

Isolation, Identification and Screening of Antimicrobial Properties of the Marine-Derived Endophytic Fungi from Marine Brown Seaweed

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Marine seaweeds are known to produce valuable medicinal compounds such as antioxidants and anticoagulants, and have been reported to display antimicrobial activity against gram positive and gram negative bacteria. Several studies have identified so-called endophytic fungi living inside their hosts as the source of active compounds. In this study marine brown seaweed, *Padina* sp., was studied with regards to their endophytic fungi to assess if they are the source of the reported antimicrobial activity. Twenty fungal isolates were isolated from *Padina* sp. collected off Talang-Talang Island, Sarawak, Malaysia. All isolates were screened for their antimicrobial properties and 11 out of 20 isolates displayed positive results. DNA was successfully extracted for five isolates and sequence analysis grouped all of them with other endophytic fungi. "Fungus 2" seems to be related to a so far uncultured endophytic fungus. "Fungus 19" showed the most promising antimicrobial properties and was chosen for further agar well assay and cytotoxicity testing. Its ethyl-acetate extract showed positive results in the agar well assay and also a cytotoxic effect on *Artemia nauplii*. The extract was screened using HPLC and showed a compound similar to a known anti-cancer compound, dihydromyricetin, which is also an anti-intoxicant, anti-inflammatory and anti-oxidative agent which may be responsible for the observed antimicrobial activity.

Key words: antimicrobial properties, endophytic fungi, Marine Brown Seaweed, *Padina* sp.

Rumput laut laut yang dikenal menghasilkan senyawa obat berharga seperti antioksidan dan antikoagulan, dan telah dilaporkan mempunyai aktivitas antimikroba terhadap bakteri gram positif dan gram negatif. Beberapa studi telah melaporkan bahwa jamur endofit yang hidup di dalam rumput laut sebagai inang adalah sumber senyawa aktif. Tujuan penelitian ini adalah mengevaluasi, apakah rumput laut coklat *Padina* sp. dengan jamur endofitnya merupakan sumber dari aktivitas anti mikroba yang dilaporkan tersebut. Dua puluh isolat jamur diisolasi dari *Padina* sp. dikumpulkan dari Pulau Talang-talang, Sarawak, Malaysia. Hasil skrining anti mikroba menunjukkan, bahwa 11 dari 20 isolat menunjukkan hasil positif. Genom DNA dari lima isolat berhasil diekstraksi dan dianalisa deret ITSnya dan dianalisa dengan kelompok jamur endofit lainnya. "Jamur 2" diduga terkait dengan jamur endofit yang tidak dapat dikultur (*unculturable*). "Jamur 19" menunjukkan sifat antimikroba yang paling menjanjikan dan terpilih untuk *agar well assay* baik dan pengujian sitotoksitas lebih lanjut. Ekstrak etil asetat dari islat ini menunjukkan hasil positif dalam *agar well assay* dan sitotoksitas terhadap *Artemia nauplii*. Hasil skrining ekstrak tersebut dengan menggunakan HPLC menunjukkan senyawa yang mirip dengan senyawa anti kanker, *dihydromyricetin*, yang juga merupakan *anti-intoxicant*, anti inflamasi, dan juga agen anti oksidan yang mungkin berkaitan dengan aktivitas anti mikroba.

Kata kunci: anti mikroba, jamur endofit, rumput laut coklat, *Padina* sp.

Endophytic fungi have attracted the interest of scientist and pharmaceutical companies, especially since the discovery of the world's first billion-dollar anticancer drug, paclitaxel (Taxol) from *Pestalotiopsis microspora*, which is an endophytic fungus living within the Himalayan yew tree, *Taxus wallichiana* (Maheswari 2006). Endophytic fungi spend their entire

life cycle within their host plant tissue without showing any apparent symptom (Strobel *et al.* 1998). They live symbiotically with the host plants, which provide them profuse nutriment and restful habitation for the survival (Firakova *et al.* 2007), while they protect their host plants from external biotic and abiotic stresses (Rodriguez *et al.* 2009). For instance, the endophytic fungi, *Curvularia protuberate* can be found on all of the non-embryonic tissues of the geothermal plant *Dichanthelium lanuginosum*. Neither the fungus nor

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the plant can tolerate temperatures above 38° C when they are grown non-symbiotically, but when grown symbiotically, they are able to tolerate temperatures up to 65° C (Márquez *et al.* 2006). In another study, a non-pathogenic mutant of an endophytic fungus, *Colletotrichum magna* (Path-1) was found living asymptotically within watermelon (*Citrullus lanatus*) and discovered to be involved in disease protection against virulent pathogens such as *Colletotrichum orbiculare* (Redman *et al.* 1999).

Marine seaweeds are known to produce valuable compounds such as antibiotics, natural antioxidants, anticoagulants, and anticancer compounds (Ganesan *et al.* 2007; Wijesinghe *et al.* 2012). They also contain high amounts of carotenoids, dietary fibres, proteins, essential fatty acids, vitamins and minerals (Foon *et al.* 2013). Besides that, Rajasulochana and team (2009) have discovered that marine brown seaweed (*Padina* sp.) extracts possess antimicrobial properties, and were able to inhibit the growth of *Bacillus megaterium*, *Staphylococcus aureus* and *Escherichia coli*. No studies have assessed the possibility of endophytic fungi in brown seaweed as the potential origin of their active compounds and the aim of this study is to look at the endophytic fungi living inside *Padina* sp. and assess them for their antimicrobial and anti-cancer potential.

MATERIALS AND METHODS

Endophytic Fungi Isolation and Purification.

Endophytic fungi were isolated from *Padina* sp., collected off Talang-Talang Island, Sarawak, Malaysia. The sample was cut into small pieces of 1 cm² in size and then surface-sterilized by immersion in 70% ethanol for 5-15 seconds. After that, the sample was immersed in sterile artificial seawater (twice) to stop the sterilization. The sample was then dried using a sterile cotton cloth and placed on a Yeast Extract Glucose Chloramphenicol Agar (YGCA) plate. One millilitre of the artificial seawater that was used to clean the sample was taken out and poured on another YCGA plate as negative control. The agar dish was then sealed with parafilm, labelled and incubated at 25 °C for 3 days.

After 3 days, growth of hyphae was observed and those hyphae were isolated using sterile plastic straws and placed on fresh YCGA dishes. This step was repeated until pure fungal colonies were obtained. Purified fungi were cultured on Potato Dextrose Agar (PDA) and incubated at 25 °C.

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

Molecular Identification. Pure fungal isolates were cultured on Potato Dextrose Agar (PDA) for 5 days and a small amount of mycelia transferred into sterile 100 µL distilled water in 1.5 mL microcentrifuge tubes by using a sterile toothpick. After that, the mixture was vortexed thoroughly for 1 min and centrifuged at 10,000 rpm for 3 min. The supernatant was discarded and 100 µL of lysis solution (TE buffer – 10 mM Tris-HCL, 1 mM EDTA, at pH 8) added to the microcentrifuge tube. The mixture was incubated at 85 °C for 20 min and centrifuged for 5 min at 5,000 rpm. Two (2) µL of the supernatant were used for the Polymerase Chain Reaction. The rest of the crude extract was stored at -20 °C until further usage.

A master mix was prepared which contained 20 µL of 10x PCR buffer, 6 µL of dNTP mixture (10 mmol L⁻¹ each), 8 µL of deionized formamide, 4 µL of MgCl₂ (25 mmol L⁻¹), 8 µL of each primer (ITS1 {5'-TCCGTAGGTGAACCTGCGG-3'} and ITS4 {5'-TCCTCCGCTTATTGATATGC-3'}; 10 mmol L⁻¹), 2 µL of Taq DNA polymerase (5 U µL⁻¹) and 144 µL of sterile distilled water in a total volume of 200 µL. Twenty (20) microliter of the master mix were transferred into a sterile 0.3 mL PCR tube together with 1 µL of the genomic DNA.

The Polymerase Chain Reaction (PCR) consisted of an initial denaturing step of 5 min at 94 °C followed by 35 cycles (50 s at 94 °C, 50 s at 54 °C, and 50 s at 72 °C), followed by a final extension step at 72 °C for 10 min. The PCR products were resolved by electrophoresis through 1% agarose gels in TAE and visualized by staining with ethidium bromide for 10 min and destaining for 15 min. The PCR products were then purified and sent for sequencing. The sequences obtained were analyzed against the NCBI (USA) database (Zhang *et al.* 2000) and a phylogenetic tree was constructed from genetic distance and bootstrap values calculated using MEGA6 (see Fig 1; Tamura *et al.* 2013).

The genus of the remaining 22 fungal isolates was identified using morphological characteristics and an overview is presented in Table 1.

Fermentation. A single cylindrical block (agar plug) of 1 week old fungal cultures was inoculated into 200 mL of potato dextrose broth (PDB) and incubated for 2 months at 25 °C, static conditions. After 2 months

of incubation, 200 mL of ethyl acetate were added and the mixture shaken overnight using a rotary shaker. The fungal biomass was filtered and crude extracts dried inside a fume hood. Dried extracts were analyzed using a High Performance Liquid Chromatography (HPLC) system (Dionex P580) coupled to PDA detector (UVD340S). Routine detection was carried out at 235, 254, 280, and 340 nm. The stationary phase was Eurospher-10 C18 (Knauer, Germany), the mobile phase consisted of MeOH and 0.02% H₃PO₄ in H₂O at the following elution gradient: 0 min, 10% MeOH; 5 min, 10% MeOH; 35 min, 100% MeOH; 45 min, 100% MeOH.

Preliminary Antimicrobial Screening (Agar Diffusion Assay). All 20 purified fungal samples were cultured on Potatoes Dextrose Agar (PDA) for 1 week before the agar diffusion assay was carried out. Three different test organisms were used in the assay, a gram positive bacterium, *Bacillus subtilis*, a gram negative bacterium, *Escherichia coli*, and yeast, *Saccharomyces cerevisiae*.

The overnight broth cultures were swapped onto a fresh PDA plate using a sterile cotton swap. A sterile plastic straw was used to transfer small segments of the purified fungal cultures onto the swapped PDA plate. Triplicates were prepared for each test organism, plates incubated at 37 °C for 24 h and the results recorded (see Table 2).

The purified “Fungus 19”, which displayed the highest activity during the agar diffusion assay, was subjected to two more tests to assess his antimicrobial activity (see below).

Cross Streaking Assay. “Fungus 19” was grown on PDA for 4 days at 25 °C. Five different types of test organisms were used in this test; a gram positive bacterium, *Bacillus subtilis*, a gram negative bacterium, *Escherichia coli*, two yeasts, *Saccharomyces cerevisiae* and *Candida albicans*, as well as a fungus, *Aspergillus niger*.

The overnight broth cultures were streaked on a PDA plate containing “Fungus 19” in the middle of the plate. The plate was then incubated at 25 °C for 3 days and results recorded (see Table 3).

Agar Well Assay. Thirty gram of agar from 15 days old “Fungus 19” were transferred to a 250 mL sterile conical flask, 150 mL ethyl acetate added and shaken for 24 h. The mixture was filtered and 75 mL of the crude extract transferred into a 100 mL beaker to air dry inside a fume hood. The weight of the beaker with and without the crude extract was measured and recorded in order to obtain the weight of the dried extract which was

utilized for the agar well assay and cytotoxicity testing.

Three different types of test organisms were used in the agar well assay, a gram positive bacterium, *Bacillus subtilis*, a gram negative bacterium, *Escherichia coli*, and a yeast, *Saccharomyces cerevisiae*. Overnight broth cultures were swapped onto a fresh PDA plate using a sterile cotton swap. A sterile plastic straw was used to create 3 well on the plate and different volumes of the extract (10, 20, and 30 µL) were loaded into the wells using a micropipette. Triplicates were prepared for each test organism. Positive control experiments were carried out using chloramphenicol (1000 ppm) for *Bacillus subtilis* and *Escherichia coli*, and decon solution for *Saccharomyces cerevisiae*; ethyl acetate as negative solvent control. The plates were incubated at 37 °C and the zones of inhibition were measured and recorded (see Table 4).

Cytotoxicity Testing (Brine Shrimp Assay). National Cancer Institute (NCI, USA) demonstrated that the Cytotoxicity Testing by using *Artemia nauplii* has a significant correlation to the growth inhibition of human solid tumour cell lines in vivo (Silva *et al.* 2006).

Serial dilutions of the dried extract were carried out and solutions of 1000, 100, 10, 1 and 0.1 ppm prepared. Ten *Artemia nauplii* (*A. nauplii*) (Carballo *et al.* 2002) were drawn through a micropipette and placed in a well containing 50 µL of artificial seawater. Fifty microliter (50 µL) of the different solutions (see above) were added to the wells to test for concentrations of 500, 50, 5, 0.5, and 0.05 ppm. Triplicates were prepared for each concentration as well as a control experiment containing 50 µL of artificial seawater with two drops of DMSO and ten *A. nauplii*. The wells were kept for 21 h at room temperature and number of alive *A. nauplii* recorded (see Table 5).

RESULTS

In total of 20 fungal isolates were isolated from one marine brown seaweed (*Padina* sp.) sample. Fifteen of the isolates were identified based on their morphology (Table 1) and five of the isolates were furthermore identified using molecular methods (Fig 1).

“Fungus 1”, “Fungus 12” and “Fungus 3” were grouped with *Aspergillus aculeatus* strain HSZ15 [GenBank accession number KJ605160; 100% similarity], fungal endophyte sp. g81 [GenBank accession number HM537058; 99% similarity] and *Cladosporium tenuissimum* isolate HSW-17 [GenBank accession number KJ475817; 100%

Table 1 Genus of 15 fungal isolates that were identified using morphological characteristics

Isolate (fungus)	Naming based on fungus morphology
4	<i>Botrydiplodia</i> sp.
5	<i>Botrydiplodia</i> sp.
6	<i>Penicillium</i> sp.
8	similar to isolate 2
9	<i>Botrydiplodia</i> sp.
10	<i>Penicillium</i> sp.
11	<i>Botrydiplodia</i> sp.
13	<i>Penicillium</i> sp.
14	similar to isolate 2
15	<i>Botrydiplodia</i> sp.
16	<i>Fusarium</i> sp.
17	<i>Botrydiplodia</i> sp.
18	<i>Botrydiplodia</i> sp.
19	<i>Penicillium</i> sp.
20	<i>Penicillium</i> sp.

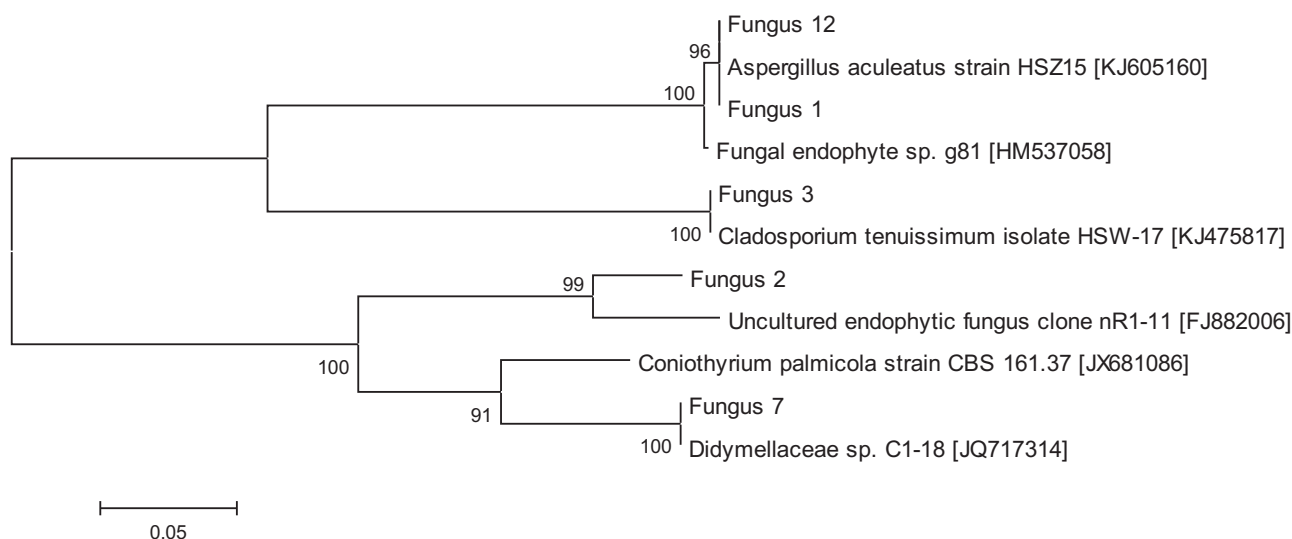


Fig 1 Internal transcribed spacer (ITS) gene-based phylogenetic tree representing amplified fungal sequences. The phylogenetic tree was generated with distance methods, and sequence distances were estimated with the neighbour-joining method. Bootstrap values ≥ 50 are shown and the scale bar represents a difference of 0.05 substitution per site. Accession numbers for the reference sequences are indicated.

similarity]. While “Fungus 2” and “Fungus 7” grouped with *Didymellaceae* sp. C1-18 [GenBank accession number JQ717314; 100% similarity] and a so far uncultured endophytic fungus clone nR1-11 [GenBank accession number JQ717314; 95% similarity] (Fig 1).

Among all 20 isolates, 45% of the fungi showed no

inhibition on the test organisms, 25% showed inhibition of gram positive bacteria, 10% showed inhibition of gram negative bacteria, and another 5% showed inhibition of both gram negative and gram positive bacteria but no inhibition on yeast (Table 2).

Our isolates seem to be able to inhibit gram positive

Table 2 Screening of antimicrobial properties of the endophytic fungi isolated from brown seaweed, *Padina* sp. from Talang-talang Island located in Kuching. Fungus 19 is highlighted in italics because it inhibited all test microorganisms. + indicates inhibition, - indicates no inhibition.

Samples	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Saccharomyces cerevisiae</i>
Surface Sterilized			
Fungus 4	+	-	-
Fungus 6	+	+	-
Fungus 8	+	-	-
Fungus 10	-	+	-
Fungus 11	+	-	-
Fungus 14	+	-	-
Fungus 16	-	+	-
Fungus 18	+	-	-
<i>Fungus 19</i>	+	+	+

Table 3 Secondary screening of antimicrobial properties (cross streaking assay) of "Fungus 19" against four different type of microorganisms. + indicate relative inhibition strength.

Test Microorganism	Inhibition
<i>Bacillus subtilis</i>	+++
<i>Escherichia coli</i>	+++
<i>Saccharomyces cerevisiae</i>	++
<i>Aspergillus niger</i>	+

Table 4 Overview of inhibition zones (in cm) for the crude extract of "Fungus 19" tested against *Escherichia coli* and *Bacillus subtilis*.

Volume (μL) (1000 ppm)	Zone of Inhibition (cm)	
	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>
30	1.07 (+/- 0.058)	0.87 (+/- 0.208)
20	0.87 (+/- 0.058)	0.80 (+/- 0.100)
10	0.63 (+/- 0.058)	0.67 (+/- 0.115)
Positive control (chloramphenicol)	2.8	3.1
Negative control (ethyl acetate)	-	-

bacteria more than gram negative bacteria (15% more) which might be due to the composition of the cell wall of the negative bacteria which is more rigid and complex (Hugo 1998). 'Fungus 19' showed to be the most promising fungal strain, which is able to inhibit all the testing microorganism including yeast (Table 2)

and was therefore targeted for secondary screening, by using cross streaking assay and agar well assay. Due to the observed wide activity, a fungus was further added as test organism for the secondary screening; *Aspergillus niger* - a fungal species that can cause chronic bilateral otomycosis (Mishra *et al.* 2004).

Table 5 Screening of cytotoxicity level of the crude extract produced by “Fungus 19”. Different concentrations (0.5 to 500 ppm), negative and positive controls are shown and mortality (in %) was calculated. The status “active”, “inactive” and “dead” indicates the mobility of the *Artemia nauplii* which are high, low and immobile, respectively.

Concentration	Well			Average	Mortality
	A	B	C		
500 ppm	all dead	2 inactive	3 inactive	2 inactive	80%
50 ppm	5 active	1 active	5 active	4 active	20%
	5 inactive	5 inactive	3 inactive	4 inactive	
5 ppm		4 dead	2 dead	2 dead	
5 ppm	all active	all active	all active	all active	0%
0.5 ppm	all active	1 dead	all active	all active	0%
		9 active			
0.05 ppm	1 dead	all active	all active	all active	0%
	9 active				
Original water from the <i>Artemia nauplii</i> tank (Negative Control)	1 dead 9 active	all active	1 dead 9 active	all active	0%
DMSO + Artificial Seawater (Negative Control)	all active	all active	1 dead 9 active	all active	0%

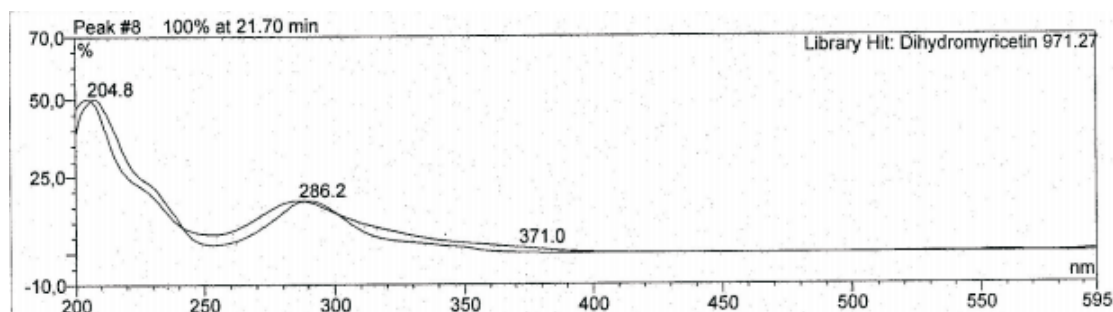


Fig 2 HPLC chromatogram of compound from “Fungus 4” and “Fungus 7” that had a similar structure to the flavonoid Dihydromyricetin

“Fungus 19” was also further assessed for its anti-cancer potency by a Brine Shrimp Assay.

The secondary screening (cross streaking) confirmed the antimicrobial properties of “Fungus 19” as it inhibited 4 test organisms, gram positive bacterium, *Bacillus subtilis*, gram negative bacterium, *Escherichia coli*, a yeast, *Saccharomyces cerevisiae*, and a fungus, *Aspergillus niger*. It showed the biggest inhibition on both gram positive and negative bacteria, followed by yeast, and the least inhibition on the fungus *Aspergillus niger* (see Table 3).

The compounds produced by the “Fungus 19” were extracted by using ethyl acetate- the most efficient

solvent for extracting extracting polar as well as nonpolar secondary metabolites from fungal cultures (Jantamas and Dusanee 2010). The crude extract of “Fungus 19” exhibited antimicrobial properties against *Escherichia coli* (inhibition zones of 0.63 – 1.07 cm, Table 4) and *Bacillus subtilis* (inhibition zones of 0.67 – 0.87 cm, Table 4). It is noteworthy that all inhibition zones were smaller than the positive control, chloramphenicol. The crude extract of “Fungus 19” did exhibit cytotoxicity against *Artemia nauplii* (Table 5).

HPLC analyses were carried out on crude extracts of all species showing activity and revealed many compounds with no known match in the database of the

Heinrich Heine Universitaet (HHU). While “Fungus 19” did show the most promising activity in the bioassays, the HPLC spectra did not reveal any compound of interest. A possible reason may be that methanol was used as solvent for the HPLC and the active compound of “Fungus 19” was not soluble in methanol. Further analyses are needed to clarify the origin of its observed activity. The spectra of “Fungus 4” and “Fungus 7”, however, did contain a compound with a structure related to Dihydromyricetin, a flavonoid (Fig 2), which has been shown to possess anti-intoxicant, anti-inflammatory, anti-oxidant, and anti-cancer activities (Nazemiyeh *et al.* 2008; Shen *et al.* 2012).

DISCUSSION

Twenty (20) fungal strains were isolated from brown seaweed (*Padina* sp.) samples, representing more than 7 genus of fungi. Phylogenetic analyses of 5 isolates revealed that all of them are related to other endophytic fungi, confirming the cleanliness and proficiency of our isolation process.

“Fungus 2” was grouped with an uncultured endophytic fungus and could be a new species as many fungi remain to be discovered by mycologists (Blackwell 2011). “Fungus 7” grouped with known plant endophytes, *Didymellaceae* sp. (Fig 1).

Members of the *Cladosporium* genus are known to produce antimicrobial agent such as cladospolide and isoclodospolide (Zhang *et al.* 2001; Dai *et al.* 2006). However, the “Fungus 3” that was grouped with *Cladosporium tenuissimum*, did not display antimicrobial properties. It could be because of the different plant hosts thus results in different production of secondary metabolites. This can be further supported by the studies done by Zhang team (2001) and Dai team (2006), both research groups isolated a *Cladosporium* sp., one from marine sponge and the other one from *Maytenus hooker*, both of the *Cladosporium* sp. results in producing different type of bioactive compounds.

Fungus 1 was grouped with *Aspergillus aculeatus* (Figure 1) which is known to produce useful multi-polysaccharide degrading enzymes such as β -glucosidase, endoglucanase, β -xylosidase, and xylanase, and is a promising producer of lignocellulose-degrading enzymes for the biomass conversion industry (Suwannarangsee *et al.* 2014). We did not assess enzymatic properties in our study, however “Fungus 1” could be analysed further in the

future. A note of caution has to add though as the same fungal species can display varying activity in different host plants as explained above for “Fungus 3”.

Only 1 out of 20 fungi, “Fungus 19”, related to *Penicillium* sp., displayed antimicrobial activity against yeast and both types of bacteria and therefore, this particular fungus was suspected to produce potential antimicrobial agents and has thus been chosen for further testing, such as cross streaking, agar well assay and cytotoxicity testing.

Penicillium sp. are known to produce a wide range of valuable bioactive compounds such as antibacterial and antifouling polyketides (Bao *et al.* 2013), cytotoxic funicone (Chen *et al.* 2014) and antifungal antibiotics (Omura *et al.* 1988). In a recent study by Subramani *et al.* (2013), a *Penicillium* sp. isolated from a marine Fijian sponge *Melophlus* sp. exhibited both antibacterial against multi-drug resistant pathogens and cytotoxicity activity against brine shrimp larvae.

In our study, the *Penicillium* sp. related “Fungus 19” ethyl-acetate extract showed to have positive results on both agar well assay and cytotoxicity testing. Besides that, the cross streaking assay showed the inhibition of the yeast and fungus. Therefore, this particular fungus can be suspected to produce some bioactive compounds that are cytotoxic and have wide antimicrobial properties or produce a variety of compounds at a sufficient level to inhibit several different test organisms.

One of the compounds produced by “Fungus 4” (related to *Botrydiplodia* sp.) is related to an anti-cancer compound, dihydromyricetin, which is also an anti-intoxicant, anti-inflammatory and anti-oxidative agent (Nazemiyeh *et al.* 2008; Shen *et al.* 2012). Zhang *et al.* (2014) have demonstrated that dihydromyricetin possesses antitumor activity against liver cancer cells without being cytotoxic to immortalized normal liver cells. “Fungus 4” was however not tested for any of these activities as it only showed antimicrobial activity against *B. subtilis* during the initial screening (Table 2). Other *Botrydiplodia* species have been reported to produce a different anti-cancer compound, fungal taxol, which is able to significantly suppress the 7, 12 dimethyl benz(a)anthracene (DMBA)-induced breast cancer in Sprague dawley rats (Pandia *et al.* 2010). Future studies should be carried out on “Fungus 4” as it seems to produce a compound with a high potential for use in pharmaceutical industries.

In conclusion, *Padina* sp. was found to be a rich source of endophytic fungi and several of the isolates found in this study showed a promising range of

activities. The extracts produced by the isolates yielded some potentially valuable compounds as well as many unknown compound and are worth further investigations.

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REFERENCES

- Bao J, Sun YL, Zhang XY, Han Z, Gao HC, He F, Qian PY, Qi SH. 2013. Antifouling and antibacterial polyketides from marine gorgonian coral-associated fungus *Penicillium* sp. SCSGAF 0023. *J Antibiot* (Tokyo). 66(4): 219-223.
- Blackwell M. 2011. The fungi: 1, 2, 3...5.1 million species? *Am J Bot*. 98(3): 426-438.
- Carballo JL, Hernández-Inda ZL, Pérez P, García-Grávalos MD. 2002. A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. *BMC Biotechnol*. 2: 17.
- Chen MJ, Fu YW, Zhou QY. 2014. Penifupyrone, a new cytotoxic funicone derivative from the endophytic fungus *Penicillium* sp. HSZ-43. *Nat Prod Res*. 18: 1-5.
- Dai HQ, Kang QJ, Li GH, Shen YM. 2006. Three new polyketide metabolites from the endophytic fungal strain *Cladosporium tenuissimum* LR463 of *Maytenus hookeri*. *Helv Chim Acta*. 89(3): 527-531.
- Ellis D, Davis S, Alexiou H, Handke R, Bartley R. 2007. Descriptions of Medical Fungi. 2nd Edition. Nexus Print Solutions. Underdale. South Australia.
- Firakova S, Maria S, Muckova M. 2007. Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia*. 62(3): 251-257.
- Foon TS, Ai LA, Kuppusamy P, Yusoff MM, Govindan N. 2013. Studies on in-vitro antioxidant activity of marine edible seaweeds from the east coastal region of peninsular malaysia using different extraction methods. *J Coast Life Med*. 1(3): 193-198.
- Ganesan P, Kumar CS, Bhaskar N. 2007. Antioxidant properties of methanol extract and its solvent fractions obtained from selected indian red seaweeds. *Bioresour Technol*. 99: 2717-2723.
- Hugo WB. 1998. Bacteria. In: Hugo WB, Russell AD, editors. *Pharmaceutical Microbiology*. 6th edition. Blackwell Science Ltd. United Kingdom.
- Jantamas J, Dusanee T. 2010. The Efficacy of Ethyl Acetate Extract of *Trichoderma* Culture Broth on Growth Inhibition and Aflatoxin Production by *Aspergillus flavus* IMI 242684. *KMITL Sci Tech J*. 10: 19-29.
- Maheswari R. 2006. What Is An Endophytic fungus?. *Curr Sci*. 90(10): 1309.
- Márquez LM, Redman RS, Rodriguez RJ, Roossinck MJ. 2006. A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *Sci*. 315: 513-515.
- Mishra GS, Mehta N, Pal M. 2004. Chronic bilateral otomycosis caused by *Aspergillus niger*. *Mycoses*. 47(1-2): 82-84.
- Nazemiyeh H, Bahadori F, Delazar A, Ay M, Topçu G, Nahar L, Majinda RR, Sarker SD. 2008. Antioxidant phenolic compounds from the leaves of *Erica arborea* (*Ericaceae*). *Nat Prod Res*. 22(16): 1385-1392.
- Omura S, Tomoda H, Kimura K, Kumagai H, Igarashi K, Imamura N, Takahashi Y, Tanaka Y, Iwai Y. 1988. Atpenins, new antifungal antibiotics produced by *penicillium* sp. production, isolation, physico-chemical and biological properties. *J Antibiot* (Tokyo). 41(12): 1769-1773.
- Pandia M, Manikandanb R, Muthumarya J. 2010. Anticancer activity of fungal taxol derived from *Botryodiplodia theobromae* Pat., an endophytic fungus, against 7, 12 Dimethyl Benz(a)anthracene (DMBA)-induced mammary gland carcinogenesis in *Sprague dawley* rats. *Biomed Pharmacother*. 64(1): 48-53.
- Rajasulochana P, Dhamotharan R, Krishnamoorthy P, Murugesan S. 2009. Antibacterial activity of the extracts of marine red and brown algae. *J Am Sci*. 5(3): 20-25.
- Redman RS, Freeman S, Clifton DR, Morrel J, Brown G, Rodriguez RJ. 1999. Biochemical analysis of plant protection afforded by a nonpathogenic endophytic mutant of *Colletotrichum magna*. *Plant Physiol*. 119: 795-804.
- Rodriguez RJ, Jr WJF, Arnold AE, Redman RS. 2009. Fungal endophytes: diversity and functional roles. *Tansley Reviews. New Phytol*. 182(2): 314-330.
- Shen Y, Lindemeyer AK, Gonzalez C, Shao XM, Spigelman I, Olsen RW, Liang J. 2012. Dihydromyricetin as a novel anti-alcohol intoxication medication. *J Neurosci*. 32(1): 390-401.
- Silva TMS, Nascimento RJB, Batista MM, Agra MF, Camara CA. 2006. Brine shrimp bioassay of some species of *Solanum* from Northeastern Brazil. *Braz J Pharmacog*. 17(1): 35-38.
- Strobel GA, Long DM. 1998. Endophytic microbes embody pharmaceutical potential. *Am Soc Microbiol (News)*. 64(5): 263-268.
- Subramani R, Kumar R, Prasad P, Aalbersberg W, Retheesh ST. 2013. Cytotoxic and antibacterial substances

- against multi-drug resistant pathogens from marine sponge symbiont: Citrinin, a secondary metabolite of *Penicillium* sp.. Asian Pac J Trop Biomed. 3(4): 291-296.
- Suwannarangsee S, Arnthong J, Eurwilaichitr L, Champreda V. 2014. Production and characterization of multi-polysaccharide degrading enzymes from *Aspergillus aculeatus* BCC199 for saccharification of agricultural residues. J Microbiol Biotechnol. 24(10): 1427-1437.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol. 30(12): 2725-2729.
- Wijesinghe WA, Jeon YJ. 2012. Exploiting biological activities of brown seaweed *Ecklonia cava* for potential industrial applications: a review. Int J Food Sci Nutr. 63(2): 225-235.
- Zhang H, Tomoda H, Tabata N, Miura H, Namikoshi M, Yamaguchi Y, Masuma R, Omura S. 2001. Cladospolide D, a new 12-membered macrolide antibiotic produced by *Cladosporium* sp.. FT-0012. J Antibiot. 54(8): 635-641.
- Zhang Q, Liu J, Liu B, Xia J, Chen N, Chen X, Cao Y, Zhang C, Lu C, Li M, Zhu R. 2014. Dihydromyricetin promotes hepatocellular carcinoma regression via a p53 activation-dependent mechanism. Scientific Reports. doi:10.1038/srep04628.
- Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA sequences. J Comput Biol. 7: 203-214.