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PHTHALATE DERIVATIVES FROM THE MARINE FUNGI *PHOMA HERBARUM* VB7

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ABSTRACT

This paper reports the antibacterial activity of marine derived fungi *Phoma herbarum*. The organism was isolated from mangrove leaves and identified by ITS sequencing. The fungal broth was prepared using PDB (potato dextrose broth) and extracted using ethyl acetate. It was then used for evaluating antibacterial activity. Compound characterization was done using TLC and GCMS spectrum. Fungi grown in Malt extract medium produced more metabolite. Phalate derivates were identified from GC MS spectrum. Based on the present study, it can be inferred that the bioassay guided fractionation and purification of *P. herbarum* may come up with potent bioactive drug.

Keywords:- Marine fungi, Antibacterial activity, *Phoma herbarum*.

INTRODUCTION

In recent years marine microorganisms have become important in the study of novel microbial products exhibiting antimicrobial (Mardernosian, 1969; Molinski, 1993), antiviral, antitumour as well as anticoagulant and cardiovascular properties. These active compounds may serve as model systems in the discovery of new drugs.

The natural environment is still the most important supply of novel drugs despite development of combinatorial chemistry, which quickly generated thousands of new chemicals (Zhang *et al*., 2010). The terrestrial environment has been mined for drugs for many years with great success. Now humans have recognized that the oceans are a rich source of natural products with potential drugs (Cooper, 2004; Incalci *et al*., 2004; Simmons *et al*., 2005). Many promising compounds of new and complicated structure types have been isolated from oceans and some have been identified as leading preclinical compounds. Interestingly, marine microalgae, cyanobacteria and heterotrophic bacteria living in association with invertebrates have been identified or strongly suspected as the true sources of many bioactive and useful constituents (Molinski *et al*., 2009; Gordaliza, 2007). Marine derived fungi have been rich sources of structurally novel and biologically active secondary metabolites which have been attractive as important sources for new chemicals in drug discovery (Li *et al*., 2009; Xia *et al*., 2007). As part of our ongoing investigations directed towards the search of biologically active natural products from marine endophytic fungi, we have studied and reported the isolation and identification of phthalate derivates by GCMS analysis.

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MATERIALS AND METHODS

Isolation of endophytic fungi

The leaves of the collected mangrove plants were washed with sterile sea water and 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. The plates were then incubated at 27°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

The pure culture isolated by the above method was grown in sabouraud's dextrose broth. The flasks were incubated in the shaker – incubator at 200rpm for 5 days. Then the mycelium and 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 ethyl acetate 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 *Vibreo cholera, Staphylococcus aureus, Salmonella typhi and Micrococcus leuteus* by agar well diffusion method. Antibacterial activity was determined using the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS)[11]. 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.

THIN LAYER CHROMATOGRAPHY

TLC was used to monitor the identity of each extracts and fractions, additionally to screen the qualitative purity of the isolated compound. It was also developed to optimize the solvent system that would be applied for column chromatography.

Analytical TLC was performed on precoated TLC plates with Si gel 60 F_{254} (0.2 mm, Merck) and RP-2 (0.2mm, Merck) using diverse solvent systems for mostly semi-polar compounds. However, solvent system containing ethyl acetate:Hexane(1:9, v/v) was mostly used, unless otherwise stated. The compound was then detected by their UV adsorbance at wavelength 254 and 366nm.

$$\text{Retention factor } (R_{f)} = \frac{\text{Distance travelled by Solute (cm)}}{\text{Distance travelled by Solvent (cm)}}$$

GC-MS Analysis

The isolated band from TLC 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 total deoxyribonucleic acid (DNA) of marine-derived fungus GIBH-Mf082 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.

RESULT AND DISCUSSION

In the present study marine fungi was isolated from mangrove plant leaves and identified as *Phoma herbarum* (Fig.1) by ITS sequencing using ITS1 and ITS4 primer and submitted to gen bank with the accession no JQ754707. 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.

The aim of this experiment was to select the most favorable medium. The fungal isolate was inoculated into 3 different broth ie malt extract broth, potato dextrose broth and czapex dox broth. Following incubation, culture filtrate was concentrated under vacuum to reduce the volume. This concentrated filtrate was extracted using ethylacetate. The solvent was next evaporated form the resulting mixture with the aid of vacuum rotary evaporator, leaving only the dry crude extract behind. This crude extract was screened for antibacterial activity. From the result (Table 1) it is clear that Malt extract broth gave maximum zone of inhibition based on antibacterial activity studies. This inhibitory effect can be noticed to use mangrove fungal extracts as a new generation of

antimicrobial agents. These results complied with mostly reported papers on bioactive compounds isolated from culture broth in several reviews (Debbab *et al*., 2010; Ebel *et al*., 2010; Valentin *et al*., 2010, 2011, 2012.

Table 1. Antibacterial activity of crude metabolite for media optimization

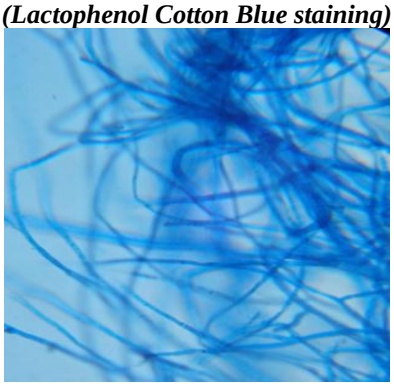
Media	Concentration (µl)	Zone of inhibition in mm			
		Vibrio cholerea	*Micrococcus leuteus*	*Salmonella thyphi*	*Staphylococcus aureus*
Malt Extract Broth	25	18	20	20	20
	50	22	20	18	21
	75	27	24	21	26
	100	27	23	20	26
Czapek-Dox Media	25	11	13	14	15
	50	13	14	15	22
	75	12	16	21	25
	100	11	15	19	22
Potato Dextrose Broth	25	18	15	15	14
	50	22	16	14	26
	75	23	21	18	26
	100	25	21	24	23

Table 2. Antibacterial activity of crude metabolite for day optimization

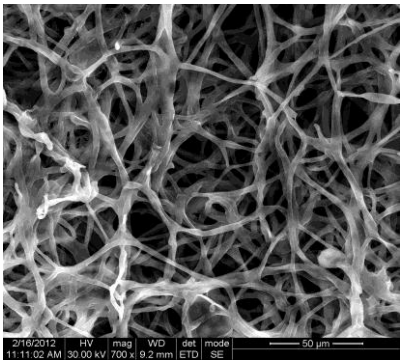
Organism	Concentration (µl)	Zone of inhibition in mm for different days						
		1	*2*	*3*	*4*	*5*	*6*	*7*
Vibrio Cholera	25	19	14	22	16	14	18	18
	50	17	16	22	19	12	20	18
	75	19	17	23	18	14	22	22
	100	18	16	19	17	16	22	22
Micrococcus leuteus	25	18	14	19	15	18	15	25
	50	24	16	19	17	20	15	24
	75	19	16	20	16	20	17	26
	100	20	16	21	17	21	18	27
Salmonella typhi	25	16	15	11	19	13	16	14
	50	16	16	13	22	14	20	15
	75	18	18	14	21	16	18	13
	100	19	17	16	24	14	18	13
Staphylococcus aureus	25	20	16	15	11	11	13	16
	50	18	19	17	12	13	11	13
	75	18	20	18	16	14	18	21
	100	19	20	17	16	12	16	16

Fig. 1. *Phoma herbarum*

Microscopic view (40x) *(Lactophenol Cotton Blue staining)*



Scanning electron microscope *view*



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. The result shows (Table. 2) shows that the fungi production is more on the sixth day. These results agreed with the previous studies (Elsa and Valentin, 2010, 2011; Valentin *et al*., 2012).

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 0.26 (Fig.2). The Chemical characteristics of active fraction was determined based on the GC MS (Fig.3) spectral data as Dibutyl phthalate (Fig.4) and Mono (2ethylhexyl) phthalate (Fig.5) with the molecular formula $C_{16}H_{22}O_4$; molecular weight 278.

Fig. 2. TLC of purified band

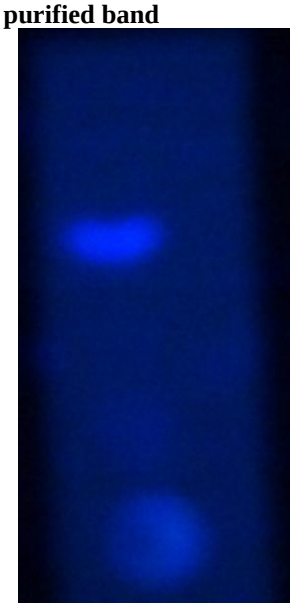
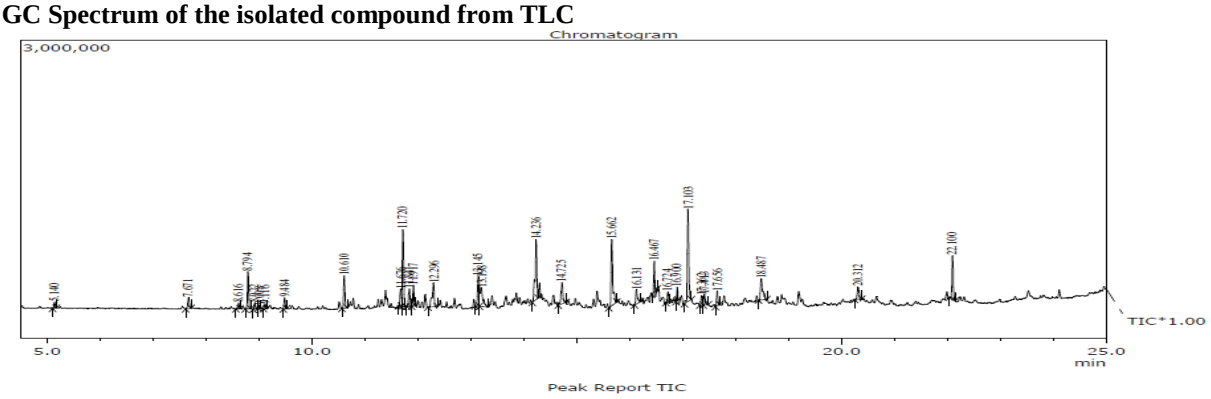


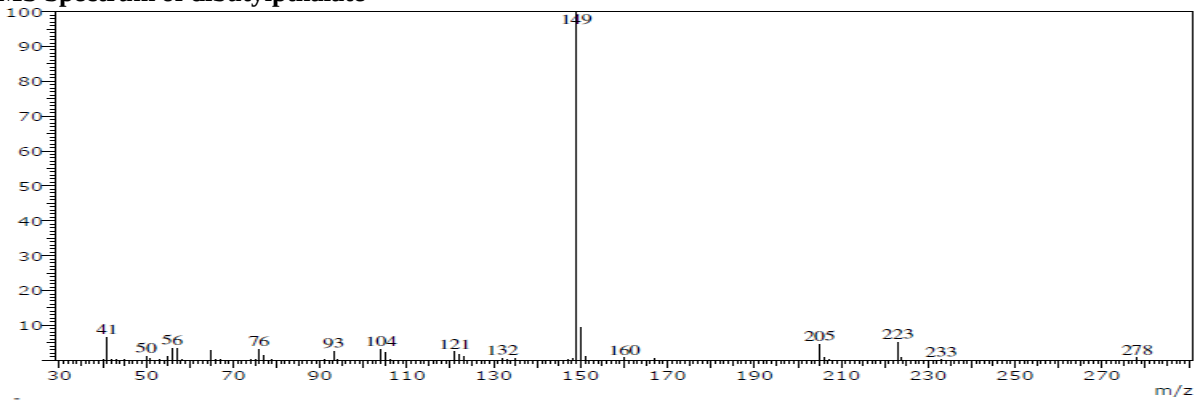
Fig 3. GC Spectrum of the isolated compound from TLC



Peak Report TIC

RT	Area	Area%	Name
17.103	1426331	10.28	Dibutyl phthalate
22.100	7910337	5.01	Mono (2ethylhexyl) phthalate

Fig. 4 MS Spectrum of dibutylpthalate



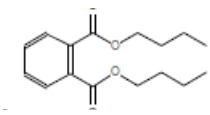
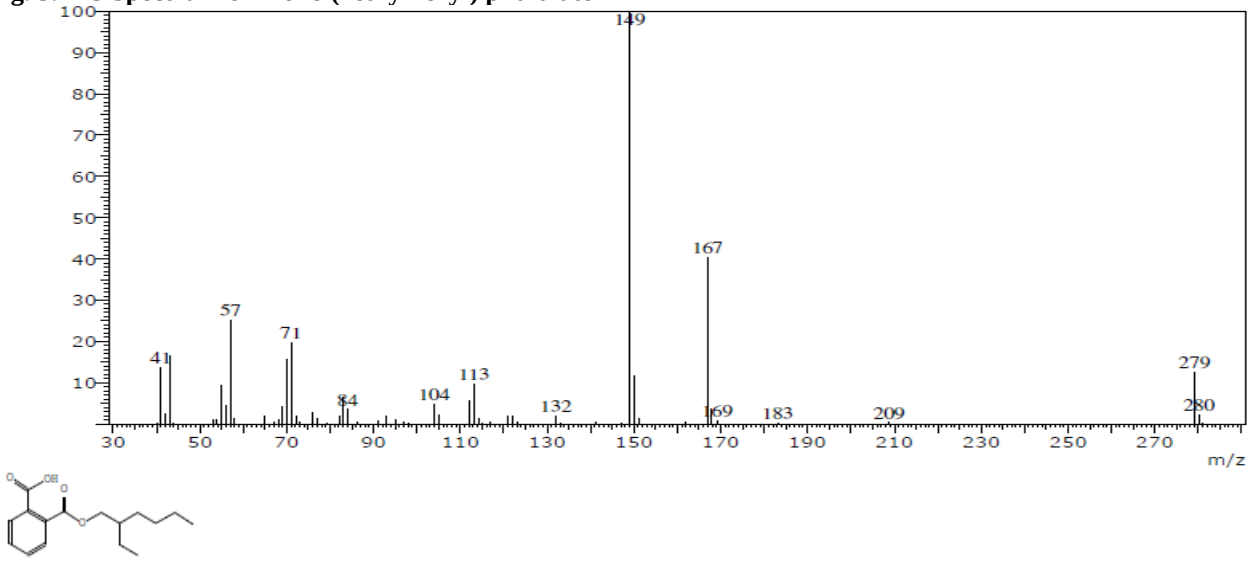


Fig. 5. MS Spectrum of Mono (2ethylhexyl) phthalate



Mangrove fungi are known as sources of new bioactive compounds (Lin et al., 2001) However, investigations on mangrove endophytic fungi metabolisms are scarce. Discovery of fungi in plant tissues opened up new possibilities in the search for metabolically active compounds. Antimicrobial properties of mangrove fungi are being increasingly reported from various parts of the world. The fungal genera *Acreomonum, Aspergillus, Fusarium and Penicillium* as 'creative species' based on the production of several bioactive metabolites. Rodrigues *et al*., 2000) considered it as another 'creative species'. Similarly, several metabolites of the marine isolate, *Aspergillus niger* showed antibacterial and antifungal potential. Novel microbial sources of Podophyllotoxin were reported from the fungi *Aspergillus fumigatus* Fresenius isolated from *Juniperus communis* L. Horstmann, *Phialocephala fortinii* isolated from *Podophyllum peltatum* and *Fusarium oxysporum* from *Juniperus recurva* Medicinal properties of different parts of mangrove plants may be fully or partially dependent on the endophytic fungi (Bandaranayak, 1998).

In conclusion, Antibacterial activity was tested using solvent ethyl acetate. The isolated fungi give maximum activity in 100ul concentration against *Micrococcus leuteus* and *Vibreo cholerae*. Thus secondary metabolites of the marine derived fungi phoma herbarum have a promising potential of antibacterial activity to be included in drug discovery.

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