A secondary metabolite with antibacterial activity produced by mangrove foliar fungus *Schizophyllum commune*

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***Abstract*--**The full value of mangrove ecosystem is often not recognized. This may be attributed to various factors. Many of the products and services provided by these ecosystems are not traded on markets with an observable value. Some of these products and services are off-site and are therefore not readily acknowledged as being related to mangrove ecosystems. So, researchers believe that mangroves and its related fauna should be explored and developed for uses, which generate directly marketable products like drugs and neutraceuticals. Mangrove fungi associated or endophytic are important and indispensable components of biodiversity. In the present study, the mangrove species *Avicennia officinalis* was selected to study the foliar fungi and its capabilities of secreting secondary metabolites. The ethylacetate filtrate was checked for antibacterial activity. We isolated three secondary metabolites by TLC from *S. commune* that could produce antibacterial compounds. And the one in the first band with an Rf value of 0.63 was identified to be Phthalic Acid ($C_{12}H_{14}O_4$) by mass spectra (MS). This is the first report on Phthalic acid produced by mangrove foliar fungus *S. commune*.

Keywords- Marine Fungi, ITS, Antibacterial activity

I. INTRODUCTION

Mankind has been benefited from nature in many different ways than one. The discovery of penicillin has initiated a new strategy in industrial drug discovery from fungi, which continues to attract scientific, and public interest. From 1989-1995 over 60% of the approved drugs and pre- NDA (New Drug Application) candidates were of natural origin given the fact that chemical natural products are incredibly distinct from other medicines with fascinating biological activity [1]. Moreover, The secondary metabolites from natural sources are good candidates for drug development because being elaborated within the living systems, they are perceived to exhibit more similarities to drugs and show more biological friendliness than totally synthetic drugs [2]. In the last three years, most new natural products described in literature have been isolated from fungi[3].

The necessity for the discovery of new lead compounds has been strongly felt since the evolution of antibiotic resistant pathogens [4]. Marine microorganisms are an inexhaustible source of new pharmacological agents. In December 2004, a drug Zinconotide, from tropical marine cone snail under the name Priatl was approved in the United States to treat severe pain in patients who require lethal analgesia. This was also given a nod by the European commission. Trabectidin branded as Yondelis was approved by the Europian Union to treat soft tissue sarcoma [5].

The uses of mangroves dates back to the 17th century as the ecosystem encompass a vast number of fungal species with the potential to produce bioactive metabolites. Be it the extensive supporting roots of *Rhizophora* or the breathing roots of *Avicennia* or the salt excreting leaves or the viviparous water dispersed seedlings, everything harbours notable potential to produce bioactive metabolites for obvious reasons of survival and propagation through 'chemical signals' to defend 'environmental clues' [6].

The present study was thus initiated to investigate the foliar fungus of *A. officinalis* and check the activity of its secondary metabolites against bacteria that play havoc on human health and characterize the compound responsible for the activity.

II. METHODOLOGY

Isolation of foliar fungi

Fresh elder leaves from mangrove species *A.officinalis* from Pichavaram mangrove forest southeast coast of India were collected, 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

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the filtrate were separately subjected to solvent extraction as follows

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.

For day optimization the fungus was grown in the malt extract medium at pH 6.2. Inoculated flasks were incubated at 27 on an incubator shaker for 8 days. The biomass production was determined each day for antibacterial activity.

Antibacterial assay

Antibacterial activity was carried out against a panel of laboratory standard pathogenic strains such as *Vibrio cholerea, Micrococcus leteus* and *Staphylococcus aureus* by agar well diffusion method.. Extracts (filtrate) were pipetted on to wells with four different concentrations (25µl, 50µl, 75µl and 100µl) . The middle well was filled with the respective solvent. . Plates were incubated at 37°C for 24 h. Antibacterial activity was expressed as the diameter of the inhibition zone (mm) produced by the extracts. Media optimization was also done with PDB, CDB and MEB.

Thin layer chromatography

In order to identify the compounds, the extract was subjected to fractionation and purification of its components. Hence TLC was performed on aluminum sheets precoated with silica gel using capillary tube (2-5µl) and allowed the plates to dry. The plate was developed in hexane: ethyl acetate (9:1v/v) and visualized under UV illuminator. The Rf value was calculated as

Retention factor (R_f) = Distance traveled by solute (cm)/ Distance traveled by solvent (cm).

Identification of components:

The compounds separated by TLC was identified using gas chromatograph (Shimadzu QP2010) equipped with a VF-5 ms column (diameter 0.25mm, length 30.0m, film thigkness 0.25µm) mass spectrum (in source 200 c; EI -70 ev). Programmed at temperature 40-650ºC with a rate of 4ºC/min. Injector flow rate was 200ºC, Carrier gas was He 99.9995% purify. Column flow rate 1.51ml/min, injection mode split, injection volume/ml.

Interpretation on mass spectrum of GC-MS was done using the database of CAS. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the CAS library. The name, molecular weight and structure of the components of the test material was ascertained.

Fungal isolation, identification

The total deoxyribonucleic acid (DNA) of mangrove fungus was extracted using the EZNA kit (Omega). 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.

III.RESULTS AND DISCUSSION

The mangrove fungus was isolated from mangrove leaves and identified as *Schizophyllum commune* (Fig.1) by ITS sequencing using ITS1 and ITS4 primer and submitted to gen bank with the accession no HQ271349. 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). The ITS region was sequenced using the primer ITS 1 and ITS 4. 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 by using the neighbor-joining algorithms from the PHYLIP package version 3.5c. The percentage of similarity between the fungus and database suggests it to be a novel strain.

Fig 1. *S.commune*

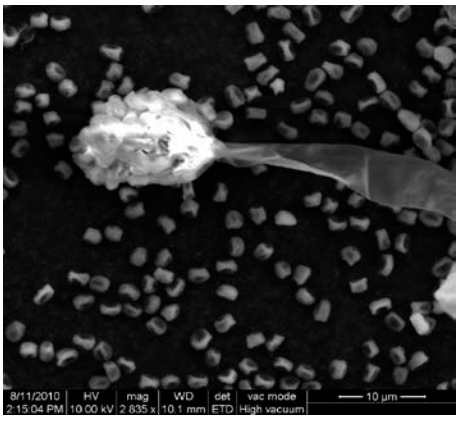

Fig. 1 Scanning electron Microscopic view

All the compounds could inhibit the growth of pathogenic bacteria such as *Vibrio cholerea, Micrococcus leteus* and *Staphylococcus aureus* with Phthalic acid showing stronger inhibition. Media optimization results show that an

appreciable inhibition in the range of 29mm – 36mm against *vibrio cholerea* was observed in Malt Extract Broth than in Potato Dextrose Broth and CDB as shown in Table 1. So, MEB proved suitable for the stability of the bioactive metabolite.

Table1: ANTIBACTERIAL ACTIVITY FOR MEDIA OPTIMISATION

Media	Concentration (µl)	Zone of inhibition in mm		
		Vibrio cholerea	*Micrococcus leteus*	*Staphylococcus aureus*
PDB	25	17	16	10
	50	21	17	17
	75	25	18	17
	100	26	20	18
CDB	25	-	15	-
	50	-	15	16
	75	-	15	15
	100	-	16	16
MEB	25	29	19	-
	50	30	23	18
	75	35	25	21
	100	36	23	-

TABLE 2: ANTIBACTERIAL ACTIVITY FOR DAY OPTIMISATION

Organisms	Conc. (µl)	Zone of inhibition in mm for different days						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Vibrio cholerea	25	17	26	23	20	24	0	31
	50	19	32	25	26	27	26	30
	75	20	28	27	26	27	26	32
	100	23	33	28	27	27	27	34
Micrococcus leteus	25	19	19	22	25	19	25	32
	50	20	22	24	31	30	31	34
	75	26	24	25	33	29	33	37
	100	21	27	26	34	33	34	38
Staphylococcus aureus	25	25	19	23	32	18	32	32
	50	26	21	24	29	21	29	34
	75	25	23	21	33	21	33	32
	100	26	27	22	34	20	34	36

The maximum secretion of metabolites from the strain was found at the 7th day of incubation as is obviously shown in Table 2 which help us conclude that this fungal strain produced metabolites with the strongest bactericide properties in relatively short culture period. The extract inhibited the growth of the test organisms in the range of 31mm – 38mm. And the inhibition was directly proportional to the concentration of the extract. With 100µl, inhibition was 34mm for *Vibrio cholerea*, 38mm for *Micrococcus leteu*s and 36mm for *Staphylococcus aureus*.

The crude extract was further purified using TLC, The Rf value was calculated as 0.63 (Fig 2). The Chemical characteristics of active fraction were determined based on the GC MS (Fig.3 and Fig 4) spectral data as phthalic acid with the molecular formula $C_{12}H_{14}O_4$ and molecular weight 222.

Fig 2 TLC of purified band

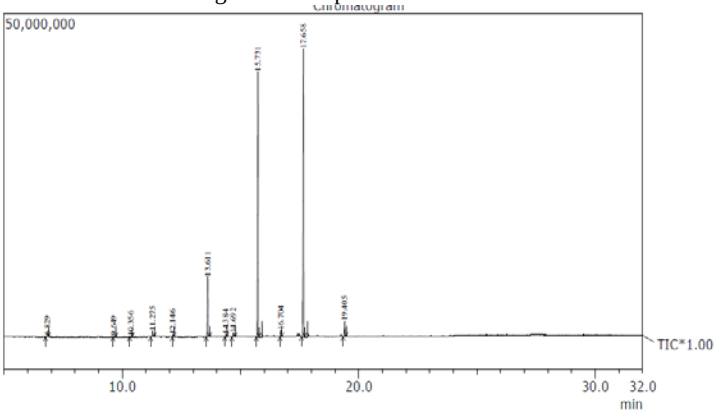

Fig 3 GC spectrum of the isolated compound

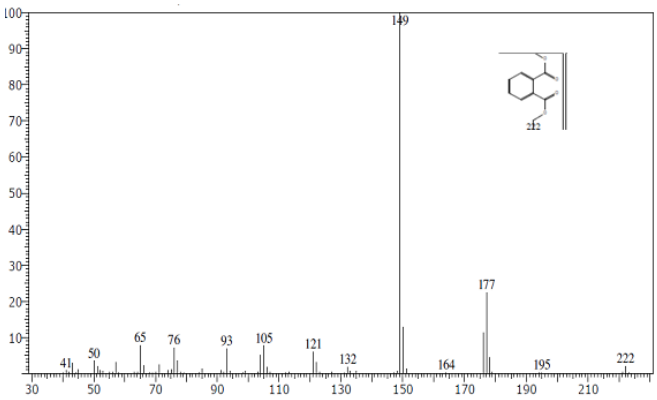

Fig 4: M S Spectrum of Phthalic Acid ($C_{12}H_{14}O_4$; Mol Weight 222)

Pharmaceutical microbiology screening programs have shown that secondary metabolites can be isolated which bind to active sites of enzyme organelles and receptors. Phthalates are reported to have antimicrobial and other pharamacological activities. Bis (ethyl hexyl) phthalate reported from

Streptomyces bangladeshiensis show antimicrobial activity against gram positive bacteria and some pathogenic fungi [7]. First occurrence of bis-(-ethylhexyl) phthalate from *Streptomyces bangladeshiensis* and naturallyoccurring dioctylphthalate showed antimicrobial activity against Gram positive bacteria were reported very recently. The presence of various bioactive compounds (identified as phthalate esters, phthalate, alkanes, esters, alcohols, sugar, sesquiterpenoids) justifies the use of the whole plant for various ailments by traditional practitioners [8]. The extract of *Gongronema latifolium* decne contains Phthalic acid, monoterpenes, and several compounds to be responsible for the activity against bacterial isolates from HIV infected patients [9]. 1, 2-Benzenedicarboxylic acid bis(2-ethylhexyl) phthalate has been isolated from a marine alga, *Sargassum weightii*, and apart from its plasticizing ability it was also found to have antibacterial effect on a number of bacteria [10] . However, the natural occurrence of phthalates in a wide variety of plants is already in the literature [11-13].

Through this study it was found that isolation of individual constituents and subjecting it to biological testing will definitely give fruitful results. Thus the objective was to identify the antibacterial compound from Schizophyllum commune by GC-MS analysis.

IV. Conclusion

In conclusion antibacterial activity was tested using solvent ethyl acetate. The isolated fungus showed maximum activity in 100ul concentration against *Micrococcus leuteus*, *Vibrio cholerae* and *Staphylococcus aureus*. Similar studies would help prompt the pharmaceutical companies to look upon fungi as prolific resources of secondary metabolites which can be used as drugs to combat human diseases.

References

[1] Baker, D.D.; Chu, M.; Oza, U.; Rajgarhia, V, The value of natural products to future pharmaceutical discovery, *Nat. Prod. Rep*., 24, (2007) 1225-1244.

[2] Shoeb M, Anticancer agents from medicinal plants. Bangladesh J Pharmacol 1 (2006) 35-41.

[3] Saleem, M.; Ali , M.S.; Hussain, S.;Jabbar, A.; Ashraf, M.; Lee, Y.S., Marine natural products of fungal origin. Nat. Prod. Rep., 24 (5) (2007) 1142- 52.

[4] Saleem, M.; Nazir, M.; Ali, M.S.; Hussain, H.; Lee, Y. S.; Riaz, N.; Jabbar, A., Antimicrobial natural products: An update on future antibiotic drug candidates. *Nat. Prod. Rep*. 27 (2010) 238-254.

[5] Mayer, A.M.S.; Glaser, K.B.; Cuevas C.; Jacobs, R.S.; Kem, W.; Little, R.D.; McIntosh, J.M.; Newman, D.J.; Potts, B.C.; Shuster, D.E., The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends in Pharm. Sci*., 31 (2010) 225-265.

[6] Elsa Lycias Joel.; Valentin Bhimba., Isolation and characterization of secondary metabolites from the mangrove plant *Rhizophora mucronata. Asian Pacific Journal of Tropical Medicine.* 3(8) *(*2010) 602-604.

[7] Al-Bari, M AA.; Sayeed, M A.; Rahman, M S.; Mossadik, M A, Characterization and antimicrobial activities of a phthalic acid derivative produced by *Streptomyces bangladeshiensis*- A novel species in Bangladesh. *Res J Med* and *Medical Sci*, 1 (2006) 77-81.

[8] M. Abdul Alim AL-Bari.; Mohammed Abu Sayeed.; M. Sazedur Rahman.; M. Ashik Mossadik, . Characterization and Antimicrobial Activities of a Phthalic Acid Derivative Produced by *Streptomyces bangladeshiensis*, A Novel Species Collected in Bangladesh. Research Journal of Medicine and Medical Sciences, 1(2) (2006) 77-81.

[9] Adeleye, I A.; Omadime, ME and Daniels F V, Antimicrobial activity of essential oils and extracts of *Gongronema latifolium* Decne on bacterial isolates from blood stream of HIV infected patients. Journal of Pharmacology and Toxicology, 3 (2011) 312-320.

[10] Sastry, V M V S and Rao G R K, Dioctyl phthalate and antibacterial compound from the marine brown alga Sargassum wightii. *Journal of Applied Physiology;* 7 (1995*)* 185-186.

[11] Perkins, E G. Characterization of the non-volatile compounds formed during the thermal oxidation of corn oil. II. Phthalate esters. Journal of the American Oil Chemists' Society, 44(3): (1967)197-199.

[12] Graham, P R. Phthalates ester plasticizers – Why and how they are used. *Environmental Health Perspectives*, 3 (1973) 3-12.

[13] Duc, N.; Dung, N.; Lyun, H L.; Hyang-Bok, L.; Dongman, K.; Junghyun, S and Eunki, K, Isolation of dioctyl phthalate with high depigmenting effect from Chinese herb *Nigella glandulifera Freyn*. *Journal of Biotechnology*, 131(2) (*2007)*S43.