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Antioxidant and cytoprotective activities of marine fungi isolated from brown seaweeds in Nha Trang Bay, Khanh Hoa Province, Central Vietnam

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ABSTRACT

This study aims to evaluate the antioxidant and cytoprotective activities of 32 fungal strains isolated from brown seaweeds collected from Nha Trang Bay, Khanh Hoa Province, Central Vietnam. These fungal extracts were screened for antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical scavenging assay. In contrast, cytoprotective activity on rat cardiomyocytes H9c2 cell line was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. The results indicated the capacity for free DPPH and ABTS radical scavenging of fungal crude extracts with 31.25% (*n* = 10) and 81.25% (*n* = 26), respectively. Among the fungal strains with high antioxidant activity, the three fungal extracts 2104NT-1.3, 2104NT-3.3, and 2104NT-7.7 increased the viability of rotenone-exposed cardiomyocyte cells by 9.9%, 15.2%, and 13.6%, respectively. Three fungal strains with significant antioxidant and cytoprotective activities were identified as *Penicillium chermesinum* 2104NT-1.3, *Aspergillus* sp. 2104NT-3.3, and *Penicillium* sp. 2104NT-7.7 based on sequence analysis of internal transcribed spacer (ITS) region. This study provided the potential fungal strains isolated from Nha Trang Bay for further investigation of antioxidant and cytoprotective compounds.

Keywords: Antioxidant activity, cytoprotective activity, *Penicillium*, *Aspergillus*, brown seaweed, marine fungi.

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INTRODUCTION

Marine-derived fungi are now considered efficient prolific sources of bioactive natural products with a unique structure and potent pharmaceutical activity [1]. Marine fungi must adapt to distinctive environments and have an outstanding secondary metabolism that differs from terrestrial strains [2]. Fungi have been obtained from virtually every possible marine habitat, including marine plants (algae, driftwood, and mangrove plants), marine invertebrates (sponges, corals, ascidians, and holothurians), and vertebrates (mainly fish) [3].

Seaweeds are one of the most prevalent sources of marine-derived fungi for chemical studies and biotechnology purposes [4]. Fungi have been reported as parasites, saprophytes, or endophytes in seaweed. Environmental factors, season, host species, and age affect fungi species diversity in seaweed [5]. Therefore, seaweed-derived fungi are of particular interest due to their ecological significance. Although the ecological role of marine fungi in association with seaweeds is still incompletely understood, the complexity and importance of these relationships are reflected in fungi's chemical and biological potential in natural product research. The role of marine algae species is to serve as a host for the fungi, and they are responsible for tremendous chemical structure diversity and expressive biological potential [6]. Besides, seaweed inhabiting marine ecosystems adapt to frequent and sporadic environmental changes such as high salinity, low oxygen content, nutrient limitation, excessively high light, and drought, which may stress fungi to produce bioactive secondary metabolites [7]. Due to the constant stresses imposed on seaweed by marine environments, their associated fungi symbionts are believed to represent a good source of structurally diverse bioactive secondary metabolites [8].

Natural products of seaweed-derived fungi have been the subject of many chemical reports, especially in the past ten years. Several new compounds were isolated and identified biological properties, including anticancer, antibiotic, antiviral, antioxidative, kinase inhibitory, immunosuppressant, and many more properties [3, 9]. The maximum number of bioactive secondary metabolites from fungi procured was in brown algae (39%), followed by red algae (28%) and green algae (23%) [8]. The comparatively short life cycle of Chlorophyceae and the slow growth of endosymbionts together might have accounted for the low fungal diversity and bioactivity in green algae [10].

Nha Trang Bay (Khanh Hoa Province, Central Vietnam) has a coastline of 103 km and possesses a diverse ecosystem. Notably, the reserves and number of seaweed species in Khanh Hoa are the highest percentage in Vietnam, predominantly brown seaweed species belonging to the genus *Sargassum*. Various compounds with new structures and remarkable biological activities have been discovered from marine-derived fungi in this sea area [11–13]. However, to our knowledge, no report is available on the cytoprotective activity of fungi isolated from Nha Trang Bay. Hence, this study evaluated the antioxidant and cytoprotective activities of brown seaweed-derived fungi from Nha Trang Bay and selected potential isolates for further investigation of bioactive compounds.

MATERIALS AND METHODS

Seaweed collection

Five species of brown seaweed (*Sargassum polycystum*, *Sargassum oligocystum*, *Padina australis*, *Hormophysa cuneiformis*, and *Turbinaria ornata*) were collected in Nha Trang Bay (12°10'N; 109°16'E, Khanh Hoa, Vietnam) in April 2021 by Scuba diving at 8–10 m depth. The specimens were placed in zip-lock plastic bags containing fresh seawater, preserved in an icebox, and transported to the laboratory. The seaweed was identified by Dr. Vo Thanh Trung, Nha Trang Institute of Technology Research and Application, VAST.

Isolation of fungi

The seaweed samples were rinsed with sterile seawater thrice to eliminate surface debris. Approximately 1 g of the sample was

homogenized with 10 mL of sterile 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 natural seawater, pH 6.0–7.0) and supplemented with streptomycin (0.03 mg/mL) [14]. After incubation at 28°C for 5–7 days, all colonies with different pigmentation and morphology were picked out by hyphal tip isolation and transferred to Sabouraud plates without antibiotics to obtain pure strain. The fungal isolates were stored in the Collection of Marine Microorganisms of the Nha Trang Institute of Technology Research and Application, VAST.

Preparation of fungal crude extract

The fungi were inoculated on a Sabouraud plate for 5–7 days. Pieces of mycelium were transferred to 500 mL Erlenmeyer flasks containing solid rice medium (20 g of rice, 20 mg of yeast extract, 10 mg of KH_2PO_4, and 40 mL of natural seawater) [15]. The fungal cultures were incubated at room temperature for three weeks. After incubation, the mycelia and medium were homogenized and extracted with ethyl acetate. The ethyl acetate extracts were concentrated in vacuo at a mild temperature of 40°C to dryness. The obtained crude extracts were stored at -20°C and used for screening biological activities.

Determination of antioxidant activity

The antioxidative effect of the fungal extracts was assessed by the DPPH and ABTS scavenging assay.

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The DPPH test is based on the ability of the stable 2,2-diphenyl-1-picrylhydrazyl free radical to react with hydrogen donors and change from purple to yellow. The analytical procedure was performed using modified methods proposed by Lee et al. (2015) [16]. Briefly, 100 µL DPPH solution (0.1 mM) was mixed with 100 µL of the sample (250 µg/mL) in 96-well plates. The reaction was allowed to take place at room temperature in the dark for 30 min. After incubation, the absorbance was measured at 517 nm using an ELISA reader. The positive DPPH free radical scavenger's standards were ascorbic acid at 200 µg/mL, and 100% methanol was used as a control. The percentage of DPPH radical scavenging activity was calculated as follows:

$$Radical\ scavenging(\%) = \left[\frac{Ac - As}{Ac}\right] \times 100$$

where: Ac and *As* were the absorbances of the control and sample, respectively. The experiment was conducted in triplicates.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assay

The ABTS reagent was prepared by mixing 5 mL of 7 mM ABTS with 88 µL of 140 mM potassium persulfate. The mixture was then kept in the dark at room temperature for 16 h to allow free radical generation and was then diluted 1:100 in methanol. 100 µL ABTS reagent was mixed with 100 µL of the sample (250 µg/mL) in a 96-well plate and was incubated at room temperature for 6 min to determine the scavenging activity. After incubation, the absorbance was measured at 734 nm using an ELISA reader [16]. The control was 100% methanol; the standard samples were ascorbic acid (200 µg/mL). The ABTS scavenging effect was measured using the following formula:

$$Radical\ scavenging(\%) = \left[\frac{Ac - As}{Ac}\right] \times 100$$

where: Ac and *As* were the absorbances of the control and sample, respectively. The experiment was conducted in triplicates.

Determination of cytoprotective activity

The cardioprotective potential of the fungal crude extracts towards the rat cardiomyocytes H9c2 cell line was measured using an MTT assay [17]. The H9c2 cells were cultivated in DMEM media with fetal bovine serum (10%)

and penicillin/streptomycin (1%). The cells were seeded in 96-well plate at concentration of 3 × 10^3 cells/well and experiments were started after 24 h. The H9c2 cells were treated with rotenone (0.1% DMSO solution) at a concentration of 10 µM for one hour. Then, the fungal crude extracts at a 10 µg/mL concentration were added for 23 h. The viability of the H9c2 cells was measured by an MTT assay, which was performed according to the manufacturer's instructions (SigmaAldrich, Munich, Germany). The experiments were performed in triplicate, presenting the results as a percentage of control data. The statistical analysis of the data with Student's t-test was evaluated using SigmaPlot 14.0 (Systat Software Inc., San Jose, CA, USA).

Identification of fungi

The selected fungi were identified based on sequence analysis of the ITS region of ribosomal DNA. Genomic DNA was isolated following the protocol proposed by Fredricks et al., (2005) [18]. The nuclear ribosomal DNA of the fungal isolate was amplified using the forward primer, ITS1 (5'-TCCGTAGGTGAACC TGCGG-3'), and the reverse primer, ITS4 (5'-T CCTCCGCTTATTGATATGC-3') [19]. Sequences of fungal ITS-rDNA regions were compared with those in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Multiple alignments of the ITS gene region were generated using MUSCLE (default conditions for gap opening and extension penalties) and implemented in MEGA 7.0 (Molecular Evolutionary Genetics Analysis). The phylogenetic tree was generated using neighbor-joining (NJ) with bootstrap analysis using 1,000 replicates.

RESULTS AND DISCUSSION

Thirty-two fungal strains were isolated from five brown seaweed samples including *S. polycystum*, *S. oligocystum*, *P. australis*, *H. cuneiformis*, and *T. ornata* (Table 1). The distinctly different isolates are based on their morphology and pigmentation.

Table 1. A list of brown seaweed samples and isolated fungal strains

Seaweed samples	Number of fungi isolated	Fungal strains
Hormophysa cuneiformis	6	2104NT-1.1, 2104NT-1.2, 2104NT-1.3, 2104NT-1.4, 2104NT-1.5, 2104NT-1.6
Sargassum oligocystum	4	2104NT-2.1, 2104NT-2.2, 2104NT-2.3, 2104NT-2.4
Turbinaria ornata	5	2104NT-3.1, 2104NT-3.2, 2104NT-3.3, 2104NT-3.4, 2104NT-3.5
Padina australis	8	2104NT-5.2, 2104NT-5.3, 2104NT-5.4, 2104NT-5.6, 2104NT-5.7, 2104NT-5.8, 2104NT-5.9, 2104NT-5.10
Sargassum polycystum	9	2104NT-7.1, 2104NT-7.4, 22104NT-7.5, 2104NT-7.6, 2104NT-7.7, 2104NT-7.8, 2104NT-7.9, 2104NT- 7.10, 2104NT-7.11

In this study, ethyl acetate extracts of 32 isolated fungi were screened for their antioxidant potential using DPPH and ABTS assays. The result showed that seaweed-derived fungi could free radical scavenging properties to varying degrees. Ten fungal extracts (31.3%) exhibited significant antioxidant activity with SC% values ranging from 50% to 70% (Table 2). Here, four fungal extracts of 2104NT-1.4, 2104NT-7.7, 2104NT-3.3, and 2104NT-1.3 showed 58.8–66.7% DPPH free radical scavenging activity as compared to ascorbic acid which showed 79.5% activity. Among the fungal extracts, the maximum scavenging activity was displayed by the fungus 2104NT-1.3, with an SC% of 66.7%.

Moreover, the tested extracts exhibited excellent free radical scavenging properties against ABTS cation compared to DPPH scavenging potential. Approximately 81.3% (*n* =

26) of the tested fungal strains recorded high activity with SC% values > 50% in the ABTS assay (Table 2). Among them, the highest scavenging activity (74.7%) was also shown by isolate 2104NT-1.3. The fungus 2104NT-1.3 isolated from seaweed *Hormophysa cuneiformis* was found to have the highest free radical scavenging capacity against ABTS and DPPH. The result showed that these seaweed-derived fungal extracts could be potential sources for further research of antioxidant compounds.

Table 2. Antioxidant activity of isolated marine fungi (SC% > 50%)

Order	Fungal strains	Radical scavenging activity (SC%) (250 µg/mL)	
		DPPH	ABTS
1	2104NT-1.3	66.7 ± 0.3	74.7 ± 0.6
2	2104NT-1.4	58.8 ± 0.5	71.5 ± 0.6
3	2104NT-3.1	50.9 ± 0.5	68.3 ± 3.2
4	2104NT-3.3	60.0 ± 0.4	72.4 ± 0.3
5	2104NT-3.5	59.3 ± 0.2	65.9 ± 0.3
6	2104NT-5.2	52.2 ± 1.3	68.9 ± 1.3
7	2104NT-5.9	51.4 ± 0.7	62.0 ± 0.3
8	2104NT-7.1	56.8 ± 0.2	71.9 ± 0.5
9	2104NT-7.7	59.8 ± 0.7	72.6 ± 0.6
10	2104NT-7.8	54.9 ± 0.5	72.4 ± 0.3
11	Ascorbic acid	79.5 ± 0.3	83.0 ± 0.2

Numerous brown seaweed-derived fungi produced antioxidant compounds and played an essential role in free radicals scavenging. The fungus *Epicoccum* sp., isolated from the brown alga *Fucus vesiculosus*, yielded 4,5,6-trihydroxy-7-methylphthalide showed 95% DPPH radical scavenging effect at 25 µg/mL [20]. In another study, the fungus *Aspergillus wentii* EN-48 from the brown algal *Sargassum* spp. produced eight secondary metabolites, including a new seco-anthraquinone (wentiquinone C), a new benzamide derivative (methyl 4-(3,4-dihydroxybenzamido) butanoate), and six phenolic compounds. These potential metabolites exhibited significant DPPH radical scavenging activity with IC_{50} values ranging from 5.2 to 99.4 µg/mL, compared to the positive control, butylated hydroxytoluene, with an IC_{50} of 36.9 µg/mL [21]. In addition, Hulikere *et al.* reported that *Cladosporium cladosporioides* isolated from seaweed *Sargassum wightii* could produce antioxidative phenolic compounds [22]. According to our previous investigation, crude extracts from marine fungal strains obtained from Nha Trang Bay could reduce DPPH and ABTS free radicals with a proportion of 61.5% and 80.3%, respectively [23]. Notably, five compounds, aspertetranone D, cycloechinulin, mactanamide, ochraceopone F, and wasabidienone E, isolated from *Aspergillus flocculosus* 01NT-1.1.5 exhibited significant antioxidant activity. Among them, cycloechinulin and wasabidienone E showed high UV absorption properties and were suggested as the natural candidates possibly used in organic sunscreen [24]. Therefore, the present results agreed with the previous reports that seaweeds would be the potential marine source for isolating fungal strains with high antioxidant activity besides sponges and sediments.

Moreover, the cardioprotective effect of the crude extracts with rat cardiomyocytes H9c2 cells against rotenone was also investigated. The data show that three fungal extracts, 2104NT-1.3, 2104NT-3.3, and 2104NT-7.7, could protect H9c2 cells against rotenone-induced cellular death. The treatment of H9c2 cells with rotenone decreased the viability of H9c2 cells by 42.7%. The three fungal extracts 2104NT-1.3, 2104NT-3.3, and 2104NT-7.7 increased the viability of rotenone-exposed cardiomyocyte cells by 9.9%, 15.2%, and 13.6%, respectively (Figure 1). Notably, all three fungal extracts also exhibited effective antioxidant activity. This

result might suggest that the extracts fungal could protect cardiomyocyte cells against the damaging influence of rotenones due to their antioxidant properties. The previous reports released that free radicals accumulate as a natural byproduct in metabolic pathways, resulting in oxidative stress in the body. The destructive effects of free radicals may cause damage to membranes, enzymes, and DNA, leading to several human diseases such as cancer, atherosclerosis, cardiovascular diseases, cataracts, rheumatoid arthritis, asthma, and neurodegenerative illness [25]. Scavenging free radicals and repairing damaged cells will increase the immune system to slow down oxidative stress in the body and prevent disease. These findings show the requirement of using antioxidants as a therapeutic intervention in addition to other protective agents.

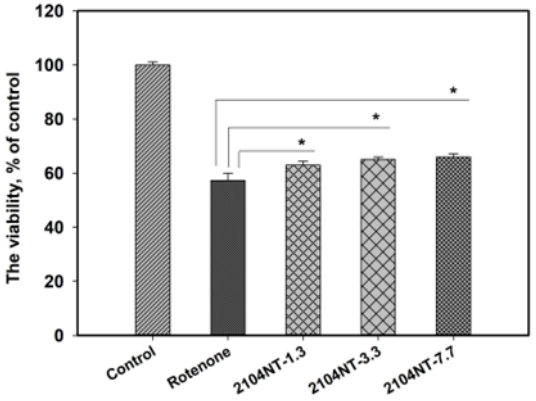


Figure 1. Cytoprotective activity of fungal extracts against rotenone toxicity in H9c2 cells. The data are presented as a mean ± standard error of mean. The asterick * indicates the significant differences with $p < 0.05$.

The importance of marine fungi as a source of pharmaceutical agents targeting protective compounds has been demonstrated through various studies [26]. The reports indicated that marine fungi from Vietnam produced significant potential bioactive metabolites as cytoprotective compounds: the compounds melatonin analog 6-hydroxy-N-acetyl-oxotryptamine, 3-methylorsellinic acid, and 8-methoxy-3,5-dimethylisochroman-6-ol from marine fungus *Penicillium* sp. KMM 4672, isolated from brown alga *Padina* sp. (Van Phong Bay), exhibited strong neuroprotective activity [27]. Similarly, the strain *Aspergillus flocculosus* from a sediment sample (Nha Trang Bay) produced diketopiperazine mactanamide protected Neuro2a cells against the damage in the 6-hydroxydopamine (6-OHDA)- and paraquat (PQ)-induced Parkinson's disease (PD) cell models [27]. Recently, gliorosein isolated from the *Lopadostoma pouzarii* strain 168CLC-57.3 (Cu Lao Cham Island) showed significant cardioprotective activity against the rotenone- and $CoCl_2$-induced damage of the cardiomyocytes H9c2 [28]. Marine fungi's metabolism has focused on survival in particularly competitive environments. For this reason, the presence of cytoprotective compounds should help fungi successfully compete in microbial communities. However, the field of marine fungi-sourced cytoprotective compounds is still in its infancy, requiring further discoveries and investigations.

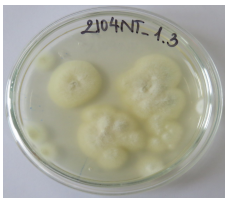
Based on the results, three strains, 2104NT-1.3, 2104NT-3.3, and 2104NT-7.7, showed significant free radical scavenging ability together with cardioprotective activity. It suggested that the cardioprotective activity of the studied fungal extracts is attributable to their pronounced antioxidant and free radical scavenging activity formed by rotenone action on rat cardiomyocyte cells. Therefore, these fungal strains were selected as promising candidates for producing antioxidant and cytoprotective agents, and fungal identification was carried out. According to the analysis of ITS gene sequences, the selected fungi were identified as *Penicillium chermesinum* 2104NT-1.3 and *Aspergillus* sp. 2104NT-3.3, and *Aspergillus* sp. 2104NT-7.7 (Table 3).

Penicillium and *Aspergillus* genera are the most ubiquitous genera of filamentous fungi and are frequently found fungi in marine environments and on Earth [29]. Many secondary metabolites with structural diversity, such as polyketides, alkaloids, terpenes, steroids, and peptides, have been isolated from these genera, and many display potent biological activities [30]. The salt tolerance, fast-growth, and ease of obtaining from many substrates could be responsible for the high

fungal diversity and bioactivity in the genera *Penicillium* and *Aspergillus* [1].

In our ongoing search to discover biologically active natural products from the Vietnam marine-derived fungi, we also found diverse biology with several fungal genera, including Aspergillus and Penicillium. *Penicillium chrysogenum* 045-357-2, isolated from a soft coral sample collected from Ca Na Bay, Ninh Thuan, produced a potent antimicrobial activity against *Bacillus cereus* and *Streptococcus faecalis* [31]. Our previous study reported that *Penicillium janthinellum* 168CLC-17.1, from a sediment sample collected from Cu Lao Cham Island, Quang Nam, exhibited anti-neuroinflammatory effects [32]. As part of our ongoing program for new metabolites among marine-derived fungi in Nha Trang Bay, we have investigated bioactive constituents from *Aspergillus niveoglaucus* 01NT.1.10.4 [33] and *Aspergillus flocculosus* [27] also possessed cytotoxic and neuroprotective activity. Previous investigations on *Aspergillus* and *Penicillium* genera indicated they are rich in bioactive secondary metabolites with various intricate structures.

Table 3. Identification of selected fungal strains

Fungal strains	Sources of isolation	Scientific name	Closest identified relative	Precent Identify
 2104NT-1.3	 *Hormorphysa cuneiformis*	*Penicillium chermesinum*	*Penicillium chermesinum* CBS 117279 (MT309662)	99%
 2104NT-3.3	 *Turbinaria ornata*	*Aspergillus* sp.	*Aspergillus* sp. CBS 141578 (MN640759)	99%
 2104NT-7.7	 *Sargassum polycystum*	*Aspergillus* sp.	*Aspergillus* sp. NRRL 4748 (EU021613)	99%

CONCLUSION

The results of our study illustrated the potential source of antioxidant and cytoprotective activities from seaweed-derived fungi in Nha Trang Bay (Khanh Hoa Province, Central Vietnam). Thirty-two fungal strains isolated from five brown seaweeds were evaluated for bioactivities, in which 31.2% (*n* = 10) strains exhibited DPPH free radical scavenging capacity, 81.2% (*n* = 26) strains exhibited ABTS free radical scavenging capacity, and 9.3% (*n* = 3) isolates showed cytoprotective activity against H9c2 cells. Three selected fungal strains were identified to belong to the genera *Aspergillus* and *Penicillium*. The data obtained here provided potential fungal strains for further investigating antioxidant and cytoprotective compounds.

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