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Contributed Paper

Marine Derived Fungi of Peninsular Malaysia – a Biochemical Perspective

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ABSTRACT

In an effort to tap into natural products harboured by marine derived fungi in Malaysia, selected marine derived endophytic and manglicolous fungi from the coastlines of Peninsular Malaysia were investigated for their antibacterial potential. Forty-one strains were isolated from marine associated plants, comprised and comprised 12 and 19 endophytic strains from *Vitex rotundifolia* and *Ipomoea pes-caprae* respectively, while 10 manglicolous strains were from decaying mangrove wood collected in Peninsular Malaysia. In preliminary experiments, a plug assay was employed to study the antibacterial activities of all 41 fungi isolates. Fifteen of the endophytic isolates and nine of the manglicolous isolates displayed antibacterial activities against at least one of the test bacteria. Based on the plug assay, the endophytic fungi from *Ipomoea pes-caprae* were shown to display higher antibacterial potential in comparison to the endophytic fungi from *Vitex rotundifolia.* In particular, *Minimidochium* sp. and *Bipolaris* sp. (ISB0014) displayed antibacterial activities against 5 or more test bacteria. Potential fungi isolates with good antibacterial activities were further analysed through a broth microdilution assay. *Minimidochium* sp. and *Dyfrolomyces rhizophorae* exhibited promising antibacterial activities with minimum inhibitory concentrations not higher than 0.5 mg/ml. Bioactivity-guided fractionation of *D. rhizophorae* extracts resulted in the isolation of fatty acids, palmitic and linoleic acid. Though ubiquitous in nature, linoleic acid is known as an essential health supplement and both fatty acids are used as biodiesel replacing conventional fuels.

Keywords: natural products, antibiotics, manglicolous fungi, endophytic fungi, bioactivity

1. INTRODUCTION

Marine natural products are known to possess a plethora of chemical variations and compounds that when harnessed, are believed to potentially exhibit a wide range

of properties,such as, antimicrobial, anticancer, antituberculosis, antiviral, antiparasitic, antihelmintic, antimalarial, antiprotozoal, anticoagulant, antiplatelet, antiinflammatory, antidiabetic, and antitumor bioactivities [1,17,19]. For the past two decades, marine and marine-related resources including plants, sponges, bacteria, cyanobacteria, algae and fungi have become an important source of novel and pharmacologically active chemical structures for the development of new drugs, antibiotics, pesticides and antifouling substances, which are made possible due to the chemical diversity of their secondary metabolites [6,7,22,32]. It is now widely accepted that marine derived fungi play a prominent role as a promising source for the discovery of novel bioactive chemical compounds, in line with the growing need of new natural products [7,28,35]. Studies have shown that marine derived fungi were a class of organisms poorly studied in comparison to its counterparts such as bacteria, plants and animals [11].

The marine ecosystem is governed by harsh biological, physical and chemical parameters which may have driven the development and evolution of novel metabolic pathways in living marine organisms, thus giving rise to chemicals with interesting structures [6,7,17]. The first report of a bioactive natural product from a marine derived fungus dates back to the 1940s when cephalosporin C was isolated from the fungus *Acremonium chrysogenum* (Thirum. & Sukapure) W. Gams collected from a sewage outlet in the Mediterranean Sea [34]. Cephalosporin C is the precursor chemical compound of the modern cephalosporin antibiotics [34]. Existing modern drugs of fungal origin include b-lactam antibiotics, griseofulvin, cyclosporine A, taxol, ergot alkaloids, and lovastatin [7,41]. To date, studies have reported some 1100 new chemical structures from marine derived fungi [6,7,11]. Forty two new compounds and 35 known compounds were discovered from marine derived fungi isolated from various substrates from the South China Sea [31]. Although there have been several reports in Malaysia on the potential of secondary metabolites of endophytic fungi, most of them originated from medicinal herbs and non-marine associated plants of Malaysia [40,43]. There is a general lack of information and few studies on the secondary metabolite chemistry of marine derived fungi from Malaysia. One of which was a study that investigated the antimicrobial properties of 152 marine derived fungi from Malaysia and successfully isolated 2,2,7-trimethyl -2H-chromen-5-ol from the marine derived fungi *Fasciatispora nypae* K.D. Hyde, a compound that was never before reported to be isolated from nature [48].

Therefore, the objective of the present study is to tap into and explore the potential for bioactive metabolites produced by marine derived fungi in Malaysia. The two host plants in the present study, *Vitex rotundifolia* and *Ipomoea pes-caprae*, are commonly found growing on the beaches of Peninsular Malaysia. There have been no studies on the bioactivities of endophytic fungi from the plant *V. rotundifolia,* while there was only one study pertaining to the bioactivity of endophytic fungi from *I. pes-caprae*. Although the sudy isolated exopolysaccharide from the endophytic fungus *F. oxysporum* from the plant *I. pes-caprae*, its antibacterial bioactivity was not tested [14]. Thus overall, there has been minimal work investigating the diversity of endophytic fungi from the leaves of *V. rotundifolia* and *I. pes-caprae*.

2. MATERIALS AND METHODS

2.1 Collection of Marine Associated Plants, Endophytic Fungi Isolation and Manglicolous Fungi Used

Vitex rotundifolia was collected from Kijal Beach, from the east coast, while *I. pes-caprae* was collected from Port Dickson Beach, from the west coast of Peninsular Malaysia. Only leaves of healthy plants were taken for the isolation of endophytic fungi. The plant materials were excised using clean scissors and placed in sterile polythene bags and transported back to the laboratory and processed within two hours of collection. Plant samples were washed with distilled water to remove surface sand and debris. The leaves and stems were then cut into 6 mm disks and surface sterilized using a series of chemical treatments [2]. Firstly, samples were soaked in 70% ethanol for two minutes followed by 4% bleach for one minute, after which they were treated again with 70% ethanol for one minute and finally rinsed thrice in distilled water. The surface sterilized plant materials were then placed on Potato Dextrose agar (PDA) without antibiotic supplement and incubated at 37°C. The agar was observed daily under the microscope for mycelia sporulation; germinating mycelia were then transferred to fresh media using a fine needle to obtain a pure fungi culture. The pure fungi cultures were then transferred to agar slants to make triplicates of each pure culture.

In this study, ten marine derived manglicolous fungal strains comprising of six genera/species that were previously isolated from the decaying wood found in mangroves of Peninsular Malaysia were screened for their antibacterial activity. All strains used in this study were obtained from the Institute of Biological Sciences (ISB), University of Malaya culture collection.

2.2 Identification of Endophytic Fungi Using Sequence Analysis of Internal Transcribed Spacer (ITS) Sequences

Endophytic fungi mycelia were harvested after an optimum incubation time of 7 days, frozen in liquid nitrogen and macerated into fine powder with a mortar and pestle. Total genomic DNA was extracted using DNeasy Plant Mini Kit (QIAGEN, Germany). The internal transcribed spacer regions of the 5.8S rRNA of the fungi were PCR amplified using primers ITS4; 5’- TCCTCCGCTTA TTGATATGC-3’ and ITS5; 5’GGAAGT AAAAGTCGTAACAAGG3’. PCR reaction mixtures of 25μl consisted of 2.5μL 1 × PCR buffer, 0.2mM dNTP, 0.2mM primer pairs, 0.5UTaq DNA polymerase and 0.1mg template genomic DNA. PCR amplifications were performed on thermo-cycler with an initial denaturation of 96°C for 5minutes, followed by 35 cycles comprising of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 90 seconds, before ending with a final extension step of 72°C for 10 minutes. PCR products were visualized on 1% TBE agarose gel. DNA sequencing was outsourced and performed by First Base Laboratories Sdn Bhd. Forward and reverse sequence chromatograms were checked for ambiguity and edited, before assembling the contig sequences for each fungal isolates using Chromas 2.33 (Technelysium Pt. Ltd.; Australia). The Basic Local Alignment Search Tool (BLAST) program at the US National Centre for Biotechnology Information (NCBI), (http://www.ncbi.nlm.gov/) was employed for species identification of the fungi isolate sequences. Fungi isolates with an ITS sequence similarity ≥ 96% were considered to be the same species [37]. The identifications must be treated with some caution

however, as many of the ITS sequence data in GenBank is wrongly named [24].

2.3 Phylogenetic Analysis

Before constructing the phylogenetic tree, the best evolutionary model for the ITS sequence alignment dataset was chosen based on Akaike Information Criterion (AIC) of the jModeltest 2.1.3 software [33]. The jModeltest result suggested the Transitional Evolutionary Model with invariable and gamma distribution (TIM2+I+G; I=0.2330; G=1.8540) for the ITS sequences. Unequal base frequencies were also observed with 0.2045, 0.2945, 0.2589 and 0.2421 for A, C, G, and T, respectively. The substitution rate matrix were [A-C] = 1.7504; [A-G] = 2.6362; [A-T] = 1.7504; [C-G] = 1.0000; [C-T] = 3.7294; [G-T] = 1.0000. Maximum Likelihood tree was constructed using Randomized Axelerated Maximum Likelihood (RAxML) web server [38], while incorporating the parameters obtained from the jModeltest analysis.

2.4 Test Bacteria

The test bacteria used comprised of five Gram positive and two Gram negative strains. The Gram positive bacteria used were *Bacillus cereus* Frankland and Frankland (ATCC 11778), *Bacillus subtilis* (Ehrenberg) Cohn. (ATCC 6051), *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC 29212), *Micrococcus luteus* (Schroeter) Cohn (ATCC 49732) and *Staphylococcus aureus* Rosenbach MTCC 96 (ATCC 9144). The Gram negative bacteria used were *Escherichia coli* (Migula) Castellani & Chalmers MTCC 443 (ATCC 25922) and *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC 27853). The test bacteria were provided by the Microbiology Department, University of Malaya.

2.5 Screening of Fungi Isolates through Plug Assay Method

The preliminary screening for antibacterial activity of marine derived fungi were carried out using a plug assay method [13,16,48]. Plates containing PDA without antibiotic supplement were seeded with 24 hour old test bacteria on Luria Broth (LB) agar and a round well of 6 mm in diameter was cut out from the centre of the agar. Plugs of actively growing fungal mycelia (6 mm) from 30 days old PDA cultures were then transferred into these wells, and incubated at 37°C for 24 hours. The diameters of the zones of inhibition surrounding the well were measured and recorded in millimetres (mm) using a ruler. An inhibition zone of 8 mm or higher indicates a positive antibacterial response. All assays were carried out in independent triplicates. Paper discs containing antibiotics penicillin and streptomycin served as negative controls.

2.6 Cultivation, Extraction and Bioassay of Fungal Secondary Metabolites

Based on the preliminary results obtained from the plug assay (Tables 1 & 2), three endophytic and three manglicolous strains were chosen for further analysis of their antibacterial potential through the broth microdilution assay following the protocols of [12]. The fungal isolates were grown on sterile PDA without antibiotic supplements in Petri-plates for 14-30 days at 25°C. Active growing fungi mycelia was then cut into 6 mm plugs and added into 250 ml conical flasks containing potato dextrose broth (PDB) and the flasks were incubated at 25°C under shaken phase of 120 rpm for 30 days. After the incubation

period, the fungal biomass was separated by filtration. The filtrate was then extracted thrice with equal volumes of ethyl acetate (1:1 v/v). The organic ethyl acetate layers were then combined and evaporated to dry the crude extract using a rotary evaporator at 25°C. The dried crude secondary metabolite was then stored at -4°C prior to the broth microdilution assay. Crude extract of the fungi was dissolved in 1% dimethyl sulfoxide (DMSO) and prepared at a two-fold concentration ranging from 2.0mg/ml to 0.0156mg/ml. The crude extract at varying concentrations was then added to a 96 well plate seeded with bacterial suspension at 5×10^5 cfu/ml. The plates were incubated at 37°C for 24 hours, after which p-Iodonitrotetrazolium violet (INT) at a concentration of 0.4mg/ml was added into each well and incubated for 20 minutes. The colour change from clear to pink indicates positive bacterial growth and a negative inhibition. The lowest concentration able to inhibit bacterial growth was recorded as the minimum inhibitory concentration (MIC) value. Antibiotics penicillin and streptomycin ranging from concentrations 2 mg/ml to 0.0156 mg/ml were used as negative controls.

2.7 Isolation of Active Constituents of *Dyfrolomyces rhizophorae*

A strain of *D. rhizophorae* was chosen for further chemical analysis based on results displayed in the broth microdilution assay. Key procedures leading to the isolation of active constituents comprised thin layer chromatography (TLC), column chromatography, nuclear magnetic resonance spectroscopy (NMR), Ultra-violet spectroscopy and Liquid Chromatography-Mass Spectrometry (LCMS). Analytical and preparative thin layer chromatography (TLC) was carried out on Merck 60 F_{254} silica gel plates (absorbent thickness: 0.25). Column chromatography was performed using silica gel (Merck 230-400 mesh, ASTM). Nuclear magnetic resonance (NMR) spectra were recorded in deuterated chloroform ($CDCl_3$) (Merck, Germany) with tetramethylsilane as an internal standard, using JEOL ECA 400MHz NMR spectrometer. Infrared IR spectra were recorded using a Perkin-Elmer Spectrum 400 FT-IR Spectrometer. Ultraviolet (UV) spectra were recorded using a Shimadzu 1650 PC UV-Vis Spectrophotometer. The Liquid chromatography-Mass spectrum-Ion trap-Time of flight (LC-MS-IT-TOF) spectra were recorded on a Ultra-fast liquid chromatography (UFLC) Shimadzu Liquid Chromatograph with a SPD-M20A diode array detector coupled to a IT-TOF The IT-TOF was operated in positive ion electrospray mode.

2.8 Bioassay Guided Fractionation of *Dyfrolomyces rhizophorae* Extract

Dyfrolomyces rhizophorae was chosen for further chemical analysis based on promising antibacterial activity against both Gram positive and negative bacteria at low MIC values. The crude ethyl acetate extract of *D. rhizophorae* (900mg) was partitioned in sequence using solvents of increasing polarity starting with *n*-hexane (3×, 0.5L), dichloromethane (3×, 0.5L) and methanol (3×, 0.5L) at room temperature, affording 3 primary extracts (110mg, 320mg, and 235mg, respectively). The active extract, identified as the *n*-hexane extract, was re-dissolved in *n*-hexane and subjected to silica gel column chromatography using a gradient elution with mixtures of *n*-hexane : ethyl acetate (100:0, 90:10, 85:15,

80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50 and 0:100 v/v), affording 7 fractions. The antibacterial activities of all 7 fractions were evaluated, whereby fractions 2 (51.1mg) and 3 (24.5mg) were found to be the most active. Final purification of fraction 2 and 3 via preparative TLC using *n*-hexane: ethyl acetate (75:25 v/v), yielded compounds 1 (3.2mg) and 2 (2.5mg), respectively.

3. RESULTS AND DISCUSSION

3.1 Identification of Marine Derived Endophytic Fungal Strains

Maximum Likelihood tree shows the clustering of isolates with their reference ITS sequences (Figure 1). Based on the strong support of the monophyletic groupings, most isolates were able to be tentatively identified to the species level. Only a few isolates were identified up to the genus or order level. A total of 31 fungi isolates were isolated from *V. rotundifolia* and *I. pes-caprae* (Table 1). *Phoma* sp. were common to both plant species while the fungi isolates *Bipolaris* sp., *Cochliobolus geniculatus* R.R. Nelson, *Curvularia* sp. and *Phoma* sp. had more than one occurrence on the same host.

3.2 Antibacterial Activities of Marine Derived Fungi

Endophytic fungi have been known to produce unique compounds with interesting bioactivities and recent years has seen a growing interest in the exploration of marine derived endophytic fungi for new and novel metabolites of pharmacological importance [7,20,22]. One example was the isolation of antibacterial indole alkaloids from the algal endophyte *Eurotium cristatum* (Raper & Fennell) Malloch & Cain [10]. In the present study, five and ten of the endophytic fungi strains from *V. rotundifolia* and *I. pes-caprae* respectively displayed positive antibacterial activity (Table 1) The broadest exhibition of antibacterial activity by the endophytic fungi from *V. rotundifolia* were by the fungal strains *Curvularia* sp. and *Paecilomyces* sp. which were active against three out of the seven test bacteria. The endophytic fungi from *I. pes-caprae*, however, displayed a wide spectrum of antibacterial potential with *Bipolaris* sp. (ISB0014) being active against six test bacteria. *Bipolaris* sp. derived from the seagrass, *Halophila ovalis*, has been known to contain a dimeric chromanone and a phthalide with antibacterial activity against *S. aureus* [3]. It was observed that only endophytic fungi from *I. pes-caprae* exhibited antibacterial activities against Gram negative bacteria, namely the strains *Bipolaris* sp. (ISB0014), *Dothideomycete* sp. (ISB0019), *Minimidochium* sp. (ISB0023) and *Penicillium* sp. (ISB0027).

Both the marine associated plants in the present study have been exploited in traditional medicine and the habitats of both plants are constantly surrounded by extreme conditions. *V. rotundifolia* has been reported to be used for cold remedies and headaches in Japan and is also used as raw material in traditional Chinese medicine [21]. It has also been reported to possess anticancer properties [23]. As for *I. pes-caprae*, it is used as an herbal drug in Mexico mainly in the treatment of kidney complaints, digestive disorders, hypertension, arthritis, rheumatism, skin infections and other inflammatory conditions. Nine antibacterial oligosaccharides were isolated from *I. pes-caprae* while there has been no reports of antibacterial compounds from *V. rotundifolia* which may reflect the host's influence on the fungi's antibacterial activities [29]. This may account for the

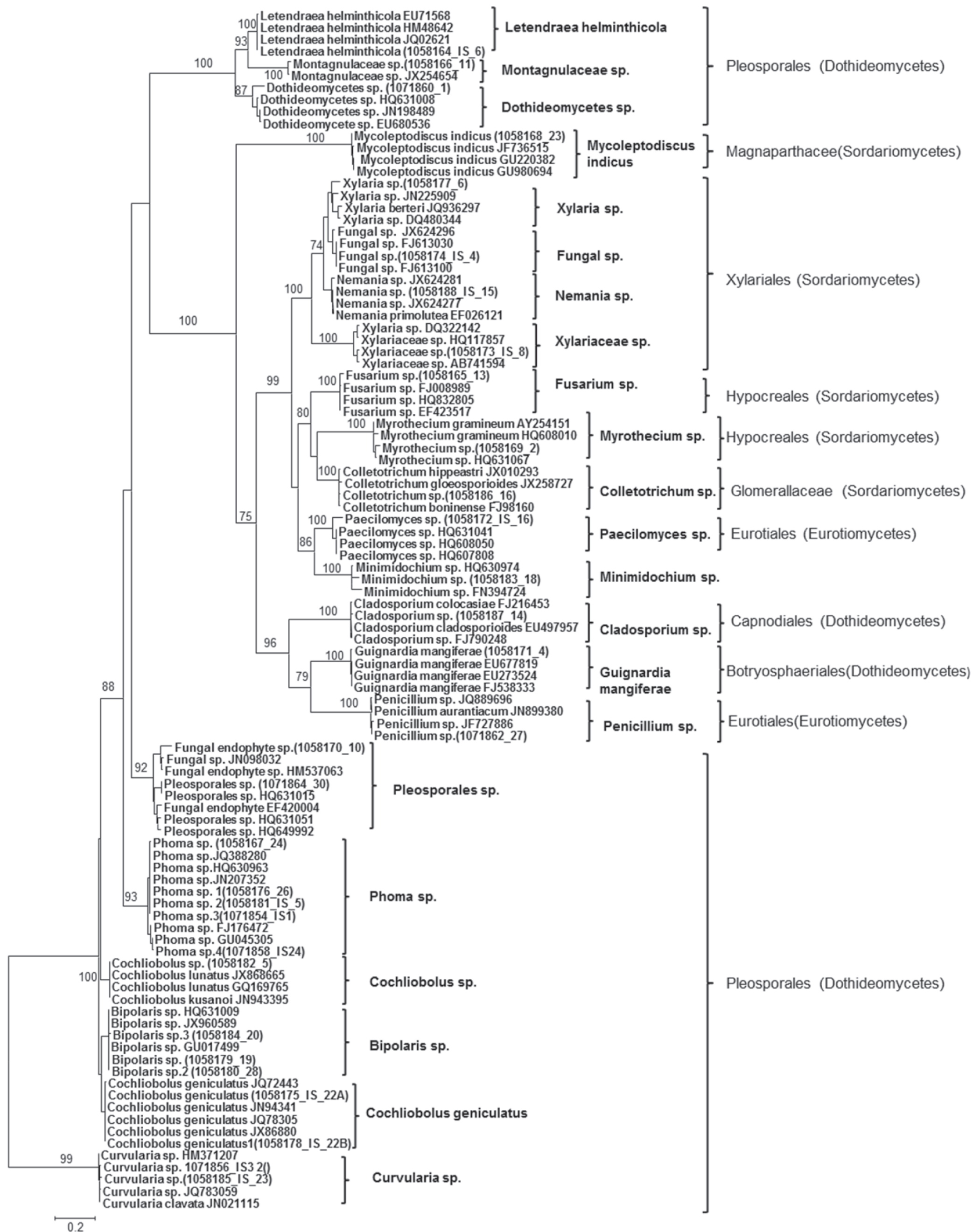

Figure 1. Maximum Likelihood tree based on ITS sequences of 31 endophytic fungi (isolate ID indicated in parenthesis) and 69 reference taxa obtained from GenBank (with accession numbers) constructed using Raxml sofware. Only bootstrap values over 70% are shown above the branches.

Table 1. Endophytic fungi and their antibacterial activities.

Endophytic fungi	Test bacteria (zone of inhibition in mm)						
Vitex rotundifolia	***S. aureus***	***B. cereus***	***B. subtilis***	***M. luteus***	***E. faecalis***	***E. coli***	***P. aeruginosa***
Cochliobolus geniculatus (ISB0001)	-	-	-	-	-	-	-
Cochliobolus geniculatus (ISB0002)	-	14.00 ± 0.00	14.83 ± 0.29	-	-	-	-
Curvularia sp. (ISB0003)	16.17 ± 0.29	14.83 ± 0.29	13.00 ± 0.00	-	-	-	-
Curvularia sp. (ISB0004)	-	-	-	-	-	-	-
Fungal sp. (ISB0005)	-	-	-	-	-	-	-
Letendraea helminthicola (ISB0006)	-	-	-	-	-	-	-
Nemania primolutea (ISB0007)	-	-	-	-	-	-	-
Paecilomyces sp. (ISB0008)	16.00 ± 0.00	16.17 ± 0.29	15.00 ± 0.00	-	-	-	-
Phoma sp. (ISB0009)	-	-	-	-	-	-	-
Phoma sp. (ISB0028)	-	-	-	8.000 ± 0.000	-	-	-
Phoma sp. (ISB0029)	10.830 ± 0.290	-	-	10.170 ± 0.290	-	-	-
Xylariaceae (ISB0012)	-	-	-	-	-	-	-
Ipomoea pes-caprae	***S. aureus***	***B. cereus***	***B. subtilis***	***M. luteus***	***E. faecalis***	***E. coli***	***P. aeruginosa***
Bipolaris sp. (ISB0013)	-	-	-	-	-	-	-
Bipolaris sp. (ISB0014)	21.000 ± 0.000	14.830 ± 0.290	10.000 ± 0.000	18.670 ± 0.290	15.670 ± 0.580	8.00 ± 0.000	-
Bipolaris sp. (ISB0015)	-	-	10.170 ± 0.290	25.000 ± 0.000	-	-	-
Cladosporium sp. (ISB0016)	-	-	-	-	-	-	-
Cochliobolus sp. (ISB0017)	-	-	-	-	-	-	-
Colletotrichum sp. (ISB0018)	-	-	-	-	-	-	-
Dothideomycete sp. (ISB0019)	10.170 ± 0.290	11.170 ± 0.290	-	10.000 ± 0.000	-	-	8.667 ± 0.236
Fungal endophyte (ISB0020)	-	-	-	-	-	-	-
Fusarium sp. (ISB0010)	-	-	-	-	-	-	-
Guignardia mangiferae (ISB0022)	-	12.33 ± 0.58	14.00 ± 0.00	-	-	-	-
Minimidochium sp. (ISB0023)	8.333 ± 0.471	8.333 ± 0.236	13.330 ± 0.580	10.830 ± 0.290	-	8.667 ± 0.236	-
Montagnulaceae sp. (ISB0024)	24.670 ± 0.290	27.330 ± 0.580	19.830 ± 0.290	24.000 ± 0.000	-	-	-
Mycoleptodiscus indicus (ISB0025)	-	-	-	-	-	-	-
Myrothecium sp. (ISB0026)	8.333 ± 0.471	8.333 ± 0.471	8.333 ± 0.236	-	-	-	-
Penicillium sp. (ISB0027)	8.000 ± 0.000	8.000 ± 0.000	-	-	-	8.667 ± 0.236	-
Phoma sp. (ISB0028)	-	-	-	8.000 ± 0.000	-	-	-
Phoma sp. (ISB0029)	10.830 ± 0.290	-	-	10.170 ± 0.290	-	-	-
Pleosporales sp. (ISB0030)	-	-	-	-	-	-	-
Xylaria sp. (ISB0031)	-	-	-	-	-	-	-

broader spectrum of antibacterial activity by the endophytic fungi from *I. pes-caprae* in the present study. Exhibition of similar bioactivities between plant and its endophytic fungi have been reported [45, 46]. Hence, the choice of endophytic fungi hosts for bio-prospecting and bioactivity studies should consider; firstly, the uniqueness of the host's environment or ecological niche, such as harsh environments that require specific survival strategies; secondly, plants that possess an ethno botanical history and thirdly, plants being exploited for their medicinal values [36,39,41].

The ability of mangrove fungi to synthesize unique secondary metabolites was considered as an adaptive response towards its harsh environment with high salinity and extreme intertidal conditions [27,44,47]. Table 2 shows that nine out of the ten manglicolous strains displayed positive antibacterial activity. The fungal strains *D. rhizophorae*, *Henningsomyces* sp. and the ISB004 strain of *Dactylospora haliotrepha* was active against four or more test bacteria with *D. rhizophorae* being the only strain active against the Gram negative bacteria *E. coli*. The antibacterial activities of some manglicolous fungi strains similar to the present study had been previously reported whereby a novel antibacterial agent corollosporine with phthalide was isolated from the marine derived fungi *Corollospora maritima* [25]. The compound corollosporine exhibited activity against *S. aureus* and *B. cereus* [30]. Metabolites enalin A and B had been previously isolated from the manglicolous fungi *Verruculina enalia* (Kohlm.) Kohlm. & Volkm.-Kohlm., from a salt lake in the Bahamas [26]. The compound enalin A is a coumaranone with known antimicrobial activities [26]. The *V. enalia* strain in the present study displayed no activity against the Gram negative *E. coli* based on the plug assay, despite previous report of antibacterial activity against *S. aureus*, *B. subtilis* and *E. coli* [48]. In the broth microdilution assay, *Dactylospora haliotrepha* (ISB003) and *Henningsomyces* sp. exhibited antibacterial activities against the Gram positive test bacteria (Table 3). However, *D. rhizophorae* was the only manglicolous fungi with antibacterial activities against four Gram positive and one Gram negative test bacteria (Table 3). Since *D. rhizophorae* displayed antibacterial potential against both bacterial Gram types, the chemistry of its metabolites was further analysed. The *D. rhizophorae* isolate used in the present study was isolated from the drift wood of *Rhizophora apiculata*.; a mangrove tree, which belongs to a genus previously explored for its antibacterial secondary metabolites [18]. Although the antibacterial potential of *D. rhizophorae* was previously unknown, another species of the same genus, *Dyfrolomyces mangrovei*, has been reported to possess antibacterial activities against *B. subtilis* and *S.aureus* [48].

3.3 Active Constituents of *Dyfrolomyces rhizophorae*

Compound 1 was isolated from sub-fraction 2 and compound 2 was isolated from sub-fraction 3, both from the *n*-hexane extract (Table 4). The gas chromatography-mass spectrum of 1 exhibited a molecular ion peak M^+ at m/z 280. Comparison of its mass spectrum with the reference spectra in the NIST 05 Library suggested that 1 was linoleic acid that was confirmed from the IR spectrum, ^{1}H NMR , ^{13}C NMR, and DEPT experiments. The absorption bands around 3276, 2922, 2847 and 1715 cm^{-1} were

Table 2. Marine derived manglicolous fungi and their antibacterial activities.

Manglicolous fungi	Test bacteria (zone of inhibition in mm)						
	SA	BC	BS	ML	EF	EC	PA
Corollospora maritima (ISB001)	-	10.333 ± 0.236	10.1667 ± 0.236	-	-	-	-
Dactylospora haliotrepha (ISB002)	10.833 ± 0.236	-	10.333 ± 0.236	-	-	-	-
Dactylospora haliotrepha (ISB003)	11.000 ± 0.408	-	10.833 ± 0.236	-	-	-	-
Dactylospora haliotrepha (ISB004)	13.000 ± 0.408	13.833 ± 0.236	14.333 ± 0.236	10.833 ± 0.236	-	-	-
Dactylospora haliotrepha (ISB005)	13.000 ± 0.408	14.333 ± 0.236	13.833 ± 0.236	-	-	-	-
Fusarium sp. (ISB006)	-	-	-	-	-	-	-
Henningsomyces sp. (ISB007)	14.667 ± 0.236	14.333 ± 0.236	13.667 ± 0.236	10.333 ± 0.236	-	-	-
Dyfrolomyces rhizophorae (ISB008)	15.833 ± 0.236	16.167 ± 0.236	15.000 ± 0.408	14.667 ± 0.236	-	8.000 ± 0.000	-
Verruculina enalia (ISB009)	11.000 ± 0.408	13.833 ± 0.236	12.167 ± 0.236	-	-	-	-
Verruculina enalia (ISB010)	10.833 ± 0.236	12.167 ± 0.236	11.000 ± 0.408	-	-	-	-

Table 3. Minimum inhibitory concentration (MIC) values of selected marine derived endophytic and manglicolous fungi in mg/ml.

Fungi	Bacteria						
	S. aureus	*B. cereus*	*B. subtilis*	*E. faecalis*	*M. luteus*	*P. aeruginosa*	*E. coli*
Endophytic fungi							
Bipolaris sp. (ISB0014)	0.500 ± 0	0.250 ± 0	NA	NA	1.000 ± 0	NA	NA
Minimidochium sp. (ISB0023)	0.125 ± 0	0.063 ± 0	0.125 ± 0	0.250 ± 0	0.250 ± 0	NA	NA
Montagnulaceae sp. (ISB00124)	2.000 ± 0	0.500 ± 0	NA	NA	1.000 ± 0	NA	NA
Manglicolous fungi							
Dactylospora haliotrepha (ISB003)	0.125 ± 0	0.125 ± 0	0.063 ± 0	NA	0.500 ± 0	NA	NA
Henningsomyces sp.	0.250 ± 0	1.000 ± 0	0.417 ± 0.15	NA	1.000 ± 0	NA	NA
Dyfrolomyces rhizophorae	0.500 ± 0	0.250 ± 0	0.052 ± 0.02	NA	0.500 ± 0	NA	0.500 ± 0

Mean ± standard deviation, n=3; NA= no activity

Table 4. Minimum inhibitory concentration (MIC) of crude, fractions and pure compounds from *Dyfrolomyces rhizophorae* in mg/ml.

Fraction/Pool/Compound	Bacteria						
	S. aureus	*B. cereus*	*B. subtilis*	*E. faecalis*	*M. luteus*	*P. aeruginosa*	*E. coli*
Crude	0.500 ± 0	0.250 ± 0	0.052 ± 0.02	NA	0.500 ± 0	NA	0.500 ± 0
Fraction n-hexane	2.000 ± 0	0.250 ± 0	0.125 ± 0	1.000 ± 0	0.500 ± 0	NA	2.0
Fraction dichloromethane	NA	NA	NA	NA	NA	NA	NA
Fraction methanol	NA	NA	NA	NA	NA	NA	NA
Sub-fraction 1	NA	NA	NA	NA	NA	NA	NA
Sub-fraction 2	0.500 ± 0	0.250 ± 0	0.250 ± 0	0.250 ± 0	0.500 ± 0	NA	NA
Sub-fraction 3	0.500 ± 0	0.250 ± 0	0.250 ± 0	NA	NA	NA	NA
Sub-fraction 4	NA	NA	NA	NA	NA	NA	NA
Sub-fraction 5	NA	NA	NA	NA	NA	NA	NA
Sub-fraction 6	NA	NA	NA	NA	NA	NA	NA
Sub-fraction 7	NA	NA	NA	NA	NA	NA	NA
Linoleic acid	1.000 ± 0	NA	0.500 ± 0	NA	NA	NA	NA
Palmitic acid	0.500 ± 0	0.250 ± 0	0.250 ± 0	NA	0.500 ± 0	NA	NA
Penicillin	0.004 ± 0	0.063 ± 0	0.063 ± 0	0.500 ± 0	0.125 ± 0	0.500 ± 0	0.125 ± 0
Chloramphenicol	0.031 ± 0	0.016 ± 0	0.016 ± 0	0.500 ± 0	0.016 ± 0	0.125 ± 0	0.008 ± 0

Mean ± standard deviation, n = 3; NA = no activity

indicative of the hydroxyl group, the aliphatic chain and the carbonyl group, respectively. The absorption band at 1686 cm^{-1} indicates the double bonds in linoleic acid. The ^{13}C NMR spectrum of 1 indicated 18 signals of which one was methyl, four olefinic carbons twelve methylenes and one quaternary carbon. The carbon resonances at δ_C 180.7 and 14.1 were attributed to C-1 and C-18, respectively. The carbon resonances at 128.1-130.2 were indicative of the olefinic carbons. The remaining signals in the highfield region between δ_C 24.8-34.3 were indicative of the methylene carbons of the aliphatic chain. The ^{1}H NMR spectrum was assigned with the aid of the HSQC spectrum, and was further confirmed by the COSY and HMBC experiments. The triplets at δ_H 2.35, 2.78 and 0.90 and the multiplet at δ_H 1.65 were respectively assigned to H-2, H-11, H-18 and H-3, the signals around δ_H 5.30-5.42 were attributed to the olefinic protons, while the signals around δ_H 1.31-2.09 were attributed to the methylene protons of the aliphatic chain. The complete NMR spectral results of 1 are presented in Table 5. The gas chromatography-mass spectrum of 2 exhibited a molecular ion peak M^+ at *m/z* 256. Comparison of its mass spectrum with the reference spectra in the NIST 05 Library suggested that 2 was *n*-hexadecanoic acid (palmitic acid), which was confirmed with the IR spectrum, ^{1}H NMR, ^{13}C NMR, and DEPT experiments. The absorption bands around 3348, 2924, 2854 and 1713 cm^{-1} were indicative of the hydroxyl group, the aliphatic chain and the carbonyl group, respectively. The ^{13}C NMR spectrum of 2 indicated 16 signals of which one was methyl, 14 methylenes and one quaternary carbon. The carbon resonances at δ_C 180.2 and 14.3 were attributed to C-1 and C-16, respectively. The remaining signals in the upfield region between δ_C 22.9-34.3 were indicative of the methylene carbons of the aliphatic chain. The ^{1}H NMR spectrum was assigned with the aid of the HSQC spectrum, and was further confirmed by the COSY and HMBC experiments. The triplets at δ_H 2.35 and 0.89 and the multiplet at δ_H 1.64 were respectively assigned to H-2, H-16 and H-3, while the signals around δ_H 1.26 were attributed to the methylene protons of the aliphatic

Table 5. The ^{1}H and 13C NMR 400Hz data of linoleic acid.

Position	δH[a]	δC	Position	δH[a]	δC
1		180.7	10	5.30-5.42 *m*[b]	128.1[g]
2	2.35 *t* (7.8)	34.3	11	2.78 *t* (6.0)	25.8
3	1.64 *m*	24.8	12	5.30-5.42 *m*[b]	128.3[g]
4	1.31-1.38 *m*[b]	29.2[e]	13	5.30-5.42 *m*[b]	130.4[f]
5	1.31-1.38 *m*[b]	29.3[e]	14	2.04-2.09 *m*[b]	27.3[d]
6	1.31-1.38 *m*[b]	29.3[e]	15	1.31-1.38 *m*[b]	29.8[e]
7	1.31-1.38 *m*[b]	29.5[e]	16	1.31-1.38 *m*[b]	22.8[c]
8	2.04-2.09 *m*[b]	27.4[d]	17	1.31-1.38 *m*[b]	31.7[c]
9	5.30-5.42 *m*[b]	130.2[f]	18	0.90 *t* (7.3)	14.1

[a]Coupling constants (J) in Hz are indicated in parentheses

[b]Overlapping signals

[c,d,e,f,g]Chemical shifts are interchangeable

chain. The complete NMR spectral results of 2 are presented in Table 6.

Palmitic acid and linoleic exhibited antibacterial activity against only Gram positive bacterial strains in this study which could be attributed to its hydrophobic nature [4]. Certain fatty acids, such as palmitic acid, have been known to display antibacterial activities against oral microorganisms [15]. The antibacterial activity exhibited by linoleic acid in the present study is similar to previous studies [8]. Linoleic acid and palmitic acid are also known to be common fatty acid constituents in essential oils [42]. The composition of 44.8% oleic acid and 33.7% of linoleic acid in argan oil has been proven to possess nutritional benefits in the reduction of atherosclerosis consequently preventing occurrences of cardiovascular diseases in humans [5]. Furthermore, fatty acids from microorganisms have also been shown to be sustainable feedstock for biodiesel production [9,49].

Table 6. The ^{1}H and 13C NMR 400Hz data of palmitic acid.

Position	δH[a]	δC
1		180.2
2	2.35 *t* (7.3)	22.9
3	1.64 *m*	32.2
4	1.26 *m*[b]	29.3[c]
5	1.26 *m*[b]	29.3[c]
6	1.26 *m*[b]	29.5[c]
7	1.26 *m*[b]	29.5[c]
8	1.26 *m*[b]	29.6[c]
9	1.26 *m*[b]	29.6[c]
10	1.26 *m*[b]	29.7[c]
11	1.26 *m*[b]	29.7[c]
12	1.26 *m*[b]	29.8[c]
13	1.26 *m*[b]	29.9[c]
14	1.26 *m*[b]	24.9[d]
15	1.26 *m*[b]	34.3[d]
16	0.89 *t* (6.4)	14.3

[a]Coupling constants (J) in Hz are indicated in parentheses
[b]Overlapping signals
[c,d]Chemical shifts are interchangeable

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