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Research Article

FUNGI FROM MANGROVE PLANTS: THEIR ANTIMICROBIAL AND ANTICANCER POTENTIALS

ELSA LYCIAS JOEL AND B.VALENTIN BHIMBA*

Department of Biotechnology, Sathyabama University, Chennai 600119. Email: bvbhimba@yahoo.co.in

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ABSTRACT

Recent research has increased our knowledge and understanding of the antimicrobial and cytotoxic effect of mangrove fungi. In a *systematic* screening effect, fungal isolates from the leaves of *Rhizophora mucronata* and *Avicennia officinalis* were investigated for antimicrobial activity and potentially active secondary metabolites. Owing to their antimicrobial and cytotoxic traits mangrove fungi are a target of research. And these traits are due to compounds synthesized in secondary metabolism of the fungi. The two selected species of study had various degrees of antimicrobial activity against the test microorganisms depending on culture conditions (stationary) and incubation time (2 to 3 days). This study also involved the testing of cytotoxic effect of the fungal extracts *Pestalotiopsis microspora VB5* at various concentrations using MTT assay. The emerging scientific evidences that go one step ahead to prove the effectiveness of secondary metabolites of mangrove fungi also suggest that mangrove fungi are far more likely to yield anticancer drugs than terrestrial sources. xic

Keywords: Fungi, Cytotoxic.

INTRODUCTION

Mangroves have long been a source of astonishment for the layman and of curiosity and interest to the scientists worldwide. Mangrove fungi also play an important role in the suppression of deleterious microorganisms and viruses that potentially cause human diseases. Most of the time viruses, bacteria and fungi act as major pathogenic organisms. The problem of antibiotic resistance is growing and the outlook for the use of antimicrobial drugs in the future is uncertain. Antibiotics are natural substances secreted by bacteria or fungi to kill other microorganisms competing for nutrients. The antibiotics used to treat people today are typically synthetic derivatives of these natural products. Scientists and the medical fraternity are dismayed to discover that some bacteria such as *Staphylococcus* and *E.coli* have become resistant through various alterations or mutations in their DNA. Scientific studies are on to develop new drugs either synthetic or natural to offer appropriate and efficient treatment to patients. According to WHO plants are the best source to obtain variety of drugs to combat pathogenic microbes. The antimicrobial properties of mangrove fungi have been investigated by a number of researchers worldwide.

Since the discovery of the world's first billion-dollar anticancer compound - paclitaxel (Taxol) - could be biosynthesized by *Pestalotiopsis microspora*, a fungus that colonizes the Himalayan yew tree, interest in studying such endophytes for their medicinal potential has grown tremendously [1]. Also, this drug is produced by many genera of endophytic fungi (*Alternaria, Fusarium, Monochaetia, Pestalotiopsis, Pithomyces and Taxomyces*) [2]. The cytotoxic and antiplatelet aggregation activity of methanol extract of Aglaia elliptifolia was also reported by some experts[3]. They provide a rich source of steroids, triterpenes. Saponins, flavanoids, alkaloids and tannins [4].

In an attempt to discover a novel substance of medicinal importance against human microbial pathogens and hep2 cancer cells, the present work aimed to evaluate antimicrobial and cytotoxic activity of the secondary metabolites obtained from *Hypocrea lixii* VB1 isolated from the leaves of *R. mucronata and A. officinalis*. The mangrove fungi remain the most important supply of novel drugs despite development of combinatorial chemistry capable of generating thousands of new chemicals in a short time.

Natural products and their analogs or molecules derived thereof comprise approximately 50% of the drugs presently used for clinical purposes. And 63% of anticancer drugs fall into this category [5,6,7]. Inspired by the abundance and diversity of mangrove fungi, the pharmacological potential of mangrove fungi have been enthusiastically pursued by researches all over. Testing of invivo growth inhibitory effect of tumour cells of animals will offer hope with regard to preclinical trials of natural anticancer drugs as extracts from several other foliar fungi isolated recently also showed appreciable activity[8].

MATERIALS AND METHODS

Isolation of foliar fungi

Fresh elder leaves from mangrove species *R. mucronata* and *A.officinalis* from Pichavaram mangrove forest southeast coast of India were collected. And thoroughly washed with distilled water to remove adhering soil particles and salts. They were grinded using distilled water and seawater in 1:1 ratio in a mortar and pestle under aseptic conditions. 1ml of the above was mixed with 10 ml of sterile water (distilled water: seawater; 1:1) to get dilution 10^{-1}aseptically.The serial dilution was repeated till 10^{-6}. From each dilution plating was done in sabouraud's agar by spread plate technique and incubated at 27^{o}c for 5 days. After 5 days, the plates were examined and the pure culture was isolated on pure agar plate.

Preparation of fungal broth culture

In order to obtain secondary metabolites the pure culture was grown in sabouraud's dextrose broth culture medium at 30^0 C for 3 days. After that a preinoculum was prepared by introducing small fragments (1cm square) of the growth culture into 250ml Erlenmeyer flasks containing sabouraud's dextrose broth and cultivated on a rotary shaker at 200rpm, 28^0 C (room temperature) for 5 days. Then the mycelium and the filtrate were separately subjected to solvent extraction as follows:

Extraction of mycelia

The fresh mycelium of each fungus was washed three times with water (distilled water**:** sea water 1:1) to remove adherent filtrate, and then plotted between folds of whattman filter paper no 1. The plotted mycelium was crushed using motar and pestle with ethyl acetate and methanol and subjected to sonication (Sartorious Labsonic) for 3-4 hours to obtain intracellular metabolites. Centrifuged at 2000-2500 rpm for 5 mins and the supernatant was used for further studies.

Extraction of the filtrate

The filtrate of each fungus was extracted several times with ethyl acetate (*v/v*) in a separating funnel. The extracts from both mycelia and filtrate were evaporated under vaccum at 50^0C till dryness. The obtained solid material was dissolved in ethyl acetate to form the crude extract and tested for bioassays.

Antibacterial assay

Antibacterial activity was carried out against bacterium *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923),

*Escherichia coli (*ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) by agar well diffusion method. Antibacterial activity was determined using the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003). Pre-warmed Mueller-Hinton agar (MHA) plates were seeded with 10^7 - 10^8 cfu suspension of test bacteria. Extracts (Biomass and filtrate) were pipetted (10 µl) onto sterile paper discs (6 mm diameter) and placed onto the surface of inoculated agar plates. Gentamicin sulphate (10 µg) was used as the positive control. Plates were incubated at 37°C for 48 h. Antibacterial activity was expressed as the diameter of the inhibition zone (mm) produced by the extracts. Then different concentrations were performed for one solvent, which showed maximum activity against the bacterial pathogens.

Cytotoxic activity (MTT assay)

Cytotoxicity of extracts at various concentrations (15- 1000 µg/ml-) was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma) assay but with minor modification, following 72 h of incubation. Assay plates were read using a spectrophotometer at 520 nm. Data generated were used to plot a dose-response curve of which the concentration of extract required to kill 50% of cell population (IC_{50}) was determined. Cisplatin (Mayne Pharma) and tamoxifen (Dynapharm), which are both established chemotherapeutics, were used for comparison. Cytotoxic activity was expressed as the mean IC_{50} (± standard deviation) of three independent experiments

GC-MS Analysis

The crude extract was quantified using gas chromatograph (Shimadzu QP2010) equipped with a VF-5 ms column (diameter 0.25 mm, length 30.0 m, film thickness 0.25µm) mass spectrometer (ion source 200^0C; EI -70 eV), programmed at temperature 40-650^0C with a rate of 4^0C/min. Injector flow rate was 200^0C; carrier gas was He 99.9995% purity, column flow rate1.51ml/min, injection mode -split.

Fungal isolation, identification

The internal transcribed spacers (ITS) of ribosomal DNA (rDNA) were amplified employing the combination of a conserved forward primer ITS1 (50- TCCGTAGGTGAACCTGCGG-30) and reverse primer ITS4 (50- TCCTCCGCTTA TTGATATGC-30). The polymerase chain reaction product is about 0.7 kb. The purified ITS rDNA was sequenced. The sequence data have been submitted to GenBank with an accession number. The sequences were aligned manually using CLUSTAL X version 1.8 with sequences of representative strains retrieved from the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank databases. Phylogenetic trees were produced using the neighbor-joining algorithms from the PHYLIP package version 3.5c.

RESULTS AND DISCUSSION

The results of the present study clearly showed that the bioactive metabolites *Pestalotiopsis microspora VB5* showed antimicrobial activity against (Table 1) tested pathogenic strains including antibiotic resistant strains. The effectiveness of the bioactive compounds of the two fungi caused the production of growth inhibition zones that appear as clear areas around the wells. However these fungal extracts showed minimal activity against some pathogenic organisms and we understand that these pathogenic strains may have some kind of resistance mechanisms eg., enzymatic inactivation, target sites modification and decreased intracellular drug accumulation [9] or the concentration of the extract used may not be sufficient. No inhibition was observed with controls, which proved that solvents could not act as antimicrobial agents. Literary references made known the fact that the antimicrobial potential of 71 endophytic fungi isolated from mangrove plants towards selected bacteria (*Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus*) were tested using ethyl acetate extracts of fungi cultivated under static conditions. All test bacteria were inhibited by a *Cladosporium* sp. isolated from the leaves of *T. populneoides* and an endophytic *Xylaria* sp. 1 isolated from *A. ilicifolius* leaves caused considerable inhibition to gram positive and gram negative bacteria[10]. Further more [11] have proved the antimicrobial potential of 14 endophytic fungi of A. ilcifolius and A. aureum, the ethyl acetate extracts of which were active against *B. subtilis, Enterococcus sp, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhi* and *Staphylococcus aureus* and fungi (*Candida albicans* and *Trichophyton metagrophytes*) [11] .

In the MTT assay appreciable activity was observed against Hep 2 cell lines that is clearly seen in the slides of three independent experiments carried out (Table 2 and Fig 1). Further research is necessary for successful separation, purification and characterization of bioactive compounds using chromatographic methods and spectroscopic techniques. Studies are being carried out to separate the individual components that are present in the fungal extracts using column chromatography the identification of its structure would suggest the development of a synthetic analogue as an efficient antibacterial or antitumour drug. As part of research program in biotechnology, Chulalongkorn University, researchers found out through a MTT assay that the crude extracts of 84 endophytic fungi had anticancer activities against A375 (human malignant melanoma), SW620 (human colorectal adenocarcinoma), Kato III (human gastric carcinoma), HepG2 (human liver hepatoblastoma) and Jurkat (human acute T cell leukemia) [10] . The GC MS analysis revealed that the active principals might be Tetradecane (6.26%) with the RT 8.606 (Fig.3)

The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). In the present study the DNA was isolated from the positive strain which showed high anti bacterial activity and the ITS region of 5.8s rRNA was amplified using specific primers ITS 1 and ITS4 and sequence was determined using automated sequencers. Blast search sequence similarity was found against the existing non redundant nucleotide sequence database thus, identifying the fungi as *Pestalotiopsis microspora VB5.* The percentage of similarity between the fungi and database suggests it as novel strain. Thus, the novel strain was named as *Pestalotiopsis microspora VB5* and made publically available in GenBank with an assigned accession number HQ823763 (http://www.ncbi.nlm.nih.gov/nuccore/HQ823763).

Although, mangrove associated fungi attract attention for their anti-inflammatory, membrane-stabilizing and extracellular enzymatic properties [12, 13] this study is unique in that it suggests that mangrove fungi also show synergistic activity against numerous pathogenic strains of gram positive and gram-negative bacteria. Further research will enable us to find some lead compounds from fungi production thereby remaining a promise to treat various forms of cancer in future. It is clear from the present study that mangrove fungi with bioactive metabolites can be expected to provide high quality biological material for high throughout biochemical, anti cancer and antibacterial screening programmes.

Table 1: Antibacterial activity of ethyl acetate extract

Pathogens	Concentration				
	std	25µl	50µl	75µl	100µl
Proteus vulagaris	16	12	18	20	18
Bacillus	-	12	16	17	16
Pseudomonas	-	17	20	24	25
Enterococcus	-	16	17	22	20
Staphylococcus	-	14	17	18	10

Table 2: MTT Assay

S. No.	Concentration µg/ml	Dilutions	Absorbance	Cell viability
1	1000	Neat	0.13	25
2	500	1:1	0.17	32.69
3	250	1:2	0.22	42.30
4	125	1:4	0.28	53.84
5	62.5	1:8	0.30	57-69
6	31.25	1:16	0.35	67.30
7	15.625	1:32	0.46	88.46
8	Cell control	-	0.53	100

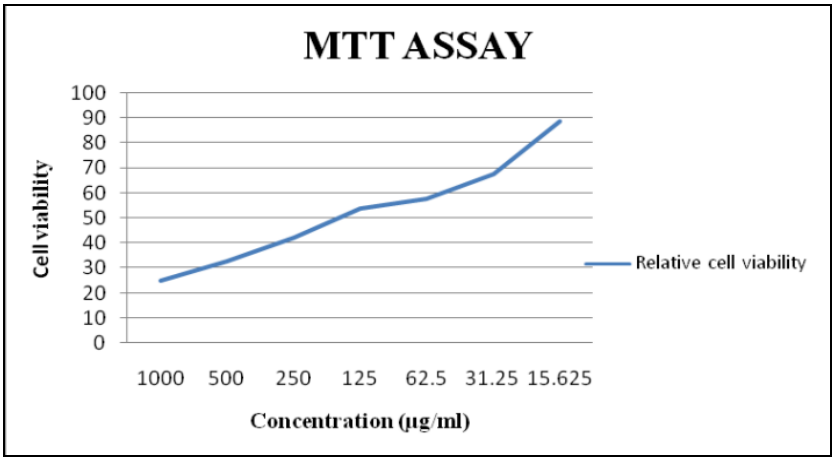
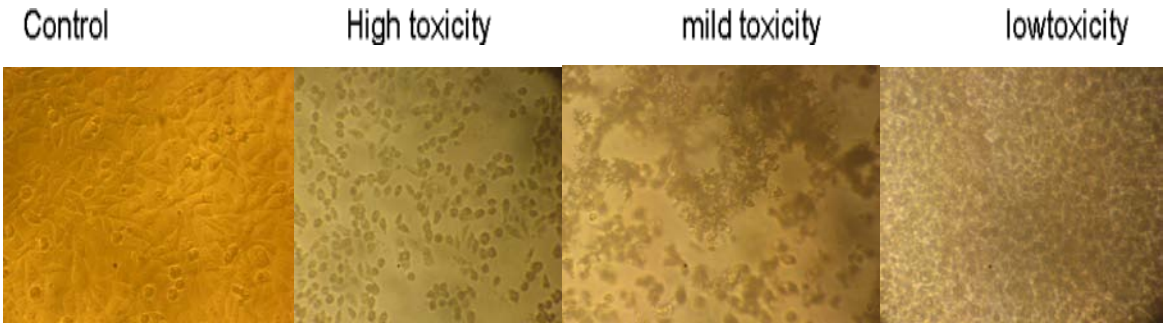


Fig. 1:



Control Metabolite added

Fig. 2: Anticancer activity of Hep2 Cell lines

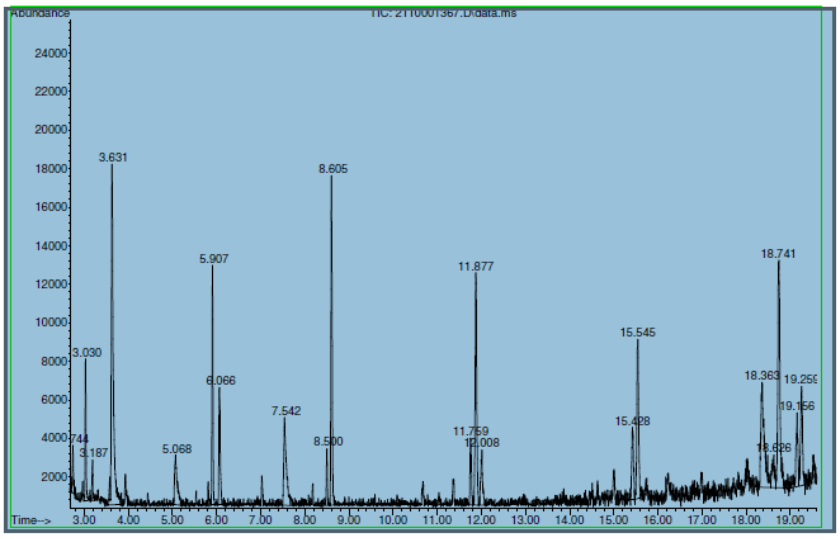


Fig. 3: GC MS spectrum

CONCLUSION

In summary we screened potent fungi with antibacterial and antitumour activity. The information stemming from the antibacterial and MTT assay will constitute the basis for development of new drugs. With the current understanding and obtained results, we can further screen and analyze the structure activity relationship of the bioactive compounds. As far as drugs from bioactive metabolites are concerned, natural products can be selected for biological screening based on ethno medical use of plants and microorganisms because many infectious diseases and viral outbreaks have been treated with plant based remedies throughout the history of mankind.

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