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RESEARCH ARTICLE

ANTAGONISTIC ASSAY OF SECONDARY METABOLITES OF MANGROVE ASSOCIATED FUNGI AGAINST FISH AND HUMAN PATHOGENS

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ABSTRACT

The mangrove endophytic fungi producing secondary metabolites were checked for the inhibitory effect against the marine fish and human pathogens. Two fungal strains were isolated in Sabouroud's Dextrose Agar and mass culture was obtained in the broth. The secondary metabolites were extracted by ethyl acetate solvent. The fungal strains were identified as *Hypocrea lixii* and *Meyerozyma guilliermondii* based on the ITS region sequence. The ethyl acetate extract showed maximum antagonistic activity in all the seven fish and human bacterial pathogens. This first step assessment of the secondary metabolites from the endophytic fungi showed their antagonistic potential to investigate further for an effective drug for fish and human pathogens.

Key words – Mangrove fungi, Antagonistic assay, Zone of inhibition, Fish and human pathogen

INTRODUCTION

Marine micro-organisms, particularly fungi, have been utilized as a source of novel bioactive secondary metabolites [1]. Several studies on fungi associated with mangrove detritus have been published elsewhere [2]. But, there are a few studies on the endophytic fungi of mangrove plants and most of them are confined to foliar endophytes [3]. Some studies showed that roots of mangrove plants are a rich source of fungal endophytes [4]. Mostly the secondary metabolites are produced as a part of defense mechanisms against the predators or competitors. After 1990's, about 272 new secondary metabolites were isolated from marine-derived fungi which led to the discovery of novel carbon skeletons providing, these fungi are a potential source of pharmaceutical leads [5]. Oh *et al*. [6] isolated diphenyl ethers, the tenellic acids A–E from the fungus *Dendrospora tenella* which showed activity against Gram-positive bacteria. Shao *et al.* [7] isolated new isoprenyl phenyl ether, 3-hydroxy-4-(3-methylbut-2-enoloxy) benzoic acid methyl ester from a mangrove fungus which exhibited anti-bacterial (*Staphylococcus aureus* and *Escherichia coli*), anti-fungal (*Fusarium oxysporum*) and anti-cancer activities. Qi *et al.* [8] isolated fungus *Cladosporium* sp. from waters surrounding the mangroves which exhibited anti-bacterial activity against larval settlement - inducing bacteria. Tao *et al.* [9] isolated and identified 86 compounds from metabolites of mangrove fungi in South China Sea which showed better anti-cancer activity by MTT assay.

The aim of the present study is to isolate and screen the mangrove associated fungi, producing secondary metabolites and check the inhibitory effects against fish and human pathogenic bacteria

MATERIALS AND METHODS

Isolation of endophytic fungi from mangrove leaves: Healthy *Avicennia* sp. leaves were collected from Vellar estuary (Lat.11˚29' N; Long.79˚46'E) and washed with sterile sea water and ground using mortar and pestle with 50% of seawater under sterile conditions. One ml of the above sample was mixed with 10ml of 50% of seawater to get dilution 10^{-1}. The serial dilution was made upto 10^{-6} under sterile conditions. From each dilution, plating was done on Sabouraud's dextrose agar by spread plate technique and incubated at 27˚ C for 5 days. After 5 days, the plates were examined and the pure cultures were isolated on sterile agar plates.

Identification of endophytic fungi: Two dominant fungi were sub-cultured in Sabouraud's dextrose agar and incubated at 27°C for 3 days. After incubation, the grown mycelium was taken with sterile scalpel and placed on clean grease-free glass slide and stained with Lactophenol Cotton Blue stain and covered with a cover slip. The prepared slide was observed under light microscope. For molecular identification the total deoxyribonucleic acid (DNA) of the fungi were extracted using the EZNA kit (Omega). The internal transcribed spacers (ITS) of ribosomal DNA (rDNA) were amplified by employing the primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTA TTGATATGC-3'). The polymerase chain reaction product was sequenced in a sequencing facility firm.

Mass culture and extraction of secondary metabolite:A seed culture of the fungal strains were prepared by inoculation of 50 ml Sabaroud's dextrose broth in a shaker (30ºC/150 rpm) for 18 h. Inoculum was transferred to 1L Erlenmeyer flask containing 500 ml Sabaroud's dextrose broth and cultured with shaking (150 rpm) for 6-8 days at room temperature. After 6 days of incubation, the medium was filtered using a 0.45µ filter paper. The filtered broths were treated with Tween 80 for destruction of spores and were filtered again. The filtrates were divided in 4×250 ml and poured in 1000 ml separating funnels and added equal volume of ethyl acetate. The organic layer was collected in separate beakers and kept in hot air oven at 40° C, until all the liquid phase in the beakers got evaporated leaving behind the crude extract. The compound from each beaker was scraped by a scalpel, weighed and collected in eppendorf tubes. These compunds were used as stock for further studies.

Terpenoid detection assay: Five ml of each extract was taken in test tubes and 2 ml of chloroform was added. This mixture was shaken gently. Concentrated H_2SO_4 (3 ml) was carefully added by the walls of the test tubes to form a layer. A reddish brown coloration of the inter face was formed to show positive results for the presence of terpenoids.

Brine shrimp toxicity assay : Forty eight hours post hatch nauplii of *Artemia salina* was used in this study. The concentrations of the tested samples were 31.25, 62.50, 125, 250, 500µg/ml. An aliquot (100 µl) of each concentration was transferred into the wells of a 96-well microtitre plate. Control wells were set up using sea water (100 µl). A suspension of nauplii containing 15 organisms (100 µl) was added into each well and the plate was covered and incubated at room temperature (26-30ºC) for 24 hours under a light source. Triplicate setup was maintained during the experiment. After incubation, the plate was examined under light microscope (100x) and the number of dead (non-mobile) nauplii in each well was counted.

Antibacterial assay for human and fish pathogens: Antimicrobial activity of crude metabolites of the two fungi was assayed by the disc diffusion method [10]. Human pathogens such as *Escherichia coli, Bacillus subtilis, Klebsiella aerogenes, Shigella flexenerii, Enterococcus faecalis, Vibrio cholerae, Shigella boydii* and fish pathogens such as *Bacillus firmus, Bacillus flexus, Bacillus vietnamensis, Bacillus cereus, Halomonas sp., Staphylococcus sp.* and *Bacillus sp.* were tested. 50µl of the metabolite extract was impregnated on each disc and then the dried discs were placed on the agar plates containing the pathogens. For positive control Ampiciline (25µg) was loaded in the discs in fish pathogen plates and Tetracycline (25µg) in human pathogen plates. For negative control 50µl sterile distilled water was used on the discs. The plates were incubated at 37°C for 24 hrs. After incubation, the plates were examined for zone of clearance around the individual discs. The area of zone of inhibition calculated and compared with positive control. Triplicate antimicrobial assay was carried out for this experiment.

FTIR analysis : Fourier Transform Infra-Red Spectroscopy analysis was performed for both the metabolite samples using Thermo Nicolet (USA). The diffuse reflectance technique was utilized in the mid-IR (400–4000 cm^{-1}) spectral region. The samples were mixed with KBr (about 200–400 mg) into a fine powder, placing the powder into the sampling cup, smoothing the powder, and compressing the powder bed into the holder using a compression gauge. The sample was placed in to light path and the spectrum was obtained by using ORIGIN (version 8.0).

Table 1: Brine shrimp toxicity assay of crude extracts

Crude extract concentration (µg/ml)	% of mortality in *Hypocrea lixii* (± SD)	% of mortality in *Meyerozyma guilliermondii* (± SD)	% of mortality in control
31.25	10 ± 2	0 ± 0	0
62.50	10 ± 3	10 ± 3	0
125	20 ± 5	40 ± 5	0
250	50 ± 5	60 ± 7	0
500	80 ± 7	70 ± 9	0

Table 2: Antibacterial activity of crude extracts against fish pathogens

	Diameter of zone of inhibition (mm ± SD)		
Fish pathogens	*Hypocrea lixii*	*Meyerozyma guilliermondii*	Positive control (Ampiciline, 25µg)
Bacillus firmus	25 ± 3	24 ± 3	24 ± 2
Bacillus flexus	16 ± 2	12 ± 2	15 ± 2
Bacillus vietnamensis	21 ± 3	23 ± 3	25 ± 3
Bacillus cereus	14 ± 2	14 ± 2	20 ± 2
Bacillus sp.	20 ± 4	16 ± 2	19 ± 3
Staphylococcus sp.	22 ± 3	20 ± 3	16 ± 2
Halomonas sp.	15 ± 2	14 ± 3	17 ± 2

Table 3: Antibacterial activity of crude extracts against human pathogens

	Diameter of zone of inhibition (mm ± SD)		
Human pathogens	*Hypocrea lixii*	*Meyerozyma guilliermondii*	Positive control (Tetracycline, 25µg)
Esherichia coli	13 ± 2	13 ± 2	24 ± 3
Bacillus subtilis	9 ± 2	8 ± 2	20 ± 2
Klebsiella aerogenes	10 ± 3	12 ± 2	25 ± 2
Shigella flexenerii	14 ± 3	11 ± 3	20 ± 3
Enterococcus faecalis	15 ± 2	9 ± 2	19 ± 2
Vibrio cholerae	12 ± 2	11 ± 3	21 ± 2
Shigella boydii	15 ± 2	14 ± 3	23 ± 2

RESULTS

In the present investigation, two fungal strains were isolated from the mangrove, *Avicennia sp* leaves and identified as *Hypocrea lixii* and *Meyerozyma guilliermondii* by ITS region sequence. The sequence obtained was subjected to BLAST and compared with the GenBank database. The sequence information was submitted to GenBank and got accession numbers (GU815343, JF730118). Both the fungi were stained with Lactophenol cotton blue and observed under light microscope at 100x (Fig.1).

Terpenoid detection assay in the crude extract results were positive. The end result appeared as a brown ring at the interface of two layers. This assay confirmed the presence of terpenoids in the crude metabolite of these two fungi (Fig. 2).

In *Artemia* toxicity assay, after 24 hr incubation, the number of each dead *Artemia* was counted under light microscope. Crude extracts of *Hypocrea lixii* and *Meyerozyma guilliermondii* showed minimum numbers of *Artemia* mortality in various concentrations. Lowest concentration of 31.25µg/ml gives very less mortality and the highest concentration of 500 µg/ml shows significantly higher lethal activities respectively (Table. 1).

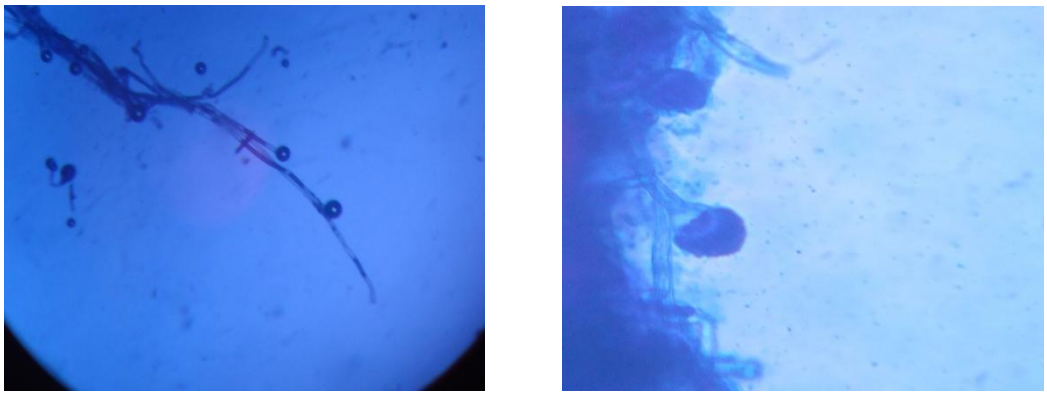
Antibacterial activity of crude extracts revealed that, significant activity was observed against the fish pathogens like *Bacillus firmus, Bacillus vietnamensis* and the remaining pathogens were also showed positive results (Table 2). Out of seven pathogens, six are represented in Fig. 3. *Hypocrea lixii* extract showed highest activity against the human pathogen such as *Shigella flexenerii,* while *Meyerozyma guilliermondii* extract was most active against *Shigella boydii.* The pathogen, *Bacillus subtilis* showed very low of zone of inhibition for both the extracts (Table 3). Out of seven pathogens, four are represented in Fig. 3. After analysis of crude compounds through FTIR, various peaks were obtained which were compared to available database.

DISCUSSION

For this study, two fungi were isolated from mangrove leaves (*Aviccenia sp*) and identified as *Hypocrea lixii* and *Meyerozyma guilliermondii*. Similar study was made by Maria *et al*. [11] by isolating 14 sp. of various fungi from various parts of plants like roots, stem, leaves, petioles etc. The crude metabolites were extracted from the potato dextrose broth using equal volume of ethyl acetate. Lu *et al*. [12] and Sun *et al*. [13] have used ethyl acetate as solvent for extraction, while Meenupriya and Thangaraj

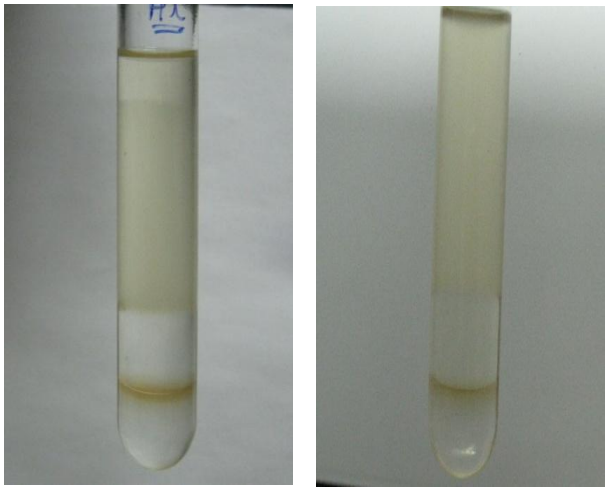
[14] used a variety of solvents of different polarities such as hexane, ethyl acetate and methanol.

In the present study, all the crude extracts exhibited promising inhibition against the fish and human pathogens. Earlier study [11] had used human pathogens as test organisms and only 8 out of 14 isolates exhibited the inhibition activity which was more than the present study. Thus it is concluded that the crude extracts have more potential against fish pathogens when compared with human pathogens. Meenupriya and Thangaraj [14] , isolated some marine derived fungi which exhibited higher activity against human pathogens like *E.coli* (42 mm) and *Staphylococcus* sp. (27 mm) as compared to the present study, there less inhibition was observed in *E. coli* (13 mm) and *Staphylococcus* sp. (21 mm). The brine shrimp lethality test was carried out for testing the potential of crude metabolites as described in earlier reports [15,16]. Positive results were obtained with 250µg/ml being the minimum concentration for the death of half of the organisms. The FT-IR peaks suggested that functional groups such as amide, peptide, siloxane, cellulose, carboxylic, phosphate, esters have the peaks at 626.87 cm^{-1}, 661.58 cm^{-1}, 1112.93 cm^{-1} etc [17], which is active in exhibiting the anti-bacterial properties in both the extracts. Meenupriya and Thangaraj [14], also studied FT-IR analysis of secondary metabolites from marine derived fungi, where they got peaks at 2522 cm^{-1}, 2227 cm^{-1} etc., which represent amine groups. While, 1660 cm^{-1} peak represents C=O stretch and 1550 represents N-H bend of amide group.



(a) **(b)**

Fig.1 Microscopic view of the fungi, (a) *Hypocrea lixii* and (b) *Meyerozyma guilliermondii* after staining with Lactophenol Cotton Blue (100x).



(a) **(b)**

Fig.2 Terpenoid detection assay shows the brown ring at the interface of two layers in (a) *Hypocrea lixii,* (b) *Meyerozyma guilliermondii* extracts.

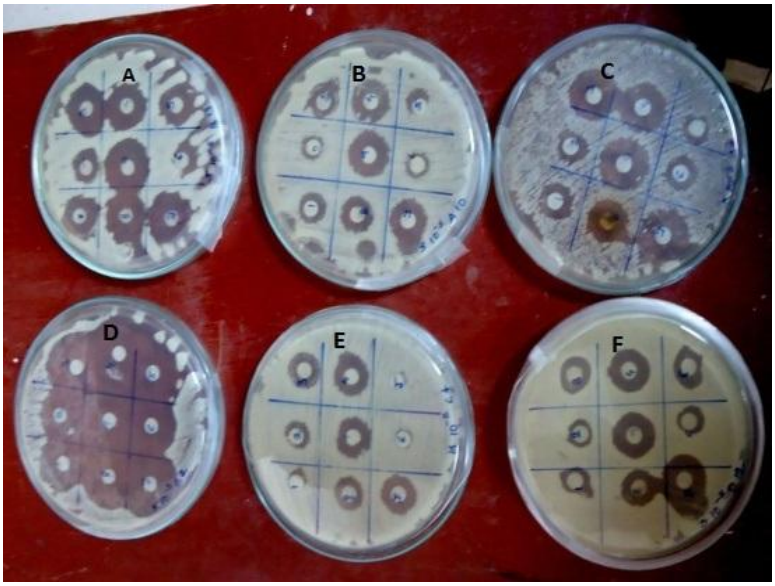


Fig.3 Activity of crude extracts of fungal strains against fish pathogens (out of seven pathogens, six are represented here). (A) *Bacillus vietnamensis* (B) *Bacillus cereus* (C) *Halomonas* sp. (D) *Bacillus firmus* (E) *Bacillus cereus* (F) *Staphylococcus* sp. First row *Hypocrea lixii* extract, Second row *Meyerozyma guilliermondii* extract and Third row Ampiciline.

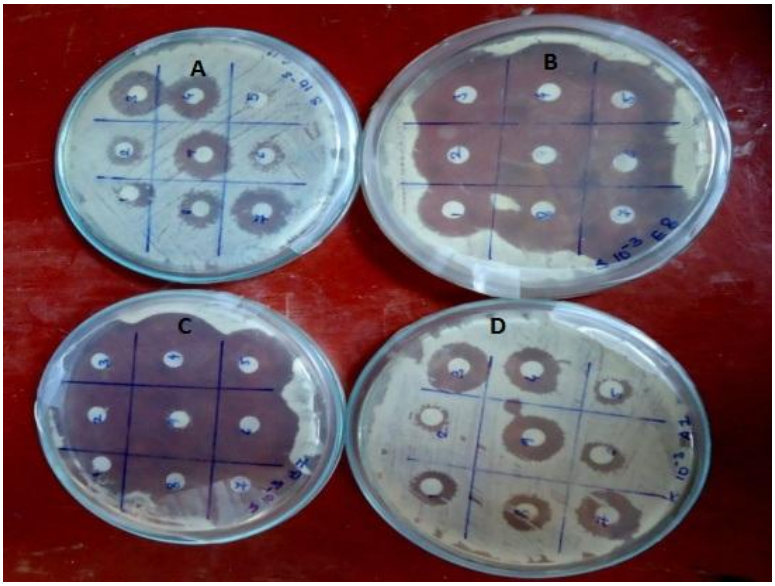


Fig.4 Activity of crude extracts of fungal strains against human pathogens (out of seven pathogens, four are represented here). (A) *Bacillus subtilis* (B) *Esherichia coli* (C) *Shigella flexenerii* (D) *Vibrio cholera.* First row *Hypocrea lixii* extract, Second row *Meyerozyma guilliermondii* extract and Third row Tetracycline.

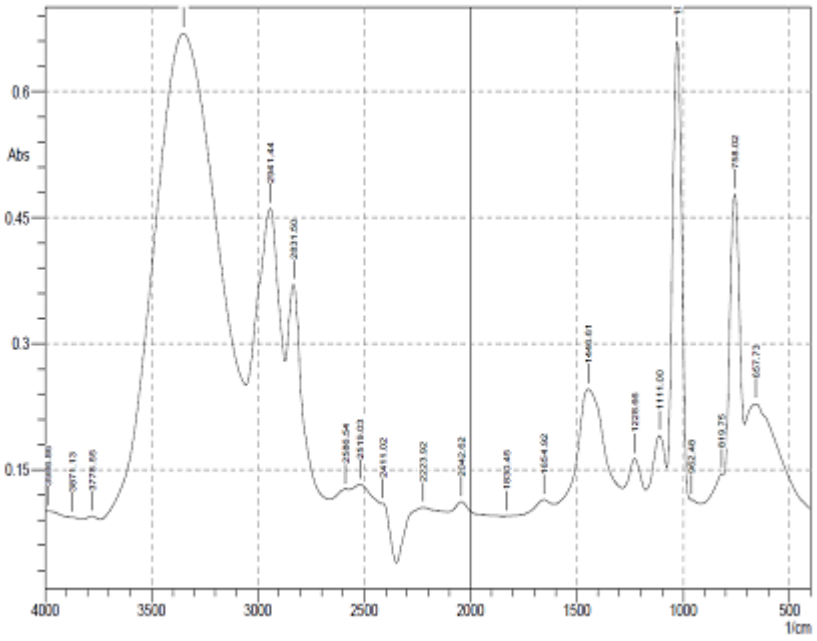


Fig.5 FT-IR Spectrum of *Hypocrea lixii* extract

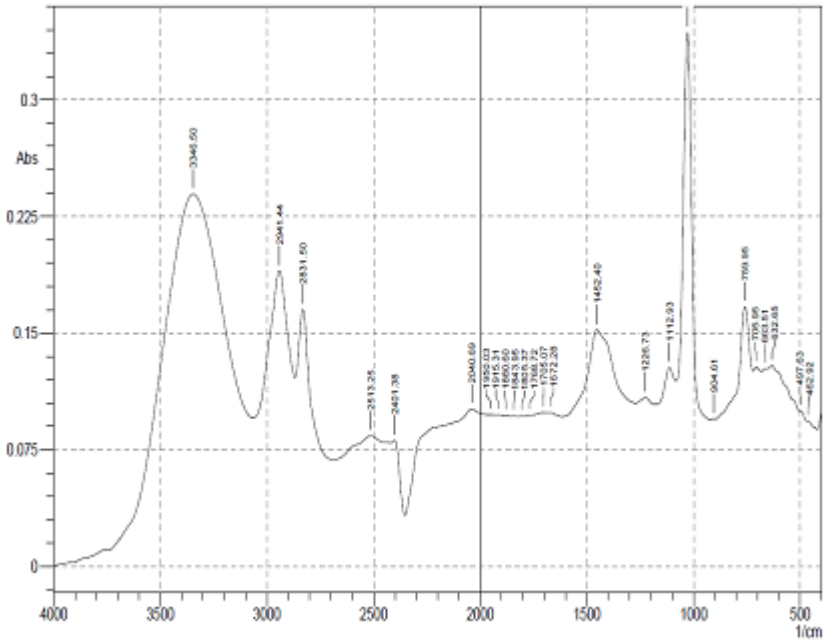


Fig.6 FT-IR spectrum of *Meyerozyma guilliermondii* extract

CONCLUSION

In conclusion, this preliminary screening of fungal endophytes revealed their potential to yield potent antibiotic compounds against fish and human pathogens. Ethyl acetate extracts of *Hypocrea lixii* and *Meyerozyma guilliermondii* showed considerable antibacterial effect indicating its possible potential for development of antibacterial drug for fish and human pathogens by further research.

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