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# Evaluation of antimicrobial and cytotoxic activity of fungi isolated from marine samples collected in Cu Lao Cham sea area, Quang Nam Province

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## ABSTRACT

Marine-derived fungi repeatedly showed potential bioactive activity against strains of pathogenic microorganisms. The objectives of this study were to isolate and identify the marine fungi strains from Cu Lao Cham, Quang Nam, Vietnam and to evaluate the cytotoxic activity of the antimicrobial and cancer cells of their crude extracts. Seven media for culturing fungi were used. Forty-four independent isolates that look like fungi were successfully obtained from 15 marine samples. Each pure fungal isolate was cultivated on a PDA medium at 25$^{o}$C for about two weeks and then extracted with ethyl acetate. All crude extracts of isolated fungi were tested for antimicrobial activity against seven ATCC pathogenic strains using the broth microdilution method and cytotoxic activity using three cancer cell lines: human liver cancer HepG2, human breast cancer MCF-7, and human lung cancer A549. Of forty-four fungal isolates, 32/44 strains showed antimicrobial activity against at least one ATCC pathogenic strain. Notably, 3/44 extracts (VH7, VH26, VH29) showed remarkable effects of antimicrobial activity against more than four pathogenic strains. The cytotoxicity results showed that 5/44 isolates (VH7, VH15, VH26, VH29, VH30) had potential activity with the percent growth inhibition of cells (% Inhibition ± SD) more than 50%. Three potential strains VH7, VH26, and VH29 were selected for further studies. Molecular identification showed that all three promising candidates belong to *Aspergillus versicolor* and their 18S rRNA gene sequences were deposited in GenBank under accession numbers PP762309 for VH7, PP767325 for VH26, and PP809383 for VH29. These potent strains are being further investigated for their ability to produce bioactive compounds.

**Keywords:** *Aspergillus* sp., antimicrobial activity, Cytotoxic, MIC, marine fungi.

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## INTRODUCTION

Marine ecosystems contain substantial concentrations of microorganisms that include fungi. Microorganisms can be multiplied to large yields by controlled fermentation methods. Marine fungi are a collection of fungi that live in marine environments; their communities are widespread throughout most ocean habitats and present on the surface water and in the deep sea. Whether marine fungi are obligate or facultative, these organisms can potentially exploit bioactive substances. Marine microorganisms, in general, and fungi, in particular, can survive and adapt to extreme conditions represents a major biological advantage. Therefore, they can produce many complex molecules with unique biologically active secondary compounds with different characteristics from terrestrial counterparts [1, 2]. According to the Mushroom Research Foundation, until 25/07/2024, the total number of marine fungi species are 2,118; distributed in 814 genera, 291 families, 110 orders, 34 classes and 10 phyla. The above include fungi documented from sporulating material in the intertidal-submerged material in the sea (drift and attached wood, leaves, fruits, pneumatophores), those isolated from seawater, deep sea, and mangrove sediments, endophytes isolated from algae, corals and submerged parts of mangrove plants and those parasitic on plankton on seagrasses (www.marinefungi.org). Aspergillus is an extensive genus and one of the most common fungi in diverse habitats. In 1729, Micheli named the fungal genus Aspergillus for the first time. They play a significant role in natural ecosystems and the global economy - data to 2024, the number of accepted species 453 for Aspergillus (https://www.myco-bank.org/). Marine Aspergillus genera are prolific producers of diverse natural products with various bioactivities such as highly cytotoxicity and antimicrobial [3, 4]. In Vietnam, several researchers focus on exploiting secondary compounds from marine microorganisms. Some valuable new compounds have been discovered, and these new compounds have shown significant biological activities [5, 6]. The Cu Lao Cham marine ecosystem is one of sixteen marine protected areas in Vietnam and has been recognized by UNESCO as a world biosphere reserve. However, this marine area's microbial resources have not been investigated and exploited. Therefore, studying and evaluating the potential antibiotic and cancer cytotoxic activity of marine microorganisms in general and marine fungi in the Cu Lao Cham marine area is considered extremely necessary.

## MATERIALS AND METHODS

### Sample collection and isolation of fungi

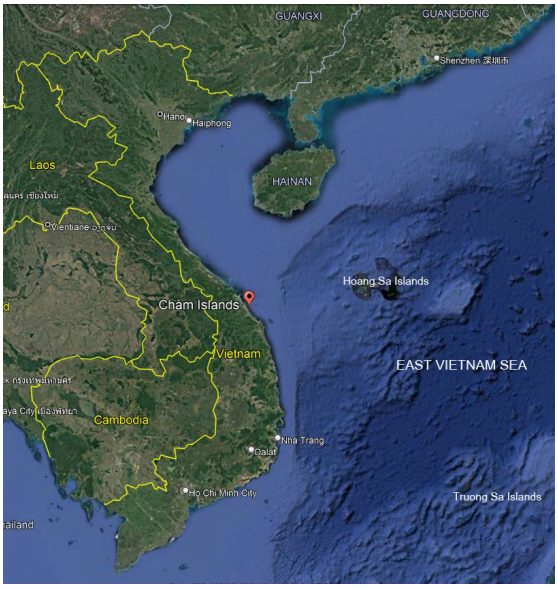



*Figure 1.* Sampling location

Samples were collected in the Cu Lao Cham sea area, Quang Nam province, Vietnam (Fig. 1), at different coordinates by SCUBA diving and pickaxe. The marine samples were collected by Nguyen Mai Anh and colleagues, Institute of Marine Biochemistry and the research group of Prof. Do Cong Thung, Institute of Marine Environment and Resources on September 25 to 29, 2016. The samples were placed in 50 mL falcon tubes or plastic boxes filled with 30% glycerol in an icebox and transported to the laboratory of the Institute of Marine Biochemistry for further analysis. In the Lab., the samples were stored at 4$^{o}$C until used. All sample boxes must be labeled with the site

name, the date and time of the sample collection, and other information [5, 6].

A 0.5 g marine sample was taken, ground, mixed separately with 4.5 mL of sterile distilled water, and homogenized by vortexing for 1 min. Then, 0.5 mL of the homogenized solution was used for serial dilution in sterile distilled marine water to $10^{-3}$. Then, 50 µL aliquots were spread over seven different selective agar media used for the isolation of fungi, including A1 (10 g soluble starch, 4 g yeast extract, 2 g peptone, 30 g instant ocean, 15 g agar); MEA (5 g malt extract, 1 g peptone, 30 g instant ocean, 15 g agar); PDA (30 g potato extract, 20 g dextrose, 5 g soluble starch, 30 g instant ocean, 15 g agar); NZSG (20 g soluble starch, 5 g yeast extract, 10 g glucose, 5 g NZ amine A, 30 g instant ocean, 15 g agar); SWA (30 g instant ocean,15 g agar); PMDA (30 g potato extract, 20 g dextrose 10 g malt extract, 30 g instant ocean, 15 g agar), CZ (30 g Saccharose, 2 g $NaNO_3$, 1 g $K_2HPO_4.3H_2O$, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 30 g instant ocean, 15 g agar); Amphotericin B was added to media and mixed well with final concentration of 0.5 µL/1 mL. Add distilled water until the volume is 100 m, pH 7.0. The plates were incubated at 30°C for 14 days. Powdered colonies were observed daily. Independent fungi colonies were picked up and streaked onto the PDA agar medium. Pure isolates were recovered, named, labeled, and stored at 4°C. For more extended storage, it was grown on nutrient broth for seven days, and glycerol was added to make the final concentration of 30%. The it was stored at -20°C [5, 7, 8].

## Preparation of crude extracts of culture broth

Fungal strains were cultivated at 28°C in sterile 1,000 mL flasks containing 500 mL PDA broth medium, shaken at 110 rpm and 28°C. After seven days of cultivation, the culture broths were filtered by filter paper (thickness 0.35–0.5 mm, particle retention 3 µm) and then extracted with 300 mL ethyl acetate (EtOAc; 5 times × 15 minutes). Extracts were then evaporated under reduced pressure (250 mbar, heating bath at 45°C) to yield crude extracts [7, 8].

## Preliminary screening of fungi for antimicrobial activity by broth microdilution method

MICs were determined by the broth dilution test according to [9] with some modifications. The method tests two-fold dilutions of multiple antimicrobial agents in 96-well disposable plastic trays. Crude extracts were diluted in DMSO (Dimethyl sulfoxide) at 1% concentration (10 mg/1 mL DMSO) and used in screening experiments for antagonistic properties against the test microorganisms. Seven pathogenic microorganisms including *three* Gram-negative bacteria (*Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Salmonella enterica* ATCC13076), and three Gram-positive bacteria (*Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC 14579) and one yeast *Candida albicans* ATCC10231. The crude extract was diluted in DMSO at a decreasing concentration range: 256, 128, 64, 32, 16, 8, 4, and 2 µg/mL for the number of experiments. The final test microbial concentration is $2 \times 10^5$ CFU/mL/well. Streptomycin was used as a positive control for bacteria and cycloheximide for the yeast *C. albicans*. All plates were incubated at 37°C with shaking at 120 rpm. After 24 h, read the MIC value as the value at the well with the lowest concentration of antimicrobial agent that completely inhibits the visible growth of a test microbial. This is confirmed by spreading the cultures on agar plates. All experiments were carried out in triplicate.

## Cytotoxicity screening of fungi extracts in cancer cell lines

Three human cancer cell lines, including human liver cancer HepG2, human breast cancer MCF-7 and human lung cancer A549 were used for determining the cytotoxic activity of extracts from all fungi strains. The antiproliferative MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium) assay was used

following the previously described protocols [5, 10]. The crude extract of the fungal sample was dissolved in 10% DMSO and diluted in a 96-well plate with cell culture medium (without FBS) to a working concentration of 100 µg/mL. All experiments were done three times, in duplicates for the data accuracy. The cells were then incubated for 48 h. At the end of the treatment step, MTT (final concentration 1 mg/mL) was added to each well, then cultured in the incubator in a humidified atmosphere (e.g., 37°C, 5% $CO_2$, 98% humidity). Check for complete solubilization of the purple formazan crystals and measure the absorbance of the samples using a microplate reader. The colored crystals of produced formazan were dissolved in DMSO (Sigma, USA), and its absorbance was measured at 540 nm using an ELx800 Microplate Reader (Bio-Tek). Camptothecin 5 µg/mL was used as a standard control, and DMSO was a negative control. A decrease in cell viability to less than 50% was noted as a positive outcome. The inhibitory rate of cell growth (*IR*) was calculated according to the following equation:

$$IR = 100\% - \left[\left(OD_{sample} - OD_{blank}\right)\right] / \left[\left(OD_{DMSO} - OD_{blank}\right)\right] \times 100$$

$OD_{sample}$ is the average *OD* value of the treated sample; $OD_{DMSO}$ as the average *OD* value of the negative control wells; $OD_{blank}$ is the average *OD* value of the blank wells with culture medium only. The TableCurve 2Dv4 software was used for data analysis and for $IC_{50}$ calculation.

## Identification of fungi

Taxonomic strain characterization is based on a combination of morphological taxonomy and uses morphology, phenotypic and molecular information to classify species. Fungal strains were cultured for 3 to 10 days on Czapek agar medium at 25°C, then observed for colony morphology, color of aerobic mycelium, and substrate mycelium. Fungi vary widely in size and shape; the hyphae and other structures combine to form an elaborate network called mycelium. Spore size, shape, and structure are used to classify and identify fungi. The shape and size of fungal hyphae and spore-forming organs, conidiophore, and ascospores were observed using a Japanese Nikon ECLIPSE 80i optical microscope.

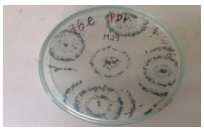
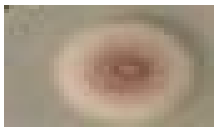
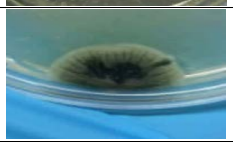
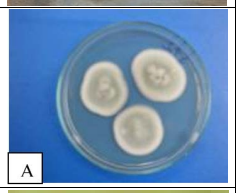
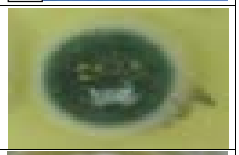
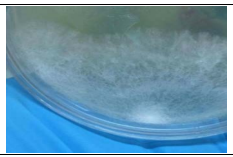
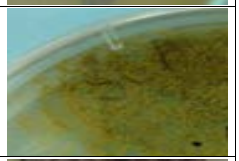
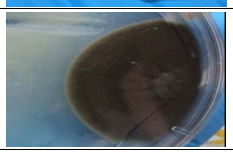
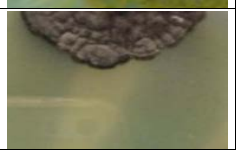
Total DNA was extracted using the Madison (USA) DNA isolation kit for molecular identification. Fungal cells were mechanically disrupted by liquid nitrogen grinding before using a DNA extraction kit. The purity and quantity of DNA solutions were checked and measured spectrophotometrically at 260 nm and 280 nm. Dissolved genomic DNA samples were stored at -20°C. Sequences of the 18S rRNA gene was amplified by using the PCR method with Taq DNA polymerase and primers $NS_3$F (5'-GCAAGTCTGGTGCCAGCAGCC-3') and $NS_8$R (5'-TCCGCAGGTTCACCTACGGA-3') [11]. The expected amplicon size is about 1.2 kb. The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 3 min followed by 30 cycles at 94°C for 1 min, primer annealing at 58°C for 1 min, and primer elongation at 72°C for 1 min 20 secs, the reaction mixture was held at 72°C for 8 min. The PCR products were analyzed using capillary electrophoresis on a DNA analyzer (ABI PRISM 3100, Applied Bioscience), and the same primers as above were used for this purpose. Gene sequences were analyzed by BioEdit version 7.2 and compared for similarity with the reference species of fungi in the GenBank database, using the NCBI BLAST. Evolutionary analysis was performed using the Maximum Likelihood method, whereas the evolutionary history was inferred using the Maximum Likelihood method and JTT matrix-based model [11–13].

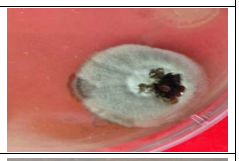
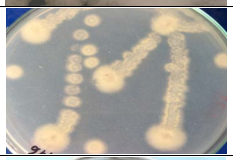
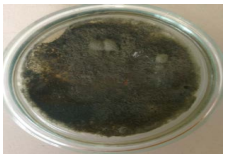
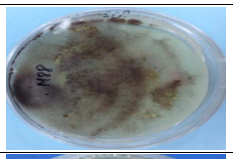
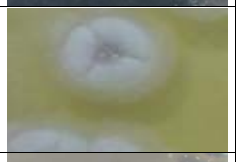
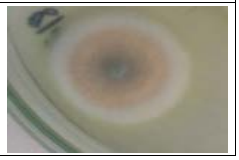
# RESULTS

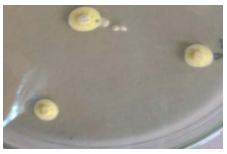
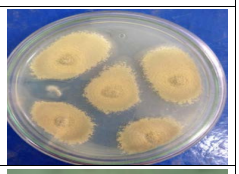
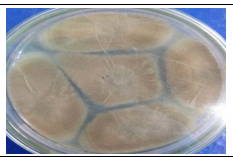
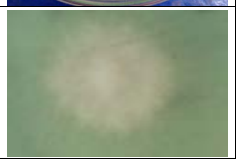
## Sample collection and isolation of fungi

By the methods of sampling and isolating marine fungi mentioned above, the details of fungal isolates are shown in Table 1.

*Table 1*. Information on coordinates, sampling depth, sample name, sample symbol and colony morphology of fungal strains isolated in Cu Lao Cham sea area, Quang Nam province

| No. | Strain name | Coordinates; Sampling depth; Sample name | Colony | No. | Strain name | Coordinates; Sampling depth; Sample name | Colony |
|---|---|---|---|---|---|---|---|
| 1 | VH01 | 15°56’34” - 108°28’55”; 10 m; Sediment; CLC12 |  | 23 | VH24 | 15°55’50” - 108°28’30”; 7 m; Mollusks; CLC5 |  |
| 2 | VH02 | 15°55’50” - 108°28’30”; 3 m; Soft coral; CLC4 |  | 24 | VH25 | 15°54’13” - 108°31’54”; 3 m; Sea hare; CLC3 |  |
| 3 | VH03 | 15°56’24” - 108°28’56”; 7 m; Sea hare; CLC15 |  | 25 | VH26 | 15°56’24” - 108°28’56”; 7 m; Seaweed; CLC10 |  |
| 4 | VH04 | 15°56’24” - 108°28’56”; 7 m; Sea hare; CLC15 |  | 26 | VH27 | 15°55’50” - 108°28’30”; 7 m; Sponge; CLC6 |  |
| 5 | VH05 | 15°56’24” - 108°28’56”; 7 m; Soft coral; CLC9 |  | 27 | VH28 | 15°56’24” - 108°28’56”; 7 m; Sponge; CLC8 |  |
| 6 | VH06 | 15°55’50” - 108°28’30”; 7 m; Mollusks; CLC5 |  | 28 | VH29 | 15°55’50” - 108°28’30”; 7 m; Sponge; CLC6 |  A |
| 7 | VH07 | 15°54’13” - 108°31’54”; 3 m; Sponge; CLC1 |  | 29 | VH30 | 15°55’50” - 108°28’30”; 7 m; Sponge; CLC6 |  |
| 8 | VH08 | 15°56’24” - 108°28’56”; 7 m; Seaweed; CLC10 |  | 30 | VH31 | 15°56’55” - 108°29’12”; 9 m; Sediment; CLC13 |  |
| 9 | VH09 | 15°55’50” - 108°28’30”; 7 m; Mollusks; CLC5 |  | 31 | VH32 | 15°54’13” - 108°31’54”; 5 m; Soft coral; CLC2 |  |
| 10 | VH10 | 15°54’13” - 108°31’54”; 3 m; Sponge; CLC1 |  | 32 | VH33 | 15°55’50” - 108°28’30”; 7 m; Sponge; CLC6 |  |

| No. | Strain name | Coordinates; Sampling depth; Sample name | Colony | No. | Strain name | Coordinates; Sampling depth; Sample name | Colony |
|---|---|---|---|---|---|---|---|
| 11 | VH11 | 15°55’50” - 108°28’30”; 7 m; Sponge; CLC6 |  | 33 | VH34 | 15°55’50” - 108°28’30”; 7 m; Sponge; CLC6 |  |
| 12 | VH12 | 15°55’50” - 108°28’30”; 7 m; Mollusks; CSCL5 |  | 34 | VH35 | 15°56’24” - 108°28’56”; 7 m; Sponge; CLC8 |  |
| 13 | VH13 | 15°56’24” - 108°28’56”; 7 m; Seaweed; CLC10 |  | 35 | VH36 | 15°56’24” - 108°28’56”; 7 m; Sponge; CLC11 |  |
| 14 | VH14 | 15°55’50” - 108°28’30”; 7 m; Sponge; CLC6 |  | 36 | VH37 | 15°56’24” - 108°28’56”; 7 m; Sponge; CLC11 |  |
| 15 | VH15 | 15°56’24” - 108°28’56”; 7 m; Soft coral; CLC9 |  | 37 | VH38 | 15°56’24” - 108°28’56”; 7 m; Sponge; CLC11 |  |
| 16 | VH16 | 15°56’24” - 108°28’56”; 7 m; Sponge; CLC8 |  | 38 | VM39 | 15°56’24” - 108°28’56”; 7 m; Sponge; CLC11 |  |
| 17 | VH18 | 15°56’24” - 108°28’56”; 8 m; Mollusks; CLC7 |  | 39 | VH40 | 15°56’24” - 108°28’56”; 7 m; Sponge; CLC11 |  |
| 18 | VH19 | 15°56’24” - 108°28’56”; 7 m; Sponge; CLC8 |  | 40 | VH41 | 15°55’50” - 108°28’30”; 3 m; Soft coral; CLC4 |  |
| 19 | VH20 | 15°56’24” - 108°28’56”; 7 m; Sponge; CLC8 |  | 41 | VH42 | 15°56’34” - 108°28’55”; 10 m; Sediment; CLC12 |  |
| 20 | VH21 | 15°54’13” - 108°31’54”; 3 m; Sea hare; CLC3 |  | 42 | VH43 | 15°55’50” - 108°28’30”; 3 m; Soft coral; CLC4 |  |

| No. | Strain name | Coordinates; Sampling depth; Sample name | Colony | No. | Strain name | Coordinates; Sampling depth; Sample name | Colony |
|---|---|---|---|---|---|---|---|
| 21 | VH22 | 15°57'21" - 108°28'52"; 17 m; Sediment; CLC14 |  | 43 | VH44 | 15°57'21" - 108°28'52"; 17 m; Sediment; CLC14 |  |
| 22 | VH23 | 15°56'24" - 108°28'56"; 7 m; Seaweed; CLC10 |  | 44 | VH45 | 15°55'50" - 108°28'30"; 3 m; Soft coral; CLC4 |  |

## Preliminary screening of fungi for antimicrobial activity by broth microdilution method

The results of screening the antibacterial activity of ethyl acetate crude extract of 44 fungal strains expressed through (MIC) values showed that: 32/44 strains had antibacterial activity against 1 to 5 strains of test microorganisms. 19/44 isolates showed anti-*C. albicans* activities. 19/44 strains were against at least 2 strains of test microorganisms. The detailed results are presented in Table 2.

*Table 2*. MIC (µg/mL) values of EtOAc extract of 44 strains

| No. | Strain | Gram + | | | Gram - | | | Yeast |
|---|---|---|---|---|---|---|---|---|
| | | *E. faecalis* ATCC29212 | *S. aureus* ATCC25923 | *B. cereus* ATCC14579 | *E. coli* ATCC25922 | *P. aeruginosa* ATCC27853 | *S. enterica* ATCC13076 | *C. albicans* ATCC10231 |
| 1 | VH1 | - | - | - | - | - | - | 256 |
| 2 | VH2 | 128 | - | - | - | - | - | 128 |
| 3 | VH3 | 128 | - | 128 | - | - | - | 128 |
| 4 | VH4 | 256 | - | - | - | - | 256 | 64 |
| 5 | VH5 | - | - | 128 | - | - | - | - |
| 6 | VH6 | 256 | - | 256 | - | - | - | 256 |
| 7 | VH7 | 32 | 64 | 128 | - | - | - | 32 |
| 8 | VH8 | 32 | - | - | - | - | - | 16 |
| 9 | VH9 | 64 | - | - | - | - | - | - |
| 10 | VH10 | 256 | 128 | - | - | - | - | 256 |
| 11 | VH11 | - | 128 | - | - | - | - | - |
| 12 | VH12 | 128 | 64 | - | - | - | - | 64 |
| 13 | VH13 | 256 | - | - | - | - | - | 256 |
| 14 | VH14 | 128 | - | - | - | - | - | - |
| 15 | VH19 | 64 | - | - | - | - | - | - |
| 16 | VH21 | - | 128 | - | - | - | - | 64 |
| 17 | VH22 | 256 | - | - | - | - | - | - |
| 18 | VH25 | - | - | - | | | - | 256 |
| 19 | VH26 | 128 | 32 | 32 | - | - | 128 | 32 |
| 20 | VH27 | 64 | - | - | - | - | - | - |
| 21 | VH28 | 128 | - | - | - | - | - | 256 |
| 22 | VH29 | 128 | 64 | 64 | - | - | 256 | 32 |
| 23 | VH30 | 256 | - | - | - | - | - | 256 |
| 24 | VH31 | 256 | - | - | - | - | - | - |

| No. | Strain | Gram + | | | Gram - | | | Yeast |
|---|---|---|---|---|---|---|---|---|
| | | *E. faecalis* ATCC29212 | *S. aureus* ATCC25923 | *B. cereus* ATCC14579 | *E. coli* ATCC25922 | *P. aeruginosa* ATCC27853 | *S. enterica* ATCC13076 | *C. albicans* ATCC10231 |
| 25 | VH34 | 256 | - | - | - | - | - | - |
| 26 | VH35 | 128 | 256 | - | - | - | - | - |
| 27 | VH36 | 256 | - | - | - | - | - | - |
| 28 | VH37 | 256 | - | - | - | - | - | - |
| 29 | VH40 | 128 | - | 256 | - | - | - | - |
| 30 | VH42 | 256 | - | - | - | - | - | 128 |
| 31 | VH44 | 128 | 256 | - | - | - | - | - |
| 32 | VH45 | 128 | - | - | - | - | - | 64 |
| Streptomycin | | 256 | 256 | 128 | 32 | 256 | 128 | - |
| Cyclohexamide | | | | | | | | 32 |

*Notes:* Positive control: Streptomycin, Cycloheximide; (-): MIC > 256 µg/mL.

## Cytotoxicity screening of fungi extracts in cancer cell lines

All crude extracts of 44 marine fungal strains were tested for cytotoxicity; however, only five strains showed inhibitory effects on at least one tested cancer cells, with a percent growth inhibition of cells ≥ 50%. Extracts from 5 fungi strains demonstrated activity against at least one of the tested cancer cell lines; the details were showed in the Table 3.

The data was collected using ANOVA using STATGRAPHICS plus 5.1. Differences in *P* values < 0.05 were considered statistically significant. A total of 44 strains of microorganisms that look like fungi were isolated from the marine samples. The details of samples and isolates were presented in Table 1. From the preliminary screening results for antimicrobial and cytotoxic activities (shown in Table 2 and Table 3), thirty-two strains were reported for significant antimicrobial activities. However, only five strains showed antagonistic activity towards cancer cell lines. Three strains (VH7, VH26, and VH29) with the best biological activity were identified and analyzed. Strain VH7 showed activity against 4/7 test microorganisms and inhibited 52.56 ± 2.41% of the human hepatocellular carcinoma HepG2 cell line. VH26 exhibited antagonistic activity against 5/7 test microbial strains. It showed significant growth inhibitory activity against cancer cell lines indicated by the recorded killing ability of the test human lung cancer A549 (60.41 ± 1.56%) and human breast cancer MCF-7 human breast cancer (69.34 ± 1.36%). VM29 strain inhibited 5/7 test microbial strains and showed human breast cancer MCF-7 cytotoxic activity of 53.46 ± 1.65%. Therefore, tree strains VH7, VH26, and VH29 were identified by microscopic and macroscopic examination and confirmed by molecular biology techniques.

*Table 3*. The cytotoxic activity of 5 bioactive strains against human cancer cell lines

| Extract of strain (100 µg/mL) | Percent growth inhibition of cells (% Inhibition ± SD) | | |
|---|---|---|---|
| | HepG2 | A549 | MCF-7 |
| VH7 | 52.56 ± 2.41 | 38.23 ± 1.21 | 37.32 ± 1.13 |
| VH15 | 52.56 ± 2.41 | 29.26 ± 2.12 | 56.32 ± 1.56 |
| VH26 | 35.67 ± 1.51 | 60.41 ± 1.56 | 69.34 ± 1.36 |
| VH30 | 23.91 ± 1.30 | 54.23 ± 1.34 | 31.26 ± 1.43 |
| VH29 | 26.34 ± 1.22 | 36.24 ± 1.23 | 53.46 ± 1.65 |
| Camptothecin* (5 µg/mL) | 85.23 ± 1.37 | 79.31 ± 1.08 | 88.26 ± 1.39 |

*Notes:* * Camptothecin: anticancer compound used as the standard.

## Identification of fungi

Morphological description of VH7 (Fig. 2): Colonies on Czapek medium grow to 2–2.5 cm/10 days at 25°C. The colony's surface is velvety, initially white, then pale green, with colorless to reddish brown secretions; The reverse side is light yellow to orange-brown. Microscopically, conidiophores, 90–750 μm × 4.5–8 μm, colorless to light brown, thick-walled, smooth. The vesicles are nearly spherical to pear or elliptical, measuring 8–19 μm. Spores with rough spines are nearly spherical to spherical, 3–3.8 μm in size, with rough spines. Conidiophores consist of simple or repeatedly verticillate phialides arranged in brush-like structures of the genus *Aspergillus.*

Morphological description of VH26 (Fig. 3): Colonies on Czapek medium grow slowly, reaching 2.5–3.5 cm/10 days at 25°C. The colony surface is flat, velvety to woolly, gray-green to brownish-green; colorless to reddish-brown secretions. The reverse side is dark yellow or straw mushroom color. The conidiophores are 140–550 μm × 3.5–8 μm, colorless to light brown, thick-walled, and smooth. The swollen phialides are clavat-shaped up to spherical, measuring 5–18 μm. Spores with rough spines are nearly or spherical, 3–4 μm in size. Hüll cells are occasionally produced, nearly spherical to spherical, 9–14 μm in size. Conidiophores consist of simple or repeatedly verticillate phialides arranged in brush-like structures of the genus *Aspergillus.*

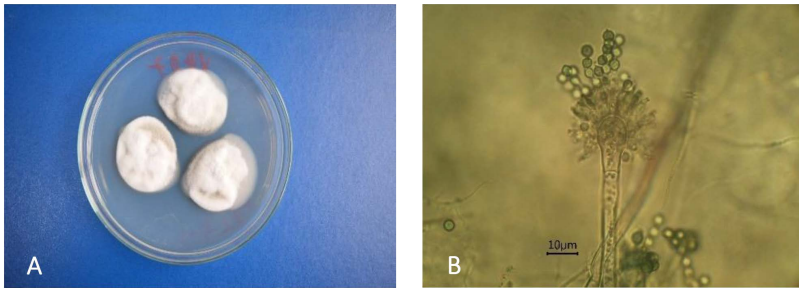



*Figure 2.* (A) Colonies on Czapek at 25°C/7 days; (B) Sporangium of VH7 isolate

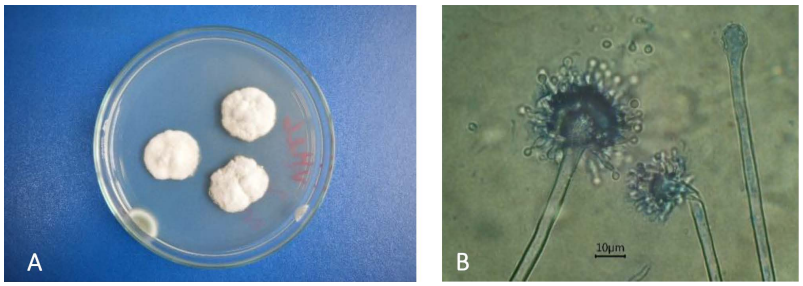



*Figure 3.* (A) Colonies on Czapek at 25°C/7 days; (B) Sporangium of VH26 isolate

Morphological description of VH29 (Fig. 4): Colonies on Czapek medium grow relatively slowly, reaching 2–3 cm/2 weeks at 25°C, folded with radial grooves and with silky and spongy aerial mycelium. The colony's surface has many spore-forming structures, initially yellow-green, gray-green, then dark green, colorless to reddish brown secretions. The reverse side is dark orange to reddish brown. Conidiophores are produced from the basal mycelium, 80–700 μm long and 3.5–9 μm wide, colorless to light yellow-brown, with thick and smooth walls. Vesicles are usually pear to subglobose in shape, size 9–20 μm. Spores are initially elliptical, then become nearly spherical or spherical, 3.5–4.5 μm in size, with sharp spines forming chains. Hüll cells are nearly spherical to spherical, 10–15 μm in size.

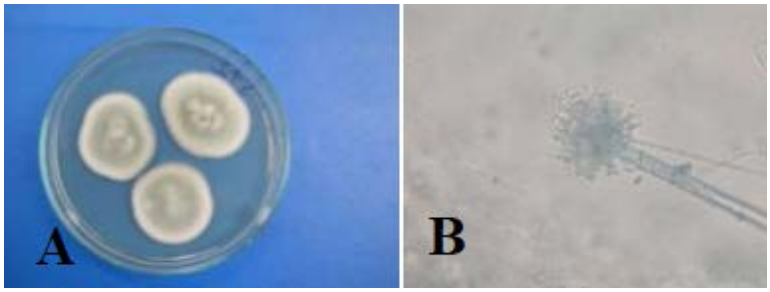



*Figure 4.* (A) Colonies on Czapek at 25°C/7 days; (B) Sporangium of VH29 isolate

Evolutionary relationships of taxa: The nearly complete 18S rRNA gene sequence of strains VH7 (1,105 bp), VH26 (1,104 bp), and VH29 (1,100 bp) were determined and compared with corresponding sequences in the Genbank database by Blast program. The result of molecular identification revealed that three potential isolates, VH7, VH26, and VH29, designated as *Aspergillus versicolor* have more than 99% similarity with *Aspergillus versicolor* NRRL 238. The evolutionary history was inferred using the Neighbor-Joining method; the optimal tree is shown in Figure 5.

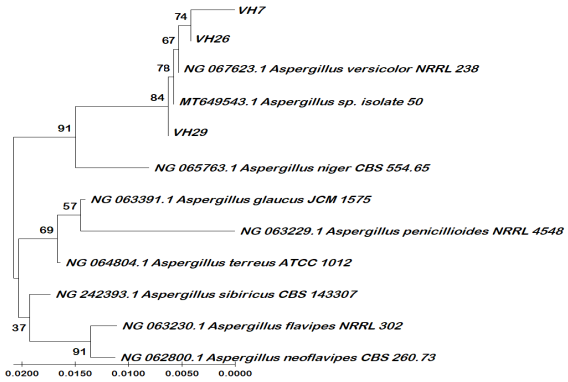



*Figure 5.* Evolutionary relationships of three promising strains with representative members of genera *Aspergillus* genus

The phylogenetic analysis involved 12 sequences of 18S rRNA gene of species of the *Aspergillus* genus. The Neighbor-Joining Method available in MEGAX was used to infer the evolutionary history and percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Nei-Gojobori method and are in the units of the number of synonymous substitutions per synonymous site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were 558 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [13]. The bar length represents 0.005 substitutions per nucleotide site. The phylogenetic tree showed three clusters in which VH7, VH26, and VH29 were grouped in the clade of *Aspergillus versicolor*. Their 18S rRNA gene sequences were deposited with the GenBank data libraries under accession numbers: PP762309 for VH7, PP767325 for VH26, and PP809383 for VH29.

## DISCUSSION

Marine fungi produce a variety of important secondary metabolites. In the review article of Shabana et al., [14] about 187 new compounds and 212 other known compounds with anti-cancer and antibacterial activities were mentioned, focusing specifically on 2011–2019 [14].

A fungus *Aspergillus sydowii* MCCC 3A00324 was isolated from deep-sea sample, produced a new compound, acremolin D, at a concentration of 20 µM, which inhibited the proliferation of Hela-S3 and K562 cell lines with inhibition rates of 30.6% and 25.1%, respectively [15].

Marine fungi, such as species from the *Penicillium* and *Aspergillus* genera, are prolific producers of a diversity of natural products with cytotoxic properties. These fungi have been successfully isolated and identified from various marine sources, including sponges, coral, algae, mangroves, sediment, and seawater. The

cytotoxic compounds derived from marine fungi can be categorized into five classes: polyketides, peptides, terpenoids and sterols, hybrids, and other miscellaneous compounds. Notably, the pre-eminent group among these compounds comprises polyketides, accounting for 307 out of 642 identified compounds. In this collection, 23 out of 642 compounds exhibit remarkable cytotoxic potency, with $IC_{50}$ values measured at the nanomolar (nM) or nanogram per milliliter (ng/mL) levels. This review elucidates the originating fungal strains, isolation sources, chemical structures, and the antitumor activity of the 642 novel natural products isolated from marine fungi. The scope of this review encompasses the period from 1991 to 2023 [16].

Cytotoxic agents are toxic substances characterized by cell death after exposure. Some bioactive secondary metabolites from the fungus *Aspergillus versicolor*, derived from a marine sponge *Petrosia* sp., showed significant cytotoxicity against five human solid tumor cell lines with low $IC_{50}$ values, and some compounds exhibited antibacterial activity against several clinically isolated [17].

One polyketides compound isolated from the deep-sea-derived fungus *Aspergillus versicolor* SH0105, displayed obvious inhibitory activity against *Staphylococcus aureus* (ATCC 27154) with the MIC value of 13.7 μM [18].

One marine fungus, *Aspergillus fumigatus* WA7S6, exhibited antimicrobial activity against *P. aeruginosa, S. aureus, A. niger*, and *Candida albicans* and cytotoxic activity against three cancer cell lines Hela, MCF, and WI-38 [19].

## CONCLUSTION

In this study, 44 fungi isolates from 12 marine samples were screened for antimicrobial and cytotoxic activity. The inhibitory effects of these strains on the growth of one pathogenic yeast, six harmful bacteria and three cancer cell lines were tested.

The crude ethyl acetate extracts obtained from the culture of 32 strains indicated promising antimicrobial activities against pathogens. Additionally, 5/44 fungi extracts showed anti-cancer potential in the HepG2, A549, or MCF-7 cells model assay with an inhibition rate of more than 50% at 100 μg/mL concentration. Three marine fungi (VH7, VH26, VH29) have been selected from this collection as they exhibit a pronounced antimicrobial and cytotoxic activity. Morphological and molecular techniques ascertained the identification of the fungi strains with the most potent antimicrobial activities. Based on the BLAST analysis on similarity, the result of isolates of 18S rRNA sequence with gene bank was found that all three potential isolates (VH7, VH26, VH29) designated as *Aspergillus versicolor* and their 18S rRNA gene sequence were deposited in GenBank. These strains are undergoing further analysis to identify bioactive metabolites which can be used for pharmaceutical applications. This report has also proven fungi strains from various marine sources.

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