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Antibacterial, cytotoxic and trypanocidal activities of marine-derived fungi isolated from Philippine macroalgae and seagrasses

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Abstract – The occurrence and bioactivities of marine-derived fungi are evaluated in this paper. A total of 16 morphospecies of marine-derived fungi (MDF) were isolated from four host macroalgae and two seagrasses and identified as belonging to the genera *Aspergillus*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Sclerotinia*, *Thamnidium* and *Trichoderma*, including five *mycelia sterilia.* Among these host organisms, the rhodophyte *Laurencia intermedia* harboured the highest number of isolated MDF. Selected MDF were then assayed and showed to inhibit *Pseudomonas aeruginosa* (8-19 mm zone of inhibition) and *Staphylococcus aureus* (6-19 mm zone of inhibition), and were cytotoxic against the brine shrimp *Artemia salina* nauplii (LD_{50}: 201.56-948.37 μg mL^{-1}). The screening led to the selection of five of the most bioactive morphospecies, all belonging to the genus *Aspergillus*. These marine aspergilli were subjected to *β-tubulin* gene sequence analysis for species identification, and to mass production in different culture media with or without marine salts, and screening of the crude culture extracts for their cytotoxic and trypanocidal activities. *Aspergillus tubingensis* cultivated in potato dextrose broth with marine salt proved to be the most cytotoxic against P388 (IC_{50}: 1028 ng mL^{-1}) and HeLa (IC_{50}: 1301 ng mL^{-1}) cancer cells. On the other hand, *A. fumigatus* cultivated in malt extract broth without marine salt was shown to be the most potent against *Trypanosoma congolense* (IC_{50}: 298.18 ng mL^{-1}). Our study therefore showed that salinity may influence the bioactivities of some species of MDF.

Key words: bioactivity, fungal natural products, marine fungi, Philippines

Abbreviations: ASW – artificial seawater; DMSO – dimethyl sulfoxide; MDF – marine-derived fungi; MEA – malt extract agar; MEAS – malt extract agar with marine salt; MEB – malt extract broth; MEBS – malt extract broth with marine salt; PDB – potato dextrose broth; PDBS – potato dextrose broth with marine salt; ZOI – zone of inhibition

Introduction

The ocean has vast biological resources, accounting for more than eighty percent of total world biodiversity, which makes it a reservoir for many kinds of organisms, including marine-derived fungi (Bugni and Ireland 2004). These marine-derived fungi (MDF) grow and possibly sporulate in the marine ecosystem (Kohlmeyer and Kohlmeyer 1979). Interestingly, many of these marine-derived fungi produce structurally diverse and bioactive compounds (dela Cruz et al. 2006a, Schulz et al. 2008, Silber et al. 2016), utilized varied substrata (dela Cruz et al. 2006b), and may play an important ecological role in marine habitats (Solis et al. 2010). In spite of their economic and ecological importance, some of the less explored MDF thrive inside healthy tissues of macroalgae, e.g. in *Sargassum thunbergii* (Miao et al. 2012), and seagrasses, such as *Cymodocea serrulata* and *Halophila ovalis* (Supaphon et al. 2013).

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Various biochemical properties with anti-infective and anti-tumor activities have also been documented in algae- and seagrass-derived marine fungi (Greve et al. 2008, Supaphon et al. 2013, 2014). For instance, *Aspergillus flavus* isolated from the green alga *Enteromorpha tubulosa* synthesized two new 5-hydroxy-2-pyrone derivatives that could induce production of cAMP on GPR12-transfected cells (Lin et al. 2008). *Penicillium chrysogenum* isolated from the red alga *Laurencia* produced the novel antifungal and cytotoxic penicisteroids A and B (Gao et al. 2011). Despite the promising chemicals that these fungi produce, there are no drugs from these fungi that have been commercially approved (Gerwick and Moore 2012). Obviously, this necessitates the need to do more research on these organisms in order to find bioactive and potentially novel chemicals for drug discovery and development. Thus, this recent study isolated marine-derived fungi in Philippine macroalgae and seagrasses, and tested their biological activities for potential chemotherapeutic uses.

Materials and methods

Screening of marine-derived fungi for biological activities

Collection of macroalgae and seagrasses

Four species of algae, belonging to Phaeophyta [*Sargassum piluliferum* (Turner) C. Agardh], Chlorophyta [*Caulerpa racemosa* (Forsskål) J. Agardh], and Rhodophyta [*Laurencia intermedia* Yamada and *Portieria hornemannii* (Lyngbye) P. C. Silva], and two species of seagrasses, *Enhalus acoroides* (Linnaeus) Royle and *Syringodium isoetifolium* (Ascherson) Dandy, were collected from the intertidal (9°19'15.82"N, 123°18'45.56"E) and subtidal (9°19'15.82"N, 123°18'46.53"E) zones of Piapi Beach, Dumaguete City, Negros Oriental, Philippines. The collected specimens were washed three times with filtered seawater (FSW) to get rid of adhering soil and epiphytes. These were placed in clean ziplock bags containing an ample amount of FSW. The samples were kept on ice and processed in the laboratory within 48 h. Identification of the host algae and seagrasses was done following detailed morphological characterization of voucher specimens.

Isolation and identification of marine-derived fungi

The thalli, roots, fronds and leaves of the collected algae or seagrasses were initially washed with sterile artificial seawater (ASW, 33 g marine salt dissolved in 1L distilled water). With a sterile razor blade, the specimens were cut into 3-5 mm explants and immersed in 70% ethanol (EtOH) for 60 s to remove surface-associated microorganisms. The explants were then washed three times with sterile ASW for 3 min and blotted dry with sterile cotton cloth. Afterwards, the explants were transferred onto petri plates pre-filled with ½-strength malt extract agar containing 33 g L^{-1} marine salt (MEAS). To prevent growth of contaminating bacteria, the culture media were supplemented with streptomycin (450 μg mL^{-1}). Six explants were placed on each culture plate. In all, five plates containing a total of 30 explants were used for each algal or seagrass species collected. Tissue printing was performed by carefully placing some of the explants over the surface of MEAS plates using sterilized tweezers to evaluate the effectivity of the surface-sterilization treatment. For sterility control, two uninoculated plates were exposed to the working environment to rule out air contamination. The culture plates were incubated for 1 week at room temperature and were monitored daily for fungal growth. Fungal colonies that grew out of the edges of the explants were subcultured on freshly prepared full-strength MEAS and purified by spore touch technique. Identification of isolates was achieved by comparing their colony, hyphal, and spore morphologies with the published literature, i.e. Klinch (2002), Raper and Fennell (1977), and Quimio (1988). To test if the isolated fungi were adapted to the marine environment, the colony extension rate (CER) for the isolates grown in MEA with or without salt was computed using the formula: [mean colony radial growth (day 7) — mean colony radial growth (day 3)] / number of days of incubation (4 days). A paired t-test was computed for the CER on MEA and MEAS to determine if the presence or absence of marine salt in the medium significantly affected the colony extension rate of the MDF.

Mass production and extraction of metabolites

Twenty-one MDF were mass produced by transferring an agar block cut from the margin of a 7-day old fungal colony onto Erlenmeyer flasks containing 100 mL malt extract broth supplemented with 33 g L^{-1} marine salt (MEBS). A total of five culture flasks were prepared for each fungal isolate and were maintained in stationary condition at room temperature for 4 weeks. Following incubation, the contents of the culture flasks coming from the same fungal isolate were pooled and extraction of secondary metabolites was performed by initially separating the broth and mycelia. The culture broth was extracted with ethyl acetate in 1:1 (v/v) proportion, while the harvested mycelia were homogenized and then soaked in methanol. The ethyl acetate and methanol extracts were concentrated *in vacuo* and stored in preweighed vials.

Screening for antibacterial activities

The test bacteria (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853) were acquired from the University of Santo Tomas Collection of Microbial Strains (USTCMS), Manila, Philippines and maintained on tryptic soy broth (TSB). Following the protocol of Quinto and Santos (2005), the 24-h old bacterial cultures were transferred to tubes containing 5 mL of 0.9% normal saline solution (NSS) and standardized to 0.5 McFarland. Then, a sterile swab was dipped on the cell suspension and streaked onto Mueller Hinton agar (MHA) plates. The crude extracts from the mycelia and culture broth were weighed and dissolved in DMSO to achieve a concentration of 50 mg mL^{-1}. The sterile Whatman paper disks (6 mm in diameter) were then impregnated with 20 μL of crude extracts and air-dried aseptically, giving a final concentration of 1 mg per disk of extract. The treated paper

disks were placed on top of the MHA plate seeded with the test bacteria. The control antibiotic was streptomycin (10 µg) while the negative control was DMSO. All culture plates (in triplicates) were incubated at 37 °C for 24 h and the zone of inhibition (ZOI) was measured in mm. The observed zone of inhibition was then compared with the controls and the reference antibiotics listed in EUCAST Ver. 7 (2017).

Screening for brine shrimp cytotoxicity

Dried cysts of *Artemia salina* were hatched in ASW (1 g cyst per liter) at room temperature with continuous illumination. ASW was prepared by dissolving 38 g of commercially available marine salt in distilled water. After hatching, the nauplii were collected with a pipette and concentrated in a vial. The testing of fungal culture extracts was conducted in 6-well microplates. The stock solution was prepared by dissolving the extracts in DMSO at 5 mg mL^{-1}. Aliquots of 0.10, 0.20, 0.40, 0.60, 0.80, and 1 mL of stock solution were diluted with 4.90, 4.80, 4.60, 4.40, 4.20, and 4 mL of brine water to make up a final concentration for the extracts of 100, 200, 400, 600, 800, and 1000 µg mL^{-1}, respectively. The negative control was DMSO. Ten brine shrimps were then carefully transferred to each well with a pipette and these were incubated with the fungal culture extracts for 24 h. The treatments and the control were tested in triplicate, and the number of nauplii that survived was counted for LD_{50} determination using probit regression analysis at 95% confidence interval.

Effects of salinity and culture media on the bioactivities of marine aspergilli

Selection and molecular characterization of marine aspergilli

The marine aspergilli were selected based on the results of the disk diffusion and the brine shrimp cytotoxicity assays. Identification of these strains was done via molecular methods. The DNA of the five marine aspergilli was extracted using the Gentorukun® (Takara Bio Inc., Ltd., Otsu, Japan) from approximately 100 µL volume of fungal mass cultured at 25 °C for 5 days on potato dextrose agar (PDA) slants. The *β-tubulin* gene was sequenced directly from PCR products using *Bt2a* (5'-ggtaaccaaatcggtgctgctttc-3') and *Bt2b* (5'-accctcagttgagtgacccttggc-3') primer pair (Glass and Donaldson 1995). The PCR conditions consisted of denaturation at 95 °C for 10 min followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were then sequenced using the Bigdye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM®3130ABI Genetic analyzer (Applied Biosystems) and the DNA sequences were edited using ATGC Ver. 4 sequence assembly software (Genetyx Co., Tokyo, Japan). Using the BLAST algorithm, the *β-tubulin* gene sequence was used to search the GenBank database at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was then constructed using the consensus sequence based on PAUP maximum parsimony analysis.

Mass production and extraction of metabolites from marine aspergilli

Five selected species of marine aspergilli were mass produced in solid rice medium and the liquid media, malt extract broth (MEB/MEBS) and potato dextrose broth (PDB/PDBS) with and without marine salt. A total of five Erlenmeyer flasks each containing 100 mL of culture media were prepared for each marine aspergillus. These were maintained under stationary conditions at room temperature for 4 weeks. For the marine aspergilli cultivated in the liquid media, the culture broth and the fungal mycelia were separated following incubation. The culture broth was extracted with ethyl acetate in 1:1 (v/v) proportion while the mycelia were homogenized and soaked in methanol. For the marine aspergilli cultivated in solid rice medium, only ethyl acetate extraction was performed. The ethyl acetate and methanol extracts were concentrated *in vacuo* and stored in preweighed vials.

Methyl thiol tetrazolium (MTT) cytotoxicity assay

Following the protocol of Takada et al. (2012), HeLa cervical cancer cells were cultured in Dulbecco's modified eagle medium (Wako Pure Chemical Industries, Osaka, Japan), containing 10% fetal bovine serum, 2 mg mL^{-1} gentamycin, and 10 mg mL^{-1} antibiotics adjusted to pH 7.0–7.4 by 1 M HCl, while P388 murine leukemia cancer cells were cultivated in Roswell Park Memorial Institute Medium-1640 (Wako Pure Chemical Industries), containing 10% fetal bovine serum, 100 mg mL^{-1} kanamycin, and 10 mM 2-hydroxyethyldisulfide. The cell lines were incubated at 37 °C under an atmosphere of 5% CO_2. Afterwards, an aliquot of 200 µL tumor cell suspension (1×10^4 cells mL^{-1}) was dispensed unto each well of the 96-well microplate and pre-incubated for 24 h. Following pre-incubation, 1 µL of marine aspergilli extracts dissolved in DMSO concentrated at 1 mg mL^{-1} was added to the cell suspension to prepare a screening concentration of 5 µg mL^{-1}. The reference chemotherapeutic drug was adriamycin. After treatment, the cells were further incubated for 72 h and to each well 50 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) saline solution (1 mg mL^{-1}) was added, followed by incubation for 3 h under the same condition to stain live cells. Finally, the culture medium was withdrawn and 150 µL of DMSO was added to dissolve cells. Using the Fusion™ α microplate reader (Packard Bioscience Company, CT, USA), the absorbance of each well was read. The IC_{50} for the most bioactive aspergilli extracts was calculated by plotting a dose-dependent curve in GraphPad PRISM 5 software (GraphPad Software Inc., CA, USA).

ATP-based luciferase viability assay for *Trypanosoma congolense*

Following the protocol of Suganuma et al. (2014), the blood stream form of *Trypanosoma congolense* IL 3000 was cultivated at 33 °C in air using Iscove's modified Dulbecco's medium (Sigma-Aldrich, Tokyo, Japan), containing 20% heat inactivated-fetal bovine serum, 60 mM HEPES,

1 mM pyruvic acid sodium salt, 0.1 mM bathocuproine, 1 mM hypoxanthine, 16 μM thymidine, 10 μg L^{-1} insulin, 5.5 μg L^{-1} transferrin, 6.7 ng L^{-1} sodium selenite, 0.0001% 2-β-mercaptoethanol, 0.4 g L^{-1} BSA, and 2 mM L-cysteine. The screening concentration for the fungal extracts was set at 2.5 μg mL^{-1}. This was prepared by initially diluting 0.5 μL of fungal crude extract (1 mg mL^{-1}) with 100 μL HMI-9. From this solution, a 50-μL aliquot was dispensed to the microplate and added to each well containing 50 μL suspension of *T. congolense* (2×10^5 cells mL^{-1}). The reference antiprotozoal drug was pentamidine. The microplates were incubated for 72 h, and subsequently, 50 μL of CellTiter-Glo® luminescent cell viability assay reagent (Promega Japan, Tokyo, Japan) was added to each well to evaluate ATP concentration. Following the incubation of the microplates for another 10 min at room temperature, luminescence was read using a GloMax®-Multi Detection System plate reader (Promega Japan, Tokyo, Japan). The IC_{50} values of the most bioactive aspergilli extracts were computed by plotting a dose-dependent curve in GraphPad PRISM 5 software (GraphPad Software Inc.). Moreover, the selectivity index (SI) of the bioactive extracts was also calculated following the formula of Koch et al. (2005): IC_{50} HeLa or P388 / IC_{50} *Trypanosoma congolense*.

Results

Occurrence of marine-derived fungi in macroalgae and seagrasses

A total of 40 MDF were isolated from algae and seagrasses collected from Piapi Beach, Philippines. These MDF belonged to 16 morphospecies from the genera *Aspergillus*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Sclerotinia*, *Thamnidium*, and *Trichoderma* (Tab. 1). Among these genera, *Aspergillus* was isolated in the highest frequency. Five of the MDF could not be identified as they remained sterile even after prolonged incubation, hence were simply designated as *mycelia sterilia*. Among the host macroalgae and seagrasses, the red alga *L. intermedia* harbored the highest number of isolates, followed by the seagrass *E. acoroides*, then the brown alga *S. piluliferum*, and the seagrass *S. isoetifolium*. Intriguingly, only one MDF was isolated from the red alga *P. hornemannii* and from the green alga *C. racemosa*.

The identified fungal genera were known to be of terrestrial origin. Hence, to assess the adaptability of the isolates in the marine environment, the MDF were cultured in MEA with or without marine salt. Of the 21 MDF tested, 17 grew much better in MEAS than in MEA (Fig. 1). Statistical analysis by one-tailed paired t-test confirmed that the mean colony extension rates in MEAS were greater than MEA and that the difference was highly significant (p-value 0.000349 < α = 0.01).

Antibacterial and cytotoxic activities of marine-derived fungi

Several MDF showed antibacterial properties and brine shrimp cytotoxicity (Tab. 2). Of the 21 MDF tested, five fungal isolates belonging to the genus *Aspergillus* and one *mycelia sterilia* inhibited *P. aeruginosa* and/or *S. aureus* with ZOI greater than 10 mm. The most bioactive crude extracts were the mycelial extract of *A. fumigatus* against *P. aeruginosa* (19 mm ZOI) and the broth extract of *A. tubingensis* against *S. aureus* (19 mm ZOI). The ZOI of the most bioactive culture extracts were comparable to the control antibiotic streptomycin (18 – 21 mm ZOI) and with some reference antibiotics listed in EUCAST Ver. 7 (2017). Interestingly, no antagonism to *E. coli* treated with the MDF culture extracts was observed. Meanwhile, of the 21 MDF tested for cytotoxicity, seven MDF, belonging to the genera *Aspergillus*, *Fusarium*, *Paecilomyces*, *Penicillium* and *Trichoderma*, proved to be cytotoxic to *A. salina* nauplii with LD_{50} ranging from 201.56-948.37 μg mL^{-1}. The most cytotoxic was *A. tubingensis*. Thus, members of the genus *Aspergillus* were noted to be generally bioactive in this study. Furthermore, there was higher record of bioactivities with the ethyl acetate extracts derived from the fungal culture broth as opposed to the methanolic extracts derived from the fungal mycelia.

Molecular characterization of marine aspergilli

The marine aspergilli that showed promising bioactivities in the previous screening were subjected to molecular characterization to validate their identity. Comparison of their *β-tubulin* gene sequences by Blast searching showed

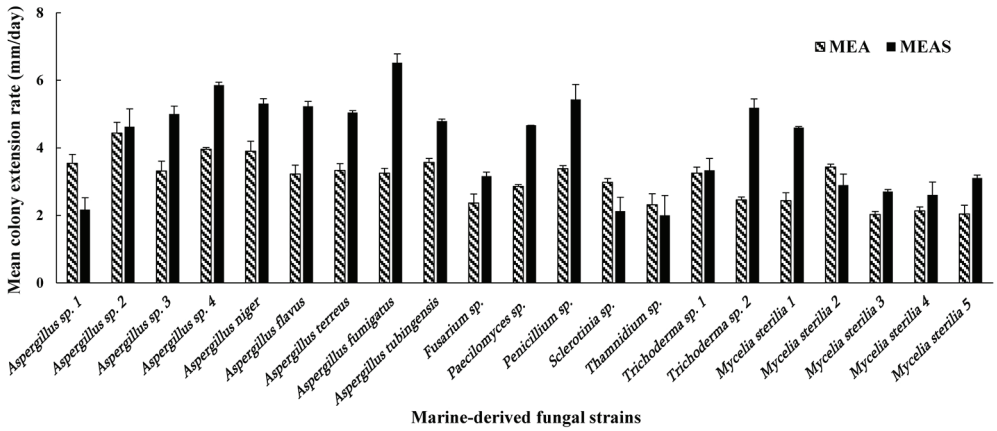
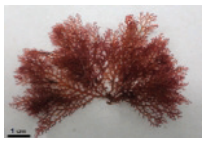
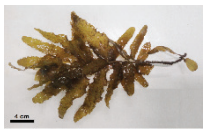
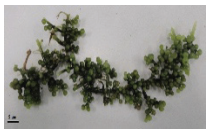
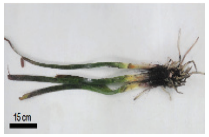


Fig. 1. Colony extension rates of marine-derived fungi grown on malt extract agar with (MEAS) or without (MEA) marine salt (n=3). Mean standard deviation is expressed in error bar.

Tab. 1. Marine-derived fungi (MDF) isolated from macroalgae and seagrasses collected from Piapi Beach, Dumaguete City, Philippines. [a]Names in bold grew better in malt extract agar with salt. Names with asterisks were isolated in more than one host alga/seagrass.

Host alga/seagrass	Substrata	Marine-derived fungi[a]	Total no. of isolated MDF
Laurencia intermedia (Rhodophyte) 	Thallus	*Aspergillus* sp. 1* ***Aspergillus* sp. 2** ***Aspergillus flavus**** ***Aspergillus terreus*** ***Fusarium* sp.*** *Thamnidium* sp. ***Trichoderma* sp. 1*** ***Trichoderma* sp. 2*** ***Mycelia sterilia* 1** *Mycelia sterilia* 2	13
Portieria hornemannii (Rhodophyte) 	Thallus	***Mycelia sterilia* 3**	1
Sargassum piluliferum (Phaeophyte) 	Frond	*Aspergillus* sp. 1* ***Aspergillus flavus****	4
	Thallus	***Aspergillus fumigatus*** ***Fusarium* sp.*** ***Trichoderma* sp. 2***	4
Caulerpa racemosa (Chlorophyte) 	Thallus	*Aspergillus niger*	2
Enhalus acoroides (Seagrass)	Root	***Paecilomyces* sp.*** ***Trichoderma* sp. 1***	4
	Leaf	***Aspergillus* sp. 1*** ***Aspergillus* sp. 3** ***Aspergillus tubingensis*** ***Mycelia sterilia* 4** ***Mycelia sterilia* 5***	6
Syringodium isoetifolium (Seagrass) 	Leaf	***Aspergillus* sp. 4** ***Paecilomyces* sp. *** ***Penicillium* sp.** *Sclerotinia* sp. ***Mycelia sterilia* 5***	6

100% similarities between the sequences of the isolated marine aspergilli and those deposited at GenBank. This homology was further supported by their phylogenetic tree constructed according to maximum parsimony analysis with bootstrap values 90% and above (Fig. 2). Molecular analysis confirmed the marine aspergilli isolates as *A. fumigatus* (AF-ST-02), *A. flavus* (AF-SL-02), *A. niger* (AF-C-01), *A. terreus* (AF-L-12) and *A. tubingensis* (SF-EL-06). All the DNA sequences were deposited at the DNA Data Bank of Japan (DDBJ) with the accession number LC176429 for *A. terreus*, LC176430 for *A. niger*, LC176431 for *A. flavus*, LC176432 for *A. fumigatus*, and LC168474 for *A. tubingensis*.

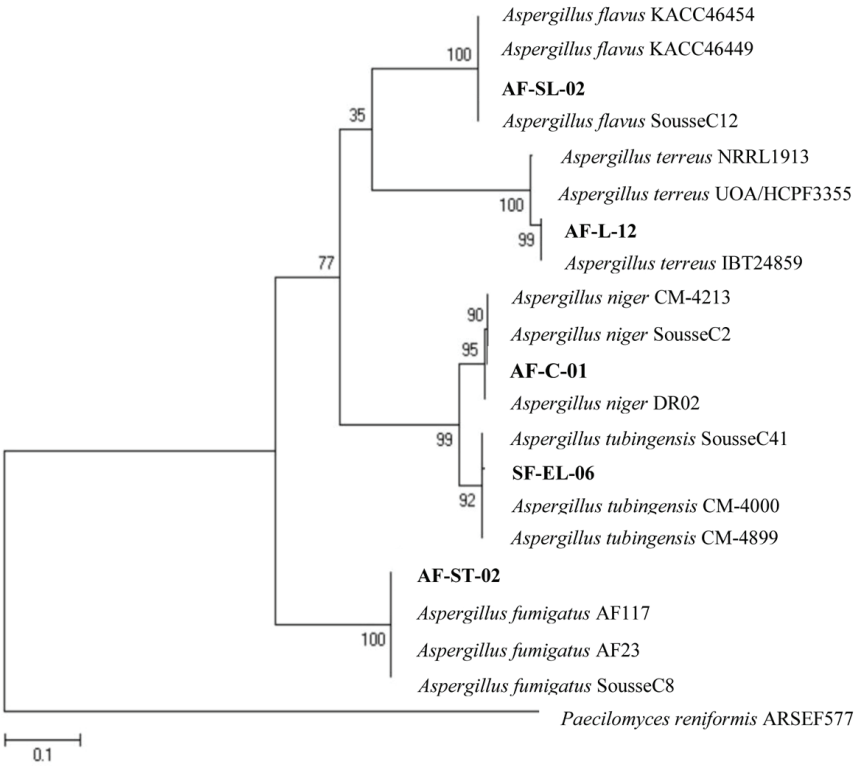


Fig. 2. Phylogenetic analysis of marine aspergilli. The consensus sequence was downloaded from GenBank and can be retrieved through the following accession numbers: *A. flavus* KACC46454 (KT354303.1), *A. flavus* KACC46449 (KT354304.1), *A. flavus* Sousse C12 (KJ136090.1), *A. terreus* NRRL1913 (EF669518.1), *A. terreus* UOA/HCPF3355 (GQ376127.1), *A. terreus* IBT24859 (FJ491707.1), *A. niger* CM4213 (FJ828913.1), *A. niger* Sousse C2 (KJ136065.1), *A. niger* DR02 (KC311845.1), *A. tubingensis* Sousse C41 (KJ136086.1), *A. tubingensis* CM4000 (KJ136086.1), *A. tubingensis* CM4899 (KJ136086.1), *A. fumigatus* AF117 (KF410677.1), *A. fumigatus* AF23 (KF410682.1) and *A. fumigatus* Sousse C8 (KJ136109.1).

Effects of culture media to the cytotoxicity of marine aspergilli

A total of 45 culture extracts were obtained from the marine aspergilli cultivated in five different media with and without marine salt. Of the marine aspergilli tested at 5 μg mL^{-1} screening concentration, *A. tubingensis* and *A. fumigatus* were shown to be the most cytotoxic against cancer cells (Fig. 3). The ethyl acetate crude extracts of *A. tubingensis* derived from the culture media supplemented with salt (MEBS and PDBS) had a percent inhibition ranging from 98–99% against P388 and from 70–76% against HeLa. The bioactivity of these extracts against P388 was comparable with the chemotherapeutic drug adriamycin, which completely inhibited the cancer cell lines. Interestingly, the crude extracts derived from the same fungus cultivated in media without salt (MEB, PDB and solid rice) provided less inhibition, which ranged from 21–76% for both cancer cell lines. For *A. fumigatus*, the ethyl acetate crude extract derived from MEB was the most active with 62.81 and 68.55% inhibition for HeLa and P388, respectively. For the other marine aspergilli, *A. niger*, *A. terreus*, and *A. flavus* showed an inhibitory activity of less than 40% for both cancer cells regardless of whether the fungi were cultivated in media with or without marine salt and of whether the extracting solvent was methanol or ethyl acetate. Based on the initial screening of 45 extracts, four candidate extracts derived from *A. fumigatus* and *A. tubingensis* were selected for IC_{50} determination (Tab. 3). In this study, only the crude culture extracts with more than 50% inhibition were considered for IC_{50} determination. The most bioactive was the ethyl acetate extract of *A. tubingensis* mass produced in PDBS with an IC_{50} at 1028 ng mL^{-1} for P388 and 1301 ng mL^{-1} for HeLa.

Effects of culture media to the trypanocidal activity of marine aspergilli

Consistent with the results of the cytotoxicity assay, *A. tubingensis* and *A. fumigatus* showed the highest trypanocidal activity at 2.5 μg mL^{-1} screening concentration (Fig. 4). The crude ethyl acetate extracts of *A. tubingensis* cultivated in media with salt (MEBS and PDBS) demonstrated an inhibitory activity of 99% comparable with the antiprotozoal drug pentamidine. In contrary, the crude extracts derived from the same fungus cultivated in media without salt (MEB, PDB and solid rice) showed lesser or no inhibitory activity that ranged from 0.53–87.75%. On the other hand, only one extract from *A. fumigatus* showed promising activity. The crude ethyl acetate extract of the same fungus in MEB exhibited an inhibitory activity of 99%, while the remaining extracts from other media provided an inhibitory activity of less than 55%. *A. niger*, *A. flavus*, and *A. terreus* gave an inhibitory activity of less than 52% regardless of culture

Tab. 2. Results of the disk diffusion and brine shrimp cytotoxicity assays of the crude ethyl acetate (EtOAc) and methanolic (MeOH) extracts from marine-derived fungi (n=3). [a]ZOI assessment: inactive < 10 mm; partially active 10–13 mm; active 14–19 mm; very active > 19 mm (Quinto and Santos 2005). [b]LD_{50} assessment: cytotoxic < 1000 μg mL^{-1}; non-cytotoxic > 1000 μg mL^{-1} (Meyer et al. 1982).

Marine-derived fungi	Test bacteria zone of inhibition – ZOI (mm)[a]						Brine shrimp assay LD_{50} (μg mL^{-1})[b]	
	Pseudomonas aeruginosa		*Staphylococcus aureus*		*Escherichia coli*			
	EtOAc	MeOH	EtOAc	MeOH	EtOAc	MeOH	EtOAc	MeOH
***Laurencia intermedia* (Rhodophyte)**								
Aspergillus sp. 1	0	0	0	0	0	0	>1000	>1000
Aspergillus sp. 2	0	0	0	0	0	0	>1000	>1000
Aspergillus flavus	13	0	10	0	0	0	836.24	948.37
Aspergillus terreus	8	0	10	0	0	0	824.71	857.75
Thamnidium sp.	0	0	0	0	0	0	>1000	>1000
Trichoderma sp. 1	0	0	0	0	0	0	834.14	>1000
Mycelia sterilia 1	0	0	0	0	0	0	>1000	>1000
Mycelia sterilia 2	13	0	11	0	0	0	>1000	>1000
***Portieria hornemannii* (Rhodophyte)**								
Mycelia sterilia 3	0	0	0	0	0	0	>1000	>1000
***Sargassum piluliferum* (Phaeophyte)**								
Aspergillus fumigatus	15	19	14	15	0	0	555.22	807.23
Fusarium sp.	9	0	6	0	0	0	834.14	863.29
Trichoderma sp. 2	11	0	9	0	0	0	>1000	>1000
***Caulerpa racemosa* (Chlorophyte)**								
Aspergillus niger	13	0	11	0	0	0	706.76	930.12
***Enhalus acoroides* (Seagrass)**								
Aspergillus sp. 3	9	0	7	0	0	0	>1000	>1000
Aspergillus tubingensis	17	0	19	0	0	0	201.56	333.27
Mycelia sterilia 4	0	0	0	0	0	0	>1000	>1000
Mycelia sterilia 5	9	0	7	0	0	0	>1000	>1000
***Syringodium isoetifolium* (Seagrass)**								
Aspergillus sp. 4	0	0	0	0	0	0	>1000	>1000
Paecilomyces sp.	0	0	0	0	0	0	>1000	875.01
Penicillium sp.	8	0	0	0	0	0	>1000	873.90
Sclerotinia sp.	9	0	9	0	0	0	>1000	>1000
DMSO (– control)	0	0	0	0	0	0	>1000	>1000
Streptomycin (+ control)	21	21	20	20	18	18	–	–

media and addition of marine salts during mass production. Furthermore, all the methanolic extracts from marine aspergilli showed weak inhibition towards *T. congolense* (less than 40%). The IC_{50} of the most bioactive crude culture extracts was determined (Tab. 3) and showed that the ethyl acetate extract of *A. fumigatus* cultivated in MEB was the most potent (IC_{50}: 298.18 ng mL^{-1}) with selectivity index ranging from 7.21–15.50.

Discussion

Marine-derived fungi are known colonizers of various organic substrates, including corals, echinoderms, seagrasses, algae, and vertebrates, and can act as endophytes, pathogens, saprobes, and parasites in the marine communities (Loque et al. 2010). In this study, 16 morphospecies of marine-derived fungi belonging to the genera *Aspergillus*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Sclerotinia*, *Thamnidium,* and *Trichoderma* including five *mycelia sterilia* were isolated from macroalgae and seagrasses (Tab. 1). Among the MDF, the genus *Aspergillus* was isolated in highest frequency and in wide distribution in brown, red and green marine algae, and seagrasses. It is well-reported that *Aspergillus* is not only isolated from terrestrial substrata, but is also widely distributed in the marine environment (Kato et al. 2007, Cui et al. 2010, Li et al. 2011). In this study, all the isolated MDF genera were observed to be terrestrial in origin. However, it is possible for terrestrial fungi to be introduced into the marine ecosystem

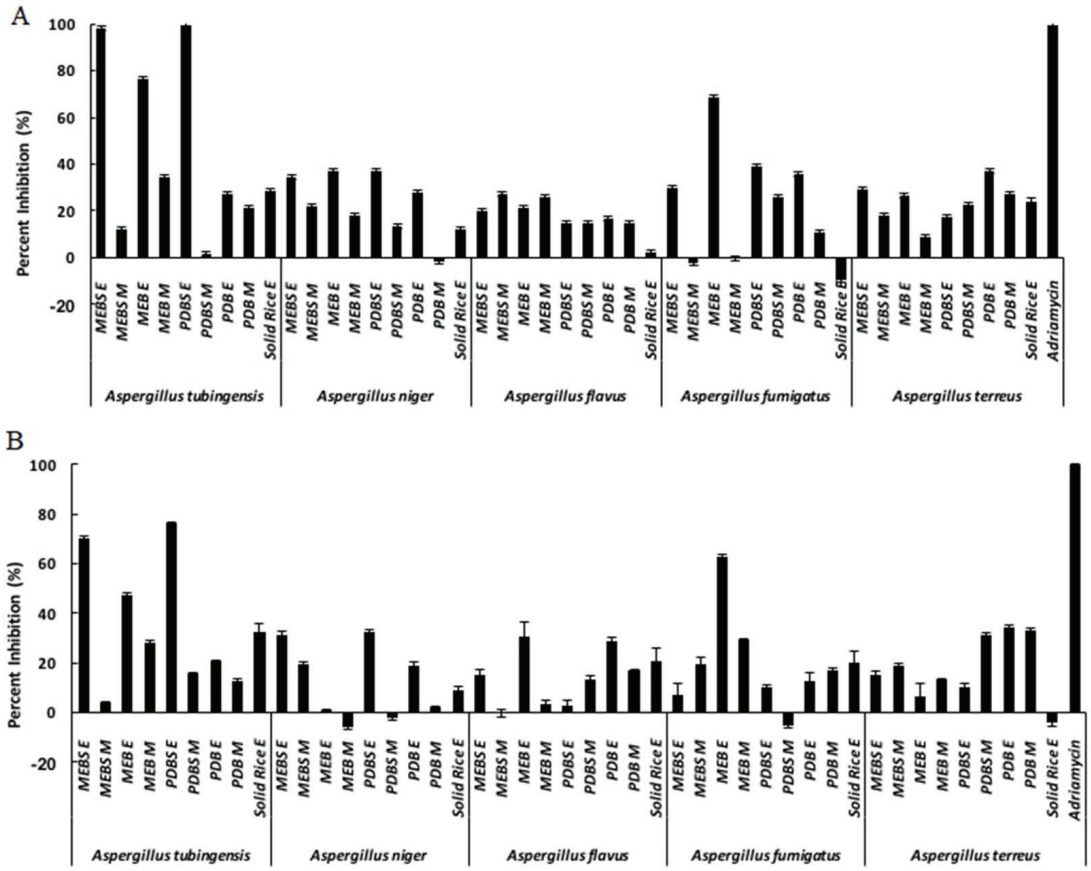


Fig. 3. Cytotoxicity profile of ethyl acetate (E) and methanolic (M) crude extracts (5 μg mL^{-1}) from marine aspergilli against P388 murine leukemia cancer cell line (A) and HeLa cervical cancer cell line (B). Marine aspergilli were previously cultivated in malt extract broth with and without marine salt (MEB/MEBS), potato dextrose broth with and without marine salt (PDB/PDBS), and solid rice. Data are average values ± SD (n=3).

Tab. 3. Median inhibitory concentration (IC_{50}) of ethyl acetate crude extracts from selected marine aspergilli. [a]Assessment of cytotoxicity: IC_{50} < 30 μg mL^{-1} signifies anticancer activity (Suffness and Pezzuto 1990). [b]Assessment of antiprotozoal activity: IC_{50} < 10 μg mL^{-1} signifies trypanocidal activity (Koch et al. 2005). [c]Assessment of SI value for trypanocidal activity: SI value > 2 signifies selectivity (Koch et al. 2005).

Marine aspergilli	Culture medium	Test organism/cell line (IC50 ng mL^{-1})			Selectivity[c] index (SI)
		P388[a]	HeLa[a]	*Trypanosoma*[b] *congolense*	
Aspergillus fumigatus	MEB	2150	4623	298.18	7.21-15.50
Aspergillus tubingensis	MEBS	2247	1168	381.16	3.06-5.90
Aspergillus tubingensis	MEB	3462	1857	2010.04	0.92-1.72
Aspergillus tubingensis	PDBS	1301	1028	485.58	2.12-2.68
Adriamycin	+ control	92.85	72.96	–	–
Pentamidine	+ control	–	–	100.45	–

and to evolve as a result of selective pressure from this new habitat (Jones 1994). As shown in Fig. 1, the colony extension rates of the isolated marine-derived fungi were greater in MEAS as opposed to MEA. This shows the adaptability of the MDF to the salinity in the marine environment. Following Kohlmeyer and Kohlmeyer (1979), the isolates were regarded as facultative marine fungi that might have originated from freshwater or terrestrial environment and undergone physiological adaptations for their survival in the marine environment. This is further supported by the ability of the MDF to grow in MEA with or without marine salt. Similar growth characteristic was observed in other marine fungi, as in the case of *Dendryphiella* (dela Cruz et al. 2006b).

The continuing prevalence of infectious diseases and the challenge to chemotherapy brought about by drug resistance call for the development of a new pipeline of drugs. Owing

to their taxonomic and metabolic diversity, marine organisms including marine fungi are now considered ideal sources of new secondary metabolites (Schulz et al. 2008). Among the tested MDF, *A. tubingensis* and *A. fumigatus* conferred the greatest antibacterial and cytotoxic properties (Tab. 2). Comparing the zones of inhibition of these crude culture extracts with reference antibiotics specified by EUCAST Ver. 7 (2017), the antibacterial property of the mycelial extract of *A. fumigatus* was more potent than netilmicin while the broth extract of *A. tubingensis* showed greater inhibition than ampicillin and benzylpenicillin. This is not surprising as the observed bioactivities of the members of the genus *Aspergillus* are well reported (Lee et al. 2013). In fact, many novel bioactive compounds have been isolated from marine aspergilli, including waikialoid A (Wang et al. 2012) and pseudodeflectusin (Ogawa et al. 2004). However, the other isolated MDF (*Fusarium*, *Paecilomyces*, *Penicillium*, *Sclerotinia*, *Thamnidium*, and *Trichoderma*) showed either no bioactivities or fewer than marine aspergilli, which further supports the idea that marine aspergilli are worthy target microorganisms for drug discovery. Interestingly, a higher record of bioactivity was observed in the ethyl acetate extracts derived from the culture broth as opposed to the methanolic extracts derived from the mycelia. This means that the bioactive metabolites must have been extracellularly produced by the MDF. Thrane et al. (2007) explained that most of the secondary metabolites, organic acids, enzymes, and other bioactive proteins are extracellularly produced by the fungi to their environment.

Based on the initial screening, the marine aspergilli *A. tubingensis*, *A. fumigatus*, *A. niger*, *A. terreus*, and *A. flavus* were selected for further testing against cancer cells and the protozoan *T. congolense*. Consistent with the previous results, *A. fumigatus* and *A. tubingensis* showed the greatest anticancer and trypanocidal activities (Tab. 3). The study reported for the first time the bioactivity of a marine-derived *A. fumigatus* against *T. congolense*. Previous studies reported the isolation of *A. fumigatus* from macroalgae, sea sediment, and holothurians, and showed to produce several cytotoxic compounds such as N-acetyltyramine (Zhao et al. 2010) and the apoptosis-inducing metabolite fumigaclavine C (Li et al. 2013). Moreover, Furtado et al. (2005) also reported that *A. fumigatus* derived from soil samples produced metabolites that are bioactive against *T. cruzi,* which causes Chagas disease. Another promising bioactive MDF is *A. tubingensis,* which was found to be cytotoxic against HeLa and P388. The isolation and the possible mode of action of its bioactive compound is fully discussed in a separate paper (Notarte et al. 2017). Nonetheless, the cytotoxicity of *A. tubingensis* is supported by Zhan et al. (2007) when a terrestrial strain derived from the rhizosphere of *Festuca paradoxa* produced metabolites that were active against several cancer cells.

The 'OSMAC (one strain-many compounds) approach', which involves manipulation of cultivation parameters, such as the media composition, is an efficient strategy for enhancing the chemical diversity of compounds that may be of interest as lead structures in conducting high-throughput biological screening (Bode et al. 2002). Wang et al. (2014) demonstrated the potential application of the OSMAC approach that induced the MDF *Ascotricha* sp. to synthesize three new caryophyllene derivatives when cultured in an oligotrophic rather than the typical eutrophic medium. In the current research, there was no general pattern on the influence of marine salt on the bioactivities of marine aspergilli, although some species were directly influenced by salinity in the production of bioactive metabolites (Figs. 3 and 4). Interestingly, cultivation in solid rice led to a weaker or loss

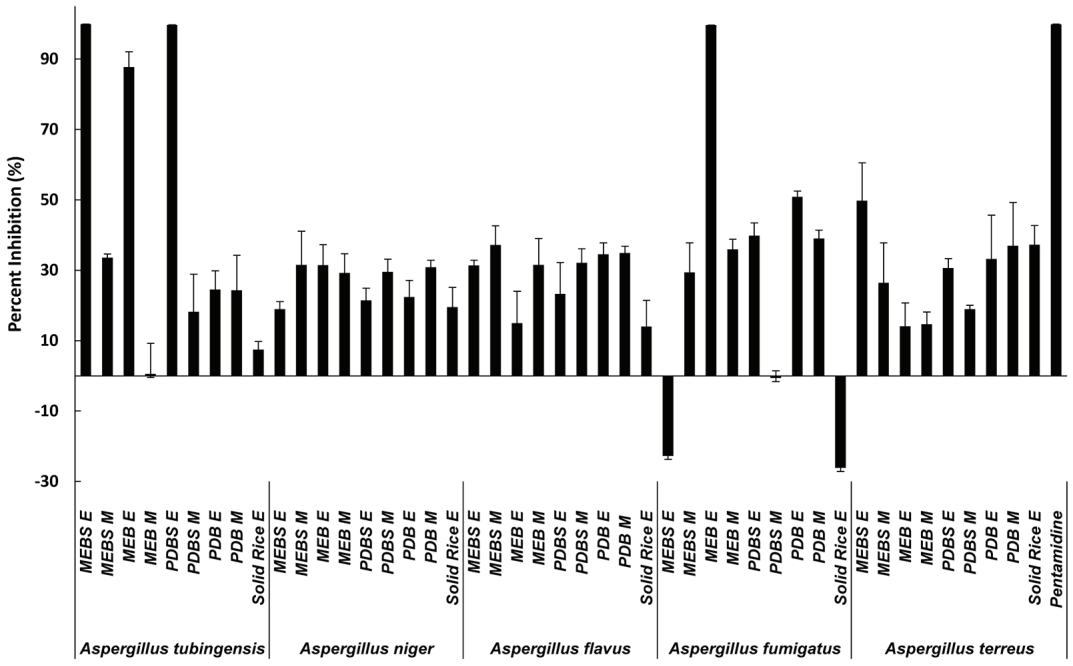


Fig. 4. Trypanocidal activity of ethyl acetate (E) and methanolic (M) crude extracts (2.5 μg mL^{-1}) from marine aspergilli cultivated in malt extract broth with and without marine salt (MEB/MEBS), potato dextrose broth with and without marine salts(PDB/PDBS), and solid rice. Data are average values ± SD (n=2).

of bioactivity for the marine aspergilli. Indeed, it is worth noting that *A. tubingensis* proved to have elicited better bioactivities against cancer cells and *T. congolense* when cultivated in the presence of marine salt regardless of the culture medium used for cultivation. This indicates that salt plays an important role in the synthesis of biologically active components present in the crude extracts of *A. tubingensis*. The crucial role of salt in the production of compounds was best demonstrated by Wang et al. (2011) when cultivation of the marine-derived fungus *Spicaria elegans* in 10% salinity led to the production of novel and chlorinated compounds that were not synthesized when the same fungus was cultured in 3% saline condition. Another interesting observation is that of *A. fumigatus,* which showed promising cytotoxic and trypanocidal activities only when cultivated in MEB without marine salt. Calvo et al. (2002) showed that the type of carbon and nitrogen source used as precursor molecules during anabolic chemical reactions affects the synthesis of certain compounds. As in the case of MEB, this culture medium contains malt extract and peptone, which served as the carbon and nitrogen sources for the production of various metabolites by *A. fumigatus*. Moreover, the production of bioactive chemicals from *A. fumigatus* without marine salt may suggest that the presence of salt could block the expression of certain biosynthetic pathways essential for the synthesis of its bioactive components. As described by Yu and Keller (2005), salinity is one factor that can regulate production of secondary metabolites.

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