Vietnam Academy of Science and Technology

Vietnam Journal of Marine Science and Technology

journal homepage: vjs.ac.vn/index.php/jmst

Antimicrobial activity assessment of crude broth fermented extracts of marine-derived fungi collected from Bai Tu Long Bay (North Vietnam)

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Received: 22 August 2023; Accepted: 23 December 2023

ABSTRACT

This study aimed to isolate and identify marine fungi from Bai Tu Long Bay and assess their antimicrobial potential. We successfully isolated twenty strains of marine-derived fungi. The crude extracts from these fungi were tested against pathogenic microorganisms. All twenty strains exhibit some degree of growth inhibition against the tested microorganisms. Notably, strains M223, M250, M253, and M256 showed significant antimicrobial activity, with MIC values equal to or lower than the positive control. These results highlight the potential of marine fungi as a rich source of antimicrobial agents, a finding of considerable importance to marine mycology and pharmaceuticals. Further analysis was conducted on four promising isolates. M253 was identified as *Hamigera avellanea*, while M223, M250, and M256 were found to belong to the *Aspergillus* genus. These isolates were then analyzed using a phylogenetic tree based on MegaX software.

Keywords: *Aspergillus*, Antimicrobial activity, MIC, Marine fungi, *Hamigera avellanea*.

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https://doi.org/10.15625/1859-3097/18868
ISSN 1859-3097; e-ISSN 2815-5904

INTRODUCTION

Marine is home to many potential microorganisms, including bacteria, fungi, viruses and etc. According to the latest report on www.marinefungi.org until 15/7/2022, 1,857 marine fungi species are distributed in 769 genera, 226 families, 88 orders, 22 classes, and 7 phyla.

Microfungi are biochemically diverse groups and are essential in terrestrial and marine environments. Harsh biological, physical, and chemical driving variables and internal properties influence the marine ecosystem. Recent years have seen a massive increase in study of diversity and function of fungi in marine habitats. Biotechnology advancements can help discover potential bioactive secondary compounds from marine fungi [1, 2].

Lately, researchers have focused on discovering marine fungi to find new compounds with biologically active properties; after that, many fungal secondary metabolites with effectiveness against bacterial infections are reported. The major bioactive compounds produced by marine fungi are alkaloids, polyketides, terpenes, isoprenoid, peptides, quinones [3]. Research on the biological activities of marine fungi in Vietnam has published some studies by the Institute of Marine Biochemistry, VAST, showing the great potential of fungi collected from Vietnam's beaches [4]. Research in marine mycology reveals a variety of fungal secondary metabolites compounds with a comprehensive range of antibacterial and antifungal activities. Specifically, in the pharmaceutical area, marine fungal secondary metabolites exhibit activities that have huge potential for medicines and agrochemicals discovery [2, 5]. This potential is the cornerstone of our research, as it could revolutionize the pharmaceutical industry. Exploiting bioactive compounds from microorganisms has the advantage that over-exploitation of macro-organisms lead to ecological imbalance, biodiversity loss, and maybe extinctions, and those extracts were obtained in limited amounts. In contrast, microorganisms can multiply in large quantities by industrial fermentation.

MATERIALS AND METHODS

Collection and processing of marine samples

The samples were collected from Bai Tu Long Bay at various locations in the North of Viet Nam, at a depth of 7–12 m. They were subdivided using disinfected scissors and placed in sterilized falcons filled with seawater. During transport to the Institute of Marine biochemistry's laboratory, VAST, the samples were kept on ice. Water depth, temperature, coordinates, and other parameters were recorded during sampling process.

The specimens were washed with autoclaved seawater to clean garbage and samples were then cut into small pieces. Aliquots of 50 µL serial homogenized, diluted solution of the marine samples were spread evenly on isolation media A1, SWA, Czapek, ISP2, PDA agar and incubated at 37^{o}C for about 2–4 weeks. The agar dishes were observed daily, and then independent fungi clone transferred to new fresh PDA media. New fungal hyphae are usually formed by the emergence of powdery or fuzzy colonies and stick firmly to the agar surface. The colony morphology characteristics were recorded: size, color, wetness, and overall surface shape [4, 6].

Fermentation, production of crude extracts

Fungal isolates were fermented in 50 L PDA media (Potato extract: 4 g/L, Dextrose: g/L) for about 14 days at 25^{o}C and 150 rpm. After 14 days, the mycelium was separated from the broth, and the extraction was crushed with ethyl acetate 5 times (5 × 35 L). The extracts were evaporated under reduced pressure to yield crude extracts. The crude extract was re-diluted in DMSO to make a stock solution at a decreasing concentration range: 256, 128, 64, 32, 16, 8, 4, and 2 µg/mL [7, 8].

Antimicrobial assay

Antimicrobial properties of fungus extracts were determined according to the modified agar dilution methods in flat-bottom 96-well, transparent microtiter plates using pathogenic three Gram-negative bacteria (*Escherichia coli*

ATCC25922 (E.C), *Pseudomonas aeruginosa* ATCC27853 (P.A), *Salmonella enterica* ATCC13076 (S.A)), and three Gram-positive bacteria (*Enterococcus faecalis* ATCC29212 (E.F), *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC 14579 (B.C)) and one yeast *Candida albicans* ATCC10231 (C.A)).

MIC assay uses broth dilution methods to determine the lowest concentration of an agent that inhibits visible growth (97–100%) of a test microorganism. The dilution yielded a starting inoculum of approximately 5×10^5 CFU/mL. MIC results were assessed after incubation for some time (16–20 h) at 37°C. Streptomycin (Sigma) and cycloheximide (Merck) were positive reference compounds for bacteria and fungi. Afterward, the turbidity intensity of each dilution concentration was measured at 650 nm by a BioTek microplate reader. Growth curves were determined by measuring turbidity, fluorescence, and Raw Data software [7, 8].

Identification of fungi and phylogenetic analysis

The isolate's DNA was extracted, and the fungi were identified up to the genus level using replication of 18s rRNA region with primer sequences: NS3F (5'-GCAAGTCTGGTGC CAGCAGCC-3') and NS8R (5'-TCCGCAGGTTCACC TACGGA-3'). PCR mix consisted of 2.0 µL fungi DNA, 25 µL Master Mix 2X, 19 µL of H_2O, and 1.0 µL of 10 pMol for each primer. The PCR products were checked on 1% agarose and then sequenced by ABI PRISM 3100, Applied Bioscience. Forward and reverse sequence chromatograms were checked for ambiguity, and edited, assembled using BioEdit version 7.2. The sequences of fungal isolates were compared with fungal 18S rRNA sequences in the GenBank database by the BLAST online program at NCBI. The phylogenetic tree was created using the Kimura 2-parametric model and using the Neighbour-Joining method in MEGA X software [9, 10].

RESULTS AND DISSCUSSION

The research team commits that using samples in this study complies with international guidelines and considers the conservation of marine resources.

Sample collection and isolation of marine fungi

Table 1. Fungi collection

No.	Samples	Organisms	Water depth	Sampling Geographic coordinates	Isolated agar medium
1	M220	Sediment	3 m	23°03’22”-107°27’30”	ISP2
2	M221	Soft coral	3 m	23°03’22”-107°27’30”	SWA
3	M222	Sea animal	7 m	21°00’55.3”-107°26’45.3”	Czapek
4	M223	Mollusca	12 m	20°58’06.4”-107°27’17.3”	PDA
5	M224	Soft coral	3.5 m	21°03’22”-107°27’30.7”	PDA
6	M225	Mollusca	12 m	20°58’06.4”-107°27’17.3”	SWA
7	M240b	Sponge	6.5 m	21°02’38.2”-107°32’38.9”	Czapek
8	M227	Seaweed	4 m	20°55’59.6”-107°26’24.7”	PDA
9	M247	Sediment	4 m	20°55’59.6”-107°26’24.7”	SWA
10	M248	Coral	7 m	21°00’55.3”-107°26’45.3”	Czapek
11	M249b	Sponge	6.5 m	21°02’38.2”-107°32’38.9”	SWA
12	M250	Crustacea	7 m	20°58’60.6”-107°33’78.2”	PDA
13	M251	Sediment	7 m	20°58’60.6”-107°33’78.2”	Czapek
14	M252	Coral	7 m	21°00’55.3”-107°26’45.3”	Czapek
15	M253	Sponge	7 m	21°00’55.3”-107°26’45.3”	PDA
16	M255	Crustacea	7 m	20°58’60.6”-107°33’78.2”	Czapek
17	M256	Sponge	7 m	20°59’21.9”-107°34’71.9”	PDA
18	M260	Sponge	7 m	20°59’21.9”-107°34’71.9”	PDA
19	M261	Sponge	7 m	21°00’55.3”-107°26’45.3”	ISP2
20	M264	Sponge	6.5 m	21°02’38.2”-107°32’38.9”	PMDA

Marine samples were taken with SCUBA diving at a depth between 3–12 m under sea level, and the water temperature ranged from 27–30^{o}C in different geographic coordinates in Bai Tu Long Bay. The colony was purified by subculturing the independent colony on selective agar several times and then transferred to Czapek culture media. There were 20 isolates successfully collected from eight locations. PDA medium was the most preferred medium for fungi isolated (35%), while the lowest amount isolated from PMDA. The detailed results are shown in Table 1.

A total of 20 isolates: 7 isolates from sponge, 2 isolates from sediment, 2 isolates from seaweed, 2 isolates from coral, 2 isolates from soft coral, 2 isolates from crustacea, 2 isolates from mollusca and 1 isolate from sea animal.

Antimicrobial assay

Screening of antimicrobial activity of 20 isolates were done by bioassay method. All in vitro experiments were repeated three times and the MIC of the extracts was consistent. MIC value corresponded to the minimum concentration of the compound that caused more than 99% test bacterial/yeast cell inhibition. The result of antimicrobial assay is presented in Table 2.

Table 2. The result of antimicrobial assay

No.	Fungal samples	Antimicrobial activity of crude ethyl acetate extracts of 20 isolates						
		Gram-positive			Gram-negative			Yeast
		E.F	S.A	B.C	E.C	P.A	S.E	C.A
MIC values (µg/mL)								
1	M220	32	-	-	-	-	-	16
2	M221	128	-	-	-	-	-	128
3	M222	64	-	-	-	-	-	16
4	M223	32	256	256	-	-	-	32
5	M224	16	-	-	-	-	-	-
6	M225	64	256	256	-	-	-	64
7	M227	64	-	-	-	-	-	-
8	M240b	32	-	-	-	-	-	64
9	M247	32	-	-	-	-	256	2
10	M248	128	-	32	-	-	-	-
11	M249b	128	-	-	-	-	-	256
12	M250	32	128	256	-	-	-	16
13	M251	32	-	-	-	-	-	32
14	M252	64	-	256	-	-	-	16
15	M253	2	16	4	256	128	128	2
16	M255	128	256	256	-	-	-	64
17	M256	16	32	16	-	128	-	2
18	M260	64	-	256	-	-	-	64
19	M261	64	256	256	-	-	-	32
20	M264	128	-	256	-	-	-	32
Streptomycin		256	256	128	32	256	128	-
Cyclohexamide		-	-	-	-	-	-	32

Notes: Streptomycin and Cycloheximide: Positive control; (-): Inactive.

As a conclusion of these experiments, twenty isolates could inhibit at least one pathogenic microorganism strain used in this study. 11/20 fungi strains displayed positive antibacterial activity against 3 to 7 test bacteria. Of the 20 isolates, M253 being the only strain active against all the test strains, including 3 Gram-negative bacteria, 3 Gram

positive bacteria and one yeast with MIC values equal to or lower than the positive control. Four strains, M223, M250, M253, and M256 combined the largest broad-spectrum with excellent efficacy against test organisms. Four isolates showed suitable activities were selected for further characterization and analysis.

Identification and phylogenetic analysis

The four potent strains were cultured on the Czapek medium for about 7 days at 25°C to observed colony morphology, substrate mycelium color, and gaseous mycelium color. A Japan's Nikon ECLIPSE 80i optical microscope was used to observe shape and size of mycelium, spore-generating organs, conidia, naked spores, and sporangia.

Description strain M223: Colonies have reached 2.5–3.5 cm diam on Czapek after 10 days at 25°C. The colony is flat, with a velvety surface, gray green to brownish-green, exudate solutions were clear and colorless, reverse side is pale yellow or mushroom color. Conidiophores 140–550 μm × 3.5–8.0 μm, colorless to light brown, thick and smooth walls. Conidia clavate to sub-globose, size 5.0–18 μm, spores near spherical or globose, size 3.0–4.0 μm, rough spines. Hüll cells are occasionally produced, spherical to globose in shape, 9–14 μm in size (Fig. 1).

Figure 1. Colony morphological characteristics of the strain M223. (A) Colonies on Czapek medium at 25°C/7 days; (B) Spore production in M223; (C) Spores × 1000

Description strain M250: Colonies attain 2.0–2.5 cm on Czapek medium. The colony's surface of the color is velvet; the center is blue green to gray green, the edge is white, and the reverse is pale yellow to orange. Exudate solutions were colorless to reddish brown. Conidiophores are 90–750 μm × 4.5–8.0 μm in size, colorless to light brown, thick and smooth walls. Conidia is nearly globose to pear-shaped or elliptical, 8–19 μm in size, spores form near spherical to spherical, size 3.0–3.8 μm, rough spines. Hüll cells are spherical to globose in shape and are 10–15 μm in size (Fig. 2).

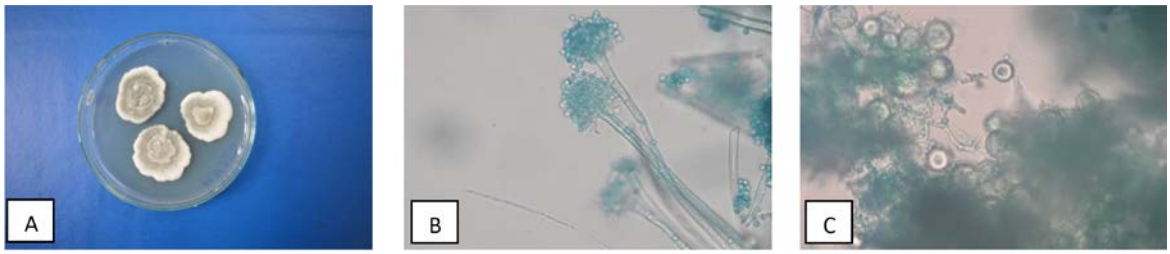

Figure 2. Colony morphological characteristics of the strain M250. (A) Colonies on Czapek medium at 25°C/7 days; (B) Spore production in M250; (C) Hüll cells × 1000

Description strain M253: Colonies grown on Czapek medium reached 2.0–3.0 cm/7 days at 25°C. Colonies are usually septate, smooth, tight, thick, or lightly porous. The mycelium is pale yellow brown to light orange or gray, with a reverse side to yellow-gray. Conidiophores

are 100–200 μm × 2.5–3.5 μm in size, but sometimes shorter, smooth, or slightly rough. Conidia is nearly globose, 6.0–9.0 μm in size, spores form near spherical size 2.2–2.5 μm, in size, smooth or rough, forming short columns (Fig. 3).

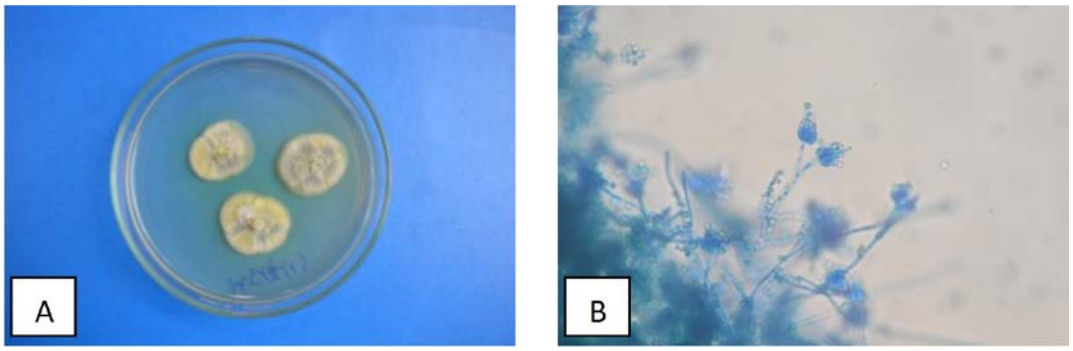

Figure 3. Colony morphological characteristics of the strain M253. (A) Colonies on this Czapek medium at 25°C/7 days; (B) Spore production in M253

Description M256 strain: Colonies are slow growing on Czapek medium, diameter is 1.5–2.0 cm after 10 days at 25°C, flat, anomalous edges are yellowish white, mossy green in the middle colorless reverse side, secretions, Hüll cells were not seen. The infertile mycelium is often rough, with pimples. Conidiophores are thick, smooth wall of 50–70 μm × 4–6 μm,. Conidia is nearly globose, 7–12 μm in diameter. Globose spores are smooth to rough, 2.5–3.5 μm in diameter (Fig. 4).

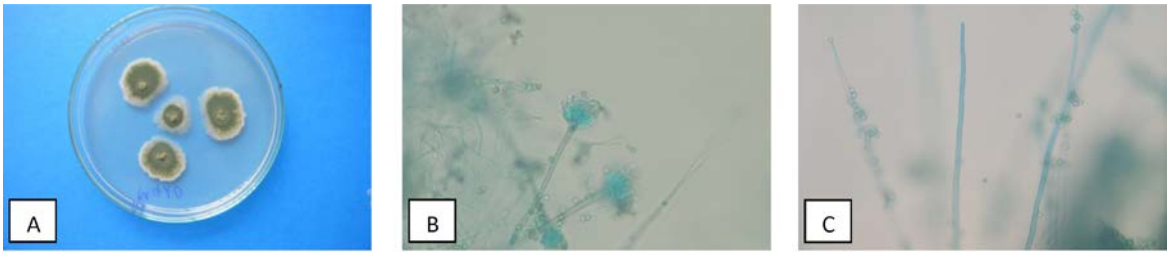

Figure 4. Colony morphological characteristics of the strain M256. (A) Colonies on this Czapek medium at 25°C/7 days; (B) Spore production in M256; (C) Infertile mycelium × 1000

The method is based on independent PCR amplification and sequencing of 18S rRNA region for molecular identification because it is conserve region for fungi. Phylogenetic analysis of the gene sequence of the strains M223, M250, M256 and related taxa revealed their more than 98% similarity to the *Aspergillus* spp. In contrast, the comparative sequence analysis revealed that the 18S rRNA sequence of M253 was highly homologous to that of the *Hamigera avellanea* (99.41%).

BLAST homology search result of the four selected fungal strains could be seen at Table 3 and the phylogenetic tree is shown by Fig. 5.

The tree shows the affiliations of the 12 fungal strains based on 18S rRNA gene sequencing and constructed by MEGA-X software. Evolutionary distances were computed using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. There were a total of 569 positions in the final dataset. The bar length represents 0.01 substitutions per nucleotide site.

The 18S rRNA sequences of the four potent strains have been registered on

GenBank under the accession numbers: M253; OR166097.1 for M250 and OR166093.1 for M223; OR166103.1 for OR166104.1 for M256.

Table 3. Homology of four potential isolates

Fungi isolate	Molecular identification (BLAST closest relatives)	Ident. percentage
M223	*Aspergillus versicolor* NRRL 238 NG_067623.1	99.67%
M250	*Aspergillus versicolor* NRRL 238	98.71%
M253	*Hamigera avellanea* CBS 295.48 NG_061105.1	99.41%
M256	*Aspergillus nidulans* ATCC 10074 NG_064803.1	99.51%

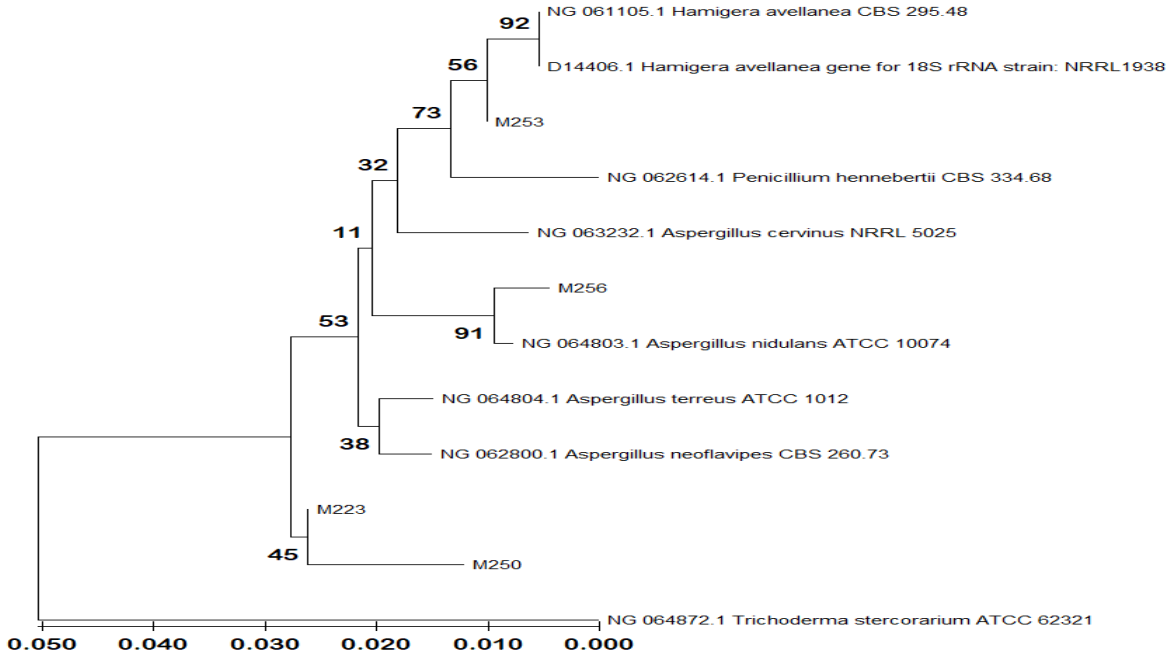

Figure 5. Evolutionary relationships of strains M2223, M250, M253 and M256

Discussions

Recently, many vital compounds from marine fungi that possess antibacterial and antifungal properties have been found. In a study by Cen et al., (2021), from deep-sea fungus, *Aspergillus* sp. CSYZ-1, one compound (3,5-dimethylorsellinic acid-based meroterpenoid) was extracted. This compound showed perfect antimicrobial activity against *H. pylori* with MIC values of around 1–4 and 2–16 µg/mL, respectively [11]. Xu et al., 2021, successfully isolated a trypacidin compound from the fungus *Aspergilus fumigatus* HX-1 associated with clams. That compound showed high *Vibrio harveyi* inhibitor activity with the minimum inhibitory concentration was 31.25 µg/mL. In a study by Machado et al., four compounds was isolated from the marine *Aspergillus flavus* KUFA1152 showed antibacterial activity against some pathogenic *S. aureus* multidrug-resistant strains, *S. aureus*, *E. faecalis* with low MIC values ranging from 4 to 16 µg/mL [6]. Masahiko Isaka et al., (2010) reported the marine fungus *Hamigera avellanea* BCC 17816 produced 14-membered macrolides. However, the biological activity of these substances has not been shown [12].

Only some articles are written clearly on marine *Hamigera avellanea*'s biological activities. The crude ethyl acetate extract of a marine fungi, *Hamigera avellanea* KUFA0732, which was collected from Thailand, exhibited antifungal activities against eleven plant

pathogenic fungi (*Alternaria brassicicola*, *Bipolaris oryzae*, *Colletotrichum capsici, C. gloeosporiodes*, *Curvularia oryzae*, *Fusarium semitectum*, *Lasiodiplodia theobromae*, *Phytophthora palmivora*, *Pyricularia oryzae*, *Rhizoctonia oryzae* and *Sclerotium rolfsii*) [13].

According to a study of Song et al., one strain *Aspergillus* sp. capable of cytotoxicity against the mouse lymphoma cell line, was isolated from sponge [14].

Many genus of fungi were found from marine habitats like *Rhodotorula*, *Aspergillus*, *Cladosporium*, *Penicillium*, *Alternaria*, *Fusarium*, *Engyodontium*, *Sistotrema*, *Schizophyllum*, *Tilletiopsis*, etc. The most common frequency are *Penicillium* and *Aspergillus* [15].

CONCLUSIONS

There were 20 marine fungi successfully isolated from Bai Tu Long Beach samples. The MIC result indicated the antimicrobial activity of the 20 fungal strains. Among 20 fungi, 4 isolates (M223, M250, M253, M256) showed good antimicrobial activity against at least 4 tested strains. The best MIC produced by the M253 strain. The lowest MIC value of 2 µg/mL was obtained with M253 broth extract against *E. faecalis* and *C. albicans*.

Genus's level and species-level identification was detected from molecular data for four candidate strains. BLAST homology search of the four fungi with the best antibacterial and antifugal properties resulted in fungus, which M253 was identified as *Hamigera avellanea*, while M223, M250, M256 belonged to *Aspergillus* genus. The four sequences were submitted to the NCBI GenBank database.

Although the marine-derived genus *Aspergillus* is widely recognized as being able to synthesize yield of biologically active natural products, but the marine *Hamigera avellanea* species has very few published reports on biological activity. The result of this study would be a good sign for further research into less common fungal strains in marine habitats.

Acknowledgement: This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 104.01-2019.306.

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