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Diversity and antimicrobial activity of endophytic fungi isolated from the seagrass *Enhalus acoroides*

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Endophytic fungi were isolated from the seagrass, *Enhalus acoroides*, collected from Trang province, Thailand. Forty-seven endophytic isolates cultured were classified into 17 phylogenetically diverse genera based on their morphology and molecular analysis of the ITS regions of the rDNA. Most common species were *Penicillium* (6 isolates), *Nigrospora* (5), and *Fusarium* (4) and 2 with unknown taxonomic affinity. Crude extracts including culture media and cells of all isolates were tested for their antimicrobial activities using a colorimetric broth microdilution method against ten potential human pathogens. Extracts from 38 isolates (80.85%) showed antimicrobial activity with minimum inhibitory concentration (MIC) values ranging from 4 to 200 µg mL^{-1}. *Nigrospora* sp. PSU-ES5 produced the most active extracts against *Microsporum gypseum* (MIC 4 to 8 µg mL^{-1}). Endophytic fungi from seagrasses such as *E. acoroides* could therefore be a good source for obtaining antimicrobial natural products.

[Keywords: Antimicrobial activity, Endophytic fungi, Seagrasses, Molecular identification, *Enhalus acoroides* **]**

Introduction

It is now well documented that endophytic fungi are a good source of bioactive natural products[1-4]. Most of the studied endophytic fungi have been isolated from terrestrial plants[5-8]. Bioactive natural products from endophytic fungi from marine plants in particular from seagrasses have been rarely studied[9-12]. Previous reports on seagrass endophytes have focused on their diversity and distribution[10,13-17].

Seagrasses are marine flowering plants that play important roles in marine ecosystems, i.e. reducing wave energy, stabilizing sand and providing a large shelter for a variety of marine animals[18-19]. They have been used in traditional medicine in India: roots of *Enhalus acoroides* have been applied as a remedy against stings from different kinds of rays and scorpions; *Cymodocea* spp. has been used as a tranquillizer for babies, or for soothing help during pregnancy and against coughs and even malaria; some *Halophila* spp. produce a strong traditional preparation that can act against malaria, skin diseases and the early stages of leprosy[20]. Recently, Ravikumar *et al.*[21] also reported antibacterial activity of a root extract from *Cymodocae serrulata* against *Klebsiella* sp., *Escherichia coli*, *Staphylococcus* sp. and

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Salmonella sp. An ethanolic extract of *E. acoroides* was shown to have strong antioxidant[23], antifeedant, antibacterial and antilarval activities[24].

Thailand is a rich source of biodiversity. Coastal areas of southern Thailand have many rich seagrass beds[22]. Twelve species representing 7 genera including *Cymodocea*, *Halodule*, *Syringodium* (Cymodoceaceae), *Enhalus*, *Halophila*, *Thalassia* (Hydrocharitaceae), and *Ruppia* (Ruppiaceae) have been reported from coastal areas of the Andaman Sea and the Gulf of Thailand. *E. acoroides* is the largest seagrass commonly found in major seagrass areas in Southeast Asia. Recently, Sakayaroj *et al*.[17] reported the diversity of endophytic fungi isolated from *E. acoroides* collected at Hat Khanom-Mu Ko Thale Tai National Park, Surat Thani province, southern Thailand. However, the largest seagrass bed in Thailand is located in Trang province, particularly around Talibong Island. Present study is to isolate endophytic fungi from *E. acoroides* collected in this area, compare the diversity of endophytic fungi of *E. acoroides* with a previous report from Surat Thani province and screen for antimicrobial active metabolites from the culture extracts of the isolated fungi against potential human pathogens.

Materials and Methods

Source and isolation of endophytic fungi

Five *E. acoroides* whole plant samples were randomly collected from Trang Province, Thailand every month for a period of one year during June 2008 – May 2009. Seagrass samples including leaves, roots, and rhizomes were surface-sterilized with 10% ethanol (3 min), 3% sodium hypochlorite (10 s), 10% ethanol (3 min), rinsed twice in sterile distilled water and dried on a sterile paper towel. Leaves were divided into 3 age groups from the outermost leaves (oldest) to the innermost leaves (youngest) as old, medium and young leaves. Each leaf age group was divided into 3 equal parts (upper, middle, and lower) as described by Sakayaroj *et al*.[17] Each of the leaf parts, root and rhizome were then cut into 6 fragments. Samples were placed onto potato dextrose-sea water agar (PDA-SW) supplemented with antibiotics (50 mg L^{-1} penicillin and 50 mg L^{-1} streptomycin). The plates were incubated at 25°C for up to 2 weeks until emergence of hyphae from the samples was observed. Endophytic fungi were subcultured onto PDA-SW without antibiotics for further purification and storage. Each pure fungal isolate was maintained on PDA-SW at 4°C and in 20% glycerol at -80°C.

Identification of endophytic fungi

All endophytic fungi were identified based on their morphology and/or analyses of the Internal Transcribed Spacers (ITS) of rDNA. Genomic DNA was extracted using the protocols described by Wang *et al*.[25] ITS regions were amplified by polymerase chain reaction (PCR) with primers ITS5/ITS4 and ITS1F/ITS4 [26-27]. Purification of the DNA fragment was performed utilizing the NucleoSpin® Extract DNA purification kit Cat. No. 740 609.50 (Macherey-Nagel, Germany), as described by the manufacturer's protocol. DNA sequencing was performed using the primers mentioned above, at Macrogen, Korea. A BLAST Search was used to search for the closest matched sequences in the GenBank database[28]. Fungal sequences in this study and other related sequences were multiply aligned using BioEdit 7.0.9[29] and the alignments were adjusted manually where necessary. Phylogenetic relationships were estimated using PAUP* v4.0b10[30]. Percentage cut-off point is 96% similarity. ITS sequences of the endophytic fungal isolates were submitted to GenBank for accession numbers.

Fungal diversity

The isolation rate (IR) and colonization rate (CR) were calculated according to Jordaan *et al*.[31] and Gond *et al*.[32], respectively using the following formulae:

%IR = (total number of isolates yielded/total number of sample segments) × 100

%CR = (number of segments of plant tissue colonized by each fungus/total number of segments of plant tissue studied) ×100

Fungal species diversity was calculated using Shannon-Wiener's index (*H*) and Simpson's

index (D). Shannon-Wiener's index $H = -\Sigma (Pi) (lnPi)$, where Pi is the relative abundance of fungal species occuring on *E. acoroides*. The Simpson's index $D = 1-\Sigma\ n\ (n-1)/N\ (N-1)$, where n is the number of individuals of a specific species and N is total number of individuals of all species.

Preparation of hyphae and extractions

Endophytic fungal cultures were grown on PDA-SW and incubated at 25°C for 3-5 days. Six agar plugs (1 cm^2) from the actively growing edge of the colony were inoculated into 500 mL Erlenmeyer flasks containing 300 mL potato dextrose broth (PDB) and incubated for 3 weeks at 25°C under stationary condition for production of antimicrobial metabolites[33]. The culture broth was filtered to separate the filtrate and mycelia. Filtrate was extracted three times with an equal volume of ethyl acetate (EtOAc) in a separating funnel. The combined EtOAc extracts were dried over anhydrous sodium sulphate (Na_2SO_4) and evaporated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator to give the BE extract. The fungal mycelia were extracted with 500 mL of methanol (MeOH) for 2 days. Aqueous MeOH layer was concentrated under reduced pressure. Distilled water (50 mL) was added to the extract and the mixture was then mixed with hexane (100 mL). Aqueous layer was then extracted three times with an equal volume of EtOAc. Hexane extract and the combined EtOAc extracts were dried over Na_2SO_4 and evaporated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator to give CH and CE extracts respectively. All crude extracts were first subjected to thin-layer chromatography (TLC) and their H^1 nuclear magnetic resonance (NMR) spectra were recorded. At least two batches of each isolate were extracted and checked for their identical TLC and NMR spectra.

Antibacterial assay

The dried endophytic fungal extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions and stored at -4°C until used. All extracts at concentrations of 200 µg mL^{-1} were screened for antibacterial activity against *Staphylococcus aureus* ATCC25923, a clinical isolate of methicillin-resistant *S. aureus* (MRSA) SK1, *Escherichia coli* ATCC25922, and *Pseudomonas aeruginosa* ATCC27853 by the colorimetric microdilution method in a 96-well microtitre plate according to Clinical and Laboratory Standards Institute (CLSI)[34] with some modifications. Microtitre plates were incubated at 35°C for 15 hours, then 10 µL of resazurin indicator (0.18%) was added to each well and examined after incubation for 2-3 hours at 35°C for the completed reaction[35]. After incubation, a blue or purple color of the wells indicated inhibition of growth (positive result) and a pink color indicated bacterial growth (negative result). The minimum inhibitory concentrations (MICs) of active crude extracts were determined by the same colorimetric microdilution method. Crude extracts were diluted using serial 2-fold dilutions with final concentrations of 0.025-128 mg mL^{-1}. Lowest concentration of extract that inhibited growth (blue or purple color) was recorded as the MIC. Concentrations of crude extract less dilute than the MIC and the MIC were streaked onto a nutrient agar (NA) plate and incubated under appropriate conditions. Lowest concentration of extract that showed no growth was recorded as the minimum bactericidal concentration (MBC). Vancomycin and gentamicin were used as standard antibacterial agents for positive inhibitory controls.

Antifungal assay

Endophytic fungal extracts were screened for their antifungal activity at a concentration of 200 µg mL^{-1} by a modification of the microbroth dilution CLSI M27-A2[36] against yeasts (*Candida albicans* ATCC90028, *C. albicans* NCPF3153, *Cryptococcus neoformans* ATCC90112, *C. neoformans* ATCC90113) and a modification of the microbroth dilution CLSI M38-A[37] against clinical isolates of *Microsporum gypseum* and *Penicillium marneffei*. Microtitre plates were incubated at 35°C for 24 hours for *C. albicans*, 48 hours at room temperature (RT) for *C. neoformans*, and 6 days at RT for *M. gypseum* and *P. marneffei*, then 10 µL resazurin indicator (0.18%) was added to each well and examined after incubation for 5 hours at 35°C for yeasts and one day for *M. gypseum* as adapted from Sarker *et al*.[35] As *P. marneffei* produces red pigment into the media,

therefore its growth was observed using a stereomicroscope. MICs of the active crude extracts were determined by the same colorimetric microdilution method using 2-fold serially diluted crude extracts (0.025-128 mg mL^{-1}). Minimum fungicidal concentrations (MFCs) of the active extracts were determined by the streaking method on Sabouraud's dextrose agar (SDA). Lowest concentration of extract that showed no growth was recorded as the MFC. Amphotericin B was used as a positive inhibitory control for yeasts and *P. marneffei* and miconazole for *M. gypseum*.

Results

Endophytic fungal isolation and identification

Forty-seven endophytic fungi were isolated from the 3,300 fragments of *E. acoroides*, among which 45 isolates were from leaves, one from root and one from rhizome (Table 1). The number of segments and number of isolates are equal in this study, so the percentage of occurrence and the isolation rate have the same values. Percentage of occurrence of endophytic fungi in the middle segment of medium leaves was the highest with value 0.56% (Fig. 1).

Only 15 isolates produced conidia and these were identified by their morphological characteristics as: *Aspergillus* sp. (1 isolate), *Cladosporium* sp. (2 isolates), *Curvularia* sp. (2 isolates), *Fusarium* sp.

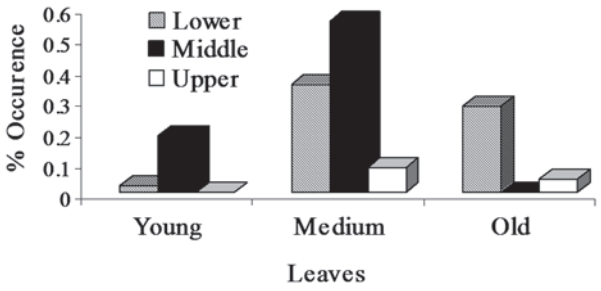

Fig. 1–The percentage of occurrence of endophytic fungi isolated from different plant segments from leaves, old, medium and young.

Table 1–The identity of endophytic fungi isolated from *Enhalus acoroides*

Genera	Code	Morphology identification	Molecular identification	% Similarity	Accession number	No. of individuals	%IR	%CR
Phylum Ascomycota								
Aspergillus						1	0.04	0.04
	PSU-ES4*	*Aspergillus* sp.	ND	-				
Bipolaris						3	0.11	0.11
	PSU-ES56#	MS	*Bipolaris* sp.	94.3	JN116619			
	PSU-ES70#	MS	*Bipolaris specifera*	96.1	JN116627			
	PSU-ES127#	MS	*Bipolaris specifera*	96.1	JN116658			
Cladosporium						2	0.07	0.07
	PSU-ES125#	*Cladosporium* sp.	ND	-				
	PSU-ES134#	*Cladosporium* sp.	ND	-				
Cordyceps						1	0.04	0.04
	PSU-ES197#	MS	*Cordyceps memorabilis*		99.5	JN116706		
Curvularia						2	0.07	0.07
	PSU-ES45#	*Curvularia* sp.	ND	-				
	PSU-ES71#	*Curvularia* sp.	ND	-				
Fusarium						4	0.15	0.15
	PSU-ES123#	MS	*Fusarium* sp.	100	JN116657			

	PSU-ES137#	*Fusarium* sp.	ND					
	PSU-ES157#	MS	*Fusarium oxysporum*		100	JN116678		
	PSU-ES158#	*Fusarium* sp.	ND					
Hypocrea lixii						3	0.11	0.11
	PSU-ES22#	MS	*Hypocrea lixii*	100	JN116598			
	PSU-ES160#	MS	*Hypocrea lixii*	98	JN116680			
	PSU-ES207#	MS	*Hypocrea lixii*	100	JN116715			
Nigrospora						5	0.19	0.19
	PSU-ES5#	*Nigrospora* sp.	ND	-				
	PSU-ES114#	*Nigrospora* sp.	ND	-				
	PSU-ES117#	MS	*Nigrospora* sp.	99.1	JN116653			
	PSU-ES136#	*Nigrospora* sp.	ND	-				
	PSU-ES151#	MS	*Nigrospora* sp.	97.7	JN116674			
Penicillium						6	0.22	0.22
	PSU-ES2#	*Penicillium* sp	ND	-				
	PSU-ES3#	*Penicillium* sp.	ND	-				
	PSU-ES21#	*Penicillium* sp.	ND	-				
	PSU-ES139#	*Penicillium* sp.	ND					
	PSU-ES159#	*Penicillium* sp.	*Eupenicillium erubescens*	100	JN116679			
	PSU-ES194#	MS	*Talaromyces flavus*	100	JN116703			
Pestalotiopsis						1	0.04	0.04
	PSU-ES148#	MS	*Pestalotiopsis* sp.	99.1	JN116671			
Phaeosphaeriopsis						1	0.04	0.04
	PSU-ES24#	MS	*Phaeosphaeriopsis* sp.		99.8	JN116691		
Simplicillium						2	0.07	0.07
	PSU-ES104#	MS	*Simplicillium lanosoniveum*	98.4	JN116646			
	PSU-ES108#	MS	*Simplicillium*	93.3	JN116649			
Trichoderma						1	0.04	0.04
	PSU-ES103#	MS	*Trichoderma* sp.	95.2	JN116645			
Hypocreales						2	0.07	0.07
	PSU-ES23#	MS	Hypocreales sp.	-	JN116599			
	PSU-ES203#	MS	Hypocreales sp.	-	JN116711			
Pleosporales						2	0.07	0.07
	PSU-ES146#	MS	Pleosporales sp.	-	JN116669			
	PSU-ES210#	MS	Pleosporales sp.	-	JN116717			
Xylariaceae						2	0.07	0.07
	PSU-ES106#	MS	Xylariaceae sp.	-	JN116648			
	PSU-ES116#	MS	Xylariaceae sp.	-	JN116652			

Phylum Basidiomycota								
Schizophyllum						2	0.07	0.07
	PSU-ES25[#]	MS	*Schizophyllum commune*	98	JN116601			
	PSU-ES49[#]	MS	*Schizophyllum commune*	98	JN116615			
Phanerochaete				-		1	0.04	0.04
	PSU-ES174[Ψ]	MS	*Phanerochaete sordida*	99.8	JN116600			
Fungal endophyte	PSU-ES147[#]	MS	Fungal endophyte	-	JN116670	1		
Unidentified fungus								
		MS	Unidentified endophytic fungi			5	0.19	0.19
Total of isolates						47		
%Isolation rate						1.42		
%Colonization rate						1.42		
Simpson's Diversity Index						0.7		
Shannon Diversity Index						3.0		

-[*], isolated from rhizome; -[#], isolated from leaves; -[Ψ], isolated from root; MS, mycelia sterilia; ND, not determined; %IR, isolation rate; %CR, colonization rate

(2 isolates), *Nigrospora* sp. (3 isolates) and *Penicillium* sp. (5 isolates). Thirty-two isolates did not produce any reproductive structure and were classified as mycelia sterilia (Table 1). These non-sporulating fungi were further identified by molecular method based on ITS sequence analyses (Table 1 and Figs. 2-3).

Phylogenetic analysis indicated that three fungal isolates were associated with the Phylum Basidiomycota (Fig. 2). Two isolates (PSU-ES25 and PSU-ES49) showed high nucleotide identity with *Schizophyllum commun*e (84.3-99.3%). An endophytic fungal isolate PSU-ES174 had an affinity with various species of *Phanerochaete*, with *P. sordida* as the most closely related taxon (71.4 - 99.8% sequence similarities). Within the most parsimonious tree of the Phylum Ascomycota (Fig. 3), three different fungal groups were classified to the Sordariomycetes (17 isolates), Eurotiomycetes (2 isolates) and Dothideomycetes (6 isolates). Fungal endophytes belonging to the Sordariomycetes had affinities within 3 orders including the Hypocreales, Xylariales and Trichosphaeriales.

Eleven isolates had affinities with the Order Hypocreales. They were identified as *Hypocrea lixii* (PSU-ES22, PSU-ES160 and PSU-ES207), *Trichoderma* sp. (PSU-ES103), *Simplicillium lanosoniveum* (PSU-ES104), *Simplicillium lamellicola* (PSU-ES108), *Fusarium* sp. (PSU-ES123), *Fusarium oxysporum* (PSU-ES157), unidentified hypocrealean species (PSU-ES23, PSU-ES203) and *Cordyceps memorabilis* (PSU-ES197).

Within the Xylariales, the endophytic fungus PSU-ES148 showed the highest similarity with a species of *Pestalotiopsis*. This fungus should be identified as *Pestalotiopsis* sp. The fungi PSU-ES106 and PSU-ES116 did not have any closely related species. Therefore, they could only be referred to be

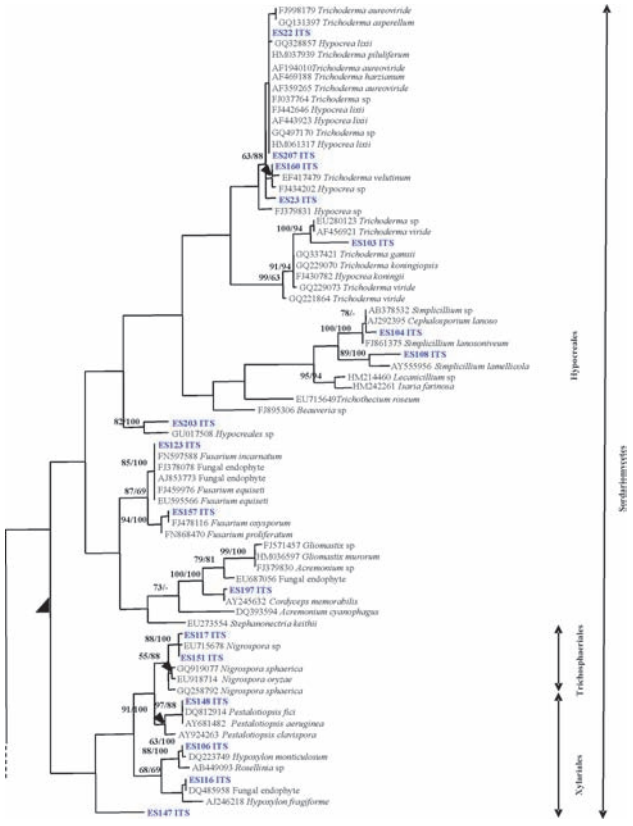

Fig. 2–Phylogenetic tree of fungal endophytes belonging to Basidiomycota based on Maximum Parsimony analysis of the ITS rDNA sequences. Length, 921 steps; consistency index (CI), 0.696; retention index (RI), 0.897; homoplusy index (HI), 0.304; rescaled consistency index (RC), 0.624, Bootstrup vulues from Maximum Parsimony (MP BS) and Neighbour Joining (NJ BS) with 500 replications are shown on the branch. MP BS values ≥ 50% are shown before the slash; NJ BS values ≥ 50% are shown after the slash.

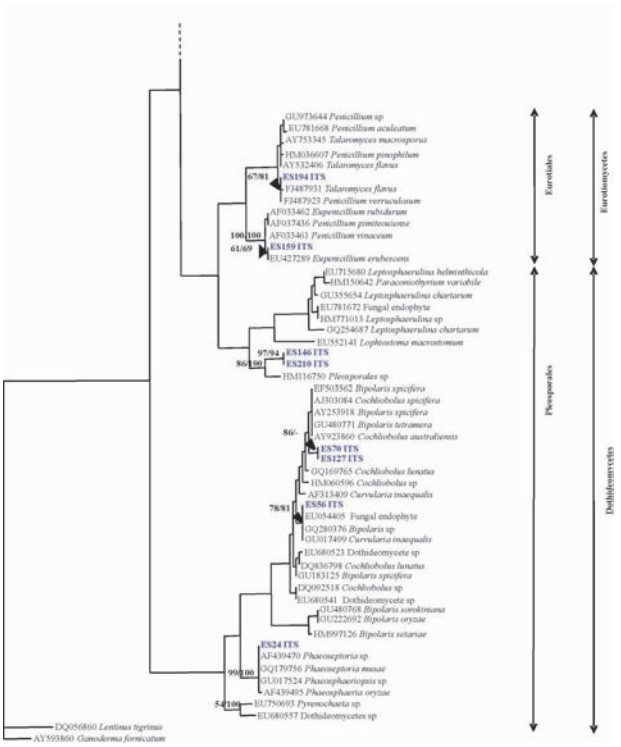

Fig. 3–Most parsimonious tree of ITS rDNA sequences of Ascomycota. Length; 1401 steps; consistency index (CI); 0279; retention index (RI); 0871; homoplasy index (HI); 0.721; rescaled consistency index (RC); 0.243. Bootstrup values from Maximum Pursimony (MP BS) and Neighbour Joining (NJ BS) with 500 replications are shown on the branch. MP BS values ≥ 50% are shown before the slash; NJ BS values ≥ 50% shown after the slash.

members of the Xylariaceae. Moreover, PSU-ES147 was tentatively identified simply as unidentified fungal endophyte, a member within family Xylariaceae but it remained alone without any related taxa.

Two isolates of endophyte assemblages (PSU-ES117 and PSU-ES151) were placed in the Trichosphaeriales. They were closely related and were identified as *Nigrospora* sp. due to their high sequence identity with several *Nigrospora* species (97.7-99.1%).

Molecular identifications confirmed that two isolates of PSU-ES159 and PSU-ES194 should be classified in the Class Eurotiomycetes, Order Eurotiales. PSU-ES159 could belong to *Eupenicillium erubescens*. *Talaromyces* and *Eupenicillium* were the sexual stages of *Penicillium* spp. while PSU-ES194 was identified as *Talaromyces flavus*. Six fungal strains (PSU-ES146, PSU-ES210, PSU-ES70, PSU-ES127, PSU-ES56 and PSU-ES24) were grouped in class Dothideomycetes. PSU-ES70 and PSU-ES127 were monophyletic and identified as *Bipolaris specifera*, while PSU-ES56 should be identified as a *Bipolaris* sp. Endophytic fungus PSU-ES24 had the closest relationships to the genus *Phaeosphaeriopsis*, therefore it can be referred as *Phaeosphaeriopsis* sp. Remaining isolates (PSU-ES146 and PSU-ES210) did not have any closest taxa. Thus they could be tentatively referred as unidentified pleosporalean

species. A summary of the identification of the endophytic fungi in the current study is shown in Table 1.

Antimicrobial assay

Three extracts (BE, CE, and CH) were obtained from each endophytic fungal isolate resulting in 141 extracts from 47 isolates. Two extracts were obtained in small amounts which were not enough for the assays, therefore a total of 139 extracts were tested for their antimicrobial activity. Screening test at a concentration of 200 µg mL^{-1} showed that ethyl acetate extracts from broth (BE) gave the highest antifungal activity against filamentous fungi (37.50%). Whereas, the ethyl acetate extracts from the mycelium (CE) showed strong activity against yeasts (42.22%) (Fig 4). Active extracts were further tested for their MICs and MBCs or MFCs against those susceptible microorganisms. Eighty-three out of 139 (59.71%) crude extracts from 38/47 isolates (80.85%) showed antimicrobial activity against at least one test microorganism with MIC values that ranged from 4 to 200 µg mL^{-1}. Seven extracts yielded strong activity with MIC values of ≤16 µg mL^{-1} (Table 3). Endophytic fungus *Nigrospora* sp. PSU-ES5 produced the best active extracts with MIC values of less than 10 µg mL^{-1}. Its CH and BE extracts had the strongest activity against *M. gypseum* (MIC 4 and 8 µg mL^{-1}, and MFC 8 and 8 µg mL^{-1}, respectively). BE from *Nigrospora* sp. PSU-ES114 showed the best activity against *S. aureus* ATCC25923 and MRSA (MIC 16 µg $mL^{-1}$and 32 µg mL^{-1}) and the CH and CE from *Fusarium* sp. PSU-ES123 inhibited *C. neoformans* ATCC90112 with an MIC of 16 µg mL^{-1} followed by the CE of *Nigrospora* sp. PSU-ES5 and BE of the unidentified fungus PSU-ES111 inhibited *M. gypseum* (MIC 16 µg mL^{-1}).

Discussion

Seagrasses are a natural resource and provide important nursery habitats for juvenile fish and other aquatic animals[19]. Association of endophytic fungi and their host plant tissue is believed to be complex[38]. There have been only a few reports on studies of the endophytic fungi from seagrasses[13-14,17], while many reports have shown the diversity of endophytic fungi isolated from terrestrial plants[39-41].

Forty-seven endophytic fungi obtained from this study belonged to the Phyla Ascomycota (93.62%) and Basidiomycota (6.38%). Molecular identification showed that 28 non-sporulating fungi were associated with three major classes: Sordariomycetes, Eurotiomycetes and Dothideomycetes. They have been reported to be the most abundant endophytic groups isolated from various plant families[42]. In this study, we also found that basidiomycetes occurred as the endophytes in *E. acoroides*. Basidiomycetous endophytes have rarely been isolated from halophytic and mangrove plants, and most of them were frequently reported from terrestrial plants[25,43]. Sakayaroj *et al*.[17] also documented an endophytic basidiomycete (*Peniophora* sp.) from *E. acoroides.*

Most of the endophytes found in our study have been previously reported from other plants such as *Aspergillus, Penicillium, Pestalotiopsis* and *Fusarium*[17,43-46]. Moreover, the most frequent endophytes isolated from *E. acoroides* included the genera *Bipolaris, Nigrospora*, an unidentified species in the Hypocreales and another unidentified species in the Pleosporales[17].

In 2008, Sun *et al*.[47] studied the diversity and ecological distribution of fungal endophyte from medicinal plants. They showed that the isolation rate and colonization rate were higher from twigs than in leaves and the colonization and isolation rates of endophytic fungi in twigs increased with age. Gond *et al*.[32] found that the endophytic fungi isolated from plants were most frequently from the bark and leaves, while they were the least from roots. Sakayaroj *et al*.[17] reported that the number of fungal isolates in the upper leaf sections of *E. acoroides* showed the highest values, which was different from the present study. We found the highest number of isolates in the middle, followed by the lower then the upper leaf sections. *Penicillium* sp. *Nigrospora* sp. *Fusarium* sp. *Bipolaris* sp. and *Hypocrea lixii* were the dominant endophytic fungi in this study. They showed an equal percentage of colonization rates and isolation rate values of 0.22, 0.19, 0.15, 0.11 and 0.11, respectively.

Table 2–The most antimicrobial crude extracts from endophytic fungi isolated from *E. acoroides* against potential human pathogens.

		MIC/MBC or MFC (µg mL^{-1})									
Fungus	Extracts	Bacteria						Yeasts		Filamentous fungi	
		Gram-positive		Gram-negative							
		SA	MRSA	EC	PA	CA1	CA2	CN1	CN2	MG	PM
Nigrospora sp.											
PSU-ES5	CH	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	4/8	-/-
	BE	64/>200	64/>200	-/-	-/-	-/-	-/-	200/>200	200/>200	8/8	-/-
	CE	64/>200	64/>200	-/-	-/-	-/-	-/-	200/>200	200/>200	16/16	-/-
Unidentifiedfungi											
PSU-ES111	BE	128/>200	128/>200	-/-	-/-	-/-	-/-	-/-	-/-	16/>200	-/-
Nigrospora sp.											
PSU-ES114	BE	16/128	32/>200	128/>200	-/-	32/128	64/128	200/200	200/200	-/-	-/-
Fusarium sp.											
PSU-ES123	CH	64/>200	-/-	-/-	-/-	-/-	-/-	16/128	64/64	-/-	200/>200
	CE	64/128	-/-	-/-	-/-	128/>200	-/-	16/128	200/200	-/-	-/-
Vancomycin		0.5/1	1/2								
Gentamicin				0.25/1	0.125/0.5						
Amphotericin B						0.125/0.25	0.125/0.5	0.125/1	0.25/2		1/2
Miconazole										1/32	

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; SA, *Staphylococcus aureus* ATCC25923; MRSA, methicillin-resistant *S. aureus*; EC, *Escherichia coli* ATCC25922; PA, *Pseudomonas aeruginosa* ATCC27853; CA1, *Candida albicans* ATCC90028; CA2, *C. albicans* NCPF 3153; CN1, *Cryptococcus neoformans* ATCC90112 (flucytosine-sensitive); CN2, *C. neoformans* ATCC90113 (flucytosine-resistant); MG, *Microsporum gypseum* clinical isolate; PM, *Penicillium marneffei* clinical isolate; CH, extract from fungal cell with hexane; BE, extract from culture broth with ethyl acetate and CE, extract from fungal cell with ethyl acetate. – no activity at 200 µg mL^{-1}.

Whereas, the dominant fungal isolates that were isolated from *E. acoroides* in the Nakhon Si Thammarat province[17] were *Cladosporium* sp., unidentified hypocrealean species and *Penicillium* sp. with isolation rates of 1.11, 3.33 and 2.80 and colonization rates of 1.11, 2.78 and 2.80. Distribution of fungal endophyte in plant tissue may be associated with many factors including age of the plant, season, nutrient levels of the surrounding water and collecting sites[13,48]. Devarajan *et al.*[15] also found low colonization densities of endophytic fungi in the seagrass *Halophila ovalis*. In addition, the colonization rate of endophytic fungi in terrestrial plants was much larger than for endophytic fungi from seagrass. However, the species diversity of endophytic fungi assemblages in seagrasses is in the same range as in other host plants. Species diversity indices of endophytic fungi from many mangrove plant species[49] were between 0.796-2.875 and from chili pepper (*Capsicum annuum*)[50] was 2.068, whereas our result was 3.0.

A combination of physical and chemical factors may affect the colonization rate of endophytic fungi in their host plants. There are many compounds in host plant tissue including alkaloids, phenolic acids, flavonoids, tannins, terpenoids, quinines, stillbenes,

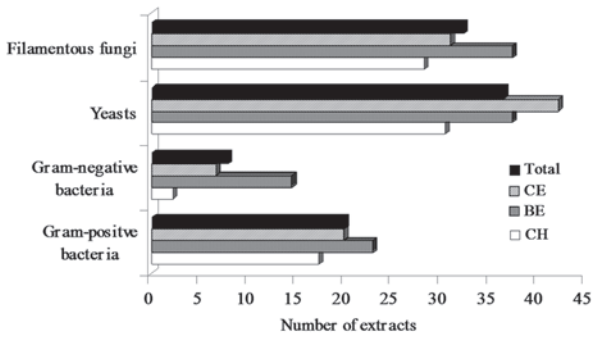

Fig. 4–Percentages of active fungal crude extracts that unhibited different microbial types

volatile and alphatic compounds. Not only do compounds in different host plant affect the fungal colonization but also the distribution of fungal endophyte in different segments such as leaves, stems, fruits, flowers and roots which habour various different fungal endophyte groups. The chemicals in host plant tissues may play important roles in the establishment of an endophytic fungal community[51].

Many antimicrobial secondary metabolites are produced from seagrasses. Methanolic extracts from three estuarine seagrass species, *Potamogeton pectinatus* L. (sagopond weed), *Potamogeton perfoliatus* L. (redhead grass) and *Ruppia maritima* L. (wigeon grass) showed antibacterial activity using a disk diffusion assay. All extracts inhibited Gram-positive and a few Gram-negative bacteria[52]. Extract from the common seagrass *Ruppia maritima* showed a higher potential for inhibition of the growth of *Lindra thalassiae* and *Fusarium* sp.[53]. An ethanolic extract of *E. acoroides* also showed antimicrobial activity[24]. We found that 50% of endophytic fungal extracts in this study showed antimicrobial activities with MIC ≤ 200 µg mL^{-1}. The number of active extracts against filamentous fungi and yeasts was higher than those active against bacteria. Crude extracts derived from *Nigrospora* sp. PSU-ES5 yielded a high antifungal activity against a clinical isolate of *M. gypseum* with MICs of 4 to 10 µg mL^{-1}, a dosage only 4 to 16 times higher than a standard antifungal drug miconazole. However, they showed better fungicidal activity (MFC 8-16 µg mL^{-1}) than miconazole (MFC 32 µg mL^{-1}). There are many reports about metabolites from *Nigrospora* sp. Griseofulvin is one of antifungal drug derived from *Nigrospora oryzae*[54] that has been used for treatment of tinea infections caused by dermatophytes including *M. gypseum*. Furthermore, the extract from *Nigrospora* sp. PSU-ES114 had a strong antibacterial activity against both strains of *S. aureus* and a moderate activity against *E. coli* and four strains of the tested yeasts. Trisuwan *et al.*[55-56] reported that nigrospotydon A, (+)- epopoxydon from *Nigrospora* sp. PSU-F5 and nigrosporapyrone A from *Nigrospora* sp. PSU-F18 had moderate activity against *S. aureus* (MIC 64-128 µg mL^{-1}). These two fungi were also isolated from marine environment. Another endophytic fungal isolate *Fusarium* sp. PSU-ES123 had strong antifungal activity against *C. neoformans* (MIC 16 µg mL^{-1}), whereas fusarubin produced by *Fusarium* sp. PSU-F135 inhibited the same strain of *C. neoformans* with an MIC of 64 µg mL^{-1} [57]. Our crude extracts exhibited stronger antimicrobial activity than those reported compounds from other marine derived fungal isolates in the same genera. Further investigation of the active compounds detected from our isolates will be made. The results from this study indicated that *E. acoroides* haboured a diversity of endophytic fungi with a high potential to become sources of antimicrobial natural products against human pathogens.

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