

## ISOLATION AND SCREENING MARINE FUNGI WITH ANTIMICROBIAL ACTIVITY FROM SAMPLES COLLECTED IN NHA TRANG BAY, VIETNAM

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### SUMMARY

The marine environment is an extremely complex ecosystem and contains a broad spectrum of fungal diversity. Marine fungi have been shown to be tremendous sources for new and biologically active secondary metabolites. The present study aims to isolate and screen antimicrobial properties of 100 fungus strains from different marine sources including seaweeds, soft corals, sponges and sediment collected at Nha Trang Bay, Vietnam. In preliminary experiments, the crude extracts of these fungal isolates with ethyl acetate were screened for their antimicrobial activity against the human microbial pathogens including *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 by the disc diffusion method. Among the 100 isolates, 59 strains exhibited antimicrobial activity against at least two tested pathogens, that 57% against *S. aureus*, 50% against *L. monocytogenes*, 49% against *B. cereus*, 45% against *S. faecalis*, 7% against *E. coli*, 5% against *C. albicans*, and only 2% against *P. aeruginosa*. The present study has revealed the presence of high numbers of marine fungi from Nha Trang waters having antimicrobial properties and they need to be investigated further for natural bioactive products.

**Keywords:** Antimicrobial activity, natural bioactive products, marine fungi, secondary metabolites, microbial pathogens

### INTRODUCTION

A potential source of novel antibacterial compounds is marine-derived fungi, which have attracted considerable attention in recent years (Liberra, Lindequist, 1995; Biabini, Laatsch 1998). Fungi that produce active metabolites have been obtained from various marine substrates, including inorganic matter, microbial communities, plants, invertebrates and vertebrates (Jones, 2011). In particular, sponges have yielded numerous fungal strains, which have been reported to produce a variety of pharmacologically active and structurally diverse metabolites (Lopez-Gresa *et al.*, 2009; Liu *et al.*, 2011; Rateb, Ebel, 2011; Zhou *et al.*, 2011). A study by Cuomo *et al.* (1995) reported that many marine fungi have good activity profiles when compared to terrestrial fungi, making them a very promising source for the isolation of bioactive

metabolites. Accordingly, this group of organisms has attracted considerable attention from natural product chemists. Numerous studies dealing with diverse and unique compounds of marine fungi have been reported, with pertinent biological activities including antimicrobial, anticancer, anti-inflammatory and antiviral properties (Bugni, Ireland, 2004; Pan *et al.*, 2008).

Vietnam has over 3,600 kilometers coastline with unique ecosystems such as the mangroves, mudflats, coral reefs, bays, lagoons and estuaries. Thus, the marine environment in Vietnam constitutes a large reservoir of undiscovered bioactive compounds. Although several investigations on the diversity and biological activities of marine fungi in North coast of Vietnam have been conducted so far, little is known regarding to this issue in the South coast, especially in Nha Trang Bay. The present

study aimed to evaluate marine fungi from Nha Trang Bay for antimicrobial activity against tested pathogens and search for new biologically active metabolites.

## MATERIALS AND METHODS

### Sample collection

Marine samples including sponges, soft corals, seaweeds and sediment were collected from Nha Trang Bay at the depth ranging of 5-10 m (12°18'N; 109°31'E). The samples were put immediately to sterile plastic bags and stored in the icebox at 4°C and transported to the laboratory for the isolation of fungi.

### Isolation of marine derived fungi

The marine organisms were rinsed three times with sterile seawater in order to remove non-attached particles, including microbes. One gram of sample was grind with 1 mL sterile sea water in a test tube, the spread on agar plate with modified Sabouraud medium (peptone 10 g, glucose 40 g, agar 18 g dissolved in 1000 mL sea water, pH 6.0-7.0) (Handayani *et al.*, 2016). Colony morphology was observed after 5-7 days incubation 28°C. Colonies of different shapes were selected then purified again to obtain pure cultures (single fungal strain). The fungal isolates were stocked in sterile seawater with 40% glycerol at -80°C in the Marine Microorganism Collection at Nha Trang Institute of Technology Research and Application (NITRA).

### Cultivation and extraction of secondary metabolites from fungal isolates

The pure isolates were cultured in agar slant containing Sabouraud medium at 28°C for 14 days then extracted by maceration with ethyl acetate for 24 h. The ethyl acetate extracts were separated from the culture medium and concentrated by using a vacuum rotary evaporator at 40°C. These crude extracts were used for screening antimicrobial activity.

### Screening for antimicrobial activity of marine fungi

Antibacterial activity of ethyl acetate extracts from marine fungi was screened against pathogens using disc diffusion assay (Becerro *et al.*, 1994). The

crude extracts were impregnated at a concentration of 100 µg/disc on to 6 mm dia sterile Whatman No1. discs and allowed to dry for solvent evaporation. Then the antibacterial activity was assessed against 7 human pathogens, including *B. cereus* ATCC 11778, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *L. monocytogenes* ATCC 19111, *S. faecalis* ATCC 19433 and *C. albicans* ATCC 10231. The test microorganisms were grown on nutrient agar media and the density of them was adjusted to standard McFarland 0.5 using a spectrophotometer at a wavelength of 625 nm. Ethyl acetate without extracts in the discs was used as negative control. The plates were incubated at 37 °C for 24 hours and results were recorded as zone of inhibition in mm.

### Identification of fungi












The fungi were identified according to its gene sequences of 28S rDNA. The genomic DNA of isolates were extracted using a FastDNA spin kit for soil (Bio 101 Systems or Q-Bio gene) by following the company's protocol. DNA was amplified using primers NL209 (5'-AAGCGCAGGAAAAGAAACCAACAG-3') and NL912 (5'-TCAAATCCATCCGAGAACATCAG-3'), purified with a GeneClean III kit (Q-Bio gene), and sequenced using the fluorescent method and a Li-COR 4200 DNA sequencer (Amodia Bioservice GmbH, Braunschweig, Germany) (Zuccaro *et al.*, 2008). For identification, sequences of fungal 28S rDNA region were compared with those in the NCBI (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>).

## RESULTS AND DISCUSSION

### Isolation of marine fungi

Totally 100 fungal isolates were obtained from various marine samples including sponges, soft corals, seaweeds and sediment. The isolation frequencies of marine fungi varied by host, with the highest isolation frequencies obtained from the brown algae *Sargassum* sp. and the sponge *Aptosuberitoides* at rates of 28% and 13%, respectively. There were 25 fungal strains isolated from 8 sediment samples at different sites in Nha Trang Bay (Table 1).

**Table 1.** List of 100 isolated marine fungal strains from different sources.

Sources	Photos of sources	Sign of fungal isolates	Number of fungal isolates
Sponge <i>Stylissa</i> sp.		01NT.1.1.1, 01NT.1.1.2, 01NT.1.1.3, 01NT.1.1.4, 01NT.1.1.5, 02VH.2.2.5	6
Sponge <i>Acanthella cavernosa</i>		01NT.1.2.1	1
Sponge <i>Aaptos suberitoides</i>		01NT.1.3.1, 01NT.1.3.2, 01NT.1.3.4, 01NT.1.4.1, 01NT.1.4.2, 01NT.1.4.3, 01NT.1.4.4, 01NT.1.6.1, 01NT.1.6.3, 01NT.1.6.4, 01NT.2.6.2, 01NT.2.6.3, 01NT.1.3.3	13
Sponge <i>Cinachyrella</i> sp.		01NT.1.5.1, 01NT.1.5.3, 01NT.1.5.4	3
Sponge <i>Rhopaloeises</i> sp.		01NT.1.7.1, 01NT.1.7.2, 01NT.1.7.3	3
Unidentified Soft coral		01NT.1.8.1, 01NT.2.4.2, 01NT.2.4.3, 01NT.2.4.4, 01NT.2.4.5, 01NT.2.5.1, 01NT.2.5.2	7
Seaweed <i>Sargassum</i> sp.		01NT.1.9.1, 01NT.1.9.2, 01NT.1.9.3, 01NT.1.9.4, 01NT.1.9.5, 01NT.1.9.6, 01NT.1.9.7, 01NT.1.9.8, 01NT.1.9.9, 01NT.1.9.10, 01NT.1.9.11, 01NT.1.9.12, 01NT.2.3.1, 01NT.2.3.2, 01NT.2.7.3, 01NT.2.8.1, 01NT.2.8.2, 01NT.2.8.4, 01NT.2.8.5, 156VN.4.0.2, 156VN.4.0.3, 156VN.18.0.2, 156VN.20.0.2, 156VN.21.0.1, 156VN.21.0.2, 156VN.21.0.5, 156VN.21.0.6, 156VN.23.0.2	28
Sediment		01NT.1.10.1, 01NT.1.10.2, 01NT.1.10.3, 01NT.1.10.4, 01NT.1.10.5, 01NT.1.10.6, 01NT.1.11.1, 01NT.1.11.2, 01NT.1.11.3, 01NT.1.11.4, 01NT.1.11.7, 01NT.1.12.1, 01NT.1.12.2, 01NT.1.12.3, 01NT.1.12.4, 01NT.1.12.5, 01NT.1.12.6, 01NT.1.18.1, 01NT.1.19.1, 01NT.1.19.5, 01NT.2.14.1, 01NT.2.14.2, 01NT.2.15.1, 01NT.2.18.1, 01NT.2.18.2	25
Sponge <i>Haliclona</i> sp.		01NT.2.1.1, 01NT.2.1.2, 01NT.2.2.1, 01NT.2.2.2, 01NT.2.2.3, 01NT.2.2.4, 01NT.2.2.6, 01NT.2.2.7	8
Unidentified soft coral		01VH.1.1.1, 01VH.1.1.2, 02VH.3.4.1	3
Unidentified sponge		02VH.3.9.1, 02VH.3.14.1, 01VH.3.25.1	3

**Screening antimicrobial activity of isolated marine fungi**

Ethyl acetate extracts of 100 fungal isolates were tested by the disc diffusion method for their antimicrobial activity. Antibacterial activity was determined in approximately 68% of all tested marine fungal strains, and was thus significantly

more prevalent than anti-yeast activity (5%). This general trend is in agreement with a similar investigation by Suay *et al.* (2000) who reported that about 70% fungal strains were active against bacteria. However, in contrast to our findings, Cuomo *et al.* (1995) and Holler *et al.* (2004) reported that the number of marine fungi inhibiting the growth of fungi was higher than those displaying

antibacterial properties. Differences in activity profiles have been shown to depend on both the amount of mycelial biomass produced and time course (Cuomo *et al.*, 1995).

**Table 2.** Antimicrobial activity of isolated marine fungi against at least 3 pathogens tested.

Strains	Zone of inhibition in mm (D-d, mm)						
	<i>B. cereus</i>	<i>C. albicans</i>	<i>E. coli</i>	<i>L. monocytogenes</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. faecalis</i>
01NT.1.1.1	7	-	-	10	-	7	7
01NT.1.1.2	8	-	-	9	-	15	9
01NT.1.1.3	8	-	-	8	-	8	9
01NT.1.1.4	-	-	-	10	-	8	11
<b>01NT.1.1.5</b>	<b>18</b>	<b>7</b>	<b>25</b>	<b>10</b>	<b>-</b>	<b>25</b>	<b>22</b>
01NT.1.2.1	-	-	-	10	-	11	9
01NT.1.3.4	8	-	-	12	-	8	8
01NT.1.4.2	-	-	-	7	-	8	9
01NT.1.5.1	-	-	-	7	-	10	9
01NT.1.5.3	11	-	7	12	-	13	16
<b>01NT.1.5.4</b>	<b>30</b>	<b>7</b>	<b>8</b>	<b>35</b>	<b>7</b>	<b>37</b>	<b>34</b>
01NT.1.6.3	13	-	-	14	-	16	10
01NT.1.9.3	7	-	-	10	-	13	10
<b>01NT.1.9.4</b>	<b>14</b>	<b>13</b>	<b>18</b>	<b>24</b>	<b>7</b>	<b>22</b>	<b>15</b>
01NT.1.9.8	7	-	-	10	-	8	9
01NT.1.9.10	-	-	-	15	-	8	10
01NT.1.9.11	8	-	-	8	-	9	-
01NT.1.11.1	9	-	-	7	-	11	11
01NT.1.11.3	15	-	14	10	-	10	-
01NT.1.12.2	17	-	-	12	-	-	13
<b>01NT.1.12.3</b>	<b>16</b>	<b>-</b>	<b>21</b>	<b>20</b>	<b>-</b>	<b>24</b>	<b>16</b>
01NT.1.12.4	9	-	-	10	-	9	7
01NT.1.12.5	7	-	-	12	-	8	8
01NT.1.12.6	10	-	-	15	-	19	12
01NT.1.19.5	8	10	-	-	-	-	12
01NT.2.1.1	8	-	-	9	-	8	-
01NT.2.1.2	7	-	-	15	-	10	10
01NT.2.2.1	11	11	-	10	-	7	10
01NT.2.2.2	8	-	-	12	-	8	-
01NT.2.2.3	8	-	-	10	-	8	7
01NT.2.3.1	8	-	-	10	-	14	-
01NT.2.4.2	7	-	-	14	-	10	8
01NT.2.4.3	12	-	-	-	-	13	13
01NT.2.4.4	11	-	-	13	-	11	10
01NT.2.8.2	-	-	-	9	-	8	9
01NT.2.8.4	9	-	-	11	-	12	11
01NT.2.8.5	12	-	-	22	-	12	12
01NT.2.14.1	10	-	9	25	-	12	14
156VN.18.0.2	14	-	-	-	-	16	10
156VN.20.0.2	16	-	-	13	-	17	15
156VN.21.0.1	15	-	-	-	-	20	12
<b>02VH.3.9.1</b>	<b>18</b>	<b>-</b>	<b>-</b>	<b>30</b>	<b>-</b>	<b>22</b>	<b>10</b>
02VH.3.25.1	13	-	-	-	-	33	15
02VH.1.1.2	21	-	-	16	-	18	18
02VH.3.4.1	10	-	-	15	-	10	7

Note: “-”: no antimicrobial activity

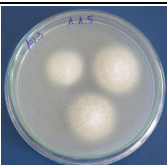
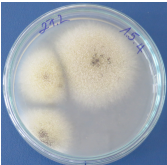
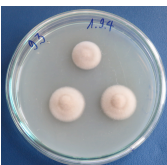
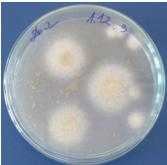



We also found antibacterial activity to be more common towards Gram-positive bacteria than Gram-negative bacteria. The results indicated that there were 50% isolates showed antibacterial activity against *L. monocytogenes*, 49% against *B. cereus* and 45% against *S. faecalis*. However, 57% fungal strains had ability to against *S. aureus*, only 7% and 2% against *E. coli* and *P. aeruginosa*, respectively (Table 2). The greater resistance of Gram-negative bacteria compared to Gram positive-bacteria was also reported by Christophersen *et al.*, (1999), Holler *et al.*, (2004) and Suay *et al.*, (2000). These differences in susceptibility towards antibioticly active secondary metabolites have been repeatedly attributed to differences in cell wall structure of Gram-positive bacteria compared to Gram-negative bacteria. The cell walls of Gram-positive bacteria are less complex and lack the natural sieve effect against large molecules (Hawkey, 1998), whereas the outer membrane and the periplasmic space presenting in Gram-negative bacteria is thought to provide an

additional degree of protection against antibiotics targeting the cell wall (Basile *et al.*, 1998).

The most promising fungal strains were 01NT.1.1.5, 01NT.1.5.4, 01NT.1.9.4, 01NT.1.12.3 and 02VH.3.9.1 those exhibited extended spectrum antimicrobial activity against most of the Gram-positive, Gram-negative bacteria and yeast. Strains 01NT.1.1.5, 01NT.1.5.4 and 01NT.1.12.3 were identified according to their 28S rDNA gene sequences with GenBank accession number MG972941.1, MH095994.1 and MH101466.1, respectively. A BLAST search results indicated that the sequences of marine fungal strains 01NT.1.1.5 and 01NT.1.12.3 are similar (100%) to the sequence of *Aspergillus flocculosus* (compared with EU021616.1). Beside, the sequence of fungus 01NT.1.5.4 was similar to the sequence of *Aspergillus niger* (GenBank accession number AM270052.1) with a 100% identity. Two of five fungal strains with highest antimicrobial activity are unidentified (Table 3).

**Table 3.** Characteristics of fungal strains demonstrating high activity to pathogens.

Fungal strains	Photos of strains	Sources of isolation	Antimicrobial activity against tested pathogens
<i>A. flocculosus</i> 01NT.1.1.5		Sponge <i>Stylissa</i> sp.	<i>B. cereus</i> , <i>C. albicans</i> , <i>E. coli</i> , <i>L. monocytogenes</i> , <i>S. aureus</i> , <i>S. faecalis</i>
<i>A. niger</i> 01NT.1.5.4		Sponge <i>Cinachyrella</i> sp.	<i>B. cereus</i> , <i>C. albicans</i> , <i>E. coli</i> , <i>L. monocytogenes</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>S. faecalis</i>
01NT.1.9.4		Seaweed <i>Sargassum</i> sp.	<i>B. cereus</i> , <i>L. monocytogenes</i> , <i>S. aureus</i> , <i>S. faecalis</i>
<i>A. flocculosus</i> 01NT.1.12.3		Sediment	<i>B. cereus</i> , <i>E. coli</i> , <i>L. monocytogenes</i> , <i>S. aureus</i> , <i>S. faecalis</i>
02VH.3.9.1		Unidentified sponge	<i>B. cereus</i> , <i>L. monocytogenes</i> , <i>S. aureus</i> , <i>S. faecalis</i>

Nowadays, the emergent drug resistance among pathogenic microorganisms, increasing the rate of microbial infections has been attracting much of public concern (Singh *et al.*, 2015). Discovering new and effective antimicrobial substances from varied natural resources, including microorganisms is an approach to overcome the problem. Therefore, further experiments need to be proceeded for the selected fungal strains to obtain bioactive natural products.

## CONCLUSION

The present study revealed that the diversity of culturable marine fungi with high antimicrobial activity could be found from various habitats in Nha Trang Bay of Vietnam. Out of 100 isolates, the proportions of strains showing activity against *S. aureus*, *L. monocytogenes*, *B. cereus*, *S. faecalis*, *E. coli*, *C. albicans*, and *P. aeruginosa* were 57, 50, 49, 45, 7, 5, and 2%, respectively. Particularly, strains namely 01NT.1.1.5, 01NT.1.5.4, 01NT.1.9.4, 01NT.1.12.3 and 02VH.3.9.1 illustrated significantly antimicrobial activity to pathogens tested. Three of these strains were classified as *A. flocculosus* and *A. niger*. Therefore, advanced studies of these potential fungal strains for bioactive secondary metabolites need to be conducted for further application.

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## PHÂN LẬP VÀ SÀNG LỌC MỘT SỐ CHỦNG VI NẤM BIỂN CÓ HOẠT TÍNH KHÁNG VI SINH VẬT TỪ CÁC MẪU THU Ở VỊNH NHA TRANG, VIỆT NAM

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### TÓM TẮT

Môi trường biển là một hệ sinh thái vô cùng phong phú với sự đa dạng của các loài vi nấm biển. Đây là một nguồn tiềm năng cho các chất chuyển hóa thứ cấp mới và có hoạt tính sinh học. Nghiên cứu này được thực hiện nhằm phân lập và sàng lọc hoạt tính kháng sinh của 100 chủng vi nấm từ các mẫu vật biển khác nhau bao gồm bọt biển, san hô mềm, rong biển và trầm tích biển được thu tại vịnh Nha Trang, Việt Nam. Ở thí nghiệm khảo sát bước đầu, dịch chiết etyl acetate thô của các chủng vi nấm này được sàng lọc hoạt tính kháng sinh đối với các chủng vi sinh vật gây bệnh cho người bao gồm *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 19111, *Streptococcus faecalis* ATCC 19433 và *Candida albicans* ATCC 10231 theo phương pháp khuếch tán trên đĩa giấy. Trong số 100 chủng khảo sát, 59 chủng thể hiện hoạt tính kháng sinh đối với ít nhất 2 chủng vi sinh vật kiểm định, cụ thể 57% kháng *S. aureus*, 50% kháng *L. monocytogenes*, 49% kháng *B. cereus*, 45% kháng *S. faecalis*, 7% kháng *E. coli*, 5% kháng *C. albicans* và chỉ 2% kháng *P. aeruginosa*. Kết quả nghiên cứu cho thấy sự hiện diện một số lượng lớn các chủng vi nấm từ vùng biển Nha Trang có hoạt tính kháng sinh và các chủng này cần được tiếp tục nghiên cứu sâu hơn về các hợp chất tự nhiên có hoạt tính sinh học.

**Từ khoá:** *Chất chuyển hoá thứ cấp, hoạt tính kháng sinh, sản phẩm tự nhiên có hoạt tính sinh học, vi nấm biển, vi sinh vật gây bệnh*