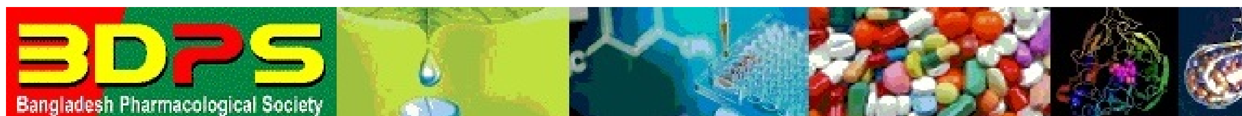

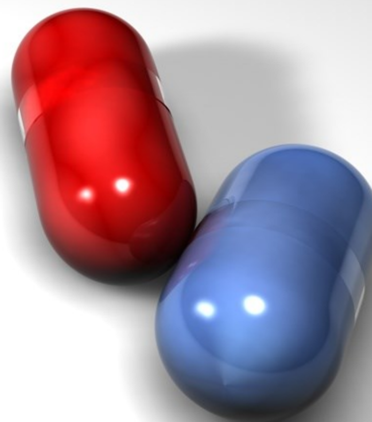

Bangladesh Journal of Pharmacology

Volume: 18; Number 1; Year 2023

Cite this article as: Madhusudan T, Chander MP. *GC-MS* analysis and *in vitro* antibacterial activities of mangrove endophytic fungi. Bangladesh J Pharmacol. 2023; 18: 36-39.

A Journal of the Bangladesh Pharmacological Society (BDPS) **Bangladesh J Pharmacol 2023; 18: 36-39**
Journal homepage: www.banglajol.info; www.bdpsjournal.org
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Global Health, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index
ISSN: 1991-0088; DOI: 10.3329/bjp.v18i1.63307

Letter to the Editor

GC-MS analysis and *in vitro* antibacterial activities of mangrove endophytic fungi

Sir,

The marine secondary metabolites are low molecular organic compounds that are synthesized during the growth phase with no specific growth link and are produced by a particular group of microorganisms with unusual chemical structures. It is well-reported that both fungi and bacteria are recognized as producers of novel chemical entities that act as drugs or their precursors (Kock et al., 2001; Bode et al., 2002). Since time immemorial it is well perceived that plants serve as the major source of therapeutic bioactive compounds against numerous forms of ailments. Several studies also have reported the production of plant secondary metabolites by endophytic fungi and it laid the path to exploiting these fungi as an alternative source for bioactive secondary metabolites (Goud et al., 2016). The bio-prospection of endophytes may lead to the discovery of natural products with tremendous therapeutic values (Kusari et al., 2012). The endophytic fungal research from the year 1987–2006 discerned about 280 new natural products followed by their identification and characterization (Zhang et al., 2008). As a result, the ecological aspect of marine-derived and mangrove endophytic fungi has drawn the attention of many researchers to exploit them with the application of biotechnology. From the previous reports about 700 new compounds were characterized from marine organisms during the past three decades (Rabet and Ebel, 2011).

Andaman and Nicobar Islands account for 13% of the total Indian mangrove area, with a relative mangrove density of 76.5%. The mangroves of the Andaman and Nicobar Islands are recognized as the best in the country in terms of density and growth (Bharathi et al., 2014). As suggested by several studies it is apparent that mangrove endophytic fungi are untapped reservoirs of novel and interesting chemical compounds and only a few studies were reported from these Islands. In context, the present study was taken up to investigate mangrove endophytic fungi and characterize their metabolites from the coast of Port Blair, South Andaman.

Healthy stilt, prop, knee, and buttress roots from 66 mangroves were collected during low tide along the coast of Port Blair, South Andaman, and brought to the laboratory in sterile bags to reduce the chance of contamination. Mangrove specimens were identified based on the keys of Naskar and Mandal (1999).

Root samples were initially rinsed under running tap water and thoroughly cleansed with autoclaved marine water to detach the adherent sediment particles. Surface sterilization of the roots was carried out by sequential immersions in 95% ethanol for 45 sec followed by 5% sodium hypochlorite for 5 min, then in 90% ethanol for 45 sec, and finally in sterile marine water for 5 min (Kjer et al., 2010). The roots were cut into small segments and placed on potato dextrose agar plates amended with chloramphenicol and streptomycin of 150 µg/mL concentration under ambient sterile conditions. Pure fungal colonies were obtained by transferring the hyphal tips onto fresh potato dextrose agar plates.

The fungal isolates were cultured by placing the mycelia plugs into a 500 mL Erlenmeyer flask comprising 250 mL potato dextrose broth and incubated at 27ºC for three weeks, shaking at 250 rpm at regular intervals. At the end of incubation, the broth culture was vacuum filtered and the filtrate was extracted thrice with equal amounts of ethyl acetate. The filtrate was subjected to rota-evaporation (Buchi 2412V0 RII, Switzerland) to obtain the metabolites. The mycelial mat was demoistured with help of Whatman filter paper and squashed with 100 mL methanol/hexane/ethyl acetate and subjected to sonification for 5 hours and centrifuged at 10,000 rpm for 30 min, supernatant was dried at 45ºC in a water bath to procure intracellular metabolites. The filtrate and mycelial crude extracts were combined weighed and stored at 4ºC for further studies. The slurry of the extracts was prepared in silica gel and dried at room temperature was loaded onto an open column chromatography glass column (100 x 2 cm) using silica gel (60-120 mesh size). The column was initially eluted with 100 ml of hexane followed by 100 mL of ethyl acetate and 100 mL of methanol respectively. Respective fractions were collected separately in a 100 mL sterile beaker and concentrated under pressure at 40°C. The following extracts were collected and stored at -40°C for further studies

The antimicrobial activity of the crude extracts and fractions from fungal isolates was determined by the

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agar well diffusion method (Rojas et al., 2006; Semerci et al., 2020 for video). GC-MS analysis of active extracts was carried out on an Alglient© 7890, which is employed for the analysis of compounds. The peaks of the compounds representing mass to charge ratio characteristics were compared with the NIST-2011 library for the identification of corresponding organic compounds.

The results presented in Table I revealed that five extracts of ethyl acetate displayed potential antibacterial activity against the tested organisms. The metabolites of *A. terreus* restricted the growth of all test pathogens and the highest activity was visualized against *S. typhi* (22.7 ± 0.6 mm) followed by *E. coli* (20.7 ± 0.6 mm) and *P. mirabilis* (20.0 ± 1.0 mm). *C. lunata* inhibited nine test pathogens, it was most active in the case of *V. fluvialis* (18.0 ± 1.0 mm) and *S. typhi* (15.0 ± 0.0 mm). The compounds of *N. clavispora* were moderately effective against tested pathogens, *S. typhi* was sensible (17.7 ± 0.6 mm) while *M. luteus*, *P. aeruginosa*, *P. mirabilis*, and *V. fluvialis* were completely resistant. *V. fluvialis* (22.3 ± 1.5 mm) was found most sensitive and *P. aeruginosa* (11.7 ± 1.2 mm) was found to be least sensitive to *P. oxalicum*. *F. equiseti* was most effective among all the endophytes against the test organisms, it was very active against *E. coli* (25.0 ± 1.0 mm) followed by *S. dysentery* (22.7 ± 0.6 mm) and *S. typhi* (22.0 ± 1.7 mm).

A total of 66 endophytic fungal isolates were isolated from varied mangrove roots. Among these 5 endophytic fungal strains that exhibited antibacterial activity were further studied. The morphological characters of those endophytic fungi were recorded. The BLAST analysis revealed that the ITS region of the endophytic fungal isolates was 98-99% similar to the existing ones in the NCBI Gene Bank and also the particular species to which the isolated endophytes fungal strains related (Table II).

GC–MS analysis of ethyl acetate extracts of five fungi revealed the presence of bioactive compounds (Table III). The most abundant compound was diisooctyl phthalate, (bis(2-ethylhexyl phthalate), hexadecanoic acid, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl). The other significant compounds detected were cyclopropaneoctanoic acid, 2-hexyl-methyl ester, megestrol acetate, 1H-indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl respectively.

The study results of the antibacterial assay, the metabolites of fungal strains are more potent towards Gram-negative bacteria as well as Gram-positive bacteria. GC-MS analysis revealed bioactive compounds from five fungal strains, and each compound has been struc-

Table I

Antibacterial activity of endophytic fungi isolated from mangrove roots

Bacterial pathogen	Activity of endophytic fungal extracts (in mm)				
	A. terreus	*C. lunata*	*N. clavispora*	*P. oxalicum*	*F. equiseti*
S. aureus	15.3 ± 0.6	11.3 ± 0.6	9.7 ± 0.6	14.3 ± 0.6	17.3 ± 0.6
M. luteus	14.0 ± 1.0	10.3 ± 0.6	_	20.3 ± 0.6	16.3 ± 1.2
E. faecalis	17.3 ± 0.6	13.7 ± 0.6	12.7 ± 0.6	12.7 ± 1.2	15.0 ± 0.0
V. cholera	20.0 ± 0.0	12.3 ± 1.2	10.7 ± 0.6	17.0 ± 0.0	21.3 ± 1.5
P. aeruginosa	15.7 ± 0.6	_	_	11.7 ± 1.2	20.7 ± 0.6
P. mirabilis	20.0 ± 1.0	12.7 ± 0.6	_	12.7 ± 0.6	18.0 ± 1.0
S. typhi	22.7 ± 0.6	15.0 ± 0.0	17.7 ± 0.6	20.7 ± 1.2	22.0 ± 1.7
S. dysentery type S	12.3 ± 0.6	9.7 ± 0.6	11.0 ± 0.0	12.3 ± 0.6	22.7 ± 0.6
Enterotoxigenic *E. coli* serotype 0115	19.3 ± 0.6	11.7 ± 1.2	11.3 ± 1.2	15.0 ± 0.0	25.0 ± 1.0
V. fluvialis	20.7 ± 0.6	18.0 ± 1.0	_	22.3 ± 1.53	20.3 ± 0.6

Table II

Gene bank accession number of endophytic fungal strains

Endophytic fungal strain	Gene bank number	NCBI DNA sequences	Sequence identity (%)
Aspergillus terrus	KY859791	FJ037754, KY053122, KR673900	99.3
Curvularia lunata	KY859790	KX610322, JX960594, FJ792584	100
Neopestalopsis clavispora	KY859789	KY810809, KC256920, KY810807	100
Penicillium oxalicum	KY952713	KX674635, LT558935, LT 5589533	99.6
Fusarium equseti	KY963137	KR364596, EV595566, KR364599	99.9

Table III

Compounds identified from the extracts of endophytic fungal strains by GC-MS analysis

Species	Compounds	Retention time (min)	Molecular formula	Molecular weight	Peak area (%)
A. terreus	Isopropyl myristate	10.3	$C_{19}H_{40}$	268.313	5.1
	Dibutyl phthalate	10.6	$C_{16}H_{22}O_4$	278.151	22.8
	Bis(2-ethylhexyl) phthalate	11.44	$C_{24}H_{38}O_4$	390.227	78
	Diisooctyl phthalate	13.35	$C_{24}H_{38}O_4$	390.277	88.5
	Squalene	14.49	$C_{30}H_{5O}$	410.391	4.8
C. lunata	1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-	9.55	$C_{18}H_{2O}$	236.156	75.2
	Hexadecanoic acid, methyl ester	11.08	$C_{17}H_{34}O_2$	270.225	78.3
	Decanedioic acid, bis(2-ethylhexyl) ester	15.11	$C_{26}H_{50}O_4$	426.370	65.1
	Cetene	16.11	$C_{16}H_{32}$	224.250	48.2
	Phenol, 2,6-bis(1,1-dimethylethyl)	17.29	$C_{14}H_{22}O$	206.167	38.4
N. clavispora	Cyclohexasiloxane, dodecamethyl	5.22	$C_{12}H_{36}0_6Si_6$	444.112	4.3
	Benzoic acid, 2-methylbutyl ester	6.42	$C_{12}H_{16}O_2$	119.112	6.5
	2-Propenamide, N,N-diethyl	10.05	$C_7H_{13}NO$	127.007	5.1
	Thiocarbamic acid	10.56	$C_{19}H_{21}NOS$	311.134	4.8
	Diisooctyl phthalate	13.48	$C_{24}H_{38}O_4$	390.227	91.2
P. oxalicum	6-Methyl-bicyclo[4.2.0]octan7-ol	9.15	$C_9H_{16}O$	140.120	5.1
	Megestrol acetate	9.37	$C_{24}H_{32}O_4$	384.321	60.5
	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2 -methylpropyl)-	10.59	$C_{11}H_{18}N_2O_2$	210.136	85.5
	Cyclopropaneoctanoic acid, 2-hexyl-, methyl ester	13.21	$C_{18}H_{34}O_2$	282.255	75.5
	Acetic acid, trifluoro-, 3,7-dimethyloctyl ester	17.52	$C_{12}H_{21}F_3O_2$	254.149	6.7
F. equiseti	Benzenesulfonyl azide, 4-methyl	12.01	$C_7H_7N_3O2S$	197.025	13.2
	Benzylamine, 4-(1-methylethyl)-N,à-diphenyl	12.25	$C_{22}H_{23}N$	301.183	15.2
	2-Butanone, 3-(4-tert-butylphenoxy)	12.35	$C_{14}H_{20}O_2$	220.146	12.2
	Diisooctyl phthalate	13.22	$C_{24}H_{38}O_4$	390.277	89.1

turally characterized so that these metabolites can act as potential pharmaceutical products or lead structures for the development of new drugs in feature. The study of endophytic fungi associated with the mangrove roots from the coast of South Andaman is the first of its kind from the islands and it should be further investigated for its application in developing novel bioactive compounds with therapeutic value.

The authors express their great gratitude for the head of the Department of Ocean Studies and Marine Biology, Pondicherry University Port Blair, and RMRC Port Blair. The authors are thanking the School of Plant Sciences, the University of Hyderabad for providing facilities to carry out GC-MS analysis.

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