0	16 ± 0.18[b]
S.typhi	0	0	0	0	0	0	8 ±0.24[b]	0	0	5± 0.17[b]	0	0	0	16± 0.17[b]
K.pneumoniae	6± 0.21[b]	0	0	0	0	0	8± 0.2[b]	6± 0.15[b]	0	6± 0.21[b]	0	0	0	17± 0.21[b]
P.aeruginosa	7 ±1.7[b]	0	0	0	0	0	12±0.23[b]	8± 0.21[b]	0	8± 0.12 [b]	0	0	0	15± 0.21[b]

Each value is the mean of three replicate determinations ± standard deviation; [a]P<0.05 – significant, [b]P<0.01 –highly significant

Table 4: Showing antifungal activity of *Ganoderma applanatum* against human pathogenic fungi

Test microorganisms	Zone of Inhibition (mm)													
	Pet. Ether Extract			Chloroform Extract			Methanol Extract			Aqueous Extract			10% DMSO (Control)	Chloramphenicol 1mg/ml (Standard)
	75%	50%	25%	75%	50%	25%	75%	50%	25%	75%	50%	25%		
C.albicans	8± 0.20[b]	6± 0.2[b]	0	6± 0.25[b]	0	0	10± 0.23[b]	6± 0.14[b]	0	5± 0.2[b]	0	0	0	18± 0.21[b]
T.rubrum	5± 0.17[b]	0	0	0	0	0	5± 0.18[b]	0	0	0	0	0	0	15± 0.17[b]
C.indicum	6± 0.17[b]	5± 0.21[b]	0	5± 0.17[b]	0	0	7± 0.18[b]	5± 0.19[b]	0	0	0	0	0	16± 0.17[b]
M.gypseum	5± 0.18[b]	0	0	0	0	0	5± 0.14[b]	0	0	6± 0.22[b]	0	0	0	14± 0.16[b]

Each value is the mean of three replicate determinations ± standard deviation; [a]P<0.05 – significant, [b]P<0.01 –highly significant

The phytochemical analysis of *G.applanatum* disclosed the presence of major phytoconstituents *viz*., saponins, steroids, phenols, glycosides, terpenoids and flavonoids. Among the four solvents used for extraction, methanol extract showed more number of phytoconstituents followed by aqueous, petroleum ether and chloroform extracts. This is in agreement with the findings of Jonathan and Fasidi [33] that, bioactive secondary metabolites of mushrooms extracted may be different depending on the extractive solvent. Different solvents have been reported to have the capacity to extract different phytoconstituents depending on their solubility or polarity in the solvent [34]. Methanol extract obtained in this study might have higher solubility for more active

antimicrobial constituents, consequently displaying the highest antimicrobial activity. Water was observed in this study to be poor solvent compared to other solvents used. This is in line with research findings that some phytochemicals are more soluble in alcohol than water [35]. This also confirmed the suggestion of Fujita *et al*. [36] who suggested that methanol was better than water as extracting solvent.

G. lucidum and other *Ganoderma* species, more often in combination with chemotherapeutic agents, have been used to treat various bacterial diseases. They have suggested that the sesquiterpenoid components play an important role in its bioactive principle[19]. Furthermore a great variety of secondary metabolites such as triterpenes, lipids, phenolics compounds, polyketides, terpenes and steroids have also been identified and characterized in mushrooms with medicinal properties [37-39]. The present findings are in accordance with other investigators who have also reported the presence of these components in the member of family Ganodermataceae [40].

Ganoderma samples were highly active against the gram positive as well as gram negative bacteria and also showed strong activities against *Candida albicans* [41], hence are a broad spectrum antibiotic. In addition, methanol extract of *G. lucidum* from India have been shown to possess efficient antibacterial activity against MRSA [42]. Extracts from *G.applanatum* and *G.pfeifferi* have been shown to possess significant antibacterial activity against *E. coli* [43-44]. Danielli [45] suggested that, the lower the MIC, the more sensitive and promising the extract. This implies that most of these higher fungi offer potential therapeutic potency against medically important bacteria. The MIC against fungi were generally high. This result confirms the observation made that the higher fungi studied possessed poor antifungal activities. The present findings of our study provides the scientific basis for the use of this macro fungus in the traditional treatment of diseases.

CONCLUSION

Demonstration of broad-spectrum antimicrobial activity by various solvent extracts of *G.applanatum* may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease chemotherapy and control. The methanol extract had promising antimicrobial activity against tested human pathogenic bacteria compared to fungi. The extract could be potential source of new antimicrobial agents; this investigation has opened up the possibility of the use of this macro fungi in antimicrobial drug development for human application and traditional medicine. The effect of these extracts on other pathogenic microorganisms, more toxicological investigations and further purification to isolate the specific active constituents need to be carried out.

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