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Antibacterial activity and bioactive compounds of a marine macroalgae endophytic fungi, *Hypoxylon monticulosum*

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ABSTRACT

Aims: This study aims to determine the antibacterial potential and identify the bioactive compounds of *Hypoxylon monticulosum* isolated from marine macroalgae *Ulva lactuca*.
Methodology and results: *Ulva lactuca* was collected from the Desaru coast, Johor, Malaysia and three endophytes were isolated following surface sterilisation. One fungal isolate was further characterised by the morphology of white, yellowish colonies and fibrous with a waxy structure indicative of a member from the genus *Hypoxylon.* Molecular identification through internal transcribed spacer (ITS) sequence analysis matches the reference sequence with more than ≥98% homology to *Hypoxylon monticulosum* AS26-D8. Minimum inhibition concentration (MIC) of the fungal ethyl acetate (EA) extract was determined against five human pathogenic bacteria. Wide spectrum antibacterial activity was noted; with MIC against *Escherichia coli* was 1.25 ± 0 mg/mL, *Bacillus subtilis* and *Enterobacter faecalis* both at 5.00 ± 0 mg/mL, and finally, both *Staphylococcus aureus* and *Klebsiella pneumoniae* were 10.00 ± 0 mg/mL, respectively. Bioassay-guided fractionation was performed using solvents of increasing polarities, producing three fractions and analysis by liquid chromatography-mass spectrometry (LC/MS) identified 128 compounds. From these, nine compounds were identified as having biological activities. Dihydrocordoin, D-pantothenoyl-L-cysteine, caffeine and Tumonoic A acid were among the compounds identified as having antibacterial properties.
Conclusion, significance and impact of study: *Hypoxylon monticulosum* from marine source has antibacterial potential owing to the compounds previously reported to display antibacterial and other biological properties. The compounds differ from those previously reported in *H. monticulosum* from terrestrial sources.

Keywords: Antibacterial potential, bioactive compounds, fungal endophytes, *Hypoxylon monticulosum*, *Ulva lactuca*

INTRODUCTION

Ulva lactuca is a green macroalgae (Chlorophyta) member that dominates the Johor coast, the southernmost part of Malaysia (Zainee *et al*., 2019). This autotrophic multicellular marine organism is home to diverse marine endophytic fungi (Zainee *et al*., 2018a; 2018b). Endophytic fungi from marine sources may provide compounds with antibacterial properties (Nisa *et al*., 2015; Zhang *et al*., 2018) especially when antibiotic-resistant pathogenic bacteria have become a serious health problem and cause millions of deaths globally (Antimicrobial Resistance Collaborators, 2022). Alternative sources of antibiotics with different modes of action hence should be explored. The alternative source should be safe and have a sustainable approach to controlling infections. In this view, we explore endophytes from *Ulva lactuca* that have the capacity to adapt to different phenotypes according to environmental parameters such as water salinity, capable of symbiosis with bacteria, provide bioactive compounds and importantly, food to the symbionts (Zainee *et al*., 2018a).

Around 60% of approved small medicinal molecules are related to natural products and 69% of antibacterial agents originate from natural products, according to Patridge *et al.* (2016) and Matsumura *et al.* (2018). Endophytes are bacteria or fungi that attack or live inside an animal or plant without causing any harm and are symptomless to their host (Petrini, 1991; Wilson, 1995). Endophytic fungi aid hosts in competing and producing host products through rehabilitating their resistance against environmental stress and protection from herbivores and pathogens (Nisa *et al*., 2015). It is suggested that endophytic fungi and hosts may possess similar biosynthesis pathways in producing secondary compounds. This is suggested upon the discovery of paclitaxel (taxol) from endophytic fungi *Taxomyces andreanne* that was due to the vertical gene transfer

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Figure 1: Location of the green macroalgae, *U. lactuca* was sampled at Desaru coast, Johor, Malaysia.

between the organisms (Soliman *et al.*, 2013). The environment that endophytic fungi live in is fairly harsh, but it does not deny the fact that they are an essential source of novel and important therapeutic secondary compounds (Deshmukh *et al.*, 2018).

Fungal endophytes from *Ulva lactuca* have been explored previously (Zainee *et al.*, 2018a) and in our laboratory, one particular genus *Hypoxylon* is interesting to be further explored. This genus was reported in other endophytes from different sources; thus, this study aims to determine the antibacterial activity of this fungal isolate and its biologically active compounds in an effort to differentiate it according to the source of isolation.

MATERIALS AND METHODS

Collection of macroalgae

Ulva lactuca is a green macroalgae (Figure 1) that was aseptically collected from the Desaru coast, Johor, Malaysia, during the low tide of the month and kept in zip-lock plastic. The macroalga was transferred back to the laboratory in a 4 °C cooler box within 24 h.

Isolation of endophytic fungus

The macroalgae as the host was surface sterilised according to the following steps: in contact with sterile seawater (30 sec), 75% ethanol (30 sec), 5% sodium hydrochloride (30 sec) and 75% ethanol (15 sec) (Zainee *et al.*, 2018b) The specimens were cut to 1 cm × 1 cm using surgical blades and left to be air-dried. The cuts were then placed on potato dextrose agar (PDA, Merck, USA) for endophyte to develop and incubated for seven days at 30 °C. Fungal colonies were sub-cultured repeatedly until pure culture was obtained.

Identification of isolated fungal endophyte

Fungal identification including colony morphology and color on both surface and reverse of PDA, hyphae morphology or mycelium, and characteristics of spore-bearing structures. Morphological species characterization was determined following the key characters described in Bridge (1985) and Yilmaz *et al.* (2014). Polymerase chain reaction (PCR) was employed to amplify the ITS gene (Figure 2) using the following primers: ITS1F (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4R (5'-TCC TCC GCT TAT TGA GC-3') (Schoch *et al.*, 2012).

Following sequencing of the amplified ITS products, sequences were edited using Sequencer software version 4.0 (Gene Codes Corp., Ann Arbour, Mich., USA). Sequence alignment was conducted using ClustalW programme version 1.8. Sequences against the existing data from the Genbank database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLASTN) programme. Phylogenetic reconstruction was calculated using the neighbour joining (NJ) algorithm and maximum parsimony (MP) methods with *Schizosaccharomyces pombe* as an outgroup. The NJ analysis was run using Molecular Evolutionary Genetic Analysis (MEGA) software, while MP analysis using Phylogenetic Analysis using Parsimony (PAUP) (Swofford, 2003) with 1000 bootstrap values respectively.

Preparation of crude extract

Fungal crude extract was prepared according to Kjer *et al.* (2010). The endophytic fungus was cultured on sterile PDA for seven days at 30 °C. This is followed by the growth of mycelium (3 × 6 mm plug) in 200 mL of potato

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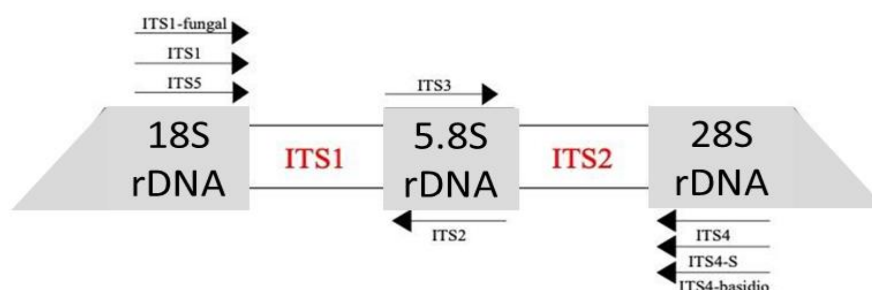

Figure 2: Primers locations in the ITS region within the ribosomal DNA.

dextrose broth (PDB, Merck, USA) at 30 ± 2 °C rotated at 100 rpm for 10 days. Fungal biomass was then separated by filtration using Whatman filter paper (Sigma Aldrich, 25 mm) and filtrate was extracted three times with ethyl acetate (EA, Systerm Chem AR) in the ratio of equal volumes to filtrate (1:1). Organic layer of the fungal EA extract was pooled together and evaporated using a rotary evaporator at 45 °C. This crude EA extract was weighed and stored at 4 °C until further use.

Determination of minimum inhibitory concentration (MIC)

Antibacterial activity of the crude fungal EA extract and later fractions was determined by minimum inhibitory concentration (MIC) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2021). Test pathogenic bacteria were obtained from Microbiology Laboratory, Faculty of Science and Technology, UKM, including *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Bacillus subtilis*. Bacterial suspension that equals 0.5 to the McFarland standard which is equivalent to 1.5×10^8 CFU/mL was prepared. Stock concentration of fungal EA extract at 10 mg/mL was prepared in 1% of dimethyl sulfoxide (DMSO, Merck, USA) followed by two times serial dilutions until 0.078 mg/mL. Test bacteria was mixed to different concentrations of extract and incubated at 37 °C for 24 h. Nutrient broth only (NB, Oxoid LTD, Basingstoke) was used as negative control and Streptomycin (2 mg/mL) acted as a positive control. Bacterial viability after treatment with extract was determined using 3-(4,5-dimethylthiazol-2-1)-2,5-diphenyl-2H-tetrazolium-bromide (MTT, Merck, USA) at 2 mg/mL as a dye. MIC value is equal to the lowest extract concentration that changed MTT to yellow, which is indicative of no bacterial growth. Bacterial growth is due to the reduction of MTT to form purple formazan crystals. The assay was replicated three times.

Bioassay-guided fractionation

The crude EA extract of *H. monticulosum* (1 g) was fractionated through sequential extraction using 10 mL of the following solvents with increasing polarity: *n*-hexane, chloroform and methanol in an orbital shaker for 7 h. Filtrates were filtered through Whatman filter paper (Sigma Aldrich, 25 mm) and were dried by evaporation in a fume hood and weighed. All fractions obtained from this procedure were tested for the antibacterial activity as above.

Liquid chromatography-mass spectrometry (LC-MS) analysis

Crude EA extract and all fractions of *H. monticulosum* were analysed on LC/MS Q-TOF for LC/MS (1290 Infinity, Brand Technologies for UHPLC coupled with 6550 iFunnel). Separation was performed using Agilent Technologies Zorbax Eclipse Plus C18 (4.6 × 100 mm, 3.5 μm) with the following elution gradient: 0-1 min, 5% B; 1-11 min, 5-100% B; 11-13 min, 95% B; 13-16 min, 5% B using mobile phase A (0.1% HCOOH in water) and mobile phase B (methanol). The injection volume was 10 μL and the flow rate was set 0.4 mL/min. Peak areas were determined with MassHunter Qualitative Analysis B.06.00 Software from Agilent Technologies. The compounds were structurally characterized from the spectral comparison with the library search of the NIST Mass Spectral Library.

RESULTS AND DISCUSSION

Isolated endophytic fungi

Isolation to single colonies of endophytic fungi was a great challenge due to the close symbiotic relationship in the macroalgae (Debbab *et al*., 2011). This is also true in this study with three fungal isolates characterised morphologically as *Trichophyton*, *Penicillium* and *Hypoxylon*. However, only *Hypoxylon* was further characterised with the morphology that appeared on PDA after an incubation period of, on average, seven days with conidia presentation is shown in Figure 3. White yellowish colonies and fibrous with the waxy structure were the diagnostic characteristics of the genus from *Hypoxylon*.

For identification by using ITS region sequence, the isolate has similarity to *Hypoxylon monticulosum* AS26-D8 (Accession number KJ774047) with at least 99.66% identity (E=0). Genetic relationship was shown in the phylogenetic analysis using the ITS sequence comparison (Figure 4). *Hypoxylon* is a genus in the Xylaraceae family with more than 130 species identified (Sánchez-Ballesteros *et al*., 2000).

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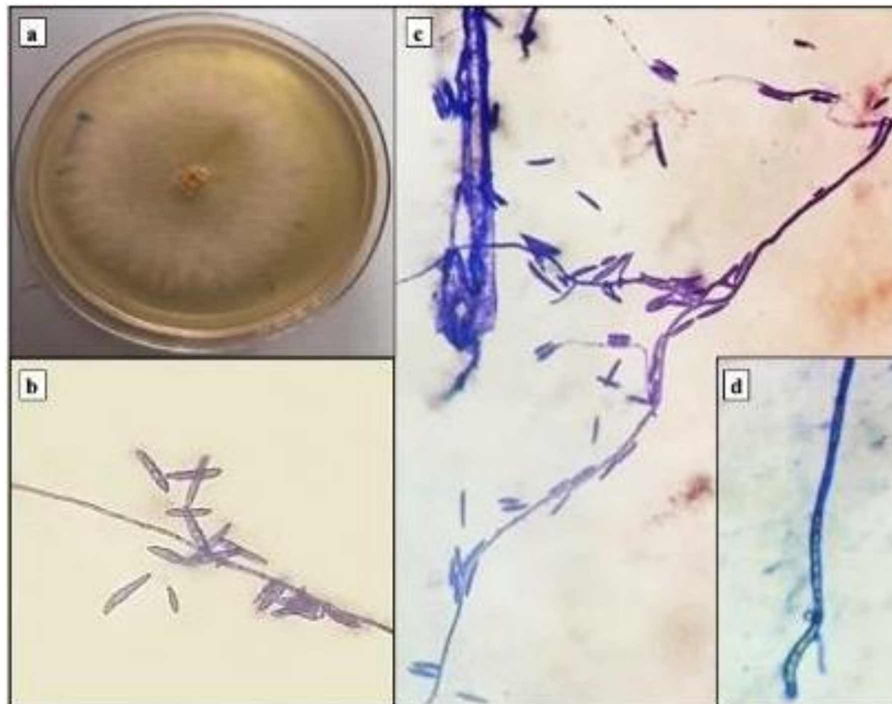

Figure 3: Microscopic and macroscopic morphology of *Hypoxylon monticulosum*. (a) Macroscopic structure-white yellowish colonies, fibrous with waxy structure; (b) Sporangium and arrangement of spores (magnification 1000×); (c) Microscopic structure under light microscope (magnification 400×); (d) Mycelial structure (magnification 400×).

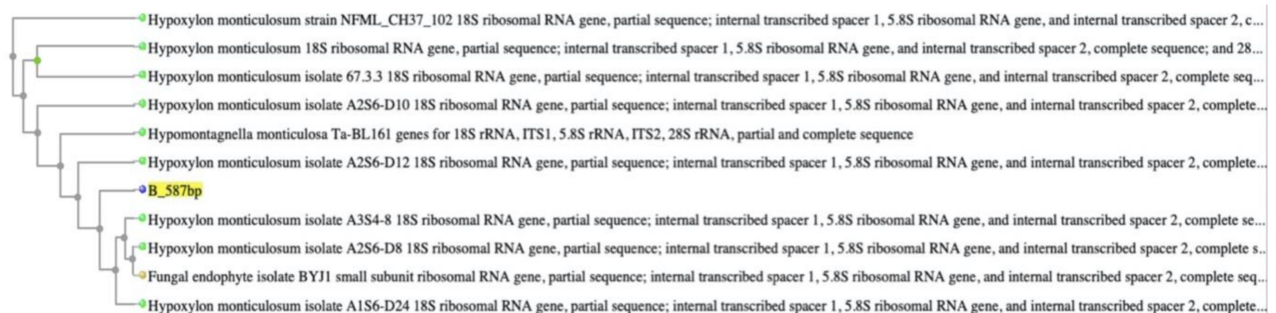

Figure 4: Phylogenetic tree of *Hypoxylon monticulosum*.

Antibacterial activity of endophytic fungi

The antibacterial assay showed that the endophytic fungal EA extract has broad spectrum activity against Gram-positive and negative test bacteria. The lowest MIC concentration of 1.25 mg/mL was against *E. coli*, 5.0 ± 0 mg/mL against *E. faecalis* and *B. subtilis,* and *S. aureus* and *K. pneumoniae* were successfully apprehended by EA extract at 10.0 ± 0 mg/mL (Table 1). Solvent sequential extraction of *H. monticulosum* EA extract afforded three fractions, *n*-hexane (FN), chloroform (FC) and methanol (FM), as shown in Figure 5. Ethyl acetate is a moderately polar solvent that allows the isolation of both polar and nonpolar compounds from the fermented broth. Following fractionation using increasing polarity solvents, FC was found to be the most active in antibacterial activity, followed by FM and FN (Table 1). To associate the antibacterial activity of the extracts and fractions, compounds can be determined by LC/MS.

Compounds isolated from *H. monticulosum* extract and fractions

Determination by LC/MS of the extract and fractions was successful in separating 128 compounds by positive-ion mode. However, only sixteen compounds have been identified from the library search according to their mass spectra (Figure 6 and Table 2). From these, nine compounds were known for their biological activities as per literature search and explained below.

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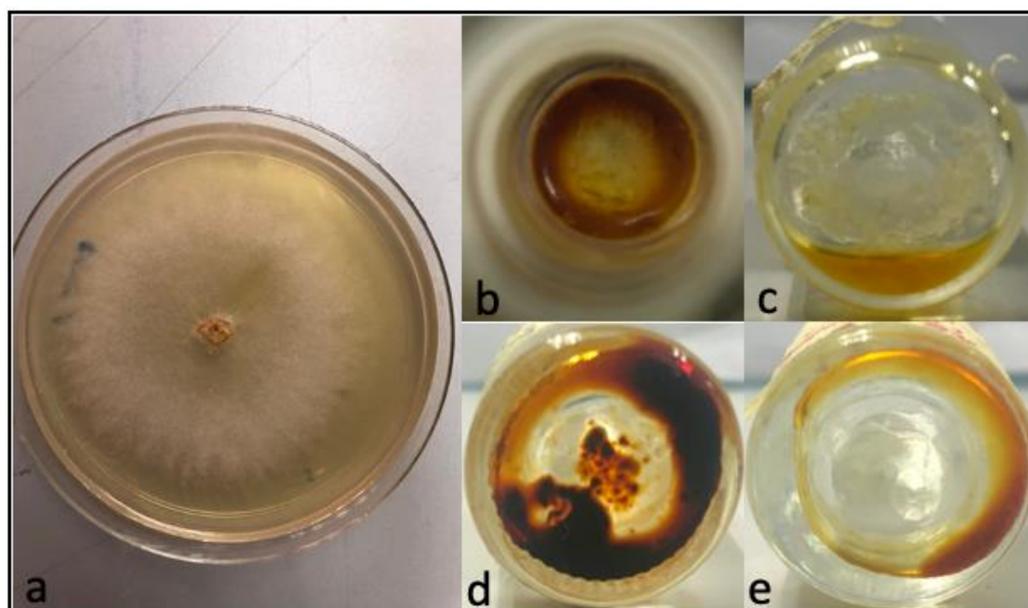

Figure 5: *Hypoxylon monticulosum* and the dried extract/fractions using different solvents. (a) *H. monticulosum* after five days of incubation; (b) EA extract of *H. monticulosum*; (c) *n*-hexane fraction (FN); (d) Chloroform fraction (FC); (e) Methanol fraction (FM).

Table 1: Minimum inhibitory concentration (MIC) values of extract and fractions of *Hypoxylon monticulosum*, nutrient broth only as negative control and streptomycin as positive control against different test bacteria.

Bacteria (Gram reaction + or -)	Minimum inhibitory concentration (MIC) (mg/mL)					
	Ethyl acetate extract	*n*-hexane fraction	Chloroform fraction	Methanol fraction	Negative control	Positive control
E. coli (-)	1.25	NA	10.0 ± 0	NA	NA	2.0 ± 0
E. faecalis (-)	5.0	NA	10.0 ± 0	NA	NA	2.0 ± 0
S. aureus (+)	5.0	NA	10.0 ± 0	NA	NA	2.0 ± 0
B. subtilis (+)	5.0	10.0 ± 0	2.5 ± 0	5.0 ± 0	NA	2.0 ± 0
K. pneumoniae (-)	5.0	NA	NA	NA	NA	2.0 ± 0

NA: No activity.

Two flavonoid members detected in *H. monticulosum* extract in this study were dihydrocordoin and xuulanin. Dihydrocordoin is part of the chalcones members, a polyphenol metabolite group that is derived from the flavonoid family. Flavonoids have been recognized as natural products with various pharmacology activities and very low toxicity, thus play a vital role in plant growth and antibacterial activity (Donnelly and Boland, 1995; Reyes-Chilpa *et al.*, 1998; Wang *et al.*, 1998; Dixon and Steele, 1999; Kopustinskiene *et al.*, 2020).

The other flavonoid detected in the form of flavan or flavanol is xuulanin. According to Hernández-Bolio *et al.* (2019), xuulanin has been reported as a novel natural product produced from the bark and root of *Lonchocarpus* spp. from the Yucatean flora. This metabolite has anti-plasmodial, anti-protozoal and cytotoxicity activities (Borges-Argáez *et al.*, 2007).

D-pantothenoyl-L-cysteine is an amino acid with an extremely weak base found in most living organisms. A derivative of this metabolite known as N-((R)-pantothenoyl)-L-cysteine is a basic component of the antioxidant glutathione. This L-cysteine derivative is one of the amino acids that formed beta-lactam and thiazolidine rings similar to the main nucleus of all penicillin structures, the first antibiotic in the world (Roach *et al.*, 1997; Keller *et al.*, 2005; Gaudelli and Townsend, 2014).

Spiredine is another metabolite found in the *H. monticulosum* fraction. It is a diterpenoid alkaloid derived from atisane hydride. There are only a few reports on this metabolite, with the first report on the isolation was from *Spirea japonica* (Gorbunov *et al.*, 1976) and later discovered from *Thalictrum sessile* (Wu *et al.*, 1988). However, Hou *et al.* (2021) have reported that there were few other derivatives found from endophyte fungi in mangrove environments, such as hypoxyterpoids (1) and (2).

Prolyl-Glycyl-Cysteine (Pro-Gly-Cys), glymidine and L-beta-aspartyl-L-aspartic acid are found in the FN fraction. There is not much information about these compounds,

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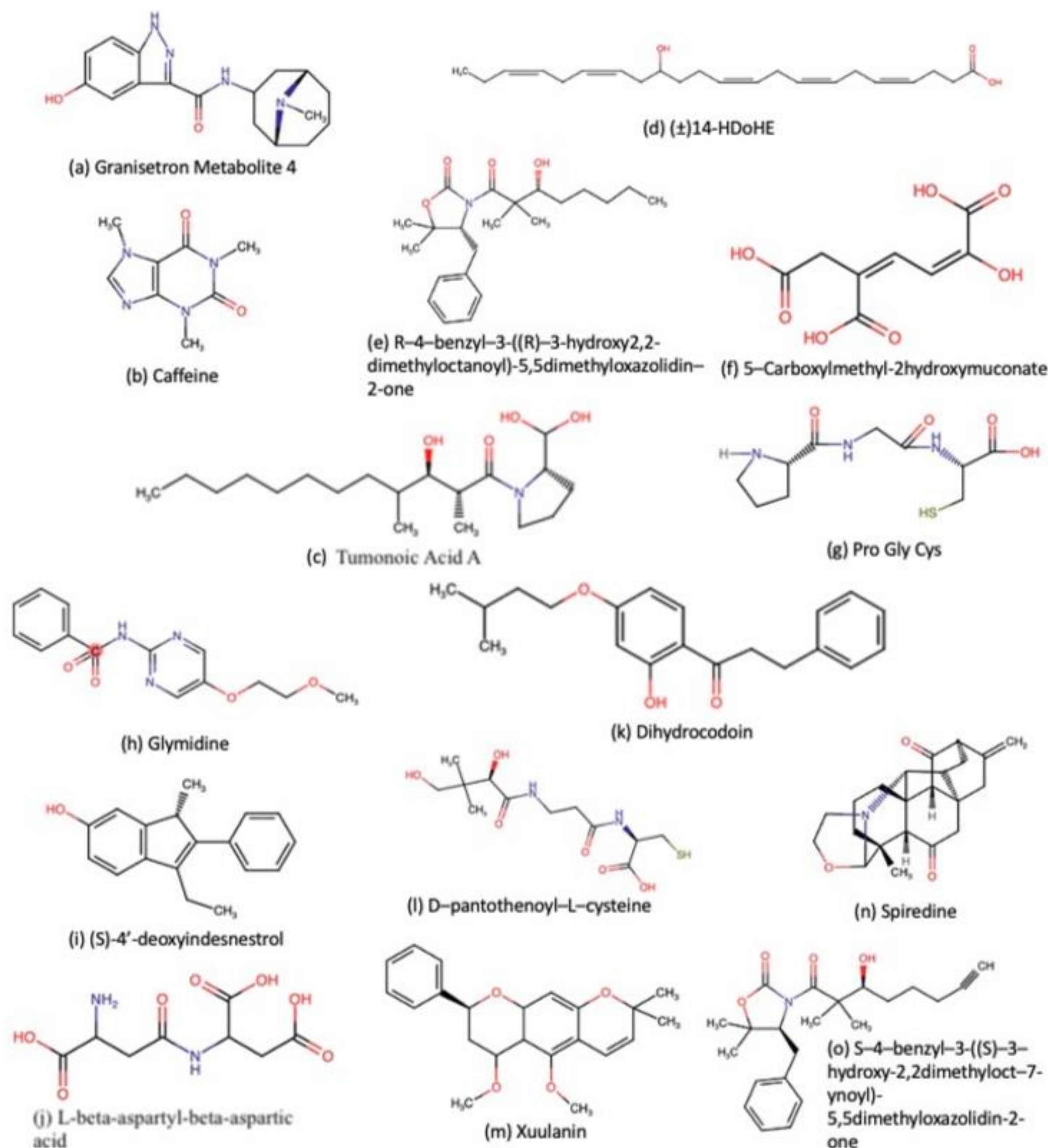

Figure 6: Selected compounds structures from LC/MS analysis of *Hypoxylon monticulosum* extract and fractions.

but according to the PubChem database (National Center for Biotechnology Information, 2002), glymidine is a metabolite derived from sulfapyrimidine and has antihyperglycemic activity. This metabolite is also able to lower blood glucose levels through the secretion of insulin from pancreatic beta cells.

Tumonoic A acid is one of the compounds found in FK. According to Harrigan *et al.* (1999), it was isolated together with Tumonoic B acid and Tumonoic C acid with no significant biological activity. Clark *et al.* (2008) reported that tumonoic A acid has antimalarial activity at a concentration of 10 mg/mL and is an N-acyl-amino acid with metabolic properties.

(±) 14-HDoHE is a type of hydroxyhexanoic acid. This hydroxy-fatty acid is produced in the green macroalgae, *U. lactuca*, after lipoxygenase enzymes cleave different polyunsaturated fatty acid substrates (Kuo *et al.*, 1997). This is an example in which the host has a similar metabolite to the endophytic fungi living in it as previously reported by Safwan *et al.* (2021). Le Cocq *et al.* (2017)

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Table 2: Mass spectrometry data obtained from LC/MS analysis of 15 selected compounds with reported biological activities in *H. monticulosum* extract and its fractions.

Name	Mass	Formula	Retention time (min)
Chloroform fraction (FC)			
(a) Granisetron metabolite 4	314.1738	$C_{17}H_{22}N_4O_2$	14.231
(b) Caffeine	194.0809	$C_8H_{10}N_4O_2$	8.446
(c) Tumonoic A acid	339.2932	$C_{19}H_{33}NO_4$	8.736
(d) (±)14-HDoHE	344.2358	$C_{22}H_{32}O_3$	9.815
Methanol fraction (FM)			
(e) R-4-benzyl-3-((R)-3-hydroxy2,2- dimethyloctanoyl) 5,5 dimethyloxazolidin-2-one	375.2391	$C_{22}H_{33}NO_4$	12.601
(f) Caffeine	194.08097	$C_8H_{10}N_4O_2$	8.455
(g) 5-Carboxylmethyl-2 hydroxymuconate	216.028	$C_8H_8O_7$	10.84
n-hexane fraction (FN)			
(h) Pro Gly Cys	275.0955	$C_{10}H_{17}N_3O_4S$	10.35
(i) Glymidine	309.0783	$C_{13}H_{15}N_3O_4S$	10.478
(j) (S)-4'-deoxyindesnestrol	258.1367	$C_{18}H_{18}O$	12.463
(k) L-beta-aspartyl-beta-aspartic acid	248.0644	$C_8H_{12}N_2O_7$	6.774
H. monticulosum ethyl acetate extract			
(l) Dihydrocodoin	310.158	$C_{20}H_{22}O_3$	11.632
(m) D-pantothenoyl-L-cysteine	322.1192	$C_{12}H_{22}N_2O_6S$	12.021
(n) Xuulanin	352.1673	$C_{22}H_{24}O_4$	12.1553
(o) Spiredine	353.1979	$C_{22}H_{27}NO_3$	12.294
(p) S-4-benzyl-3-((S)-3-hydroxy-2,2dimethyloct-7-ynoyl)-5,5 dimethyloxazolidin-2-one	371.2117	$C_{22}H_{29}NO_4$	12.637

have stated in their report that the relationship between the endophyte fungus and the host plant symbiotically has increased its ability to mimic the variety of chemical compounds of the host and it is believed to be the reason for the production of biologically active compounds when the relationship between the endophyte fungus and the host exists.

Caffeine can be found in both FC and FM fractions, is a type of methylxanthine alkaloid and antagonist receptor towards adenosine (Labbe and Nolan, 1987). In this study, FC was active against four bacteria except for *K. pneumoniae,* but FM does not show antibacterial activity. Hence, caffeine as the metabolite with antibacterial activity is not strongly supported as observed in the FC or FM fractions. Daglia *et al.* (2002) have previously reported that caffeine has no antibacterial activity against *Streptococcus mutans*, but in contrast, Ibrahim *et al.* (2006) stated that caffeine is active against pathogenic bacteria, *E. coli* O157:H7. This contrasting observation, hence, needs to be confirmed with further evaluation of the other compounds that are available along with caffeine and the type of bacteria that may be sensitive to it.

Although several compounds isolated from *H. monticulosum* are not reported to have antibacterial activities, some of them are associated with other biological activities. S-4-benzyl-3-((S)-3-hydroxy-2,2dimethyloct-7-ynoyl)-5, 5 dimethyloxazolidin-2-one and R-4-benzyl-3-((R)-3-hydroxy2,2-dimethyloctanoyl) 5,5 dimethyloxazolidin-2-one, are marine and natural product inhibitor compounds of cathepsin B which is a cysteine protease that can cause brain disorders (Phan *et al.*, 2022).

Granisetron metabolite 4, is in a class of medication called 5-HT3 receptor antagonists where it works by blocking serotonin, a natural substance in the body that causes nausea and vomiting. In addition, 5-carboxylmethyl-2-hydroxymuconate is a bacterial isomerase enzyme that shares characteristics analogous with multifunctional pro-inflammatory protein macrophage migration inhibitory factors, which is implicated in the tumorigenesis, angiogenesis and metastasis of many cancer phenotypes (O'Reilly *et al.*, 2016). On the other hand, L-beta-aspartyl-beta-aspartic acid is a hybrid peptide, which is a potential biomarker in the consumption of anatidaes (Anatidae), chickens (*Gallus gallus*), domestic pigs (*Sus scrofa domestica*) and milk (cow) (Jandke and Spiteller, 1986).

Endophytic fungi in macroalgae are influenced by the hosts and the environment. Antagonistic interaction in endophytic fungi of macroalgae is common. Active antagonism exhibited by the species of endophytic fungi in macroalgae indicated that they could be promising sources of metabolites. Fungi from the genus *Hypoxylon* have been identified as the main producer of potential bioactive compounds and it is the most prominent member in the Xylaraceae family that can be found either in the terrestrial or ocean ecology (Stadler *et al.*, 2006; Stadler *et al.*, 2008; Lutfia *et al.*, 2021). Cheng *et al.* (2020) revealed three new compounds from *H. monticulosum* that were isolated from the terrestrial plant *Litsea akoensis* var. *chitouchiaoensis*, which were hypoxyloamide, 8-methyoxynaphtalene-1,7-diol and hypoxylonol. These compounds were not found in this study, suggesting differences in compounds produced by the marine *H. monticulosum.* The difference is suggested

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to be due to differences in the fungus host, habitat and environment from which it was isolated. This fact is supported by Samanta *et al.* (2021) that observed the production of secondary compounds by endophytic fungi may vary with different habitats of hosts where they grow and accommodate. More than 200 compounds have been identified from *Hypoxylon* species only, according to Tan and Zou (2001).

CONCLUSION

This study has demonstrated that *U. lactuca* hosts a fungal endophyte, *H. monticulosum* with potential broad-spectrum antibacterial activity. Compounds from the marine endophytic fungus extract with its fractions revealed pharmacologically active compounds with antimicrobial properties and other biological activities. The compounds were different from *H. monticulosum* isolated from terrestrial sources. These preliminary results expressed the potential of *H. monticulosum* in new sources of therapeutic agents of antimicrobial treatments as further determination and characterisation are worthwhile for these marine endophytic fungi.

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