World Applied Sciences Journal 10 (8): 861-866, 2010
ISSN 1818-4952

Antibacterial and Antifungal Activities of Culture Filtrate Extract of *Pyrofomes demidoffii* (Basidiomycete)

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Abstract: In this study cultures of twelve basidiomycetes were screened for antimicrobial activity. Among them the culture filtrate extract of *Pyrofomes demidoffii* produced the strongest antimicrobial activity. The objectives of this study are to assess submerged growth of this fungus and to determine its antimicrobial activity. Hundred ml of yeast extract, malt extract and glucose medium were inoculated with culture of *P. demidoffii.* All flasks were incubated at 28°C on a rotary shaker set at 120 revolutions per minute. Growth and antimicrobial activity of the culture were studied by harvesting the culture at five days interval. Mycelial dry weight was determined by dry oven method while the antimicrobial activity of the culture filtrate extract was determined by disc diffusion assay method. In submerged growth of the fungus mycelial dry weight increased from the 10th to the 20th days of growth and showed no appreciable change up to the 30th days of growth. Antimicrobial activity reached its maximum on the 20th days of growth and was similar up to the 30th days of growth. The culture filtrate extract of *P. demidoffii* inhibited the *in vitro* growth of an array of bacteria and fungi of medical importance. The extract was more active against test bacteria than test fungi.

Key words: Heart rot fungus, Polypore, Culture filtrate extract, Submerged culture, Tropical basidiomycetes

INTRODUCTION

In the past several years, a large number of antimicrobial compounds have been discovered, of which only small proportion of them are in clinical use [1]. Though major successes have been achieved against bacterial infections, emergence of drug resistance bacterial strains have made treatment of bacterial diseases more difficult [2-8].

Some important antifungal drugs of microbial and synthetic origin have also been discovered for the treatment of fungal infections. However, the existing antifungal drugs fail to achieve the desired results. Toxicity to the host [9], narrow spectrum of activity [10-12], difficulty in drug administration [9], development of drug resistance [12-17] and recovery of fungi after treatment [9] are the major limitations of the existing antifungal agents.

Drawbacks of the presently available antibacterial and antifungal agents have initiated the interests of many academic institutions and pharmaceutical industries a search for new, better active, less toxic and cheaper antibacterial and antifungal agents [12].

It has been pointed out that chemical synthesis and a search for natural products from living organisms (microorganisms and higher plants) are the two sources of new biologically active compounds [18]. Though many new biologically active metabolites have been isolated and characterized from basidiomycetes (higher fungi) [18-20], this group of fungi are still among the less extensively screened groups of microorganisms. Basidiomycetes particularly the tropical ones have been little investigated from the viewpoint of bioactive secondary metabolites [21-23]. In the present work fifteen Ethiopian basidomycetes, *Pyrofomes. demidoffii* (Lev.) Kotl. et Pouz. *Schizophyllum commune* Fr. ex Fr. *Ganoderma australe* (Fr.) Pat, *Fomitopsis penicola* (Swartz:Fr.) Karst, *Lentinus edodes* (Berk) Pegler, *Pleurotus ostratus* (Jacq.) Quel. *Pleurotus sajor caju* (Fr.) singer, *Laetiporus sulpherous* (Bull.:Fr.) Murr, *Perinnioporia telephora* (Mont)Ryv. *Fomitiporia Pseudopunctata* (A. David et.al) Fiasson, *Fomitiporia aethiopica* (Decock, Bitew et Castillo) sp. nov, *Fomitiporia tenuis* (Decock, Bitew et Castillo) sp. nov, *Coniophora bimacrospora (*Callier, Bitew et Castillo and Decock) *sp. nov, Tyromyces ethiopica*

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(Bitew and Ryvarden) sp. nov, *Tyromyces cinereobrunneus* (Bitew and Ryvarden) sp.nov, were screened for antimicrobial activity. Of these the culture filtrate extract of *P. demidoffii* produced the most effective antimicrobial activity. In this paper, submerged growth, antimicrobial activity and the most susceptible test organism of the culture filtrate extract of *P. demidoffii* are presented.

MATERIALS AND METHODS

Culture of *P. demidoffiil*: This study was conducted in the School of Medical Laboratory Sciences, Addis Ababa University. Basidiocarp of the fungus was collected from Menagesha forest situated 30 km South West of Addis Ababa, Ethiopia. Culture of the fungus was obtained from fresh basidiocarp collected from *Juniperus procera Endl.* which is one of the indigenous trees of Ethiopia as follows:- Pieces of fungal materials from the basidiocarp were transferred aseptically onto 3% malt extract agar (Oxoid, Basingatoke, Hampshire, England) medium containing chloramphenicol (Karnataka antibiotics and pharmaceutical, India) and gentamacin (Karnataka antibiotics and pharmaceutical) at a concentration of 16 µl /ml and 5 µl/ml respectively. Culture plates were incubated at 25°C until young hyphae emerged, from which pieces of agar culture blocks were transferred to the same medium with out antibiotic to obtain pure culture. Culture of the fungus was maintained as Ethiopian culture collection ECC 75 on malt extract agar slant and was preserved as agar plugs in glycerol (10%, v/v) at minus 80°C in the School of Medical Laboratory Sciences, Addis Ababa University.

Submerged Growth of *P. demidoffii*: Sterilized 100 ml of yeast extract malt extract glucose medium, consisting of 4 g yeast extract (Sigma Chemicals, Germany), 10 g of malt extract (Sigma) and 4 g of glucose (Aldrich, Germany) per liter in 250 ml capacity of unbaffled Erlenmeyer flasks were inoculated aseptically with eight 5 mm culture blocks of the fungus. All flasks were then incubated at 28°C on a Bûhler rotary incubator shaker (Johanna Otto GMBH, Germany) set at 120 revolutions per minute (rpm). Growth (mycelial dry weight) and antimicrobial activity were studied by taking aliquot of culture medium at five days interval. Mycelial dry weight was measured by dry oven method. The antimicrobial activity of the culture against time was studied by agar disc diffusion assay using *Staphylococcus aureus* (ATCC 25923) as follows: The culture filtrate was extracted with an equal volume of ethyl acetate (Aldrich) after it was dried over anhydrous Na_2SO_4 (BDH). The ethyl acetate extract was concentrated under reduced pressure (Rotavapour, Buchi, Model 140). A maximum of 50 µl of the culture filtrate extract dissolved in methanol (Merk) was adsorbed on 6 mm diameter antibiotic assay discs (Aldrich). The discs were air dried and placed on test plates seeded with the bacterium. All test plats were incubated at 37°C for 24 hours and diameter of zone of inhibition produced by the culture filtrate extracts against the bacterium was measured by using a metal caliper.

Evaluation of the Crude Extract Against an Array of Bacteria and Fungi: Since the antimicrobial activity of the crude extract against *S. aureus* reached its maximum and remained the same between the 20th and 30th days of submerged growth (Fig. 1), 500 ml unbaffled Erlenmeyer flasks of yeast extract malt extract and glucose medium were inoculated with eight 5 mm agar culture blocks to produce enough extract for further study. All flasks were incubated as indicated above. All cultures were then harvested between the 20th and 30th days of submerged growth and the culture filtrate was extracted and concentrated as indicated above. The extract so obtained was used for the determination of antimicrobial activity against an array of bacteria and fungi.

Impregnation of Antibiotic Assay Discs: One milligram of the culture filtrate extract obtained between the 20th and the 30th days of submerged growth of the fungus was dissolved with 1 ml of methanol. A maximum of 50 µl of the culture filtrate extract was adsorbed on 6 mm diameter antibiotic assay discs. The discs were air dried before being placed onto test plates seeded with test bacteria and fungi.

Test Microorganisms and Assay Plates: In vitro antimicrobial activity tests were performed using an array of bacteria and fungi that include *Bacillus cereus* (clinical isolate), *S. aureus* (ATCC 25923), *S. aureus (clinical isolate), S. epidermidis* (clinical isolate), *Streptococcus pyogenes* (clinical isolate), *S. pneumoniae* (clinical isolate), *Escherichia coli* (ATCC 25922), *Klebisella pneumoniae* (clinical isolate), *Proteus mirabilis* (clinical isolate), *Pseudomonas aeruginosa* (ATTC 27853), *Salmonella species* (Clinical isolate), *Aspergillus fumigatus* (ATCC 13697), *Aspergillus niger* (ATCC 10535), *Candida albicans* (clinical isolate),

C. tropicalis (clinical isolate) *and Rhodotrula species* (clinical isolate) obtained from the Ethiopian Health and Nutrition Research Institute. Four to five bacterial and yeast colonies were inoculated into 5 ml of soyabean casein digest broth (Oxoid) and Sabouruad dextrose broth respectively and test bacteria were incubated at 37°C while yeasts were incubated at 30°C for 12 hours. The turbidity of the broth culture was then equilibrated with similar broth to match that of 0.5 McFarland standard to obtain approximately the organism number of 1x 10^6 colony forming unit (CFU) per ml. In brief, a loop full (4 mm diameter) of test yeasts and bacteria other than *Streptococcus* species were applied to the center of sterile Sabouruad dextrose and Muller Hinton (Oxoid) agar plate medium respectively. Species of *Streptococcus* were applied to the centre of a plate agar medium of blood agar base (Oxoid) to which 10% sheep blood has been added. Then, the test organisms applied to agar media were evenly spread using dry cotton swabs.

Test mycelial fungi were inoculated onto Sabouruad dextrose plate agar medium and incubated at 30°C until they produced spores. Spores were then harvested with sterile Sabouruad dextrose broth and filtered. Then, a heavy spore suspension (1-2 $\times 10^4$ spores/ ml) of test fungi was suspended in Sabouruad dextrose plate agar medium prior to pouring into sterile glass plates. The number of spores per ml of Sabouruad dextrose broth was determined by Neubauer counting chamber.

Antimicrobial Activity Assays: The antimicrobial activity of the culture filtrate extract of *P. demidoffii* against test bacteria and fungi was determined by standard disc diffusion assay method. Antibiotic assay discs impregnated with the compound were placed on test plates seeded with test bacteria and fungi. All test bacteria and yeasts were incubated at 37°C and yeast at 30°C for 24 hours. Mycelial fungi were incubated at 30°C for 48. Diameter of zone of inhibition produced by the culture filtrate extract against each test organism was measured with metal caliper.

RESULTS

The time course of submerged culture of *P. demidoffii* was followed, by measuring the antibacterial activity of the culture filtrate extract against *S. aureus*. As shown in Fig. 1, mycelial dry weight increased from day 10 to day 20 of growth and showed no appreciable change up to day 30. However, the mycelial dry weight decreased afterwards. The antimicrobial activity started from the 10th day of growth and reached its maximum on the 20th day of growth. Antimicrobial activity of culture filtrate extract of the fungus was the same from the day 20 to day 30 of submerged growth of the fungus. However, after the 30th days of growth, a decrease in antimicrobial activity of the fungus was observed.

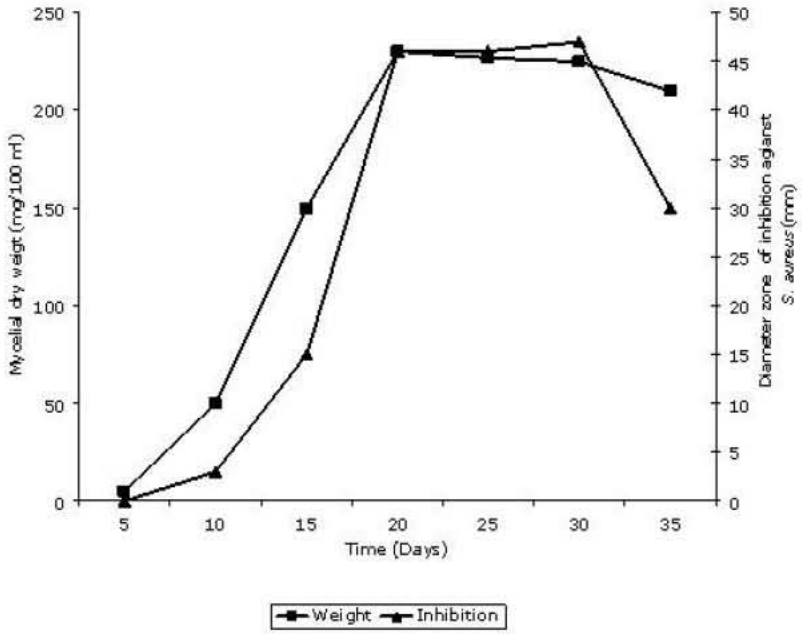

Fig. 1: Submerged culture of *P. demidoffii*

Table 1: Antibacterial activity of the culture filtrate extract *P. demidoffii* (disc diffusion assay)

	Diameter Inhibition Zone (mm) µg /disc		
Test organism	30	40	50
Gram positive bacteria			
B. cereus (clinical isolate)	17	20	23
S. aureus (ATCC 25923)	46	46	47
S. aureus (clinical isolate)	35	38	46
S. epidermidis (clinical isolate)	39	41	45
S. pyogenes (clinical isolate)	30	32	35
S. pneumonia (clinical isolate)	27	31	33
Gram negative bacteria			
E. coli (ATTC 25922)	20	23	27
K. pneumoniae (Clinical isolate)	20	24	25
P. mirabilis (Clinical isolate)	46	47	48
P. aeruginosa (ATTC 27853)	15	15	17
Salmonella sp. (clinical isolate)	18	20	25

Table 2: Antifungal activity of culture filtrate extract of *P. demidoffii* (disc diffusion assay)

	Diameter Inhibition Zone (mm) µg/ disc		
Test organism	30	40	50
Mycelial fungi			
A. flavus (ATCC 13697)	-	-	10
A. niger (ATCC 10535)	-	-	8
Yeasts			
C. neoformans (clinical isolate)	21	23	25
C. albicans (clinical isolate)	-	12	20
C. tropicalis (clinical isolate)	-	7	11
Rhodotrula sp (clinical isolate)	-	5	9

- no inhibition

The culture filtrate extract of the fungus inhibited the *in vitro* growth of all bacteria tested (Table 1). The *in vitro* growth of all gram-positive bacteria tested was strongly affected by the culture filtrate extract of the producing fungus. Among gram positive bacteria staphylococci were the most susceptible. Fifty microgram per disc of the culture filtrate extract produced about 47, 46 and 45 mm diameter of zone of inhibition against *S. aureus* (ATCC 25923), *S. aureus* (clinical isolate) *and S. epidermidis* (clinical isolate) respectively. Among gram positive bacteria *Bacillus cereus* was the least susceptible.

The *in vitro* growth of an array of gram negative bacteria tested was also affected by the culture filtrate extract of the fungus. Among gram negative bacteria *P. mirabilis* was the most susceptible. Fifty micro grams per disc of the producing fungus produced an inhibition zone diameter of 48 mm.

The crude extract also exhibited antifungal activities on both yeasts and mycelia fungi (Table 2). The activity against yeasts was better than against mycelial fungi tested. Among fungi, *C. neoformans* was the most susceptible.

DISCUSSION

In this study, the antimicrobial activity of culture of *P. demodoffii* was studied in submerged culture. In submerged culture of the producing fungus, the antimicrobial activity of the culture filtrate extract was low up to the 20th days of growth. However, antimicrobial activity was the highest between the 20th and 30th days of growth and this indicates that to obtain a good yield of the active principle the culture must be harvested between the 20th and 30th days of submerged growth. The highest antimicrobial activity was demonstrated when the fungus completed its growth. In batch cultures, high level of secondary metabolites are usually produced only after most of the cellular growth has occurred [24]. Depletion of one or more growth limiting substrates has been reported to arrest growth and initiate secondary metabolite synthesis [25]. It has been observed that antimicrobial activity of the fungus was almost linear between the 20th and 30th days of growth followed by decline in its activity after day 30. In previous work [26] depletion of the precursors of the secondary metabolite, irreversible decay of one or more secondary metabolite synthetase and feedback effect of the metabolite against its production have been suggested as the most probable explanation for secondary metabolite synthesis cessation.

The results of this study indicated that the culture filtrate extract of *P. demidoffii* contained biologically active substance that inhibited the *in vitro* growth of a wide range of bacteria and fungi of medical importance. Culture filtrate extract of *P. demidoffii* was more active against gram positive bacteria than gram negative bacteria. Variations in susceptibility to the culture filtrate extract of the fungus between gram negative and gram positive bacteria shown in this study could partly be attributed to differences in chemical composition of their cell wall.

The study has also shown that the culture filtrate extract of *P. demidoffii* exhibited antifungal activity. However, the *in vitro* antifungal activity of the culture filtrate extract of *P. demidoffii* against a number of disease causing yeasts and aflatoxin producing mycelial fungi was not as promising as that of its activity against test

bacteria. The known aflatoxin producing fungi, *A. flavus* and *A. niger* were less susceptible to the culture filtrate extract of *P. demidoffii.* In conclusion the culture filtrate extract inhibited the *in vitro* growth of an array of bacteria and fungi belonging to twelve genera and this may indicate that the extract has a wide spectrum of activity.

Strong inhibition of the *in vitro* growth of standard and clinical isolates of bacteria and fungal human pathogens by the culture filtrate extract of the fungus indicated that the product has a wide spectrum of activity. Thus the extract appears to be promising to develop new antibacterial and antifungal drug. To this end, to compare the potency of the active principle of the crude compound with the existing antibacterial and antifungal drugs, to elucidate its structure and study its mode of action purification of the active principle from the crude extract is in progress.

ACKNOWLEDGMENTS

The author is very grateful to the Ethiopian Health and Nutrition Research Institute for free provision of test organisms. I also thank Prof. Leif Ryvarden for confirming the identity of basediomycetes used in this study.

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