academicJournals

Vol. 9(5), pp. 265-274, 4 February, 2015
DOI: 10.5897/AJMR2014.7045
Article Number: 0A5F17D50443
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antimicrobial potential of *Ganoderma* spp. fruiting bodies and cultured mycelium

Pradip Kumar Sharma[1]*, Sushil Kumar Shahi[2], Pradeep Kumar Sharma[1], Mahesh Kumar[3], Ramakant Lawaniya[4] and Mandeep Balhara[4]

[1]Department of Microbiology, Chaudhary Charan Singh University Meerut, India.
[2]Department of Botany, Guru Ghasi Das Vishwavidhyalaya Bilaspur, India.
[3]Department of Microbiology, J. V. College Baraut Baghpat, India.
[4]Dairy Microbiology Division, National Dairy Research Institute, Karnal, India.

Received 2 August, 2014; Accepted 22 December, 2014

Present investigation focuses on the antimicrobial potential of cultured mycelium extracts over fruiting bodies of *Ganoderma* spp. Minimum inhibitory concentration (MIC) of ethanolic extracts of the fruiting body of *G. lucidium* against multidrug resistance pathogens was evaluated using broth dilution method. MIC of crude ethanolic extracts of fruiting body of *G. lucidium* was 0.78 mg/ml for *Salmonella typhi,* 3.12 mg/ml for *Salmonella typhimurium*, and 1.56 mg/ml for *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans;* while MIC of the cultured mycelium crude extract was 0.39 mg/ml for *S. typhi,* 0.78 mg/ml for *S. typhimurium, S. aureus, P. aeruginosa* and *C. albicans*. Their activities were found to be pH tolerant and thermostable. Additionally, both types of the extracts inhibited heavy doses of inoculum. The extracts showed broad spectrum antimicrobial activity. MIC used for *Escherichia coli, Klebsella pneumoniae, Staphylococcus saprophyticus, Enterococcus faecalis* and *Proteus vulgaris* was 1.56 mg/ml for the fruiting body extract (crude) and 0.78 mg/ml for the cultured mycelium crude extract; while MBC used for *E. coli, Klebsella pneumoniae, Staphylococcus. saprophyticus, Enterococcus faecalis* and *Proteus vulgaris* was 3.12 mg/ml and 1.56 mg/ml for the fruiting body extract (crude) and cultured mycelium crude extract, respectively. Cultivated mycelium is a continuous source for obtaining antimicrobial compounds compared to fruiting bodies, and manipulation of media components may result in enhanced production of desired compounds. The findings are interesting from a commercial point of view. Therefore, there is potential possibility to establish research and development of antimicrobial compounds from 'indigenous' *Ganoderma* spp.

Key words: Antimicrobial activity, *Ganoderma* spp., fruiting body, cultured mycelium.

INTRODUCTION

Antimicrobial drugs, derived from different kinds of microorganisms, have long been used for prophylactic and therapeutic purposes. However, using the same antimicrobial for a long period may cause resistance in

*Corresponding author. E-mail: pradeep.pkrn@gmail.com.

microorganisms. Natural products have played an important role in medicine since the 1940s (for example Penicillins) and continually serve as an important source and inspiration to produce new drugs (Newman et al., 2003). Continuous search for novel chemical compounds by expanding natural products search is essential to combat the adaptability of infectious microbes and to keep pace with the ever-increasing need for new drugs to cure various diseases (Dobson, 2004).

Parallel to this is a scenario wherein microorganisms have developed resistance to many antibiotics. After a very short period of using penicillin in clinical practices in the 1940s, drug resistance started to develop in Staphylococci, and today some strains have developed resistance to more other expressions. This may result in very tough conditions, as development of new drugs is very slow (Henry and Frank, 2009).

There have been a significant number of bacteria becoming resistant to antimicrobial drugs (Lorenzen and Anke, 1998), and this is partly due to the misuse and overuse of current antibiotics (Monroe and Polk, 2000). Antimicrobial drug resistance is of major economic concern, as it affects physicians, patients, health care administrators, pharmaceutical producers and the public (McGowan, 2001).

Since the discovery of Penicillin from the fungus *Penicillium* (Fleming, 1929), there has been much focus on the production of antibacterial agents from the filamentous fungi within this division (Florianowicz, 1998; Larena and Melgarejo, 1996; Mayordomo et al., 2000; Rodrigues et al., 2000). Fungal fruiting bodies, fungal mycelium or the culture fluid in which the mycelium is cultivated may be explored for biological activity. Some advantages of using filamentous fungi over plants as sources of bioactive compounds are often that the fruiting body can be produced in much less time; the mycelium may also be rapidly produced in liquid culture and the culture medium can be manipulated to produce optimal quantities of active products (Anand and Chowdhry, 2013).

A number of reviews have been described for the bioactive substances, medicinal effect and health benefits of Ganoderma species (Chang, 1995; Chen and Miles, 1996a). It has been examined predominantly for bioactive triterpenes and polysaccharides. Fungi within the same genus can produce a different variety of secondary metabolites as a result of stress (Pointing and Hyde, 2001) or from exposure to different environmental conditions (Kim et al., 2003). In addition, there have been reports of different Ganoderma species possessing different biologically active compounds (Gao et al., 2000; Mothana et al., 2003; Smania et al., 2003).

Cultivation of fungi for fruiting body production is a long term process, taking one to several months, depending upon the species and substrates. In contrast, production of fungal mycelium would allow acceleration in the growth and to obtain high yield of biomass with constant composition (Edelstein and Segel, 1983; Grigansky et al., 1999).

The focus of this investigation was to evaluate the potential of antimicrobial compounds to fight multi-drug resistant microorganisms, that originate from mycelium. Cultured mycelium of fungi is an ideal source for the production of antimicrobial compounds. However, the difference in the effect of the substances isolated from two stages of growth of fungi (mycelium and the fruiting body) must be ascertained.

MATERIALS AND METHODS

Collection of fruiting bodies

Four different fruiting bodies of wood rotting *Ganoderma* were collected from Directorate of Mushroom Research, Solan Himachal Pradesh designated as, *G. applantum*, (GF 1*) G. lucidium* (GF 2), *G australe* (GF 3) and *G. sinense* (GF 4). Fruiting bodies were dried and specimen were submitted to Bioresourec Tech Lab, Dept. of Microbiology, Chaudhary Charan Singh University Campus, Meerut.

Mycelial culturing

The mycelium of the fungus, *Ganoderma,* was isolated by discharged spore method (Crittenden et al., 1995). Fruiting bodies were washed in a turbulent flow of tap water in order to remove surface contamination. Individual bodies were cut off and attached to the underside of the Petri plate lids with the help of petroleum jelly. Petri plates containing modified malt agar were then inverted over the lids and basidiospores were allowed to discharge onto the agar medium. Plates were incubated at 27°C in BOD incubator and observations were recorded periodically over a week period. Mycelium of germinating spores was transferred to fresh modified malt agar medium and culture was maintained at 4°C for further use.

Crude extraction from fruiting bodies and cultured mycelium

Metabolites of *Ganoderma* fruiting bodies and the mycelial mat, extracted in organic solution, were further used to investigate their antimicrobial activity against multidrug resistant pathogenic microorganisms. For extraction of metabolic compounds, acetone, benzene, chloroform, ethyl alcohol, ethyl acetate and methanol organic solvent were used. Fruiting bodies were washed with fast flowing tap water followed by 70% ethyl alcohol and dried with hot air oven at 40°C. Air dried fruiting bodies were ground with a pestle and mortar. Out of this, 1 g powder was accurately weighed in a 30 ml black cap bottle and 10 ml of each solvent, described above, was added to it. The extracts were sonicated for 30 min and then left overnight at room temperature. Isolated fruiting bodies were cultured on malt extract agar medium. Mycelium from 21 days old culture was used for extraction. Mycelium was removed with the help of water bath at 40°C and dried at 40°C in hot air oven. Dried mycelium was ground with pestle and mortar. 1.0 g of ground fungal mycelium powder was extracted in 10 ml of organic solvent. The extracts were sonicated for 30 min and then left overnight at room temperature. The extracts were filtered over Whatman No. 1 filter paper and both extracts were dried by evaporating the organic solvents. Equal amount of DMSO was added and sterilized by membrane filtration using polypropylene housing nylon membrane filter compatible with solutions containing DMSO with 0.45 µm pore size filters (Pall life science). The extracts were stored at 4°C until use (Figure 1).

Figure 1. Fruiting body (A) and cultured mycelial mat (B) of *Ganoderma* spp and its extracts.

Procurement and maintenance of test micro-organisms

Antimicrobial effect of the extracts of *Ganoderma spp.* on certain strains of clinically important microorganisms was investigated. The test microorganisms were procured from Collection of Bioresource Type Culture (CBTC), Department of Microbiology, Chaudhary Charan Singh University, Meerut; they are: *Salmonella typhimurium* - CBTC 174, *Salmonella typhi*- CBTC 175, *Pseudomonas aeruginosa* -CBTC 162, *Staphylococcus aureus* -CBTC 330, *Candida albicans* -CBTC 119 and ATCC including *E. coli (ATCC 11775), K. Pneumoniae (ATTC 27736), E. faecalis (ATCC 29212)* and *P. vulgaris* (ATCC 6380). All test microorganisms used in the study were maintained on nutrient agar slants. The slants were inoculated and incubated at 37°C for 24 h and then stored at 4°C; while the yeast strain was maintained on malt extract agar, incubated at 30°C for 48 h and then stored at 4°C.

Preparation of microorganisms

All bacterial strains and the yeast *C. albicans* were inoculated in 5 ml Brain Heart Infusion (BHI) broth and incubated overnight at 37°C. The turbidity of the actively growing culture was adjusted with sterile saline (0.85%) to obtain turbidity optically comparable to that of 0.5 McFarland standard.

Antibiotic susceptibility profile of test microorganisms

Antibiogram of various antibiotics was performed using Kirby Bauer disc diffusion assay according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (Lehrer et al., 1991; NCCLS, 2001), with slight modifications. Approxamately, 40 antibiotics and six antifungal drugs were used to check out the susceptibility profile of the test pathogens used in the study. 20 ml of sterile molten Muller Hinton Agar (MHA) containing 50 µl of test microorganism suspension (0.5 Mcfarlad Standard) was poured into plates. After solidification, the antibiotic discs were applied. Plates were incubated in a BOD incubator for overnight at 35°C for test pathogens. Zone of inhibition was measured using Hi antibiotic zone scale™ by resting plates lid down on a black non-reflecting surface. The experiment was performed in triplicates. Results were expressed in terms of susceptible if zone of inhibition ≥ 12 mm including disc diameter, intermediate if zone of inhibition was 12 mm and resistant if zone of inhibition was < 12 mm (Bell et al., 2009).

Antimicrobial activity of the extracted crudes of fruiting bodies

First to screen out the best fruiting body extract, qualitative antimicrobial activity of the extracts was evaluated by employing agar well diffusion method (Lehrer et al., 1991). Optical density of 0.5 Mcfarland standard was checked by using plate reader perkin elmer Victor 3 at 600 nm. This O.D. was used as ref O.D. to set the OD of test microorganisms in sterile 10 mM potassium phosphate buffer of 6.8 pH. 20 ml of sterile MHA containing 50 µl of inoculum suspension was poured and plates were incubated at 37°C for half an hour to get solidify; then, 4 wells of 5 mm diameter were prepared in the 90 mm Petri plate containing test microorganism with the help of a sterile cork borer. 100 µl extracts of different fruiting bodies and their respective organic solvent were filled into the wells of agar plates. Plates were incubated at 35°C for 24 h. Inhibition zone was measured using Hi antibiotic zone scale™. The above experiment was repeated in triplicate. The inhibition zones were compared with those of reference discs.

Determination of minimum inhibitory concentration (MIC)

MIC is the lowest concentration of a test material at which no visible growth occurs. To determine the minimal inhibitory concentration, broth dilution method using Microtiter (plate 96 well) was followed (Sarker et al., 2007). This assay incorporates resazurin [2-(4-iodophenyl)-3-(4-nitrophenyl)-5- phenyltetrazolium chloride (INT, Sigma)] as indicator of any microbial growth.

In this method, 96 well sterile micro-titer plates were labelled and 100 µl volume of crude extracts from fruiting bodies and mycelium was transferred into the first column of the plate. All wells except first and 11^{th} were filled with 50 µl of nutrient broth. 11^{th} column was kept blank for discard. 50 µl of test extract was taken from first well of the first column and serially diluted. This resulted in 50 µl of the test material in serially descended concentration.

To each well, except first and 11th columns, 10 µl of resazurin (0.5 mg/mL in alcohol) and 30 µl of nutrient broth were added. Finally, 10 µl of bacterial suspension (0.5 Mcfarlad Standard) was added to each well, ensuring a final volume of 100 µl in them (except last row which had saline instead). A broad spectrum of antibiotic and antifungal was used as positive control. For negative control, DMSO was used instead of extracts.

Micro-dilution plates were incubated at 35°C for 12 h for bacteria and at 30°C for 24-48 h for *C. albicans*. All experiments were performed in triplicates, and colour change from purple to pink or colourless was noted as positive signal for growth and the lowest concentration at which colour change appeared was considered as MIC. The average of three values was calculated.

Determination of minimum microbicidal concentration (MMC)

MBC is the concentration of extract at which the growth is totally

inhibited due to death of microorganism. It was determined from broth dilution MIC tests by sub-culturing to agar media without antibiotics. The result was that both the extracts were static to all test microorganisms since growth was observed at MIC for al organisms. To determine the MBC for each set of wells in the MIC determination, a loop-full of broth from those wells that did not show any bacterial growth as well as control wells was streaked on nutrient agar plates. The plates were incubated at 35°C for 24 h. After incubation, the concentration at which no visible bacterial growth occurred was noted as the MMC. A broad spectrum of antibiotic and antifungal was used as positive control. For negative control, DMSO was used instead of extracts.

Effect of pH, temperature and inoculum density on antimicrobial activity of extracts

To study the pH effect on the antimicroboal activity of the extract, 2 ml of different extracts was constituted in test tubes and pH of extracts were adjusted to 3.0, 5.0, 9.0 using 0.1 N HCl and 0.1 N NaOH solutions, respectively (Doughari, 2006). Then the inhibitory activity of the extracts was studied against the test organisms by agar well diffusion method. Observations were recorded after overnight incubation at 37°C. Extracts with pH 7.0 were considered as control.

To study the temperature effect, 2 ml of different extracts was taken in test tubes and treated for 1 h at 4°C in the refrigerator, and at 60 and 100°C in water bath (Doughari, 2006). Then, the inhibitory activity of the extracts was studied against the test organisms by agar well diffusion method. Observations were recorded after incubation at 37°C for all the bacteria and 25 yeasts.

Effect of inoculum density of the test pathogens on antimicrobial activity of the *Ganoderma* extracts was carried out using procedure outlined by Dixit and Dikshit (1982). The requisite quantity of extracts was mixed in pre sterilized Muller Hinton Broth. Microbial suspension of 0.5 O.D. of the test pathogens was aseptically inoculated in culture tubes containing MHB of MICs of the extracts. A negative control (without inoculum) and a positive control (without extract) were run parallel. The O.D. of microbial suspension was increased progressively up to 1.0 O.D. in multiple of 0.1 O.D. The absence of microbial growth after overnight incubation at 37°C showed the potential of the extracts to inhibit heavy inoculum of the test pathogen. Average value of the three replicates was recorded. The tests were performed to determine the increased inoculum density at MICs of the extracts using poisoned food technique, with slight modification (Grover and Morre, 1962).

Statistical analysis

Results obtained were expressed as mean. Statistical analysis was performed by two-way ANOVA and P values below 0.05 were considered significant.

RESULTS AND DISCUSSION

Antibiotic susceptibility profile of test microorganism

Antibiotic susceptibility profile of the test microorganisms was carried out to ensure whether the test microorganisms, included in the study, are resistant or susceptible to the drugs being used. The sensitivity of four bacterial test organisms was tested against 40 antibiotics and of one fungal pathogen against six antifungal drugs. *Salmonella Typhimurium* showed resistance towards 41% of antibiotics tested including amikacin, ampicillin, cephotaxim, gentamycin, penicillin and tetracycline and was sensitive to 53.8% antibiotics including chlorempenicol, gatifloxacin, levofloxacin, methicillin penicillin. *Salmonella Typhi* was found resistant to 76.9% of antibiotics tested including ampicillin, carbenicillin, chloramphenicol, cephotaxim, methicillin, novobiocin, penicillin and streptomycin while sensitive to only 23 % of drugs including amikacin, ciprofloxacin, gatifloxacin, levofloxacin and norfloxacin.

P. aeruginosa was found resistant against 74.3% of antibiotics tested including ampicillin, carbenicillin, chloramphenicol, erythromycin, levofloxacin, naldixic acid, penicillin, streptomycin and tetracycline as no zone of inhibition against these antibiotics was observed; while it was found sensitive to a few 20.52% of antibiotics including amikacin, gatifloxacin. *S. aureus* was found resistant to 48.7% abundantly used antibiotics tested including ampicillin, ciprofloxacin, cephatoxim, gentamycin, norfloxacin, streptomycin and tetracycline while it was found sensitive to 35.89% of antibiotics including amikacin, chloramphenicol, co-trimoxazole, erythromycin and levofloxacin. On the other hand, *C. albicans* showed resistance to 66.6% antifungal drugs tested including itraconazole, fluconazole, Nystatin and amphotericin-B while it was found to be sensitive to ketaconazole and intermediate to clotrimazole.

Minimum zone of inhibition was shown against amphotericin B. These results showed that all the test microorganisms are resistant to most of the drugs used in the market (Figure 2). Prasad and Wesely (2008) found 10 samples of *S. aureus* resistant to 16 drugs commonly available in the market. Standard procedures in repression of harmful microorganisms are applications of antibiotics, but too much use of antibiotics and the ability of microorganisms to develop resistance to them have resulted in their decreased effects on microorganisms. MRSA is responsible for worldwide outbreaks of nosocomial infections. However, the pharmaceutical arsenal available to control MRSA is very limited now (Li et al., 2008).

Antimicrobial screening of *Ganoderma* extracts

To find the best fruiting body and the best solvent system, extracts of different *Ganoderma* spp. fruiting bodies were prepared in six different organic solvents and were screened for antimicrobial potential towards test microorganisms using agar well diffusion method. Tests were carried out in triplicates along with controls.

G. applantum fruiting body extracts showed maximum zone of inhibition (overall mean value) with Benzene (11.50) extract followed by chloroform (10.84), ethyl alcohol (8.97), ethyl acetate (7.9) and acetone (2.84) extracts. Although, no inhibition was found with methyl alcohol extract. *S. Typhi* was found to be most sensitive with maximum zone of inhibition (14.25) followed by

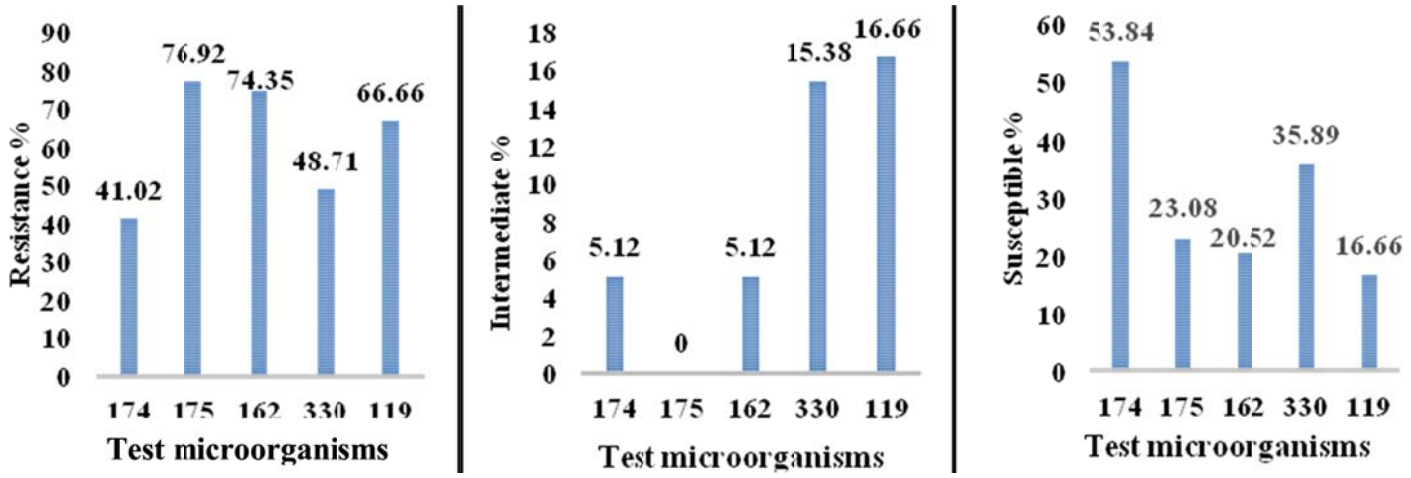

Figure 2. Antibiotic susceptibility profile of test organisms.174- *S. typhimurium*; 175- *S. typhi*; 162- *P. aeruginosa*; 330- *S. aureus*; 119- *C. albicans.*

Table 1. Zone of inhibition (mm) of *G. applantum* extracts against test pathogens.

Ganoderma applantum	**Acetone Ext**	**Benzene Ext**	**Chloroform Ext**	**Ethyl acetate Ext**	**Ethyl Alcohol Ext**	**Methyl Alcohol Ext**	**Overall mean**
S. typhimurium	0.00	20.67	0.00	0.00	12.33	0.00	5.41^{r}
S. typhi	0.00	21.67	19.67	26.33	18.33	0.00	14.25^{p}
P. areuginosa	14.67	15.67	13.67	13.67	14.67	0.00	11.97^{q}
S. aureus	0.00	0.00	21.33	0.00	0.00	0.00	3.47^{s}
C. albicans	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Overall mean	2.84^{e}	11.50^{a}	10.84^{b}	7.90^{d}	8.97^{c}		

Different alphabets in the rows and column indicates significant different mean, where (n_1=15, n_2=18 and n_3=3, P <0.05).

Table 2. Zone of inhibition of *Ganoderma lucidium* extracts against test pathogens.

Ganoderma lucidium	**Acetone Ext**	**Benzene Ext**	**Chloroform Ext**	**Ethyl acetate Ext**	**Ethyl Alcohol Ext**	**Methyl Alcohol Ext**	**Overall mean**
S. typhimurium	14.33	18.33	18.33	21.67	29.67	13.67	19.16^{p}
S. typhi	0.00	17.33	21.67	28.33	30.67	0.000	16.16^{r}
P. areuginosa	0.00	0.00	15.00	13.67	27.33	0.000	9.16^{t}
S. aureus	10.67	11.67	15.33	21.33	26.33	10.67	15.825^{s}
C. albicans	17.33	16.33	13.67	21.33	29.67	13.33	18.44^{q}
overall mean	8.276^{e}	12.54^{d}	16.61^{c}	21.076^{b}	28.54^{a}	7.34^{f}	

Different alphabets in the rows and column indicates significant different mean, where (n_1=15, n_2=18 and n_3=3, P <0.05).

Pseudomonas aeruginosa (11.97), *S. Typhimurium* (5.41) and *S. aureus* (3.47) and no zone of inhibition was found against *Candida* spp. (Table 1).

In case of *G. lucidium* fruiting body extracts, maximum zone of inhibition was found with ethyl alcohol (28.52) extract followed by ethyl acetate (21.07), chloroform (16.60), benzene (12.54), acetone (8.27) and methyl alcohol extracts (7.34) while *S. Typhimurium* (19.15) was found to be most sensitive with maximum zone of inhibition followed by *C. albicans* (18.43), *S. Typhi* (16.15), *S. aureus* (15.82) and *P. aeruginosa* (9.15) mm zone of inhibition (Table 2).

In *G. australe* fruiting body extracts, maximum zone of inhibition was found with ethyl alcohol (31.96) extract

Table 3. Zone of inhibition of *Ganoderma australe* extracts against test pathogens.

Ganoderma australe	Acetone Ext	Benzene Ext	Chloroform Ext	Ethyl acetate Ext	Ethyl Alcohol Ext	Methyl Alcohol Ext	Overall mean
S. typhimurium	0	17.33	21.33	21.00	30.67	17.67	17.85_q
S. typhi	0	0.00	21.33	21.67	33.67	23.67	16.57^r
P. areuginosa	0	0.00	13.67	13.33	29.33	13.67	11.51^t
S. aureus	10.33	16.67	16.33	20.67	31.67	0.00	15.79^s
C. albicans	0	14.00	24.33	30.67	35.33	27.67	21.85^p
Overall mean	1.90^f	9.43^e	19.23^c	21.30^b	31.96^a	16.36^d	

Different alphabets in the rows and column indicates significant different mean, where (n_1=15, n_2=18 and n_3=3, P <0.05).

Table 4. Zone of inhibition of *Ganoderma sinense* extracts against test pathogens.

Ganoderma sinense	Acetone extract	Benzene extract	Chloroform extract	Ethyl acetate extract	Ethyl alcohol extract	Methyl alcohol extract	Overall mean
S. typhimurium	0	0.00	0.00	0.000	29.67	0.00	4.84^s
S. typhi	0	20.67	18.67	31.00	23.33	0.00	15.51^p
P. areuginosa	0	0	13	12.67	14.67	14.67	9.07^r
S. aureus	12.67	0	0	0	0	15.33	4.57^t
C. albicans	0.00	0	16.67	13	22.67	12.00	10.62^q
Over all mean	2.42^f	4.02^e	9.55^c	11.22^b	17.95^a	8.28^d	

Different alphabets in the rows and column indicates significant different mean, where (n_1=15, n_2=18 and n_3=3, P <0.05).

followed by ethyl acetate extract (21.30), chloroform (19.23), methyl alcohol (16.36), benzene (9.43) and acetone (1.90) extracts. *C. albicans* was found to be most sensitive with maximum zone of inhibition (21.84) followed by *S. Typhimurium* (17.84), *S. Typhi* (16.57), *S. aureus* (15.79) and *P. aeruginosa* (11.51) mm zone of inhibition (Table 3).

In *G. sinense* fruiting body extracts, maximum zone of inhibition was found with ethyl alcohol (17.95) extract followed by ethyl alcohol (11.22), chloroform (9.55), methyl alcohol (8.28), Benzene (4.02) and acetone (2.42) extracts. *S. Typhi* was found to be most sensitive with maximum zone of inhibition (15.50 mm) followed by *C. albicans* (10.62 mm), *P. aeruginosa* (9.06 mm), *S. Typhimurium* (4.84 mm) and *S. aureus* (4.56 mm) zone of inhibition (Table 4).

Multidrug resistant *Staphylococcus aureus* is effectively inhibited by methyl extract of *Ganoderma* fruiting body (Prasad and Wesely, 2008). Beyond this, Klaus and Niksic (2007) also concluded that hot water extract of different parts of fruiting body, spore broken cell wall, hyphae, and powdered spores was found effective against various pathogens. Methyl australate and its corresponding acid were active against fungi and gram positive bacteria and the methyl ester also was active against gram negative bacteria (Fatima et al., 2007). On the basis of the above observations, it was concluded that fruiting body of *G. lucidium* and *G. australe* have potential antimicrobial property among all while in terms of solvent systems only ethyl alcohol was the most suitable solvent. So, only the ethanolic extracts of fruiting bodies of *G. lucidium* and *G. australe* along with their mycelium extract were evaluated further for their antimicrobial activity against test microorganisms.

Agar well diffusion of ethanolic extracts of fruiting bodies and cultured mycelium of *G. lucidium* and *G. australe*

After screening of effective fruiting bodies along with suitable solvent, the fruiting bodies were used to obtain the mycelium using spore discharge method. Activity of ethanolic extracts of mycelial extracts of *G. lucidium* and *G. australe* was found in terms of zone of inhibition using agar well diffusion assay. In *G. lucidium* cultured mycelial (CM) extracts, maximum zone of inhibition was found with ethyl alcohol (38.01) extract followed by chloroform (26.48), ethyl acetate (26.28), benzene (20.55), acetone (13.61) and methyl alcohol extract (10.95). *S. typhimurium* was found to be most sensitive with maximum zone of inhibition (27.92) followed by *C. albicans* (26.15), *S. aureus* (24.26), *S. typhi* (20.81) and *P. aeruginosa (*14.26 mm) (Table 5).

In *G. australe* cultured mycelial extracts, maximum zone of inhibition was found with ethyl alcohol (34.83)

Table 5. Zone of inhibition (mm) of cultured mycelium (CM) extracts of *G. lucidium* against test pathogens.

Cultured mycelium from fruiting body 2	Acetone extract	Benzene extract	Chloroform extract	Ethyl acetate extract	Ethyl Alcohol extract	Methyl alcohol extract	overall mean
S. typhimurium	24.67	25.67	28.33	29.33	39.00	22.67	27.93^{p}
S. typhi	0.00	31.33	31.33	28.33	36.00	0.00	20.82^{s}
P. areuginosa	0.00	0.00	24.67	23.67	39.33	0.00	14.26^{t}
S. aureus	20.67	21.33	26.00	30.67	38.33	10.67	24.26^{r}
C. albicans	24.67	26.33	24.00	21.33	39.33	23.33	26.15^{q}
Overall mean	13.62^{d}	20.55^{c}	26.49^{b}	26.29^{b}	38.02^{a}	10.952^{e}	

Different alphabets in the rows and column indicates significant different mean, where (n_1=15, n_2=18 and n_3=3, P <0.05)

Table 6. Zone of inhibition (mm) of cultured mycelium (CM) extracts of *G. australe* against test pathogens.

Cultured mycelium from fruiting body 3	Acetone extract	Benzene extract	Chloroform extract	Ethyl acetate extract	Ethyl alcohol extract	Methyl alcohol extract	Overall mean
S. typhimurium	0	27	21.33	22.33	36.00	26.33	21.89^{q}
S. typhi	0	0	24.00	23.33	35.33	24.33	17.56^{s}
P. areuginosa	0	0	24.67	23.67	35.67	24.33	17.78^{s}
S. aureus	20.33	26.333	26.33	23.33	34.33	0.00	21.50^{r}
C. albicans	0	24.33	26.67	30.67	34.33	30.67	24.17^{p}
Overall mean	3.76	15.23^{d}	24.30^{b}	24.36^{b}	34.83^{a}	20.83^{c}	

Different alphabets in the rows and column indicates significant different mean, where (n_1=15, n_2=18 and n_3=3, P <0.05)

extract followed by ethyl acetate (24.36), chloroform (24.29), methyl alcohol (20.83), benzene (15.23) and acetone extracts (3.76). *C. albicans* was found most sensitive with maximum zone of inhibition (24.16 mm) followed by *S. typhimurium* (21.89), *S. aureus* (21.50), *P. aeruginosa* (17.78) and *S. typhi* (17.55) (Table 6).

The essential oil of *G. japonicum* mycelium has significant activity. It is a potential source that can be used as a natural antibiotic (Liu, et al., 2009). This study reports the antimicrobial activity of the essential oil of cultured mycelia of *G. japonicum* that showed 24, 20, 23, 27, 25 and 29 mm zone of inhibition against *E. coli, P. vulgaris, S. typhi, S. aureus, B. subtilis* and *Candida* spp. Respectively. This study also included some clinical isolates with zone of inhibition from 19 to 25 mm. Many of the compounds extracted from both the mycelium and fruiting body of *Ganoderma* spp. have been found to exhibit biological activity (Gao et al., 2003; Lin et al., 2003; Mothana et al., 2003). Modern medicinal research and clinical applications showed that *G. japonicum* mycelium had similar effects with wild fruiting bodies of *G. japonicum* in the treatment of respiratory diseases (Wang et al., 2004). Therefore, it can be concluded that mycelial extract has more antimicrobial activity than that of fruiting body extracts as depicted in Tables 5 and 6. Mycelial extract of *G. australe* was also found to be active against the test organisms, but mycelial extract of *G. lucidium* was more effective than it. Although the extracts from fruiting body also exhibited antimicrobial activity, the cultured mycelium exhibited higher antimicrobial activity than it. So for the analysis of MIC and MBC only the *G. lucidium* extract was taken into consideration.

Minimum inhibitory concentration of ethanolic extracts of *G. lucidium* and its cultured mycelial extract

MIC is the lowest concentration of extract in which no visible microbial growth is observed. The MIC of fruiting body (FB) extract was 3.12 mg/ml of crude extract for *S. Typhimurium,* 0.78 mg/ml of crude extract for *S. Typhi* and 1.56 mg/ml of crude extract for *Pseudomonas spp., S. aureus* and *C. albicans while* MIC of mycelial extract was 0.39 mg/ml of crude extract for *S. Typhi,* and 0.78 mg/ml of crude extract for *S. Typhimurium, Pseudomonas* spp*., S. aureus* and *C. albicans* (Table 7). The above results show that mycelial extracts have more potential antimicrobial activity than the fruiting bodies, as evident in the MIC (Table 7).

Broad spectrum activity of the extracts was also evaluated and MIC of the fruiting body extract (crude) was 1.56 mg / ml against *E. coli, K. pneumoniae, E. faecalis* and *P. vulgaris* while 0.78 mg/ml of crude extract of cultured mycelium was effective (MIC) against these pathogens (Table 8). Fatima et al. (2007) has reported that MIC of australic acid (2.0 mg/mL) was effective against *E. coli, P. aeruginosa*; 1.0 mg/ mL effective

Table 7. Minimum inhibitory concentration and Minimum bactericidal (or microbicidal) concentration of *G. lucidium* fruiting body extracts and cultured mycelium (CM) extracts of *G. lucidium* against test pathogens.

Microorganism	Minimum inhibitory concentration (mg/ml)		Minimum microbicidal concentration(mg/ml)	
	Fruiting Body extract	Mycelial extract	Fruiting body extract	Mycelial extract
S. typhimurium	3.12	0.78	6.25	1.56
S. typhi	0.78	0.39	1.56	0.78
P. areuginosa	1.56	0.78	3.12	1.56
S. aureus	1.56	0.78	3.12	1.56
C. albicans	1.56	0.78	3.12	1.56

Table 8. Minimum inhibitory concentration of fruiting body of *G. lucidium* and its mycelial extract against other pathogenic microorganisms.

Microorganism	Minimum Inhibitory concentration (mg/ml)		Minimum Bactericidal concentration(mg/ml)	
	Fruiting body extract	Mycelial body extract	Fruiting body extract	Mycelial extract
E. coli	1.56	0.78	3.12	1.56
K. pneumoniae	1.56	0.78	3.12	1.56
E. faecalis	1.56	0.78	3.12	1.56
P. vulgaris	1.56	0.78	3.12	1.56

against *S. aureus* and 0.25 mg/mL against *B. cereus*. MIC of methyl australate (2.0 mg/mL) was effective against *E. coli, P. aeruginosa*, *S. aureus* and 0.25 mg/mL was effective against *B. cereus.* Liu et al., (2009) reported the antimicrobial activity of essential oil of cultured mycelia of *G. japonicum* and reported MIC and MBC of 2.05 mg/mL against *E. coli, S. typhi, B. subtilis*, 4.11 mg/mL against *P. vulgaris* and 1.03 mg/mL in case of *S. aureus*. Shah et al. (2014) reported the antibacterial activity of *G. lucidum* collected from Gujrat. MIC of the crude extract in different solvents (0.625 to 2.5 mg /mL) was reported against *B. cereus, S. aureus, E. aerogens, E. coli* and *P. aeruginosa*. The results correlate with the literature and clearly show that both extracts have good potential against a variety of pathogens.

Minimum bactericidal (or microbicidal) concentration of ethanolic extracts of *G. lucidium*

MBC was determined by using broth dilution method and growth was checked by sub-culturing to agar media. Both the extracts were found static to all test microorganisms since growth was observed at MBC for all organisms.

The MBC of fruiting body extract was found to be 6.25 mg/ml of crude extract for *S. Typhimurium,* 1.56 mg/ml of crude extract for *S. Typhi,* and 3.12 mg/ml of crude extract for *Pseudomonas spp., S. aureus* and *Candida spp*. while MBC of mycelial extract was 0.78 mg/ml of crude extract for *S. Typhimurium,* 1.56 mg/ml of crude extract for *S. Typhi, Pseudomonas spp., S. aureus* and *C. albicans* (Table 7). Also, MBC of the fruiting body extract (crude) was 3.12 mg / ml against *E. coli, K. pneumoniae, E. faecalis* and *P. vulgaris* while it was 1.56 mg/ml of crude extract of cultured mycelium against the test pathogens. So it can be concluded that the extract is highly effective against the test microorganisms and shows that the MBC of mycelial extracts has same pattern with MIC.

Effect of pH, temperature and heavy inoculum on antimicrobial activity of *G. lucidium* extract

Ethanol extract of *G. lucidium* fruiting body as well as cultured mycelium showed little or no variation in diameter of zone of inhibition after conditions at different temperatures, pH and heavy doses of inoculum. Extracts were treated at 4, 60 and 100°C for 1 h and subjected to test for inhibitory activity. It indicates that the extract is thermostable. Further effect of pH on the activity of the extracts was also evaluated and found that the extracts were well effective at various pH levels. No growth was observed after heavy inoculation of test organisms even

against heavy inoculum up to 1.0 OD in all the test organisms.

G. lucidum, one of the oldest salutary remedies known for more than 3000 years, has become a subject of interest in many contemporary research papers. Standard procedures in repression of harmful microorganisms are applications of antibiotics, but too much usage of antibiotics and the ability of microorganisms to develop resistance to them result in their decreased effects on microorganisms. MRSA is responsible for worldwide outbreaks of nosocomial infections. However, the pharmaceutical arsenal available to control MRSA is very limited at this time (Jiao, 2003; Li et al, 2008). Multidrug resistant *S. aureus* is effectively inhibited by methyl extract of *Ganoderma* fruiting body (Prasad and Wesely, 2008). Beyond this, Klaus and Niksic (2007) also concluded that hot water extract of different parts of fruiting body, spore broken cell wall, hyphae, and powdered spores was effective against various pathogens.

Mycelium from effective fruiting body was cultivated on solid agar surface and the biomass was subjected to investigation of antimicrobial activity. Initially, the isolate was cultivated for 21 days on a standard fungal cultivation medium, MEA, to produce mycelia biomass. This biomass was extracted using organic solvent ethyl alcohol.

Antimicrobial investigations were then performed on the extract and an overall analysis of the active extract was performed. After all only one extract with ethanol was found to be most effective against all the test pathogens under study. The essential oil of *Ganoderma japonicum* mycelium has significant activity. It is a potential source that can be used as a natural antibiotic (Dandan et al., 2009).

Conclusion

Early in this investigation, to determine whether the extracts possessed any biological activity, antibacterial screening on fruiting bodies extracts was performed by agar well diffusion method to identify which isolate contained the greatest antimicrobial activity. Once activity was confirmed, further analysis was performed on the agar cultivated mycelium of *G. lucidium* and *G. australe*. This study demonstrates that cultivated mycelium is a more valuable source of antimicrobial compounds and with this outcome, culture medium manipulation may increase the possibilities of more antimicrobial or bioactive substance production. As this investigation is of a current interest, it should be continued with more microorganisms and numerous chemically defined fractions derived from cultured mycelium of *Ganoderma.* Findings from this investigation are interesting particularly from a commercial point of view, as mycelial products offer standardized quality and whole year production. In this way, potential possibilities exist to establish research and development of antimicrobial compounds from 'indigenous' *Ganoderma* spp.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGMENTS

This work was carried out as an academic project. The author is grateful to Dr. P. K. Sharma, Head, of Department of Microbiology and Dr. S K Shahi, Assistant Professor, Department of Microbiology, CCS University, Meerut for their cooperation and their valuable suggestions during the work.

REFERENCES

Anand N, Chowdhry PN (2013). Anti-tumour potential of active compounds (polysaccharides) of wild mushrooms from Rajouri Dist. of Jammu & Kashmir, India. Int. J. Pharm. Sci. Res. 4(5):1743-1746.

Bell SM, Pham JN, Fisher GT (2009). Antibiotic susceptibility testing by the CDS method. A Manual for Medical and Veterinary Laboratories. The Antibiotic Reference Laboratory. Fifth Edition, South Eastern Area Laboratory Services Randwick NSW 2031 Australia ISBN 978-0-646-51681-3.

Chang ST (1995). Ganoderma - The leader in production and technology of mushroom nutriceuticals. In Proc 6th Int Symp Recent Adv Ganoderma lucidum Res (ed. B. K. Kim, I. H. Kim and Y. S. Kim). The Pharmaceutical Society of Korea, Seoul, Korea. pp. 43-52.

Chen AW, Miles PG (1996a). Biomedical research and the application of mushroom nutriceuticals from Ganoderma lucidum. In Proc 2nd Int Conf. Mush. Biol. Mush. Prod. (ed. D. J. Royse). University Park, Pennsylvania. pp. 161-175.

Crittenden PD, Davis JC, Hawksworth DL, Campbell FS (1995). Attempted isolation and success in culturing of a broad spectrum of lichen forming and lichenocolous fungi. New Phytol. 130:267-297.

Dandan L, Zheng H, Zhigang L, Wenjuan T, Liang L (2009). Chemical composition and antimicrobial activity of essential oil isolated from the cultured mycelia of *Ganoderma japonicum.* J. Nanjing Med. Univ. 23(3):168-172.

Dixit A, Dikshit SN (1982). Cedrus oil-A promising antifungal agent. Indian Perfum. 26: 216-227.

Dobson CM (2004). Chemical space in biology. Nature 432:824-828.

Doughari JH (2006) Antimicrobial activity of *Tamarindus indica Linn*. Trop. J. Pharma Res. 5:597-603.

Edelstein L, Segel LA (1983). Growth and metabolism in mycelial fungi. J. Theor. Biol. 104:187-210.

Fatima E, Smania A, Delle F, Rosendo AY, Paulert R, Smania A (2007). Antimicrobial activity of methyl australate from *Ganoderma australe*. Rev. Bras. Farmacogn. 17(1):14-16.

Fleming A (1929). On the antibacterial action of a *Penicillium* with special reference to their use in the isolation of *B. influenzae*. Br. J. Exp. Pathol. 10:226-236.

Florianowicz T (1998). *Penicillium expansum* growth and production of patulin in the presence of benzoic acid and its derivatives. Acta Microbiol. Pol. 47:45-53.

Gao XX, Fei XF, Wang BX, Zhang J, Gong YJ, Minami M, Nagata T, Ikejima T (2000). Effects of polysaccharides (FI0-b) from mycelium of Ganoderma tsugae on proinflammatory cytokine production by THP-1 cells and human PBMC (I). Acta Pharmacol. Sin. 21:1179-1185.

Gao Y, Zhou S, Huang M, Xu A (2003). Antibacterial and antiviral value of the genus *Ganoderma* P. Karst. species (Aphyllophoromycetideae): A review. Int. J. Med. Mushrooms 5:235-246.

Grigansky AP, Solomko EF, Kirchoff B (1999). Mycelial growth of medicinal mushroom *Hericium erinaceus* (Bull.:Fr.) Pers. in pure culture. Int. J. Med. Mushrooms 1:81-87.

Grover RK, Morre JD (1962). Toxicometric studies of fungicides against brown rot organisms *Sclerotinia fructicola* and *S. lexa*. Phytopathology

52:876-880.
Henry FC, Frank RD (2009). Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat. Rev. Microbiol. 7:629-641.
Jiao M (2003). The analysis of drug resistance and bacteria of acute respiratory tract infection in children. Acta Universitatis Medicinalis Nanjing. 23: 82-84.
Kim SW, Hwang HJ, Xu CP, Sung JM, Choi JW, Yun JW (2003). Optimization of submerged culture process for the production of mycelial biomass and exo-polysaccharides by *Cordyceps militaris* C738. J. Appl. Microbiol. 94:120-126.
Klaus A, Niksic M (2007). Influence of the extracts isolated from *Ganoderma lucidum* mushroom on some microorganisms. Proc. Nat. Sci. 113:219-226.
Larena II, Melgarejo P (1996). Biological Control of Monilinia laxa and *Fusarium oxysporum f. sp. lycopersici* by a Lytic Enzyme-Producing *Penicillium purpurogenum*. Biol. Control 6:361-367.
Lehrer RI, Rosenman M, Harwig SSSL, Jackson R, Eisenhauer P (1991). Ultrasensitive assay for endogenous antimicrobial polypeptides. J. Immunol. Methods 137:167-173.
Li S, Xiao GY, Zhou GP, Cai JY, Huang YP, Li Q (2008). The analysis of the drugs used by the patiants with MRSA infection. Modem Hosp. 8:19-21.
Lin SB, Li CH, Lee SS, Kan LS (2003). Triterpene-enriched extracts from *Ganoderma lucidum* inhibit growth of hepatoma cells via suppressing protein kinase C, activating mitogen-activated protein kinases and G2-phase cell cycle arrest. Life Sci. 72:2381-2390.
Liu D, Hua Z, Liua Z, Yanga, Tub W, Lia L (2009). Chemical composition and antimicrobial activity of essential oil isolated from the cultured mycelia of *Ganoderma japonicum*. J. Nanjing Med. Univ. 23(3):168-172.
Lorenzen K, Anke T (1998). Basidiomycetes as a source for new bioactive natural products. Curr. Org. Chem. 2:329-364.
Mayordomo I, Randez-Gil F, Prieto JA (2000). Isolation, purification, and characterization of a cold-active lipase from *Aspergillus nidulans*. J. Agric. Food. Chem. 48:105-109.
McGowan JEJ (2001). Economic impact of antimicrobial resistance. Emerg. Infect. Dis. 7:286-292.
Monroe S, Polk R (2000). Antimicrobial use and bacterial resistance. Curr. Microbiol. 3:496-501.
Mothana RAA, Awadh Ali NA, Jansen R, Wegner U, Mentel R, Lindequist U (2003). Antiviral lanostanoid triterpenes from the fungus *Ganoderma pfeifferi*. Fitoterapia 74:177-180.
National Committtee for Clinical Laboratory Standards-NCCLS (2001). Performance Standards for Antimicrobial Susceptibility Testing; 11th Information Supplement. Approved Standard M2-A7 and M7-A5. Wayne, PA, U.S.A.
Newman DJ, Cragg GM, Snader KM (2003). Natural products as sources of new drugs over the period 1981-2002. J. Nat. Prod. 66:1022-1037.
Pointing SB, Hyde KD (2001). Bio-exploitation of filamentous fungi, First edition. Fungal Diversity Press, Hon g Kong, China. p. 219.
Prasad Y, Wesely WEG (2008). Antibacterial Activity of the bio-multidrug (*Ganoderma lucidum*) on multidrug resistant *Staphylococcus aureus* (MRSA). Adv. Biotech 9-16.
Rodrigues KF, Hesse M, Werner C (2000). Antimicrobial activities of secondary metabolites produced by endophytic fungi from *Spondias mombin*. J. Basic Microbiol. 40:261-267.
Sarker SD, Nahar L, Kumarasamy (2007). Microtiter plate-based antibacterial assay incorporating resazurin as an indicator of cell growth and its application in the in vitro antibacterial screening of phytochemicals. Methods 42(4):321-324.
Shah P, Modi HA, Shukla MD, Lahiri SK (2014). Preliminary phytochemical analysis and antibacterial activity of *Ganoderma lucidum* collected from dang district of Gujarat, India. Int. J. Curr. Microbiol. Appl. Sci. 3(3):246-255.
Smania EFA, Della MF, Smania AJ, Yunes RA, Cuneo RS (2003). Antifungal activity of sterol and triterpenes isolated from *Ganoderma annulare*. Fitoterapia 74:375-377.
Wang L, Wang YH, Zhang KC (2004). A Study on the effect of 5 kinds of Chinese traditional medicines on submerged fermentation of *Ganoderma lucidum* and its fermented broth on chronic bronchitis. Edible Fungi China 5:39-40.