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Antimicrobial activity of four wild edible mushrooms from Darjeeling hills, West Bengal, India

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Abstract: The present study was carried out to evaluate the antibacterial and anticandidal activity of ethanolic and ethyl acetate fraction of fruit bodies from *Meripilus giganteus, Entoloma lividoalbum, Ramaria aurea* and *Pleurotus flabellatus* on selected six bacterial pathogens such as *Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus* and one fungal strain *Candida albicans*. For antimicrobial test, disk diffusion technique was used and the zone of inhibition of microorganisms was measured in mm. The ethanolic fraction of the fruit body of *P. flabellatus* and *M. giganteus* showed potential antimicrobial activities against the selected strains. Ethanolic and ethyl acetate fraction were effective against both bacterial and fungal pathogens but ethanolic fraction was better than ethyl acetate fraction. The results provided evidence that the studied mushrooms might indeed be potential sources of natural antimicrobial agents.

Keywords: Antibactrial, anticandidal, disk diffusion method, ethanolic fraction, ethyl acetate fraction, wild edible mushroom.

INTRODUCTION

Over the last decade, it has become clear that antibiotics are losing their effectiveness as pathogens evolve resistance against them, a problem compounded by the fact that new drugs only rarely reach the market[1]. In recent years, multiple drug resistance in human pathogenic microorganisms has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This situation forced scientists for searching new antimicrobial substances from various sources, which are the good sources of novel antimicrobial chemotherapeutic agents[2].

Natural products are considered to be a fundamental source of new chemical diversity and an integral component of today's pharmaceutical compendium. Mushrooms are not only prized for their splendid tasteful flavor, they could also be served as a good healthy supplement too. Recently use of mushrooms with therapeutic properties has drawn a great deal of attention because it demonstrates the efficiency against numerous diseases even when consumed as food. Because of unique geoclimatic variations, the state West Bengal becomes a treasure house for the luxuriant growth wild edible mushrooms. Several extracts and purified compounds of wild edible mushrooms from this state were explored for their medicinal possibilities and have shown strong

antioxidant[3-8], antimicrobial[9,10], cardioprotective[11], hypoglycemic[12], hepatoprotective[13-15] nd apoptogenic[16,17] activity.

The purpose of the present study was to investigate the antimicrobial properties of four wild edible mushroom of Darjeeling hills viz. *Meripilus giganteus, Entoloma lividoalbum, Ramaria aurea* and *Pleurotus flabellatus.* In this paper, we report the results of such studies in order to orient future investigations towards the finding of new, potent, safe antimicrobial compounds.

MATERIALS AND METHODS

Collection and identification of edible mushrooms

Wild edible mushroom specimens (*Meripilus giganteus, Entoloma lividoalbum, Ramaria aurea* and *Pleurotus flabellatus*) were collected from the different areas of Darjeeling during the month of May to September 2012. Information of edibility were gathered by discussion and direct interview with local people and by direct observation on the way different mushrooms were being collected and used. Damaged, infected and very young fruit bodies were avoided. Collected different samples were kept separately. The morphological and ecological features were noted and color photograph of the materials were taken during field trips. After the specimens were brought to the laboratory, the macroscopic and microscopic (Olympus research microscope) properties were determined. Then the specimen were identified according to[18-20]. The voucher specimens have been deposited in the Department of Botany, St. Joseph's college, Darjeeling, West Bengal.

Extraction Procedure

Fresh mushrooms were randomly selected into three samples of 150 g each and air-dried in an oven at 40˚C for 48 h. Powdered basidiocarps (100 g) were extracted with 80% ethanol at room temperature overnight and was repeated 4 times, and then freeze-dried (Ethanolic fraction). The residual fraction was dissolved in distilled water in a boiling water bath for 4 h. The aqueous phase was evaporated and reduced to half its volume and then mixed with 99% ethanol (1:4, v/v), and the precipitated fraction were separated from aqueous phase and discarded. The aqueous phase was then evaporated to remove the ethanol and mixed with ethyl acetate (2:1, v/v). The upper ethyl acetate layer was then evaporated and lyophilized (Ethyl acetate fraction) (Figure 1). The freeze-dried extracts were separately reconstituted in Dulbecco's phosphate-buffered saline (DPBS) to a concentration of 10 mg/ml). These stock solutions were sterilized by filtration through a 0.45 µm pore membrane and kept in the dark at 4˚C for further use[21].

Antimicrobial activity

Test organisms

All the test microorganisms *Staphylococcus aureus* MTCC CODE 96, *Proteus vulgaris* MTCC CODE 426, *Candida albicans* MTCC CODE 183, *Bacillus cereus* MTCC CODE 1306, *Escherichia coli* MTCC CODE 68, *Pseudomonas aeruginosa* MTCC CODE 8158, *Bacillus subtilis* MTCC CODE 736, were obtained from the culture collection of the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.

Screening of antimicrobial activity by disk diffusion method

Antimicrobial activities of ethanolic and ethyl acetate fraction of different edible mushrooms were determined by the agar-disk diffusion method[22]. The bacterial pathogens mentioned above were incubated at 37±0.1^{o}C for 24 h by inoculation into Nutrient broth. *C. albicans* was incubated in Yeast extract (YE) broth at 28±0.1^{o}C for 48 h. Nutrient Agar (NA) and YE Agar (20 ml) were poured into each sterilized Petri dish (90 mm diameter) after injecting cultures (100 µl) of bacteria and yeast and distributing medium homogeneously. For the investigation of the antibacterial and anticandidal activity, filter paper disks (6 mm in diameter) were impregnated with each of the fraction in order to reach the final levels of 100 µg/disk. The impregnated disks were air-dried before being placed on the Petri dishes with the test microorganisms. The plates were incubated at 37±0.1^{o}C for bacterial pathogen and 28±0.1^{o}C for fungal pathogen and the inhibition areas were measured in mm using clear graduated ruler. Studies performed in triplicate and the inhibition zones were compared with those of reference discs[23]. Standard antibiotic discs Streptomycin (10 µg/disc), Tetracycline (30 µg/disc), Ampicillin (10 µg/disc), Nystatin (100 µg/disc) and Clotrimazole (10 µg/disc) were used as positive control.

Statistical analysis

Five disc per plate and three plates were used, and each test was run in triplicate and zone of inhibition was determined in millimeters. All the results were statistically expressed as the mean ± SD.

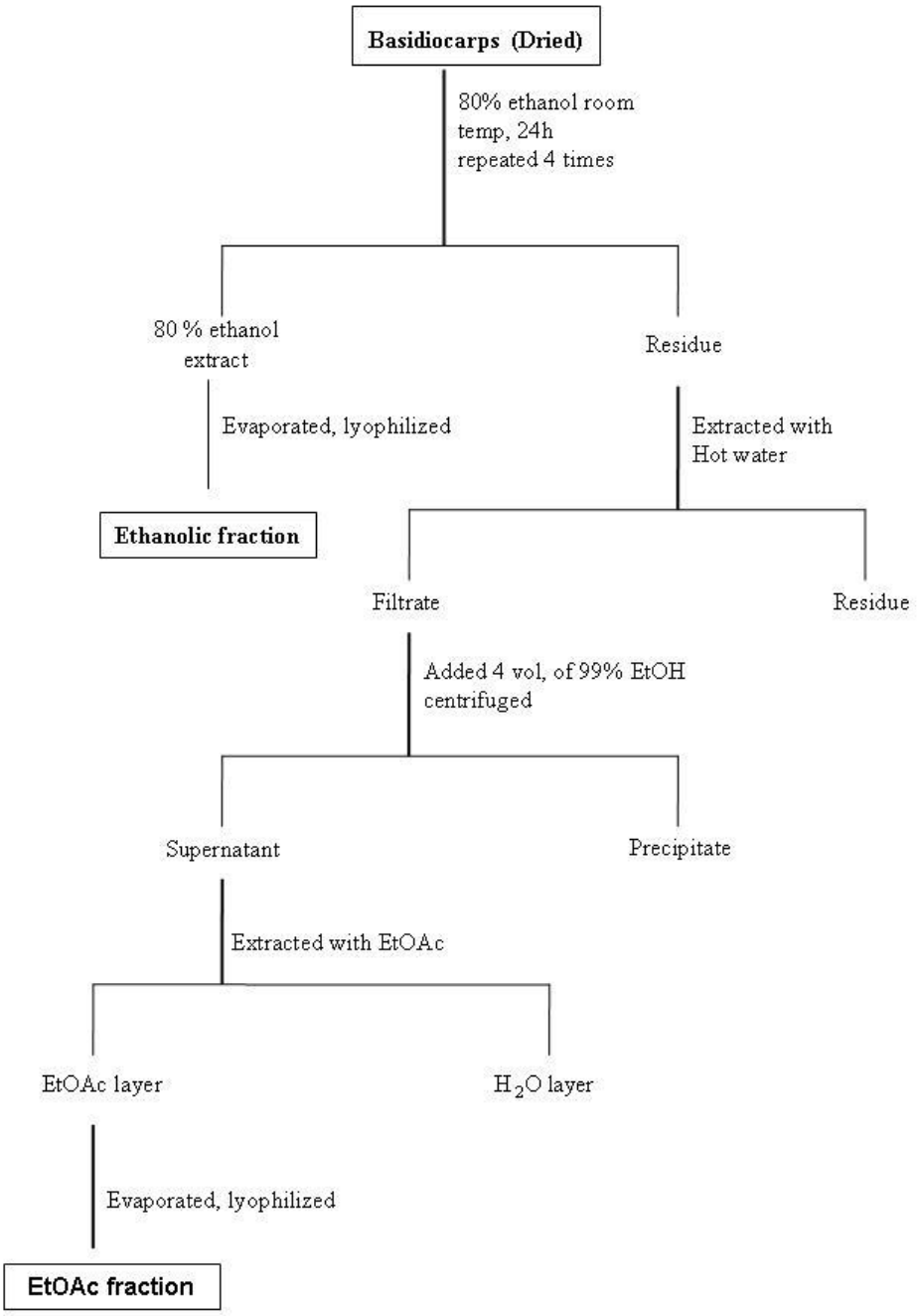

Figure 1. Flow chart for the preparation of Ethanolic and Ethyl acetate fraction

RESULTS AND DISCUSSION

Antibiotic resistance among microbes urgently necessitates the development of novel antimicrobial agents such as alternate therapies using natural products. Many pharmaceutical substances with potent and unique health-enhancing properties have been isolated from medicinal mushrooms and distributed worldwide[24]. Mushroom based products either from the mycelia or fruiting bodies are consumed in the form of capsules, tablets or extracts[25]. It has been reported by many workers that fruit bodies of different mushrooms like *Lactarius* sp.[26, 27]; *Fomitopsis* sp.[28]; *Boletus* sp.[29]; *Pleurotus tuber-regium*[30]; *Lactarius deliciosus*, *Sarcodon imbricatus* and *Tricholoma portentosum*[31]; *Russula delica*[32]; *Pleurotus eryngii* var. *ferulae*[33]; *Infundibulicybe geotropa*, *Lactarius controversus*, *Lactarius delicious* and *Phellinus hartigii*[34]; *Lactarius indigo*[35] and *Stereum ostrea*[36] contain a wide range of antimicrobial activity, our present results strengthened the outcomes of earlier works done by others. In the present study, the ethanolic and ethyl acetate fraction of the selected four macrofungi (*M. giganteus, E. lividoalbum, R. aurea* and *P. flabellatus*) were tested against three Gram-negative (*P. vulgaris, E. coli, P. aeruginosa,*), three Gram positive (*B. subtilis, B. cereus, S. aureus*) bacteria and a Yeast (*C. albicans*) by the disc diffusion method which showed variable zones of inhibition (Figure 2 and 3).

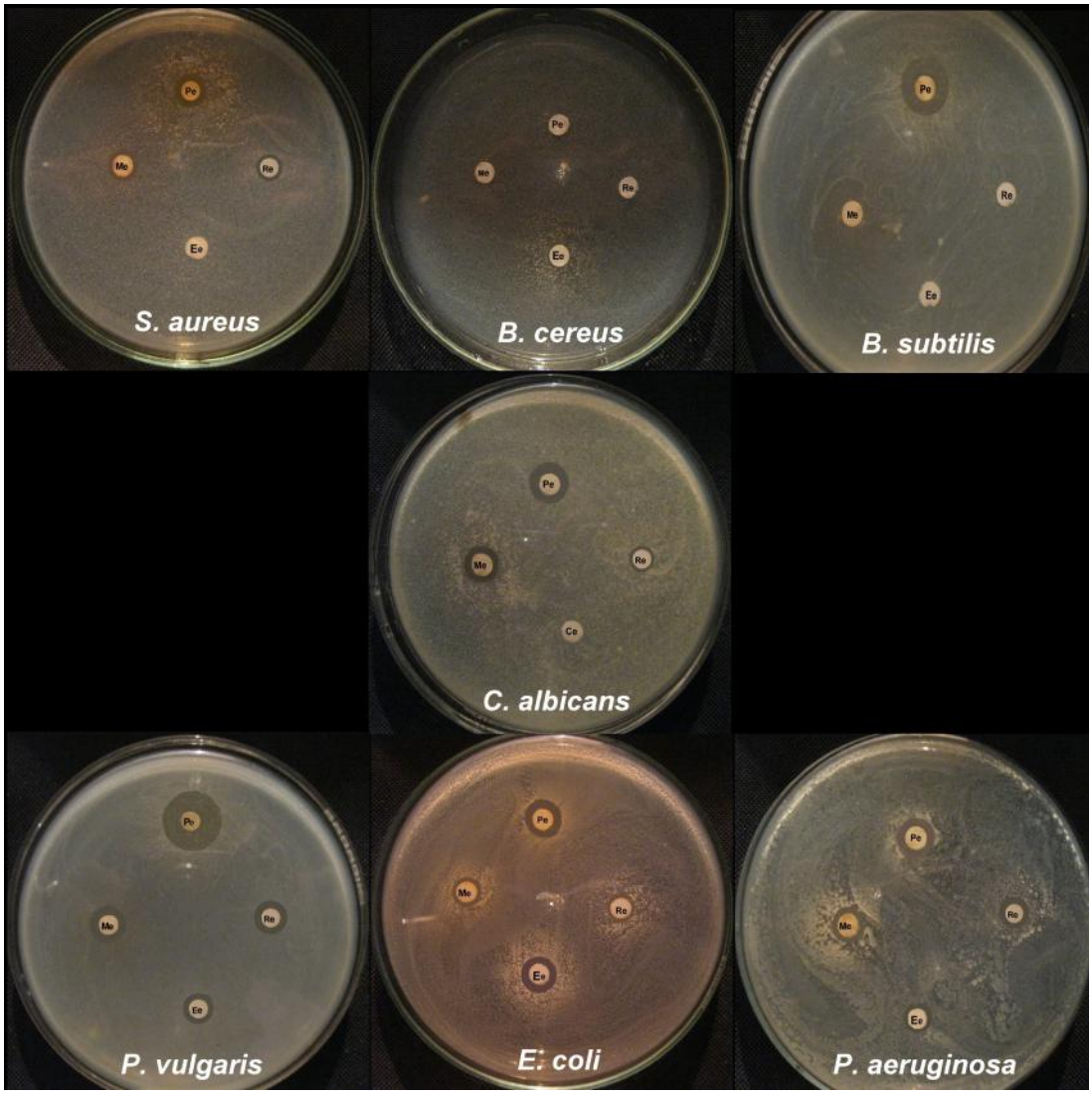

Figure 2: Antimicrobial activity of ethanolic extract of different mushrooms against six bacterial and one fungal pathogen

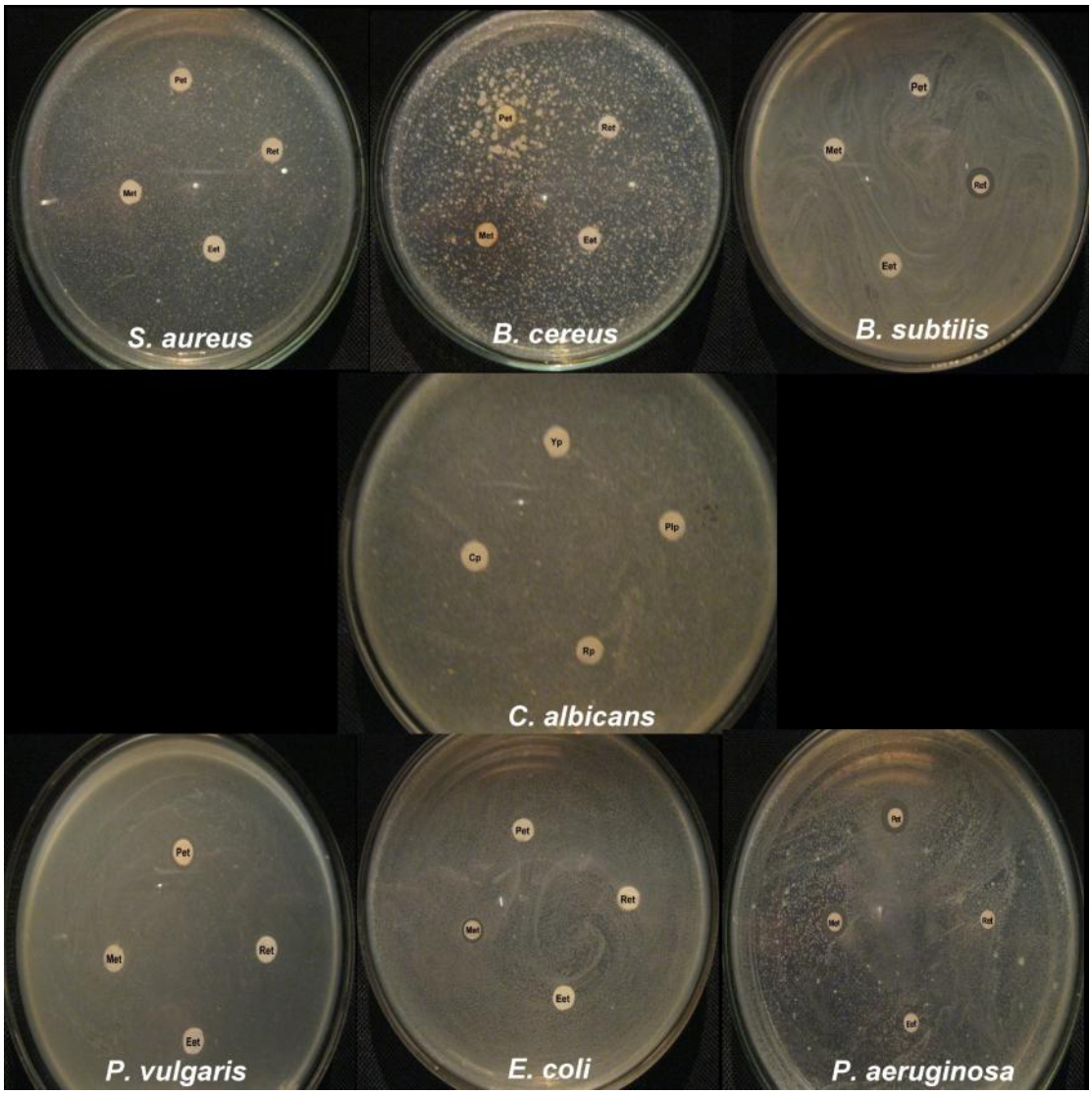

Figure 3: Antimicrobial activity of ethyl acetate fraction of different mushrooms against six bacterial and one fungal pathogen.

All the extracts showed different degree of antimicrobial activity at a concentration of 100µg/disc against the test pathogens (Table 1). The antimicrobial activities were comparable with those of commonly used antibiotics (reference disk) against the test pathogens. Ethanolic and ethyl acetate fraction were reveled different degree of effectiveness against both bacterial and fungal pathogens. All the Gram negative bacteria showed different degree of sensitivity towards all the ethanolic extracts. The maximum activity 17.29 mm were recorded from ethanolic fraction of *P. flabellatus* against *P. vulgaris* followed by 12.41 mm against *P. aeruginosa* and minimum 8.05 mm against *E. coli* from ethanolic fraction of *M. giganteus*. The ethyl acetate fraction of *R. aurea* showed its highest activity (9.79mm) against *B. subtilis* and *M. giganteus* extract showed moderate activity against *E. coli* and *P. aeruginosa.* The ethyl acetate fractions recorded negative for most of the bacterial pathogen as well as anticandidal activity was also recorded negative. The zone of inhibition against fungal pathogens ranged between 8.15 - 11.46 mm in ethanolic fraction. The maximum activity (11.46mm) was recorded from the extract of *P. flabellatus*, and minimum (8.15mm) from *R. aurea* against *C. albicans* (Table 1).

Table 1: Antimicrobial activity of different mushroom extracts. Values are mean ± SD of three separate experimental sets.

Mushroom extract/Standard disk	Test Pathogens (Inhibition zones in mm)						
	P. vulgaris	*E. coli*	*P. aeruginosa*	*B. subtilis*	*B. cereus*	*S. aureus*	*C. albicans*
P. flabellatus #	17.29±0.08	11.40± 0.06	12.41± 0.06	15.65 ± 0.10	-	10.68± 0.02	11.46 ± 0.09
R. aurea #	9.42±0.06	8.34±0.06	10.49±0.04	-	-	8.23±0.08	8.15±0.07
E. lividoalbum #	9.14±0.04	10.43±0.07	8.28±0.06	-	-	-	-
M. giganteus #	9.32±0.06	8.05±0.16	10.12±0.02	-	-	8.88±0.04	10.41±0.15
P. flabellatus *	7.89±0.04	-	8.11±0.06	9.79±0.04	-	-	-
R. aurea *	-	-	-	-	-	-	-
E. lividoalbum *	-	-	-	-	-	-	-
M. giganteus *	-	8.14±0.16	8.42±0.02	-	-	-	-
Streptomycin	-	18	-	13	17	20	NT
Tetracycline	21	-	-	-	28.5	24	NT
Ampicillin	-	14	13.5	-	-	-	NT
Nystatin	NT	NT	NT	NT	NT	NT	-
Clotrimazole	NT	NT	NT	NT	NT	NT	-

Notes: (#) ethanolic faction; (*) ethyl acetate faction; (-) Negative; (NT) not tested.

CONCLUSION

In general, reference discs are more active than ethanolic fraction of four different mushrooms but microorganisms get resistant to the antibiotics after sometime. Therefore, extracts of mushroom may be used as source of antimicrobial agents for safe and lacking side effects. Our results show that the basidiocarp of four wild edible mushrooms having potential antimicrobial activity which may have diverse therapeutic activities as well as makes it necessary to perform further studies in that regard by isolating and characterizing the molecules responsible for the observed activities.

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