

Original research paper

Evaluation of antifungal activity of the extracts of wild fruiting bodies and cultured Basidiomycete macrofungi- *Pleurotus sapidus* and *Pleurotus flabellatus* on several azole-resistant *Candida* spp.

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Abstract

Medicinal mushrooms are widely used as traditional medicinal ingredients for treatment of various diseases and related health problems. Most of the medicinal extracts from mushroom are different forms of polysaccharides which strengthens the immune system with little or no side effect. Pleurotus (Oyster mushroom) represent one of the world's greatest untapped resources of nutritious food. Pleurotus are rich in protein, minerals, and vitamins, and they contain an abundance of essential amino acids. Two Pleurotus mushroom fruiting bodies as well as their cultured mycelium were subjected to extraction in different solvents. After a prescreening of various extracts, MICs of acetone and ethanol extracts of both was determined against several azole resistant Candida spp. The fruiting body and mycelial extracts were compared with regard to their effectiveness with the result that fruiting body extracts hold more potential than the later. This concludes that the Candida sp. which nowadays gaining resistance to the azoles, can thereby be tested for their susceptibility to two Pleurotus spp. extracts which needs to be refined with reference to the different compounds in order to exploit their full potential. Another important point to be considered is that Pleurotus comes in the category of edible mushrooms thus the compounds extracted from the different fractions can be assumed to be nontoxic.

Key Words: *Multidrug resistance, Candida spp. Macrofungi, Pleurotus spp.*

1. Introduction

In recent years, multi drug resistance in pathogenic microorganisms has developed due to misuse of antimicrobial drugs commonly used in the treatment of infectious diseases. This situation has forced scientists to search for new antimicrobial substances from various sources as novel antimicrobial chemotherapeutic agents (Karaman *et al.*, 2003). The scientific community, in search of new therapeutic

alternatives, has studied many kinds of mushrooms and has found variable therapeutic activity such as anti-carcinogenic, anti-inflammatory, immune-suppressor and antibiotic, among others. Edible mushrooms are nutritionally gifted fungi (most of the Basidiomycetes) growing naturally on the trunks, leaves and roots of trees as well as decaying woody materials (Chang and Miles, 1992; Stamets, 2000; Lindequist *et al.*, 2005).

Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds (polyketides, flavonoids, terpenes and steroids). Phenolic compounds were found to have antioxidant activity in the inhibition of LDL oxidation (Acharya *et al.*, 2005). Phenolics are one of the major groups of nonessential dietary components that have been associated with the inhibition of atherosclerosis and cancer (Williams and Iatropoulos, 1997). Flavonoids have been proven to display a wide range of pharmacological and biochemical actions, such as antimicrobial, antithrombotic, antimutagenic and anticarcinogenic activities (Cook and Samman, 1996; Kandaswami and Middleton, 1997; Sahu and Green, 1997).

Pleurotus (*Oyster mushroom*) represent one of the world's greatest untapped resources of nutritious food (Mizuno, 1999). *Pleurotus* are rich in protein, minerals, and vitamins, and they contain an abundance of essential amino acids. The interest in the use of mushrooms and mushroom derived formulations for food and medicine is increasing across the world (Chen, 1992). In 2000, Wang first identified a novel ubiquitin-like protein from *Pleurotus* that inhibits HIV-1 reverse transcriptase activity. Cultivation of mushroom for fruiting body production is a long term process taking 1 to several months, depending upon the species and substrates. In contrast, production of mushroom mycelium in submerged culture would allow acceleration in the growth and to obtain high yield of biomass with constant composition. Hence, cultured mycelium of mushroom is an ideal source for the production of antimicrobial compounds. However, the difference effect of the substances isolated from two stages of growth of mushroom (*i.e.* mycelium and the fruiting body) must be ascertained. In the present study, after pre-screening of different extracts in six solvents minimum inhibitory concentration of both types of extracts was a determined using ethanol and acetone solvent system.

2. Materials and Methods

2.1 Source of macrofungi

Mushroom fruiting bodies of *Pleurotus sapidus* and *Pleurotus flabellatus* were collected from Biocontrol Laboratory of Sardar Vallabh Bhai Patel University of Agriculture and Technology, Modipuram, Meerut

(India) in April, 2008 and stored at 4 °C in refrigerator until use.

2.2 Source of Test microorganisms used and their maintenance

Five strains of *Candida* spp used were as follows: MTCC 224 and MTCC 226 of *Candida albicans*, MTCC 225 and MTCC 227 of *C. guilliermondii*, and MTCC 228 of *C. krusei*. These organisms were obtained from Microbial Type Culture Collection (MTCC) IMTECH, Chandigarh (INDIA) and stored on malt extract medium at 4 °C in refrigerator and sub cultured at regular intervals of 48 h until use.

2.3 In vitro mycelial culture of mushroom

Spore discharge method (Crittenden *et al.*, 1995) was followed to get the mycelial culture of mushroom on malt extract agar media. Cultures were incubated at 27 °C in incubator and examined periodically over a week period. Germinating spores were transferred to fresh medium and examined daily. After about two week growth culture were used for further investigation.

2.4 Extracts of fruiting body and mycelial cultured fungi

Dried fruiting body of *P. sapidus* and *P. flabellatus* were crushed using pestle and mortar to fine formed powder. 1 gm powder of each mushroom fruiting body powder was extracted in 10 ml of ethanol and acetone separately. In same way, isolated fungus culture, grown on malt extract agar medium, of 15 day old, were used for extraction. Dried mycelia were ground, using pestle and mortar, to fine powder. 1 gm powder of each mushroom fruiting body powder was extracted in 10 ml of ethanol and acetone separately. The mixtures were sonicated for 30 minutes then left at room temperature overnight. The extracts were filtered over Whatman No.1 filter paper, and the filtrates were sterilized by membrane filtration using 0.45µm pore size filters.

2.5 Antibiogram

The Kirby Bauer disc assay was used for testing 5 antifungal drugs (Fluconazole, Kitoconazole, Itraconazole, Clotrimazole, and Nystatin) against the five *Candida* spp. for profiling their resistance pattern according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (NCCLS, USA, 1997). Zone of inhibition was measured to the

nearest whole millimeter by Hi Antibiotic Zone Scale™.

2.6 Determination of Minimal Inhibitory Concentration (MIC) by Microtiter plate based antimicrobial assay

Resazurin assay utilizing microtiter-plate method (Sarkar, et al., 2007) was used to achieve more accuracy in the determination of the minimal inhibitory concentration (MIC) value of natural products including crude extract against various *Candida* strains. A final concentration of 5×10^5 cfu/ml of test microorganisms was adopted for this assay. A sterile 96 well microtiter plate was labeled. A volume of test material (10 mg/ml) was pipetted into the first row of the plate, from A1 to H1. All other wells (A2 to H2 till A10 to H10) contain 50 μ l of nutrient broth. Serial dilution was performed using micropipette. Tips were discarded after use such that each well had 50 μ l of the test material in serially descending concentrations. To each well, from A2 to H2 till A12 to H12, 10 μ l of resazurin indicator solution was added followed by addition of 30 μ l of nutrient broth. Finally, 10 μ l of test microorganism suspension (5×10^6 cfu/ml) was added to each well ensuring a final volume of 100 μ l in them. Controls were also put together having 50 μ l of solvents of analytical grade (ethyl acetate and acetone) and DMSO in place of extract.

The plates were prepared in replicates (both for fruiting body and mycelium) and placed in incubator set at 130 rpm for 24 hours at 27 °C for *Candida*. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of two values was calculated and that was the MIC for the test material and test organism strain.

3. Results

The resistance pattern of given five strains of *Candida* spp. was investigated against five antifungal drugs (Fluconazole, Ketoconazole, Itraconazole, Clotrimazole, and Nystatin), Ketoconazole and Fluconazole were found to be most effective with the zone of inhibition of 29 and 33 mm, respectively, against *Candida albicans* MTCC 224. The minimum zone of 14 mm was found due to Itraconazole against this species. *Candida guilleirmondi* MTCC 225 was found very less sensitive against all the antifungal agents used. The maximum zone was formed of 17 mm due to Ketoconazole. This species found

resistance against Fluconazole and Itraconazole. The inhibition zone due to Ketoconazole and were formed of 28 and 32 mm, respectively against *Candida albicans* MTCC 226. Nystatin and Itraconazole were found very less effective and formed inhibition zone of 10 and 13 mm, respectively (Plate 4.4C; fig 1). *Candida guilleirmondi* MTCC 227 showed the inhibition zone of 28 and 35 mm with the antifungal Ketoconazole and Fluconazole, respectively. Nystatin and it showed the zone of inhibition of 14 and 13 mm, respectively and showed the minimum effect on this species. *Candida krusei* MTCC 228 was found very less sensitive against all the antifungal used. The maximum zone was formed of 16 mm due to Ketoconazole.

On the above observations, *Candida guilleirmondi* MTCC 225 and *Candida krusei* MTCC 228 were showed the very less sensitivity against the all five antifungal. The minimum inhibitory concentration of ethanol extract against *C. albicans* (MTCC 224) and *C. krusei* was found to be the least i.e. 6.25×10^4 mg/ml and thus the extracts were most effective against them. The minimum inhibitory concentrations of both the extracts were found to be the same against all the test microorganisms i.e. 6.25×10^4 mg/ml thus the extracts were equally effective against them.

Amongst the extracts the minimum inhibitory concentration for acetone extract was the least for *C. albicans* (MTCC 224), *C. guilleirmondi* (MTCC 225), *C. guilleirmondi* (MTCC 227) and *C. krusei* (MTCC 228) i.e. 12.5×10^4 mg/ml, and for ethanol extract least against *C. krusei* MTCC 228) i.e. 6.25×10^4 mg/ml and thus the extracts were most effective against them (Table 1).

Amongst the extracts the minimum inhibitory concentration for acetone extract was the least for *C. albicans* (MTCC 224) and *C. krusei* (MTCC 228) i.e. 6.25×10^4 mg/ml, and for ethanol extract least against *C. albicans* (MTCC 226) and *C. krusei* and thus the extracts were most effective against them.

4. Discussion

P. sapidus & *P. flabellatus*, mushroom are primarily consumed for its nutritive value and used industrially as a bioremediator (Solomko and Eliseeva, 1988; Fountoulakis et al., 2002; Tsioulpas et al., 2002). The present study has further revealed the antimicrobial activity of the metabolite of the macrofungus extracted with Acetone, Ethanol. The observed phenolic and tannin constituents of *Pleurotus* may also elicit antibacterial activity as found in many medicinal plants with mechanisms of action characterized by cell membrane lysis, inhibition of protein synthesis, proteolytic enzymes and microbial adhesins (Cowan, 1999). Therefore, there is no doubt that the future determination of the structure and functions of the phenolic and terpenoid constituents of both mushrooms would further validate its antimicrobial potentials. Antimicrobial activities showed by *Pleurotus flabellatus* and *P. sapidus* may be due to the presence of bioactive substances in these fungi and the solubility of those active compounds in the solvent used. Mushrooms have been reported for its extensive use in medicine for curing variety of ailment or diseases. (Stametes, 1993; Mau et al. 1998; Oso, 1977). The presence of bioactive substances in these mushrooms is in accordance with the work of Benedict and Braddy (1972) who reported isolation of bioactive bases from antibiotics producing mushrooms. It was observed that most of the test organism showed resistance to mushroom extracts in methanol, chloroform and benzene. The resistance may be due to the presence of antibiotics resistance genes that may be located on plasmids of these organisms as a result of mutation that might occur in these organisms (Roland, 1984). The results obtained from the antibiotic susceptibility tests, showed that *Candida albicans* (MTCC 224) exhibited a high resistance to most of the antifungal used (Co-trimoxazole, Nystatin, Itraconazole, Ketoconazole and Fluconazole).

5. Conclusion

The fruiting body and mycelial extracts were compared as regards to their effectiveness with the result that fruiting body extracts hold more potential than the later. This concludes that the *Candida sp.* that are nowadays are gaining resistance to the azoles can thereby be tested for their susceptibility to the two spp. of *Pleurotus* extract which needs to be refined as regards to the different compounds in order to exploit their full potential. Another important point to be considered is that *Pleurotus* comes in the category of edible mushrooms thus the compounds

extracted from the different fractions can be assumed to be non-toxic.

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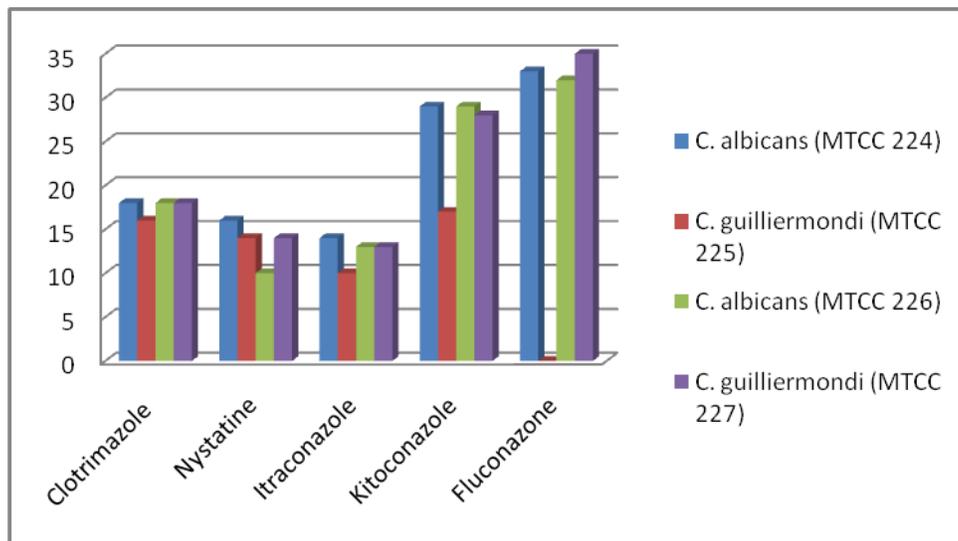


Fig 1. Antibiogram of different *Candida* species against different antifungal drugs

Table 1: Comparative analysis of Minimum inhibitory concentration of fruiting body extracts and mycelium extracts of *Pleurotus flabellatus* and *Pleurotus sapidus* against various *Candida* spp

Test microorganisms	Fruiting body extracts (mg/mL)				Mycelium extracts (mg/mL)			
	<i>Pleurotus flabellatus</i>		<i>Pleurotus sapidus</i>		<i>Pleurotus flabellatus</i>		<i>Pleurotus sapidus</i>	
	Acetone extract	Ethanol extract	Acetone extract	Ethanol extract	Acetone extract	Ethanol extract	Acetone extract	Ethanol extract
<i>C. albicans</i> (MTCC 224)	12.5×10 ³	6.25×10 ⁴	6.25×10 ⁴	6.25×10 ⁴	12.5×10 ³	25×10 ²	6.25×10 ⁴	12.5×10 ³
<i>C. guilleirmondi</i> (MTCC 225)	25×10 ²	25×10 ²	6.25×10 ⁴	6.25×10 ⁴	12.5×10 ³	12.5×10 ³	12.5×10 ³	12.5×10 ³
<i>C. albicans</i> (MTCC 226)	12.5×10 ³	12.5×10 ³	6.25×10 ⁴	6.25×10 ⁴	25×10 ²	25×10 ²	12.5×10 ³	6.25×10 ⁴
<i>C. guilleirmondi</i> (MTCC 227)	12.5×10 ³	12.5×10 ³	6.25×10 ⁴	6.25×10 ⁴	12.5×10 ³	12.5×10 ³	12.5×10 ³	12.5×10 ³
<i>C. krusei</i> (MTCC 228)	12.5×10 ³	6.25×10 ⁴	6.25×10 ⁴	6.25×10 ⁴	12.5×10 ³	6.25×10 ⁴	6.25×10 ⁴	6.25×10 ⁴