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Evaluation of cytotoxic activity of marine fungi isolated from sponges in Nha Trang bay

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ABSTRACT

Marine fungi are evaluated as a potential source for new natural compounds with bioactivities of pharmaceutical values. In this study, 66 fungal strains, isolated from 37 sponge samples in Nha Trang bay, were determined for cytotoxic activity against two human cancer cell lines, including cervical cancer (Hela) and breast cancer (MCF-7) cells using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The results showed that 46.9% (31/66) strains exhibited cytotoxic activity against both test cancer cell lines. Based on the internal transcribed spacer (ITS) gene sequences analysis, five isolates with significant anticancer activity were identified as *Aspergillus* sp. 1901NT-1.2.2, *Talaromyces* sp. 1901NT-1.39.3, *Aspergillus subramanianii* 1901NT-1.40.2, *Phoma* sp. 1901NT-1.45.1, and *Penicillium* sp. 1901NT-2.53.1. Our finding indicated that the sponge-derived fungi in Nha Trang bay might be a potential source for anticancer compounds and need further study to discover new anticancer drugs.

Keywords: Cytotoxic activity, marine fungi, anticancer compounds, sponges.

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INTRODUCTION

Cancer is one of the leading causes of mortality among humans in many countries and is increasing rapidly due to the quality of food sources and the living environment [1]. In 2020, approximately 19.3 million new cancer cases were reported globally, resulting in about 10 million deaths [2]. New medicines with novel mechanisms of action are desperately needed; therefore, much research has been done on new anticancer therapies derived from natural sources, including plants, microorganisms, and marine creatures [3]. Mainly, marine fungi offer the potential for discovering novel products that might help with cancer prevention and therapy [4, 5].

Marine fungi are considered a group of microorganisms with high diversity that can be isolated from various sources, including seawater, sediments, or associated with seaweeds, corals and sponges [6]. The studies have estimated there are more than 10,000 marine fungal species, of which the majority belong to the *Ascomycota* and *Basidiomycota* phyla [7]. In particular, the number of fungal strains obtained from sponges accounts for a high proportion. The reports demonstrated that marine fungi account for up to 50% of the host's biomass and can be easily obtained from the internal tissues of sponges [8]. Moreover, microorganisms living in symbiosis with sponges are diverse and capable of biosynthesizing many bioactive compounds due to their essential roles in host metabolism [9]. With high species diversity and wide distribution, sponges are considered a valuable source to acquire many marine fungal species capable of generating new secondary metabolites [10]. Interestingly, many studies demonstrated that most of the medicinally useful secondary metabolites obtained from sponges are biosynthesized by symbiotic microorganisms [11]. In recent years, over a thousand secondary metabolites isolated from marine fungi have a variety of medical applications, with several serving as anticancer agents [12, 13].

Nha Trang bay is assessed to have a diverse marine ecosystem with numerous flora and fauna living in symbiosis, including 89 species of sponges present [14]. Therefore, this study was conducted to evaluate the cytotoxic activity of sponge-derived fungi in Nha Trang bay and select potential isolates for further investigation of anticancer compounds.

MATERIALS AND METHODS

Sponges samples

The sponge samples were collected in Nha Trang bay ($12^{o}10'$N; $109^{o}16'$E) by SCUBA diving at 8–10 m. The specimens were placed in zip-lock plastic bags containing fresh seawater, preserved in an icebox, and transported to the laboratory.

Fungal isolation

Specimens of sponges were rinsed with sterile seawater three times to remove contaminants and sediments. Approximately 1 g of sponge sample was homogenized with 10 mL of pure seawater using a sterile pestle and mortar. Then 0.1 mL of the suspension was spread on Petri dishes containing modified Sabouraud agar (peptone 10 g, glucose 40 g, agar 18 g dissolved in 1,000 mL seawater, pH 6.0–7.0) [15]. About 100 mg/L of amoxicillin was added to the solution to avoid bacterial growth. After three days of incubation at 28^{o}C, morphological fungi were observed and checked daily for four weeks. Each fungal isolate was individually picked and continuously transferred onto a new Sabouraud plate without antibiotics until a pure colony was obtained. Mycelial plugs of these fungal strains were cut and stored in 40% glycerol at -80^{o}C to maintain a fungal collection.

Fungal cultivation and extraction

Each fungal strain was cultivated in a 500 mL Erlenmeyer flask containing rice medium (rice 20 g, yeast extract 20 mg, KH_2PO_4 20 mg, seawater 40 mL). The fungi inoculated on the Sabouraud plate for 5–7 days were aseptically cut into small pieces (1 cm × 1 cm) and transferred into flasks. After three weeks of incubation at 28^{o}C, the metabolites produced by each fungal isolate, released into the culture, and retained in the

mycelial biomass were extracted with ethyl acetate (200 mL/flask) for 24 h. The ethyl acetate extracts were separated from the culture medium by collecting supernatant solvents and concentrated using a vacuum rotary evaporator at 40^{o}C [16]. The obtained crude extracts were kept at -20^{o}C for bioassays and further analyses.

Screening of cytotoxic activity of crude extracts from isolated fungi

The fungal extracts were evaluated for their cytotoxic activity against two cancer cell lines, including cervical cancer (Hela) and breast cancer (MCF-7), based on the MTT method [17]. The cells were cultured in DMEM medium (Biolot, St. Petersburg, Russia) containing 10% fetal bovine serum (Biolot, St. Petersburg, Russia) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37^{o}C in a humidified atmosphere with 5% (v/v) CO_2. Cell suspensions (5×10^3) were incubated with the fungal extracts (100 µg/mL) prepared in dimethyl sulfoxide (DMSO) for 24 h. The absorbance of the converted formazan was measured using a Multiskan FC microplate photometer (Thermo Scientific, USA) at λ = 570 nm with background subtraction at λ = 630–690 nm. The experiments were performed in triplicate, and the results were presented as a percent of control data.

Fungal identification

The genomic DNA of the selected fungal strains with significant bioactivities was extracted using a NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA) following the manufacturer's instructions. The DNA (50–300 ng/µL) of each strain was used as a template for polymerase chain reaction (PCR), using ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'- TCCTCCGCTTATTGATA TGC-3') primers [18]. The fungal internal transcribed spacer regions sequences were compared with related sequences in the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSIONS

This study evaluated ethyl acetate extracts of 66 fungal strains isolated from 37 sponge samples in Nha Trang bay for cytotoxic activity against two cell lines, including cervical cancer (Hela) and breast cancer (MCF-7). The results showed that 46.9% (31/66) of the fungal isolates exhibited growth inhibition against both test cancer cell lines. These fungal strains were able to inhibit more than 50% viability of the tested cell lines compared with the control sample. Besides, a higher number of fungal extracts exhibiting cytotoxic activity towards MCF-7 cells (74.2%, 49/66) than those for Hela cells (51.5%, 34/66) was observed (Table 1). Among the fungal strains showing significant cytotoxic activity, five isolates were identified based on ITS gene sequence analysis, including *Aspergillus* sp. 1901NT-1.2.2 (MN577307), *Talaromyces* sp. 1901NT-1.39.3 (OL719301), *Aspergillus subramanianii* 1901NT-1.40.2 (MN577309), *Phoma* sp. 1901NT-1.45.1 (MN585773), and *Penicillium* sp. 1901NT-2.53.1 (OL719302). The ITS sequences from five isolates shared 99–100% similarity with known fungal strains in GenBank. The fungal strains 1901NT-1.2.2, 1901NT-1.39.3, 1901NT-1.45.1, and 1901NT-2.53.1 shared 99% similarity with the fungi belonging to genera *Aspergillus*, *Talaromyces*, *Phoma*, and *Penicillium*, respectively. The fungus 1901NT-1.40.2 showed 100% similarity to *Aspergillus subramanianii* NRRL 5170. The phylogenetic tree based on rDNA-ITS sequences of the selected marine fungal strains and related sequences from GenBank was constructed using neighbor-joining (NJ) with 1,000 bootstrap replicates and implemented in MEGA 7.0 (Figure 1).

Many marine natural products have been shown to have anticancer activity *in vitro* against a variety of tumor cell lines, including renal, lung, prostate, bladder, melanoma, osteosarcoma, mammary, and lymphoid cancer-derived cell lines. Furthermore, most reports on the mechanism of action of marine products in inhibiting tumor growth *in vitro* and *in vivo* suggested that it was mediated by tumor cell apoptosis, necrosis, and lysis. Sponge-

derived fungi have been found to have significant anticancer activity in *in vitro* and *in vivo* models and are evaluated as vital oceanic resources [4]. According to a previous study, 46.2% of fungal strains isolated from sponge *Neopetrosia chaliniformis* in Mandeh island, Indonesia, recorded cytotoxic activity against human colon carcinoma cells (WiDr) [19]. Among them, the fungus *Aspergillus nomius* ND06 exhibited activity against test cancer cells but did not affect the growth of normal cells (Vero). In another report, 5/19 fungal strains obtained from 3 sponge species, including *Tedania anhelans*, *Myxilla arenaria*, and *Callyspongia fibrosa* from Kerala, India, were also reported have effective cytotoxic activity against human lung cancer cells (NCI-H460). All five active isolates belong to the genera *Aspergillus* and *Pencillium* [20]. Another study also showed that the fungus *Aspergillus versicolor* obtained from the sponge *Petrosia* sp. exhibited significant cytotoxic activity against five cancer cell lines, including A-549, SK-OV-3, SK-MEL-2, XF-498, and HCT-15 [21]. These results have confirmed that sponge-derived fungi play an essential role in searching for anticancer compounds and drug development.

Among the fungi, *Aspergillus* spp. and *Penicillium* spp. have received much attention due to their ability to produce various classes of secondary metabolites such as alkaloids, terpenoids, xanthones, sterols, and anthraquinones with anticancer properties against a variety of cancer cells [22, 23]. In this study, all five identified fungal strains belong to the phylum *Ascomycota*, of which three isolates were classified as belonging to the genera *Aspergillus* and *Penicillium*. In our previous study, five new sesterterpenes from the marine fungus *Aspergillus flocculosus* showed potent cytotoxicity against six cancer cell lines (HCT-15, NUGC-3, NCI-H23, ACHN, PC-3, and MDA-MB-231) with GI_{50} values ranging from 0.14–2.01 μM [24]. Besides, antimicrobial and antioxidant activities were also reported from sponge-derived fungi in Nha Trang bay [25], describing cytotoxic activity of sponge-derived fungi from Nha Trang bay. The screening results indicated that marine fungal strains isolated from sponges in Nha Trang bay would be a potential source for extensive research on marine natural products to contribute to the new chemical classes of anticancer agents and offer a promising opportunity for the discovery of a new generation of anticancer drugs used in clinical trials.

Table 1. Cytotoxic activity of ethyl acetate extracts of isolated fungal strains

Oder	Fungal strains	% cell viability	
		HeLa	MCF-7
1	1901NT - 1.2.2	21,8±2,8	16,8±0,7
2	1901NT - 1.32.1	47,3±22,1	25,0±0,9
3	1901NT - 1.37.1	13,5±1,5	15,0±0,4
4	1901NT - 1.37.4	11,5±0,7	11,5±0,2
5	1901NT - 1.39.2	45,9±3,8	39,5±3,7
6	1901NT - 1.39.3	42,0±5,6	25,7±1,6
7	1901NT - 1.40.1	17,3±0,5	13,5±0,3
8	1901NT - 1.40.2	20,9±2,0	12,5±0,3
9	1901NT - 1.40.3	47,6±8,6	46,2±4,4
10	1901NT - 1.40.4	12,0±1,9	14,6±0,5
11	1901NT - 1.42.1	29,6±4,4	18,4±1,3
12	1901NT - 1.44.1	12,8±0,7	37,4±7,6
13	1901NT - 1.45.1	35,2±6,0	27,5±1,8
14	1901NT - 1.45.2	16,3±4,0	21,8±1,8
15	1901NT - 1.45.4	38,4±9,7	20,3±1,1
16	1901NT - 1.50.1	14,9±2,8	24,3±0,4
17	1901NT - 1.71.5	21,7±4,4	16,1±3,0
18	1901NT - 2.2.1	47,9±13,2	25,7±2,1
19	1901NT - 2.2.2	18,8±2,7	10,7±0,4
20	1901NT - 2.9.1	12,2±0,2	48,2±4,8
21	1901NT - 2.11.3	39,8±6,3	39,8±1,8
22	1901NT - 2.24.1	18,4±2,2	11,2±0,8
23	1901NT - 2.31.2	42,2±3,4	30,3±0,4
24	1901NT - 2.35.3	15,4±2,1	21,5±1,6
25	1901NT - 3.13.4	11,7±0,3	12,4±0,2
26	1901NT - 2.45.2	32,3±2,6	33,0±5,2
27	1901NT - 2.45.3	11,2±1,0	19,8±1,1
28	1901NT - 2.45.5	31,3±9,9	30,2±2,4
29	1901NT - 2.50.2	11,4±0,8	12,9±0,8
30	1901NT - 2.51.4	38,6±1,7	35,6±2,0
31	1901NT - 2.53.1	25,9±2,5	26,2±1,1

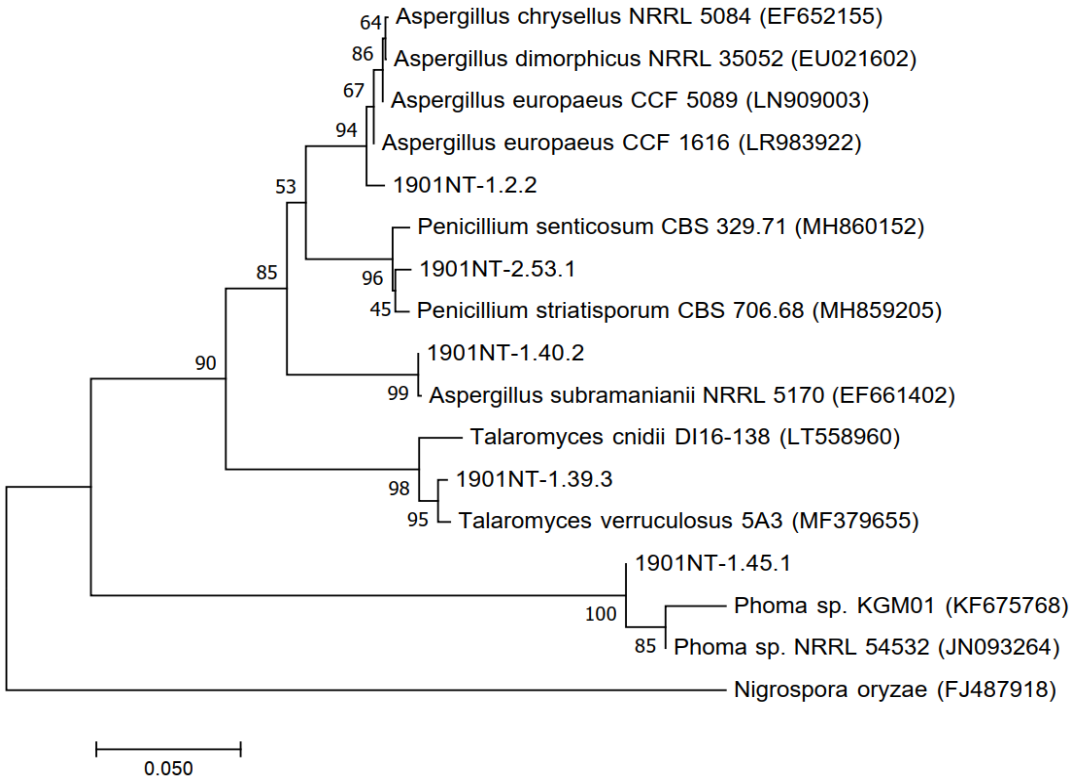

Figure 1. Phylogenetic tree based on rDNA-ITS sequences of selected sponge-derived fungi isolated in Nha Trang Bay and related sequences from GenBank

CONCLUSION

The study revealed that sponge-derived fungi in Nha Trang bay are a potential source for discovering natural substances with cytotoxic activity against Hela and MCF-7 cancer cell lines. Among 46.9% of fungal strains showing activity, 5 isolates 1901NT-1.2.2, 1901NT-1.39.3, 1901NT-1.40.2, 1901NT-1.45.1, and 1901NT-2.53.1 were identified belong to four genera *Aspergillus*, *Penicillium*, *Phoma* and *Talaromyces*. Our findings offered opportunities for further study to discover anticancer compounds from marine fungi with significant cytotoxic activity.

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