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ANTIMICROBIAL ACTIVITY OF MARINE FUNGI ISOLATED FROM THE SON TRA PENINSULA, DA NANG, VIETNAM

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ABSTRACT: Marine fungi have become an important source of bioactive natural products. The present study was concerned with the screening of antimicrobial activity from 73 fungal strains isolated from various marine habitats collected from five different localities at the Son Tra Peninsula, Da Nang, Vietnam. For the first step of screening, ethyl acetate extract of each fungal isolate was prepared and their antimicrobial activity against the human microbial pathogens was investigated using the disc diffusion method. The panel of human microbial pathogens used were *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 19111, *Streptococcus faecalis* ATCC 19433 and *Candida albicans* ATCC 10231. Among 73 fungal isolates, 29 exhibited antimicrobial activity against at least two tested pathogens. The proportion of the fungal isolates having anti-microbial activity against *B. cereus, S. faecalis, L. monocytogenes*, *S. aureus*, *E. coli*, *C. albicans* and *P. aeruginosa* were 42, 33, 31, 22, 7, 5 and 3%, respectively. Further investigations to isolate and characterize the anti-microbial components in the extracts are needed.

Keywords: Antimicrobial activity, natural products, marine fungi, microbial pathogens.

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INTRODUCTION

Marine fungi collected from various marine habitats, such as sponges, soft corals, animals, sea grasses and algae produce various biologically active metabolites (Biabini et al., 1998; Liberra and Lindequist, 1995). This group of organisms has attracted considerable attention from natural product chemists, and diverse and unique compounds of marine fungi with pertinent biological activities including antimicrobial, anticancer, anti-inflammatory and antiviral properties have been reported (Bugni and Ireland, 2004; Pan et al., 2008).

An increasing number of fungi living in distinctive environments, such as endophytic fungi from mangrove and marine fungi, are being investigated for their bioactivities to discover new antimicrobial compounds. Since the last decade, the number antibacterial and antifungal compounds found from marine fungi have been rapidly increasing, and marine fungi is considered a potential source of natural antibiotics (Singh et al., 2015). In Vietnam, although several investigations have been conducted so far for the diversity of marine fungi, virtually not so much information is available on their biological activity. Therefore, the current study was undertaken to evaluate marine fungi from the Son Tra Peninsula, Da Nang, for their antimicrobial activity against a panel of microbial pathogens.

MATERIALS AND METHODS

Sample collection

Marine samples including sponges, soft corals, seaweeds and sediments were collected from four different sites, Huc Lo (16°11' N; 108°31' E), Bai Nom (16°10' N; 108°29' E), Bai But (16°09' N; 108°28' E) and Hon Sup (16°08' N; 108°26' E), of the Son Tra Peninsula at the water depth ranging 8-15 m. The samples were placed in the polythene bags, stored in the icebox at 4-8°C and transported to our laboratory for the isolation of fungi.

Isolation of marine fungi

The collected marine organisms were rinsed with sterile seawater three times in to remove non-attached bacteria and 1 g of each sample was ground with 1 mL sterile seawater in a test tube. Then 0.1 mL of suspension was spread on modified Sabouraud agar (peptone 10 g, glucose 40 g, agar 18 g dissolved in 1000 mL sea water, pH 6.0-7.0) (Handayani et al., 2016). Morphological observation was performed after incubation for 5-7 days at 28°C. The fungal isolates were stocked in 40% glycerol in seawater at -80°C, as the Marine Microorganism Collection, Nhatrang Institute of Technology Research and Application (NITRA).

Screening for antimicrobial activity of marine fungi

The pure isolates, cultured in slant Sabouraud agar at room temperature for 14 days, were macerated with ethyl acetate for 24h for extraction. The ethyl acetate extracts were separated from the culture medium by collecting supernatant solvents and concentrated using a vacuum rotary evaporator at 40°C. Antimicrobial activity of the crude extracts were screened using a paper disc diffusion assay (Beccerro et al., 1994). In brief, the crude extracts were impregnated at 100 µg/disc concentration onto 6 mm diameter sterile Whatman no1. discs and allowed to dry for solvent evaporation. Then the antimicrobial activity was assessed against 7 human pathogens including Gram-positive bacteria (*B. cereus* ATCC 11778, *S. faecalis* ATCC 19433, *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 19111), Gram-negative bacteria (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) and yeast (*C. albicans* ATCC 10231). The pathogens were grown on nutrient agar and the turbidity of microbe suspensions was adjusted to 10^8 cells/mL using a spectrophotometer at a wavelength of 625 nm. Ethyl acetate without extracts on the disc was used as a negative control. The plates were incubated at 37°C for 24 hr and the results were recorded as the diameter (mm) of the zone of inhibition.

Identification of marine fungus by ITS gene analysis

The selected fungi were identified based on sequence analysis of ITS region of ribosomal DNA. Fungal DNA was extracted using the procedure described by Fredricks et al. (2005) with slight modifications. Briefly, the mycelial powder was transferred to a 1.5 mL Eppendorf tube containing 400-500 µL TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.3); an equal volume of phenol solution was added to the tube. After brief mixing, the mixture was centrifuged at 12,000 *g* for 10 min at 4°C. The aqueous phase was transferred to a new microtube and sequentially extracted with phenol solution and chloroform. RNA in the aqueous phase was removed using RNase. The sample was extracted again with phenol solution and chloroform. Finally, DNA was precipitated by adding two volumes of ethanol. The DNA pellet was washed with 75% ethanol and resuspended in 50-100 µL of sterile water. The resulting genomic DNA was used as a template to amplify fungal ITS-rDNA fragments using the primers ITS1 (5'-TCCGTAGGTGAACCTGC G-3') and ITS4 (5'-TCCTCCGCTTATTGATA TGC-3') (White et al., 1990).

Sequencing analyses were performed on an ABI 3730 XL (Applied Biosystems) automated sequencer using the ITS1 and ITS4 primers for PCR templates or universal plasmid primers (T3 and T7) for plasmid templates. Sequence data were edited with Chromas Lite, version 2 (Technelysium). For preliminary identification, sequences of fungal ITS-rDNA regions were compared with those in the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). Fungal ITS-rDNA sequences in this study and the matched sequences from GenBank were edited and

aligned with Seq-Man and Megalign (DNASTAR Package). The aligned sequences were imported into PAUP 4.0b10 (Swofford, 2002).

RESULTS AND DISCUSSION

Isolation of marine fungi and screening for their antimicrobial activity

A total of 73 fungal isolates were obtained from various marine sources including sponges, soft corals, seaweeds and the sediment from the Son Tra Peninsula. Among 73 isolates, 19 were isolated from sponges, 17 from soft corals, 32 from seaweeds and only 5 from sediments (table 1).

Table 1. A list of marine sources and the number of fungi isolated

Sources and collected sites	Number of samples collected	Number of fungi isolated
Huc Lo (16°11' N; 108°31' E) Sponges		
Soft coral	3	7
Seaweed	1	2
	1	3
Bai Nom (16°10' N; 108°29' E)		
Sponge	1	4
Soft corals	3	5
Seaweeds	4	6
Sediment	2	2
Bai But (16°09' N; 108°28' E)		
Sponges	3	6
Soft corals	5	8
Seaweeds	4	10
Hon Sup (16°08' N; 108°26' E)		
Sponge	1	1
Soft corals	3	3
Seaweeds	7	13
Sediment	2	3
A total number of isolates	40	73

Using the disc diffusion assay screening, crude extract of 73 marine fungi isolates were tested for their antimicrobial activity. Of 73 marine fungi, 32 isolates displayed activity against bacteria or yeast, with the majority of them being active against *B. cereus* (31 strains), *S. faecalis* (24 strains), *S. aureus* (16 strains) and *L. monocytogenes* (23 strains). Only 4 fungal extracts showed antiyeast against *C. albicans* (table 2). The crude extracts of 32 strains exhibited different levels of inhibitory activity against pathogens indicating the presence of multiple fungal metabolites with antibacterial property.

Antimicrobial activity of the fungal extracts was more common against Gram-positive bacteria than Gram-negative bacteria. The proportion of isolates showing antimicrobial activity to Gram-positive bacteria, Gram-negative bacteria and yeast was 38% (28/73), 6% (5/73), and 5% (4/73), respectively. This observation was corroborated with that of Christophersen et al. (1999), Holler et al. (2000) and Suay et al. (2000). Such different susceptibility of gram-positive and gram-negative bacteria against fungal antimicrobial activity have been repeatedly explained to the different cell wall structure of gram-positive and gram-negative bacteria. The cell walls of gram-positive bacteria are less complicated and lack the natural sieve effect against large molecules (Hawkey, 1998). In contrast, the outer membrane structure and the periplasmic space present in Gram-negative bacteria are

thought to provide an additional degree of protection against antibiotics targeting the cell wall (Basile et al., 1998).

In this study, the fungal strains 168ST.16.1, 168ST.35.2 and 168ST.51.1 (figure 1), isolated from seaweeds *Padina* sp., *Actinotrichia fragilis* and *Caulerpa* sp., respectively, exhibited broad spectra of antimicrobial activity against most of Gram-positive, Gram-negative bacteria and yeast with high inhibition zone. Xu et al. (2015) also reported that seaweeds are one of the most common materials for the isolation of fungal strains producing antibacterial and antifungal compounds.

Table 2. Antimicrobial activity of marine fungi against pathogens

Microbes	Antimicrobial activity (zone of inhibition in mm)						
	B. cereus	*S. faecalis*	*S. aureus*	*L. monocytogenes*	*E. coli*	*P. aeruginosa*	*C. albicans*
168ST.01.1	8	8	-	-	-	-	-
168ST.02.1	9	7	10	10	-	-	-
168ST.02.2	16	12	20	21	-	-	-
168ST.03.2	10	9	-	-	-	-	-
168ST.06.1	9	10	10	17	-	-	-
168ST.09.1	7	8	-	-	-	-	-
168ST.09.2	12	10	23	22			
168ST.09.3	22	20	21	27	-	-	8
168ST.09.4	10	9	28	17			
168ST.11.1	11	14	25	26	21	-	-
168ST.14.2	9	9	10	16	-	-	-
168ST.15.2	20	17	-	33	26	-	-
168ST.16.1	**35**	**34**	**32**	**29**	**34**	**19**	**17**
168ST.23.1	12	10	-	-	8	-	-
168ST.26.1	14	11	-	-	-	-	-
168ST.34.1	7	-	-	-	-	-	-
168ST.34.2	9	-	-	-	-	-	-
168ST.35.1	9	-	-	10	-	-	-
168ST.35.2	**22**	**23**	**24**	**25**	**31**	**14**	**13**
168ST.36.1	13	10	-	13	-	-	-
168ST.36.3	8	11	-	12	-	-	-
168ST.40.1	9	-	13	10	-	-	-
168ST.40.2	9	10	10	10	-	-	-
168ST.45.1	13	9	8	12	-	-	-
168ST.49.2	-	-	-	9	-	-	-
168ST.51.1	**28**	**26**	**32**	**34**	**31**	**18**	**15**
168ST.54.1	9	-	-	11	-	-	-
168ST.54.2	8	7	-	14	-	-	8
168ST.54.3	7	-	-	9	-	-	-
168ST.56.1	9	-	15	15	-	-	-
168ST.56.3	10	9	-	-	-	-	-
168ST.59.2	20	17	18	21	-	-	-

"-": No active against pathogens

The analysis of the ITS gene sequences is an important tool for accurate indentification of fungal species (Wiese et al., 2011). The strain 168ST.16.1, phenotypically similar to *Aspergillus* spp., showed

100% sequence identity (540/540 bp) to a reference sequence of *A. flocculosus* in GenBank (NCBI accession no. EU021616.1). Two other strains (168ST.35.2 and 168ST.51.1) having high antimicrobial activity were not identified yet.

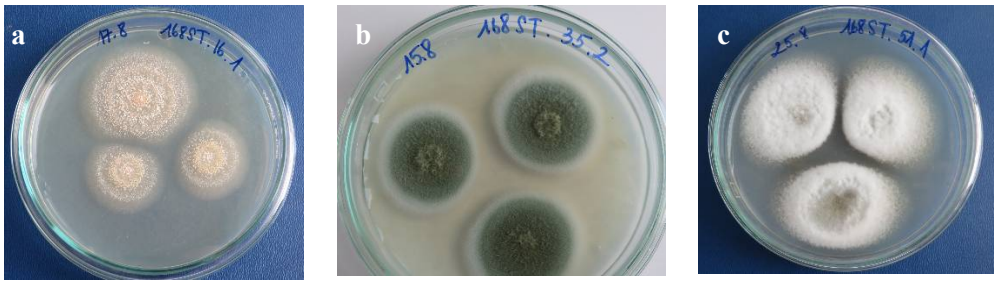

Figure 1. Colonies of marine-derived fungi 168ST.16.1 (a), 168ST.35.2 (b) and 168ST.51.1 (c).

The genus *Aspergillus* has more than 100 species, and belongs to the Ascomycota division, Deuteromycota subdivision, Hyphomycetes class, Moniliales order, Moniliaceae family (Feitosa et al., 2016). The fungal species are widely found in nature and diversified in marine ecosystems. They are well known to produce antimicrobial and anticancer compounds, bio-surfactants, etc. Thus, the *Aspergillus* fungi have been considered as an important source of natural products useful for exploration in medicine, agriculture and industry (Petersen et al., 2015). However, among genus *Aspergillus*, *A. flocculosus* has not been investigated extensively either for its chemistry or for its activity. Therefore, the strain *A. flocculosus* 168ST.16.1 need to be studied in future for new bioactive compounds.

CONCLUSION

In the present study, we have highlighted the potential antimicrobial activity of fungal isolates obtained from various marine sources in the Son Tra Peninsula, Da Nang, Vietnam. Among 73 marine fungi, 29 isolates have antimicrobial activity against at least two pathogens tested. Three strains (168ST.16.1, 168ST.35.2 and 168ST.51.1) isolated from seaweeds illustrated significant antimicrobial activity to all pathogens tested. Advanced studies of these potential fungal strains for bioactive secondary metabolites are needed for further application.

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