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Research Article

Saline Tolerant of Marine Endophytic Fungi from Teluk Kemang Malaysia – A Rich Source of Bioactive Material

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ABSTRACT

Endophytic fungi have been reported to have the potential as an alternative source for active metabolites in drug discovery. In a recent study, bioactive compounds were isolated from marine endophytic fungi in Malaysia. However, marine endophytic fungi were not identified. In this study, therefore the 18 endophytic fungi that were isolated from eight marine seaweeds collected from Teluk Kemang Negeri Sembilan, Malaysia were identified and evaluated for their antimicrobial activity. Out of 18 marine endophytic fungi, 11 of them were successfully identified based on internal transcribed spacer (ITS) sequencing. Of the 11, six marine endophytic fungi (MV, CN, CS1, CS2, ED1, PA1) identified were *Aspergillus* sp, whereas the other marine endophytic fungi isolates (UF, ED2, PA2) had sequences that were similar to *Exophiala dermatitidis*, *Diaporthe pseudomangiferae, Arthrinium xenocordella, Phanerochaete carnosa,* and *Psathyrella purpureobadia* respectively. A significant antifungal activity against three pathogenic fungi was exhibited by using the disc diffusion method. Eight extracts (CN, CN1, MV, MV1, ED1, ED11, ED2, ED21, PA7, PA71) exhibited antifungal activity ranging from 6.5 mm ± 0.71 mm to 12mm±1.41 (p<0.05) against *Candida albicans* and *Trichophyton rubrum*. The fungicidal effect of CN1 and ED11 extracts was detected at a lower concentration tested (0.625mg/mL) and the diameter of zone inhibitions for these two extracts (CN:9.0 mm ± 0.00 and ED11: 10.5 mm ± 0.71) were even bigger when compared to Amphotericin B (7.5mm ± 0.71). This study also showed that the salinity (additional 3% sea salt) influenced the growth, spore production, and antifungal properties of the marine endophytic fungi. Marine endophytic fungi isolated from the selected seaweeds in the present study, therefore represent a promising source of antifungal and warrant further detailed investigation.

Key words: Antifungal, identification, marine endophytic fungi, salinity, seaweed

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INTRODUCTION

The marine environment offers a vast source of bioactive compounds from many marine organisms. This leads to the discovery of new and potential bioactive compounds with many therapeutic values on various diseases (Karthikeyan *et al*., 2022; Fouillaud *et al*., 2016). Endophytic fungi are microorganisms that live within plant tissue without causing any harm to the host plant (Jeewon *et al*., 2013). Marine endophytic fungi might be host-specific, housing novel natural bioactive compounds that are useful in drug discovery. Isolated marine endophytic fungi in this study have been reported to have great bioactivities potential such as antioxidant, antimicrobial, anticancer, anti-inflammatory as well as wound healing properties (Ariffin *et al*., 2011; Ariffin *et al*., 2014; Aqilah *et al*., 2018). Though seaweeds have been well-recorded as a good bioactive compound source, not many studies source provide holistic data on the marine endophytic fungi derived from seaweed (Flewelling, 2015).

Identification of endophytes traditionally depends on the morphological characteristic of the fungi as it is easy to be observed and recorded. However, this method is not practical to be applied to non-sporulating fungi (Jeewon *et al*., 2013). Furthermore, morphological characterizations of endophytic fungi will only allow the identification of

samples up to the genus level (Tibpromma *et al*., 2018). Therefore, confirmation of the species of marine endophytic fungi has to be made through molecular characterization (Zakaria *et al*., 2016). However, although molecular identification provides extensive information on genetic analysis, morphological identification is still vital to obtain more comprehensive data on the identification of marine endophytic fungi (Jayasiri, 2015). In this study, endophytic fungi isolated from eight seaweeds (*Gracilaria arcuata zanardini, Gracilaria coronopifolia* (J. Agardh), *Chaetomorpha minima* (F.S Collins & Harvey), *Caulerpa sertularioides, Acantophora spicifera* (M. Vohl), *Padina minor* Yamada*, Enteromorpha compressa* and *Caulerpa lentifera)* were used to investigate the impact of salinity on the growth, morphological and spore production. This is the first investigation into the variety and abundance of fungal endophytes derived from marine algae in Teluk Kemang Negeri Sembilan and screening for their antifungal properties.

MATERIALS AND METHODS

Isolation of marine endophytic fungi

Endophytes were isolated as described by Strobel (2003) from eight local seaweeds collected from Teluk Kemang Negeri Sembilan Malaysia. Three of the seaweeds belonged to red seaweeds (*Gracilaria arcuata* Zanardini, *Gracilaria coronopifolia* J. Agardh, *Acantophora spicifera* M. Vohl), four were green seaweeds (*Chaetomorpha minima* F.S Collins & Harvey, *Caulerpa sertularioides*, *Enteromorpha compressa,* & *Caulerpa lentifera*) and one was brown seaweed which is *Padina minor,* Yamada. Seaweed was washed under running water, sterilized with 75% ethanol for 1 min and household bleach (5% sodium hypochlorite, NaOCl) for 3 min, drained, and immersed in 75% ethanol again for the 30 s. Finally, the samples were rinsed with sterile water and cut aseptically into 1 cm long segments. The cut segments were incubated on potato dextrose agar (PDA, Oxoid, Basingstoke, UK) supplemented with chloramphenicol (10 gL^{-1}) Sigma-Aldrich, St Louis, MO, USA) at 28 °C until mycelia were observed. Pure cultures were isolated, subcultured on PDA free of antibiotics, and incubated for 30 days at 28 °C before extraction. The stock cultures were maintained at the Marine Pharmaceutical Research Group (MaReG) laboratory, Puncak Alam, Malaysia (Ariffin *et al*., 2011).

Effects of artificial sea salt on the growth of marine endophytic fungi

The marine endophytic fungi were subcultured on PDA with different artificial sea salt (AS) levels (0%, 1%, & 3% AS), and the optimal growth was determined. The diameter of the marine endophytic fungi growth was observed and recorded after 7 days of incubation at 28 °C. The longest and shortest diameters of the colony were measured and the mean was recorded as the size of the colony following Huang *et al*. (2011).

Identification of marine endophytic fungi

Morphological identification

The identification of the isolated fungi was based on the description of colonies and morphological structures, and types of conidiophores described in Descriptions of Medical Fungi by Ellis *et al*. (2007).

Molecular identification: DNA extraction and PCR amplification

Six replicates of pure marine endophytic fungi isolates were subcultured for seven days scraped out from the plate and subjected to DNA extraction using the automation kit Maxwell® 16 DNA purification kit (Promega, USA). Amplification of cDNA was performed by Polymerase Chain Reaction (PCR) using ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'TCCTCCGCTTATTGATATGC-3') as primers. The PCR mixture with a total reaction volume of 50 µL contains 5 µL of 10× buffer, 1 µL dNTP, 2 µL of each primer, 0.25 µL *Taq* DNA polymerase, and 2 µL of DNA template. The amplification cycle condition set on the thermocycler was: initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C, 1 min annealing at 50 °C and 1 min of extension at 72 °C with a final extension at 72 °C for 10 min. The DNA fragment amplified from the ITS region was observed by electrophoresis on 1% (w/v) agarose gel with a size of DNA approximately between 500-700 base pairs.

The amplified PCR products were examined by electrophoresis in 1.5% agarose gels in TAE buffer, purified using a PCR clean-up kit (Promega USA), and sequenced. Sequences were manually edited and aligned using BioEdit v 7.0.5 and matched with DNA sequences from GenBank using the BLAST software (BLASTN) at the National Center of Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov). The sequences were deposited in the GenBank (Hazalin *et al*., 2013).

Extract preparation

The extraction method was carried out according to the procedure explained by Ariffin *et al*. (2011). The endophytic fungi on PDA (about 150 plates) were macerated and transferred to a conical flask filled with 100% ethyl acetate and the resultant mixture was stirred overnight at room temperature. The extract was filtered through No 1 filter papers, 20–25 mm in diameter (Whatman, Maidstone,

UK,), after which sodium sulfate (3-4 g) was added to further remove the aqueous layer within the extract. The sodium sulfate was removed by filtration before the organic phase was dried by rotary evaporation. The resulting extracts were weighed and dissolved in methanol (0.08 g/mL) and used to determine the biological activity. The extracts were stored at -18 °C

Determination of antimicrobial activity

Marine endophytic extracts were screened for antimicrobial activity using the disc diffusion method (CLSI 2020). The microorganisms used were *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Salmonella enterica* (NCTC 4444) and *Salmonella typhimurium* (NCTC 13348), *Aspergillus niger* (ATCC 16404), *Candida albicans* (ATCC 10231) and *Trichophyton rubrum* (ATCC 40051). Cultures of bacteria were cultivated on Mueller-Hinton agar (MHA) plates. One or two overnight-grown colonies were suspended in a test tube containing nutrient broth. The turbidity (expressed as optical density; OD) of the bacterial suspensions was measured with an optical spectrophotometer (λ = 545 nm) and adjusted to McFarland 0.5. A sterilized cotton swab was immersed in the resulting suspension, and a lawn of bacteria was applied to the agar plates. Meanwhile, 200 µL of fungi that have been standardized to McFarland 0.5 were seeded into pre-warmed Sabouraud Dextrose Agar (SDA, Oxoid). Sterile paper discs (6 mm diameter, Oxoid) were placed onto the surface of the agar plate. and 10 microliters of the extract (each at 0.625 mg mL^{-1}, 1.25 mg mL^{-1}, 2.5 mg mL^{-1}, 5 mg mL^{-1}, & 10 mg mL^{-1} in 0.5% of methanol) were loaded on sterile paper discs. Streptomycin (10 mg, Oxoid) and amphotericin B (10 mg, Bristol-Myers Squibb, Avenue Park, NY, USA) were used as positive controls, and 0.5% methanol as a negative control. The plates in duplicates were incubated at 37 °C (24 h) for bacteria and 28 °C (72 h) for fungi. The diameters of inhibition zones (mm) were measured.

Statistical analysis

The comparison of the colony growth rate with different AS concentrations was performed using a repeated-measures t-test. The results were expressed as means ± SD and analyzed using SPSS version 17.0 statistical software. *p* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Eight different seaweeds were sampled from Teluk Kemang Port Dickson Negeri Sembilan. A total of 18 endophytic fungi were isolated from the collected seaweed samples. *Padina minor Yamada* hosted seven different types of fungal endophytes implying the highest community diversity as compared to other seaweeds, followed by *Caulerpa lentifera* (3), *Caulerpa sertularioides & Acantophora spicifera (*M. Vohl) (2), and one fungal isolate each from the seaweeds *Enteromorpha compressa, Gracilaria arcuata zanardini, Gracilaria coronopifolia* (J. Agardh), *Chaetomorpha minima* (F.S Collins & Harvey). From the morphological observation, six marine endophytic fungi (MV, CN, UF, CS1, CS2, ED1) showed similar morphological characteristics. Green conidia can be seen distributed along with brown pustules scattered throughout the plate. PA1 had brown-black conidia with a white basal layer growing throughout the plate. PA2 and PA3 showed whitish mycelium in which the yellow spore production can be seen after five days of cultivation. PA4 has whitish mycelium with green spores that can be seen from the center of the agar plate. However, no spore production was observed from PA5, PA6, and PA7. The growth of endophytic fungi was affected by different concentrations of AS. The fungi displayed different types of characteristics mainly faster growth, more sporulation, and looked fluffier. The colony diameters of the seven marine endophytic isolated from *Padina minor Yamada* and grown in a high concentration of AS gave larger diameters (Table 1). Endophytic fungi (PA1, PA2, PA3, & PA4) exhibited more spores on PDA 3% AS plates (Figure 1a). Moreover, the other three marine endophytic fungi (PA5, PA6, & PA7) showed fluffier

Table 1. The colony diameters of marine endophytic fungi isolated from *Padina minor Yamada* grown at three concentrations of artificial sea salt; 0%, 1%, and 3%

Fungi	Marine Endophytic Standardized colony diameter/ cm ±SD[1]		
	0%	1%	3%
PA1	4.18(±0.45)*	4.79(±0.43)*	5.33(±0.00)*
PA2	4.20(±0.34)*	4.53(±0.52)*	5.20(±0.00)*
PA3	3.49(±1.31)*	5.09(±0.36)*	5.55(±0.00)*
PA4	4.41(±0.65)*	5.45(±0.05)*	5.43(±0.00)*
PA5	4.57(±0.18)*	5.03(±0.00)*	5.25(±0.00)*
PA6	2.21(±0.26)*	4.07(±0.63)*	4.89(±0.16)*
PA7	2.02(±0.38)*	3.65(±0.43)*	4.42(±0.54)*

*Significant differences
[1]Values are means ± SD of three experiments each in duplicates

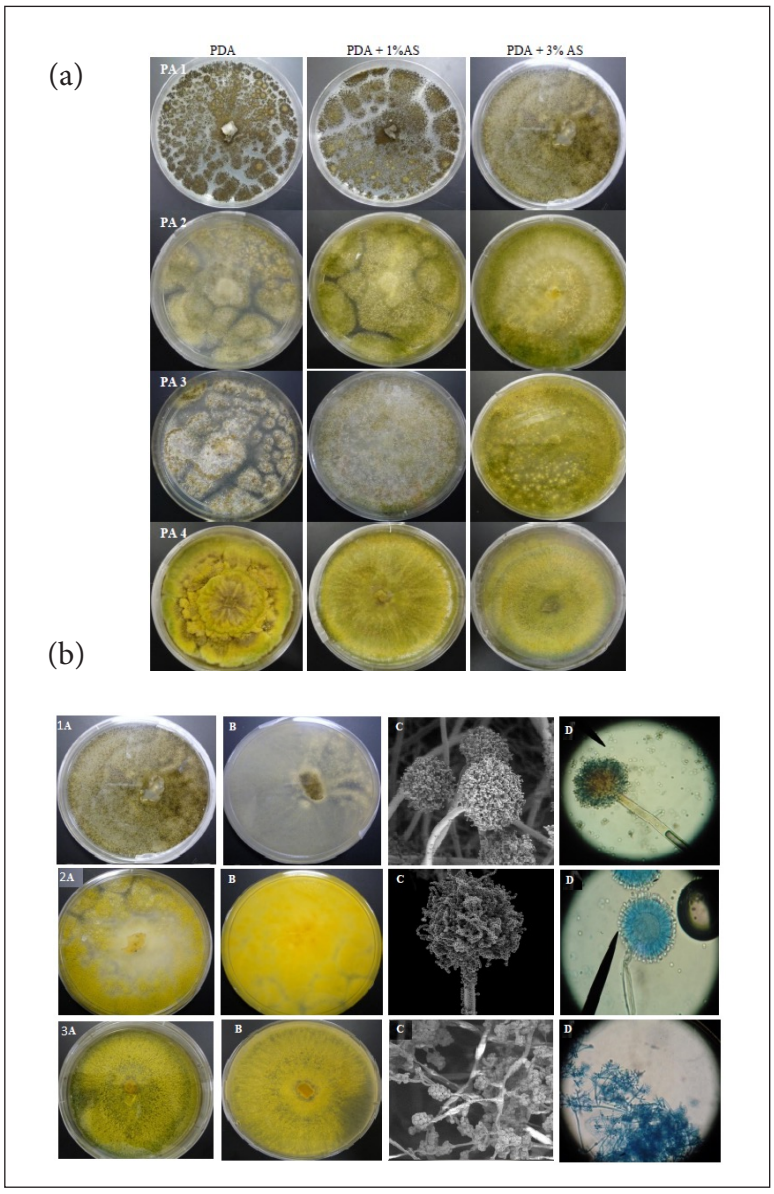

Fig.1. a) The differences in colony appearance of marine endophytic fungi at 7 days of incubation. **b)** Morphological characteristics of isolate PA1 (1A), PA2 (2A) and PA4 (3A). The front of colonies (A), the reverse (B), observation of conidia under Environmental Scanning Electron Microscope (ESEM) at 1000× (C), and observation of conidia under Light Microscope (LM) at 100× (D).at 28°C on PDA media and PDA supplemented with 1% and 3% of AS.

white mycelium that was distributed well on PDA with 3% AS plates compared to the other two plates (PDA & PDA1% AS). Figure 1b shows the morphology as observed under a light microscope and ESEM.

Marine endophytic fungi seem to require a saline environment to mimic their original habitat to grow well. In this study, the colony's diameter was significantly larger by the increased AS concentration (p<0.05). The growth rates of marine endophytic fungi increased gradually as the concentration of AS increased from 1% to 3%. The results obtained in this study were to previous findings by Kerzaon *et al*. (2008). The presence of AS can encourage conidial production and the proliferation of marine endophytic fungi. Due to the presence of ions such as calcium chloride, potassium chloride, sodium sulfate, sodium chloride, and magnesium chloride, artificial salt has a higher ability to imitate the environment of the sea than sodium chloride. The best salinity condition for marine endophytic fungi growth and conidiogenesis was found at 3% AS and is supported by a report by Huang *et al*. (2011). The genus and species of marine endophytic fungi can be further confirmed using molecular techniques which are highly sensitive and specific (Sette *et al*., 2006).

To confirm the identification of marine endophytic fungi, a molecular characterization

study was conducted. Eleven of the marine endophytic fungi isolates were able to be identified through the molecumethods. The sequence of each strain along with the accession number including the host plant are listed in Table 2. Out of 18 fungi isolates, 11 isolates were successfully identified. A BLAST search for similar ITS region in GenBank showed that five isolates (MV, CN, CS1, CS2, ED1) had 99% similarities with *Aspergillus fumigatus* and one isolate (PA1) showed 99% identity sequence with *Aspergillus niger*. Isolate UF, ED2, and PA2 exhibited 99% similarities with *Exophiala dermatitidis*, *Diaporthe pseudomangiferae,* and *Psathyrella purpureobadia* respectively. The *Aspergillus* spp. seem to be the most commonly identified seaweeds in this study. (Table 2). Previously, many of the marine endophytic fungi failed to sporulate, therefore making identification through morphological method difficult. In this study increased salinity (3%) in the growth agar was proven to enhance the growth and sporulation of isolates that might be useful during the identification process. The DNA sequence of the ITS region will, therefore, provide more reliable data on the divergence as well as the taxonomic identity of the marine endophytic fungi. The molecular identification will also provide additional information to further validate the morphological identity.

In this study, 18 marine endophytic fungi isolates were studied to identify their genus level. *T. rubrum* was used as an internal control in this study. Based on the morphological characteristics, more than half of the isolates were similar to *Aspergillus* spp. This was also proven through the molecular method in which six of the isolates were identified as *Aspergillus* spp. Five of them were *Aspergillus fumigatus* and one of them was *Aspergillus niger. Aspergillus* spp is the most common fungal genus and is abundant among the algal species (Kamat *et al*., 2020). The genus *Aspergillus* has been known to be a major contributor to the secondary metabolites of marine fungal origin (Wei *et al*., 2010). In the report, *Aspergillus* sp produced phenolic bisabolene-type sesquiterpenoids and showed cytotoxic activity against human lung carcinoma lines. Metabolites from *Aspergillus* spp have extensively contributed to the pharmaceutical industry (Abdel-Motaal *et al*., 2010).

Another marine endophytic fungus that has been identified is *Exophiala dermatitidis which* was isolated from *Chaetomorpha minima*. *Exophiala* sp has the potential in reducing the adverse effects of oxidative stress in crop plants when having mutual interactions with them (Khan *et al*., 2012). This shows that endophytic fungi enhance plant growth and might be useful in agriculture. Meanwhile, the genus *Diaporthe* sp was isolated from *Acantophora spicifer*a and was previously studied to possess two novel active metabolites of benzopyranones derivatives which are diaportheone A and diaportheone B. Both of the benzopyranones derivatives show inhibition of growth against *Mycobacterium tuberculosis* (Bungihan & Franzblau, 2011). Moreover, this genus was investigated for its cytotoxic activity and antimicrobial property (Agusta, 2006). *Diaporthe* sp are distributed worldwide in many ecosystems. They are regarded as potential sources for producing diverse bioactive metabolites (Xu *et al*., 2021). *Arthrinium* sp., a marine endophytic fungus that was isolated from a species of *Padina Minor*, is also known to produce secondary active metabolites. A study has been conducted to examine the antibacterial and antioxidant properties of *Arthrinium* sp (Pansanit & Pripdeevech, 2018). According to the findings, *Arthrinium* sp. is capable of inhibiting the growth of *Micrococcus luteus* and *Pseudoaltermonas piscida* (Zhang *et al*., 2013).

Table 2. The identification of 11 marine endophytic fungi isolated from eight Malaysian seaweeds

Host plant	Sample ID	Closest GenBank Match	E value	Identity (%)	Accession No.
Gracilaria arcuata Zanardini	MV	*Aspergillus fumigatus*	0.0	99	NR_121481.1
Chaetomorpha minima F.S Collins & Harvey	UF	*Exophiala dermatitidis*	0.0	99	NR_121268.1
Gracilaria coronopifolia J. Agardh	CN	*Aspergillus fumigatus*	0.0	99	NR_121481.1
Caulerpa sertularioides	CS1	*Aspergillus fumigatus*	0.0	99	NR_121481.1
	CS2	*Aspergillus fumigatus*	0.0	99	NR_121481.1
Acantophora spicifera (M. Vohl)	ED1	*Aspergillus fumigatus*	0.0	99	NR_121481.1
	ED2	*Diaporthe pseudomangiferae*	0.0	99	NR_111858.1
Padina minor Yamada	PA1	*Aspergillus niger*	0.0	99	NT_166520.1
	PA2	*Psathyrella purpureobadia*	0.0	88	NR_119670.1
	PA7	*Arthrinium xenocordella*	1e-164	91	NR_120274.1
Caulerpa lentifera	CR3	*Phanerochaete carnosa*	6e-159	89	NW_006767655.1

All 18 endophytic extracts seem to exhibit better antifungal activity when compared to the antibacterial effect. Out of 18, five marine endophytic fungus extracts (CN, MV, PA7, ED1, & ED2) had a notable antifungal effect against *C. albicans* and *T. rubrum* (Table 3). These extracts,

Table 3. Antifungal activity of marine endophytic fungi extracts against pathogenic fungi

Fungi	Time, h	[mg/mL]	Diameter of inhibition zone (mm)				
			AN	CA		TR	
				PDA	PDA 3%	PDA	PDA 3%
CN	48	10.00	-	8.0 ± 0.00	8.0 ± 0.00	-	12.0 ± 1.41
		5.000		7.5 ± 0.71	-	-	11.0 ± 1.41
		2.500	-	-	-	-	9.50 ± 0.71
		1.250	-	-	-	-	9.50 ± 0.71
		0.625	-	-	-	-	9.00 ± 0.00
	72	10.00		8.0 ± 0.00	8.0 ± 0.00	-	11.0 ± 2.82
		5.000		-	-	-	10.0 ± 0.00
		2.500	-	-	-	-	9.50 ± 0.71
		1.250		-	-	-	9.50 ± 0.71
		0.625		7.5 ± 0.71	-	-	9.00 ± 0.00
MV	48	10.00	-	-	8.5 ± 0.71	-	10.0 ± 1.41
		5.000		-	7.0 ± 0.00	-	7.0 ± 1.41
		2.500		-	7.0 ± 0.00	-	-
		1.250		-	6.5 ± 0.71	-	-
	72	10.00		-	8.5 ± 0.71	-	9.5 ± 0.71
		5.000	-	-	7.0 ± 0.00	-	7.0 ± 1.41
		2.500		-	7.0 ± 0.00	-	-
		1.250		-	6.5 ± 0.71	-	-
PA7	48	10.00	-	8.5 ± 0.71	8.5 ± 0.71	-	11.0 ± 0.00
		5.000		8.0 ± 0.00	-	-	
	72	10.00	-	8.5 ± 0.71	8.5 ± 0.71	-	10.5 ± 0.71
		5.000		8.0 ± 0.00	-	-	-
ED1	48	10.00		7.0 ± 0.00	9.0 ± 1.41	-	11.5 ± 0.71
		5.000		-	7.5 ± 2.12	-	10.0 ± 1.41
		2.500		-	7.0 ± 1.41	-	7.0 ± 1.41
		1.250	-	-	-	-	10.5 ± 3.54
		0.625		-	-	-	10.5 ± 0.71
	72	10.00		7.0 ± 0.00	9.0 ± 1.41	-	11.5 ± 0.71
		5.000		-	7.5 ± 2.12	-	10.0 ± 1.41
		2.500		-	7.0 ± 1.41	-	7.0 ± 1.41
		1.250	-	-	-	-	10.5 ± 3.54
		0.625		-	-	-	10.5 ± 0.71
ED2	48	10.00		-	9.5 ± 0.71	-	-
		5.000		-	9.0 ± 1.41	-	-
		2.500		-	7.5 ± 0.71	-	-
		1.250		-	7.5 ± 0.71	-	-
	72	10.00	-	-	6.5 ± 0.71	-	-
		5.000		-	8.5 ± 0.71	-	-
		2.500		-	6.0 ± 0.00	-	-
		1.250		-	6.0 ± 0.00	-	-
AMP	48		11.5 ± 0.71		9.5 ± 0.71		11.0 ± 0.00
	72		11.5 ± 0.71		8.0 ± 0.00		7.50 ± 0.71

[1]Values are means ± SD of three experiments, each in duplicate, [mg/mL] extract concentration, - indicates no inhibition.PDA, Potato Dextrose Agar, PDA 3%; PDA with 3% of artificial sea salt, AN, *Aspergillusniger;* CA, *Candida albicans;* TR, *trichophytonrubrum.*

however, did not inhibit *A. niger*. The zone inhibition diameters of these endophytic fungi against the two pathogenic fungi that were examined were equivalent to the positive control (Amphotericin B) (Table 3). Stronger antifungal capability, particularly against *T. rubrum*, was produced due to the effects of 3% sea salt that was added to PDA. Both these pathogens are dermatophytes that cause skin infections in humans. It can also cause vaginitis, as well as oral infection in babies and AIDS patients (Egusa *et al*., 2008). The five extracts from the present study, which showed notable fungicidal activity, deserve further study for their potent mechanisms of action. Contrary to antifungal activity, these extracts were not potent when tested against pathogenic bacteria.

CONCLUSION

The 18 endophytic fungi isolated from eight local seaweeds found in this study were chiefly identified using their microscopic characteristics and molecular methods. Out of 18 isolates, 11 isolates were identified through morphology characteristics, and the non-sporulating fungi (PA5, PA6, & PA7) were identified and further confirmed using molecular methods. The morphology, growth rate, conidia production, and antifungal activity were significantly affected by increasing salinity concentration (3% of artificial sea salt). Five of the isolates (CN, MV, ED1, ED2, & P7) showed promising antifungal properties. The inhibition produced was equivalent to that of the commercial drug, Amphotericin B. The isolates potentially have valuable compounds and worth further investigation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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