Journal of Medicinal Plants Research Vol. 5(4), pp. 527-532, 18 February, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Anti-*Staphylococcus aureus* activity of *Pisolithus albus* from Pune, India

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Accepted 2 February, 2011

In vitro **studies were conducted to investigate the anti-staphylococcal activity of crude extracts of two strains of *Pisolithus albus* collected from different hosts in the vicinity of Pune University Campus, Pune, India. Organic solvents of increasing polarities were used for extraction of secondary metabolites. In addition, bioactive components of known antimicrobial activity were isolated and tested against thirty strains of clinical isolates of methicillin resistant *Staphylococcus aureus.* Strong activity was exhibited by ethyl acetate and methanol extracts while aqueous extract showed weak activity. All strains were resistant to the chloroform extract. Maximum effectiveness was recorded for sesquiterpenoids extract (zone diameter: 16.67 to 31.5 mm). Triterpenoids and diterpenoids extracts displayed weak to moderate activity. Polysaccharides fractions IIIa and IIIb were weakly effective while fractions I and II did not show activity. Minimal inhibitory concentration was determined to be in a range of 0.62 to 1.2 mg/ml.**

Key words: *Pisolithus albus, Staphylococcus aureus*, methicillin resistant antimicrobial activity.

INTRODUCTION

Macrofungi have long been used as a valuable food source and as traditional medicines around the world since ancient times, especially in Japan and China. In recent decades, interesting compounds of different biogenetic have been isolated from basidiomycetes and were found to have antibacterial, antifungal and antiviral activities.

Mushrooms are rich sources of antibiotics. In these, the cell wall glucans are well-known for their immunomodulatory properties, but few medical practitioners are aware that many of the externalized secondary metabolites – extracellular secretions by the mycelium combat bacteria (Kupra et al., 1979; Benedict and Brady, 1972) and viruses (Eo et al., 1999; Brandt and Piraino, 2000). Interestingly, some mushrooms and their components are target specific in their antibiotic properties, whereas others have broader effects.

With an increasing number of bacteria developing resistance to commercial antibiotics, such as methicillin resistance *Staphylococcus aureus* (MRSA), a high number of scientists around the world have gained interest in the investigation of natural materials as sources of new antibacterial agents.

Review of literature demonstrates that mushrooms, similar to plants, have a great potential for the production of useful bioactive metabolites and that they are a prolific resource for drugs. The responsible bioactive compounds belong to several chemical groups, very often they are polysaccharides or triterpenes. One species can possess a high variety of bioactive compounds, and therefore of pharmacological effects.

Pisolithus is cosmopolitan in both tropical and temperate regions, it is widely distributed globally (Marx, 1977) and forms ectomycorrhizal (EcM) associations with a broad range of woody plants including members of Myrtaceae, Mimosaceae, Pinaceae, Fagaceae, Cistaceae, Dipterocarpaceae and Caesalpiniaceae.

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Isolates of *Pisolithus* are some of the most commonly used in forestry, with growth stimulation reported for several tree species including *Eucalypts*, pines and acacias (Garbaye et al, 1988; Marx, 1977; Duponnois and Ba, 1999).

The triterpene pisosterol has been shown to have antitumor activity against seven tumor cell lines, especially leukemia and melanoma cells (IC50 of 1.55, 1.84 and 1.65 µg/ml for CEM, HL-60 and B16), respectively (Montenegro et al., 2004). Also, literature survey indicates that many components such as diterpenoids, triterpenoids, sesquiterpenoids; various fractions of polysaccharides like fractions I, II, IIIA , IIIB and ergesterol possess antimicrobial activity (Mizuno, 1999; Hwang et al., 2000; Smith et al., 2000).

The rising concern around the world about what is commonly perceived as "superbugs", MRSA is an organism that has dominated headlines in medical journals for three decades. In recent years, fears about the organism have migrated to international newspaper headlines. The public have genuine concerns about the "level" of MRSA in the institutions that deliver healthcare and the consequences of the organism for patients.

In view of the current global problem of antimicrobial resistance, and in light of the bioactive potential possessed by a number of medicinal mushrooms against a wide spectrum of microorganisms including MRSA, this study was undertaken to investigate the antimicrobial activity of *Pisolithus albus* against thirty strains of clinical isolates of MRSA *in vitro.*

MATERIALS AND METHODS

Fruit body of *P. albus* (Cooke and Mass.) M.J. Priest were collected during rainy season from *Acacia auriculiformis* and *Eucalyptus globules*, outside Joshi gate, Pune university campus, Pune, India, The strains of *P.albus* (Cooke and Mass.) M.J. Priest were identified at Agarkar Research Institute (ARI), Pune, India and a voucher specimen was deposited under accession numbers AMH No. 9294 at ARI.

Mushroom samples were milled to a fine powder (Hammer Mill, Basant, India). Twenty gram-portions of the mushroom samples were extracted with 100 ml of different solvents successively (chloroform, ethyl acetate, methanol, water) with aid of intermittent warming (Harborne,1988), then stored at room temperature in the dark for 24 h. The process was repeated for a second time, the two portions were pooled together; filtered and concentrated to dryness in a rotary evaporator **(**Medica Instrument MFG Co., India). The condensate was dissolved in Dimethyl SulfOxide (DMSO) and stored at 4°C.

Test microorganisms

A total of 26 clinical isolates of MRSA were collected from different hospitals in Mumbai (National Burns Hospital Airoli, Thane) and Pune (Joshi hospital). Two standard strains (National Chemical Laboratory) and two strains of methicillin sensitive MSSA (GenomBiotech, Pune) were also included in the study for comparison.

All strains of *S. aureus* collected on nutrient agar slant were identified on KB004 HiStaph^{TM} Kit (HiMedia, Mumbai). All strains of *S. aureus* were subjected to antimicrobial susceptibility testing employing the Kirby-Bauer method (Bauer et al., 1966). Four antibiotics namely methicillin (5 mg), vancomycin (5 mg) and linezolid (30 mg) and mupirocin (5 mg) procured from HiMedia, Mumbai were used to evaluate the susceptibility of the strains.

Bioassay of crude extracts

Antimicrobial activity of 40 mg/ml of each *P. albus* extract was evaluated by the well agar diffusion method (Stoke and Ridgway, 1980). Wells of 7 mm in diameter were made on each plate using sterile cork borer. Using a micropipette, 50 µl of the crude extract was introduced into each well. All plates were carried out in triplicates and control experiments were also set up by adding different solvents as well as DMSO. Plates were incubated at 37°C for 24 h. The antibacterial activity of the extracts were expressed as the diameter of the inhibition zones (in mm) which appeared on the incubated plates. The plates were kept for one week at room temperature to observe the overgrowth in the zone diameter.

Extraction of bioactive components

The strain of *P. albus* that displayed more effective activity was further examined for its bioactive components like diterpenoids, triterpenoids, sesquiterpenoids and various fractions of polysaccharides viz., I, II, IIIA and IIIB. For extraction of these fractions, the methods described by Harborne (1988), Mizuno (1999), Huie and Di (2004), Smith et al. (2000) and Hwang et al. (2000) were adopted.

Bioassay of bioactive components

All isolated bioactive components (diterpenoids, triterpenoids, sesquiterpenoids, and polysaccharides I, II, IIIA and IIIB) were bioassayed for antimicrobial activity against all strains of *S. aureus.*

Minimal inhibitory concentration (MIC)

The MIC was determined by using the microdilution method (Eloff, 1998), using (12 × 8 wells) microtitre plates. Solutions containing 40 mg/ml sesquiterpenoid were prepared. Aliquots (50 µl) of the sesquiterpeoids crude extract of *P. albus* and 200 µl of the inoculum were pipetted to the well labeled as A. Only 100 µl of each inoculum of the strains was added to the wells labelled B–H. The inoculum and crude extract in well-A were mixed thoroughly before transferring 100 µl of the resultant mixture to well B. The same procedure was repeated for inoculum mixtures in wells B–H. Standard vancomycin was used as the reference drug (2-8 µg/well). Methanol served as negative control. Plates were incubated for 24 h at 37°C. Bacterial growth was determined after addition of 50 µl *p*-iodonitrotetrazolium violet (0.2 mg/ml, Himedia, Mumbai).

Effect of temperature on the activity of sesquiterpenoids

The effect of temperature on antimicrobial activity of sesquiterpenoids was evaluated by incubating aliquots of 1 mg/ml at 0.0, 4.0, 20.0, 30.0, 40, 50, 60, 70, 80, 90 and 100 for 30 min and 121°C for 20 min. Antimicrobial activity was determined by the disk diffusion method against two strains of MRSA. Each paper disk (Oxoid, England) was impregnated with 10 µl of sesquiterpenids extract using nutrient medium seeded with 10^6 cfu/ml of overnight growth of the respective strain of bacteria. The results were

compared with that of the control (extract at zero time).

Effect of pH on the activity of sesquiterpenoids

Antimicrobial activity of sesquiterpenoids extract of *P. albus* was evaluated at different pH values by the disk diffusion method against two strains of MRSA. The pH values of the extract were changed by using the following buffers: acetate (pH 3.0, 4.0, 5.0), phosphate (6.0, 7.0, 8.0), Tris Hcl (pH 9.0) and carbonate – bicarbonate (pH 10.0, 11.0). An aliquot (50 µl) of sesquiterpenoids (1 mg/ml) was added separately to 50 µl of each buffer, after one hour incubation at 37°C, 10 µl of each solution was used to impregnate each paper disk. Disks were placed on surface of nutrient agar plates seeded with respective strain of bacteria. Methanol was used as control. All plates were incubated at 37°C for 24 h, then evaluated for antimicrobial activity (Munimbazi and Bullerman, 1997).

Effect of shelf life of the activity of sesquiterpenoids

The effect of time on antimicrobial activity of sesquiterpenoids extract of *P. albus* was evaluated by storing two extracts at room temperature and 4°C, respectively, for a period of 12 months. Results were compared with the activity of fresh extracts.

RESULTS

Antimicrobial activity of 40 mg/ml of different extracts of *P. albus* fruiting bodies hosted by *Eucalyptus globules* were tested against 30 strains of MRSA and MSSA. Chloroform extract did not show activity towards any of the bacterial strains used in this investigation whereas aqueous extract showed weak activity (7.0 to 11.75 mm). Maximum activity was exhibited by ethyl acetate (7.0 to 21.25 mm) with a mean of 14.54 ± 3.23 mm. High zone diameters ranging between 20.0 to 21.25 mm against four strains (13.33%) of the target organisms were displayed by ethyl acetate extract. However, one strain of bacteria showed resistance and some weak activities with zone diameters as low as 9.67 to 10.0 mm were also observed. Methanol extract showed highly moderate activity against most of the test strains (15.25 to 18.33 mm) with a mean of 16.94 ± 0.77 mm (Table 1).

Various bioactive components of known antimicrobial activity were bioassayed against the target bacteria. Polysaccharide I and II did not show antimicrobial activity and therefore, are not represented. However, other bioactive components of *P. albus* viz., polysaccharide fractions IIIa and IIIb showed weak to moderate activity ranging from 9.25 to 14.75 mm and 8.75 to 11.75 mm, respectively. Moreover, triterpenoids and diterpenoids exhibited weak activity against these strains with zone diameters ranging between 8.33 to 16.5 mm (mean 12.27 ± 1.74 mm) for triterpenoids and 7.0 to 13.0 mm (9.96 ± 2.16 mm) for diterpenoids. Nonetheless, 10 strains (33.33%) were resistant to diterpenoids. On the contrary, strong activity with zone diameters ranging between 16.67 and 31.5 mm (mean 22.59 mm ± 4.47) were recorded for sesquiterpenoids (Table 2). MIC for sesquiterpenoids of *P. albus* was in the range of 0.62 to 1.2 mg/ml.

Effects of temperature, pH and time on sesquiterpenoids

Heat treatment of sesquiterpenoids extracts at different temperatures showed drastic change in their activity at high temperatures against the test strains. Sesquiterpenoids extracts retained their activity up to 80°C, but a sharp drop in activity was noticed at temperature 90 and 100°C, and a complete loss of activity was observed at 121°C.

Treatment of sesquiterpenoids extracts at different pH values displayed moderate activity at a low pH value (4.0) and exhibited strong activity at pH 6.0 to 8.0 against one of the strains tested. Antimicrobial potential of sesquiterpenoids extracts was not significantly affected following storage at 4°C for a twelve – month period. On the contrary, the stability of sesquiterpenoids extracts showed a considerable decrease upon storage at room temperature for a period of twelve months as compared to samples which were maintained at 4°C.

DISCUSSION

Although the genus *Pisolithus* is cosmopolitan in both tropical and temperate regions and forms ectomycorrhizal associations with a wide range of woody plants, reports about antimicrobial activity of *Pisolithus* spp. are very scanty in the literature; most research has been focused on its ectomycorrhizal associations with plant hosts, genetic variability, taxonomic position, management, soil fertility and erosion control.

Nevertheless, Shrestha et al. (2005) reported similar results from *Pisolithus* spp. against a spectrum of Gram negative and Gram positive bacteria. The metabolites of *Pisolithus* sp. in concentration of 10 mg/ml showed high zone of inhibition against *Salmonella typhi, Kleibsiella* sp., *Bacillus sp., Escherichia coli* , *Pseudomonas aeruginosa*, *Agrobacterium tunifaciens* and low zone of inhibition against *S. aureus* and *Shigella dysenteriae* in agar well diffusion method. However, on review of the literature there appears to be no such investigations reported on the antimicrobial potential of *Pisolithus albus* extracts against MRSA species and therefore, the findings here are the first report of the antimicrobial activity of a *Pisolithus* species against clinical MRSA isolates. Treatment of sesquiterpenoids extracts at high temperature may lower the antimicrobial effectiveness of this mushroom. Our results agree with the findings of those obtained by Hirasawa et al. (1999) who obtained similar gradual decrease in the antimicrobial effectiveness of the extracts of *L. edodes* as temperature

Table 1. Antimicrobial activity (mm) of various crude extracts of *P. albus* fruiting bodies against different strains of *S. aureus.*

Strain code	Solvents		
	Ethyl acetate	Methanol	Water
MRSA-1	21.25 ± 1.38	17.5 ± 0.95	8.75 ± 0.69
MRSA-2	20.0 ± 1.55	17.0 ± 0.95	9.75 ± 0.8
MRSA-3	20.0 ± 1.19	17.0 ± 1.44	8.75 ± 1.07
MRSA-4	20.25 ± 1.84	16.0 ± 1.63	8.5 ± 0.76
MRSA-5	16.5 ± 1.7	15.8 ± 0.85	8.67 ± 0.74
MRSA-6	17.75 ± 1.54	16.5 ± 1.41	10.75 ± 1.07
MRSA-7	12.5 ± 0.81	16.75 ± 0.8	10.8 ± 1.06
MRSA-8	13.75 ± 1.1	16.25 ± 1.28	10.25 ± 1.57
MRSA-9	17.33 ± 0.74	17.0 ± 1.11	8.5 ± 0.76
MRSA-10	15.33 ± 1.24	16.0 ± 1.41	9.0 ± 0.81
MRSA-11	9.67 ± 0.37	18.33 ± 1.97	8.5 ± 0.76
MRSA-12	16.0 ± 0.64	16.67 ± 1.1	8.75 ± 0.69
MRSA-13	14.67 ± 0.68	17.33 ± 1.69	10.0 ± 1.29
MRSA-14	12.33 ± 1.24	17.33 ± 1.1	8.75 ± 1.07
MRSA-15	15.33 ± 1.06	17.33 ± 1.1	9.75 ± 0.55
MRSA-16	16.0 ± 1.41	17.67 ± 0.94	8.8 ± 0.37
MRSA-17	7.0 ± 0	16.67 ± 1.51	9.5 ± 0.95
MRSA-18	14.0 ± 1.0	17.0 ± 1.04	8.0 ± 0.0
MRSA-19	11.0 ± 1.29	18.33 ± 2.05	7.0 ± 0.0
MRSA-20	14.67 ± 1.24	16.67 ± 0.94	8.5 ± 0.76
MRSA-21	14.67 ± 1.59	17.0 ± 0.64	9.75 ± 0.55
MRSA-22	13.33 ± 0.94	17.67 ± 0.94	8.5 ± 0.76
MRSA-23	14.0 ± 1.15	17.0 ± 0.76	10.33 ± 0.74
MRSA-24	10.0 ± 1.04	18.33 ± 2.28	9.67 ± 1.24
MRSA-25	12.33 ± 0.94	18.0 ± 1.55	9.0 ± 0.81
MRSA-26	15.33 ± 1.28	17.33 ± 1.69	8.33 ± 0.47
MRSA-GBT	11.5 ± 1.22	15.75 ± 1.34	9.5 ± 0.76
MSSA- GBT	12.0 ± 1.04	16.0 ± 1.41	10.0 ± 1.29
ATCCNo. 29737	13.25 ± 1.4	17.0 ± 0.64	10.5 ± 1.7
ATCCNo. 9144	14.5 ± 1.25	15.25 ± 1.07	11.75 ± 0.25
Mean	14.54	16.94	9.28
SD	3.23	0.77	0.97

SD: standard deviation, n = 6.

was elevated. Moreover, our results support the findings of Sharifi (2006) who reported similar decrease in antimicrobial activity of sesquiterpenoids extracts isolated from *Phellinus merrillii* against *Candida albicans* and *Proteus mirablis*. The temperature resistance may be an indication that the phytoconstituents are thermoresistant. It is a known fact that the loss of antibacterial activity of natural products by heating may be due to volatilization and/or the physical and chemical changes that take place during heating (Durairaj et al., 2009).

In the present study, treatment of sesquiterpenoids extracts at different pH values showed a wide range of activity; it displayed moderate activity at a low pH value (4.0) and exhibited strong activity at pH 6.0 to 8.0 against one of the strains tested. Exhibition of maximum zones of inhibition at pH 6.0 to 7.0 indicates that they exert their highest effect at optimal pH value for bacterial growth. However, since this is the first report on the antimicrobial activity of *P. albus*, therefore, no comparison can be made at present. Further investigation is suggested.

Antimicrobial potential of various sesquiterpenoids extracts was not significantly affected following storage at 4°C for a twelve – month period. On the contrary, the stability of sesquiterpenoids extracts showed a considerable decrease upon storage at room temperature for the same period as compared to samples which were maintained at 4°C. These results support the findings of Sharifi (2006) who reported similar decrease in

Table 2. Antimicrobial activity of various bioactive components of *P. albus* against different strains of *S. aureus*.

Strain code	Zone of inhibition in response to various bioactive components (mm)				
	Poly IIIa	Poly IIIb	Sesquiterpene	Triterpene	Diterpene
MRSA-1	11.67 ± 0.74	8.75 ± 0.69	22.5 ±1.25	13.0 ±1.04	12.0 ± 0.81
MRSA-2	10.33 ± 1.1	11.5 ± 1.25	20.67 ± 1.37	10.67 ± 1.1	11.33 ± 1.37
MRSA-3	10.33 ± 1.24	9.33 ± 0.79	19.33 ± 1.97	11.0 ± 0.57	7.0 ± 0.0
MRSA-4	9.25 ± 0.9	10.75 ± 0.38	18.67 ± 1.79	10.33 ± 1.1	11.0 ± 1.41
MRSA-5	10.33 ± 0.94	11.67 ± 0.74	20.67 ± 1.1	12.67 ± 0.37	11.0 ± 0.81
MRSA-6	9.75 ± 0.9	11.0 ± 1.0	24.67 ± 1.59	11.0 ± 1.0	7.0 ± 0.0
MRSA-7	10.25 ± 1.34	9.0 ± 1.0	20.33 ± 1.24	13.67 ± 0.74	11.0 ± 0.95
MRSA-8	11.25 ± 1.28	9.75 ± 0.8	17.33 ± 1.1	10.5 ± 0.95	7.0 ± 0.0
MRSA-9	12.33 ± 0.47	10.0 ± 0.81	28.67 ± 0.74	10.5 ± 0.81	12.0 ± 0.81
MRSA-10	11.0 ± 1.0	10.67 ± 1.1	18.33 ± 1.24	11.67± 0.74	10.33 ± 1.1
MRSA-11	10.67 ± 1.1	10.5 ± 0.76	19.33 ± 2.0	13.5 ± 0.91	7.0 ± 0.0
MRSA-12	14.75 ± 0.98	10.75 ± 0.9	25.33 ± 1.37	14.0 ± 1.04	13.0 ±1.04
MRSA-13	10.67 ± 1.1	10.75 ± 0.38	27.5 ± 1.25	12.67 ± 0.68	11.33 ± 1.37
MRSA-14	12.5 ± 1.77	10.67 ± 0.74	19.33 ± 1.97	14.33 ± 1.24	11.33 ± 1.37
MRSA-15	10.75 ± 0.69	10.0 ± 1.52	29.67 ± 0.74	10.67 ± 1.1	7.0 ± 0.0
MRSA-16	9.33 ± 0.79	10.75 ± 0.8	17.0 ± 1.15	12.33 ± 0.37	11.67 ± 1.1
MRSA-17	12.0 ± 1.41	10.25 ± 0.69	20.5 ± 2.06	14.33 ± 0.94	11.67 ± 1.24
MRSA-18	10.0 ± 1.0	10.75 ± 0.38	19.0 ± 1.82	11.5 ± 0.76	10.67 ± 1.1
MRSA-19	9.75 ± 0.8	10.67 ± 1.1	21.33 ± 1.97	12.67 ± 0.37	7.0 ± 0.0
MRSA-20	10.25 ± 0.69	10.25 ± 0.9	26.0 ± 1.82	9.0 ± 0.57	7.0 ± 0.0
MRSA-21	10.75 ± 0.69	9.75 ± 0.55	29.67 ± 1.1	8.33 ± 0.47	7.0 ± 0.0
MRSA-22	9.33 ± 0.89	9.5 ± 0.81	18.0 ± 1.29	13.67 ± 3.26	7.0 ± 0.0
MRSA-23	10.5 ± 0.81	10.33 ± 0.74	16.67 ± 2.13	12.0 ± 1.0	7.0 ± 0.0
MRSA-24	11.75 ± 1.07	11.25 ± 0.55	22.3 ± 1.68	11.33 ± 0.94	11.0 ± 1.0
MRSA-25	9.5 ± 0.81	11.67 ± 1.1	17.67 ± 1.69	14.33 ± 1.24	11.33 ± 1.37
MRSA-26	12.25 ± 0.75	11.75 ± 0.69	21.5 ± 2.29	11.67 ± 1.1	10.67 ± 1.1
MRSA-GBT	12.33 ± 0.37	11.33 ± 1.49	28.33 ± 0.47	13.25 ± 1.14	13.0 ± 0.81
MSSA- GBT	12.67 ± 0.68	10.0 ± 1.0	26.0 ± 1.29	14.67 ± 1.06	11.33 ± 1.37
ATCC No.29737	10.67 ± 0.74	10.75 ± 0.69	31.5 ± 0.5	16.5 ± 1.11	11.67 ± 0.74
ATCC No. 9144	12.33 ± 0.68	9.75 ± 0.8	30.0 ± 1.73	12.5 ± 1.08	11.67 ± 0.94
Mean	10.97	10.46	22.59	12.27	9.96
SD	1.24	0.76	4.47	1.74	2.16

SD: standard deviation, n = 6.

antimicrobial activity of sesquiterpenoids from *P. merrillii* and *Phellinus fastuosus* against *P. mirabilis* and *C. albicans*. The strain of *P. albus* collected from *Acacia auriculiformis* did not exhibit pronounced activity against majority of MRSA strains and therefore, data are not presented.

Conclusion

The present study showed that mushroom extracts from *P. albus* are promising antimicrobial agents which can be employed alone or in combination with commercial antibiotics to combat several diseases causing pathogenic bacteria including the multi-drug resistant MRSA. However, further investigation and evaluation of the antimicrobial potential of *P. albus* as well as other species which are indigenous to India are needed. Ectomycorrhizal fungi not only can be used as biofertilizers, but also they could be used for human drugs such as antibiotic and antifungal for many human diseases.

ACKNOWLEDGEMENTS

The authors wish to gratefully acknowledge the assistance and cooperation of Dr. Pradhan (Joshi

Hospital, Pune), Dr. D. V. Gokahle (NCL, Pune), Dr. Anand Joshi (National Burns Hospital Airoli, Thane) and Dr. Bikash (GenomeBioTech, Pune), for providing test microorganisms.

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