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Research Article

The Chemotaxonomic Identification Using Structure Types of Secondary Metabolites and Their Bioactivities of Bornean *Litophyton arboreum*

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ABSTRACT
The structure types and bioactivities of secondary metabolites derived from *Litophyton arboreum*, distributed in Sepanggar Bay, Sabah, Malaysia, were investigated as additional tools for establishing their species identification. As a result, a total of two secondary metabolites (alismol (**1**) and 10α-methoxy-4ß-hydroxy guaian-6-ene (**2**)) were isolated from Bornean soft coral *L. arboreum*. Their structures were elucidated based on spectroscopic data analysis and the antifungal activities of compounds **1** and **2** were determined. In addition, the compound **2** showed highest antifungal activity against *Haliphthoros milfordensis*. As a result of comparison with previous literature, significant variations were observed in relation to structure types of secondary metabolites and bioactivities. Information from this study gives additional evidence of chemotaxonomic significance and baseline data for effective selection of suitable lead pharmaceuticals.

Keywords*:* Soft coral, *Litophyton arboreum*, Sesquiterpene, Chemotaxonomy, Antifungal activity.

Introduction
Recently, natural products from marine resources have increasingly been recognized to have an important role for human health and a vital component of healing practices (Carroll et al., 2022). The soft corals produce an unprecedented diversity of secondary metabolites with a wide range of biological activities and are considered the largest remaining reservoirs of undiscovered natural molecules (Nurrachma et al., 2021). However, despite increasing attention, the soft coral species are particularly difficult to identify because there are still few tools specific to the classification group required to evaluate gene flow at the population level (Fujita et al., 2012, Metwally et al., 2020). Correctly identifying soft coral species that have significant potential as

drugs for human use is the first basic step in any development of marine natural products. Among them, the genus of *Litophyton* (phylum Cnidaria, class Octocorallia, order Alcyonacea, family Nephtheidae), which produces many secondary metabolites such as diterpenoids, sesquiterpenoids and steroids, is attracting attention due to their wide diversity of biological activities such as anti-cancer, anti-HIV and antiproliferative (Ellithey et al., 2013; Ghandourah et al., 2015; Yang et al.,2020) properties.

However, despite the development of chemistry research, taxonomical knowledge is still lacking and often confusing. It has come to a stage where many researchers have isolated interesting compounds but could not reproduce them since identification problems are still not resolved. Generally, their identification is characterized by morphological features such as polyps arranged on the terminal branches only, colour and external form, by the arrangement of sclerites and their DNA barcoding (McFadden et al., 2009; Santhanam, 2020). However, soft corals identification using these methods is quite difficult and the classification is often re-described (McFadden et al., 2014; Imahara et al., 2017). Actually, in 2007, it was reported that the genus *Litophyton* should be morphologically synonymous with the genus *Nephthea* (van Ofwegen, 2016). Hence, a simple and highly reliable new identification method is required and the soft corals secondary metabolites can be suitable as a taxonomic marker since they produce secondary metabolites that show variations by species (Aratake et al., 2012).

The existence of many genus *Litophyton* (synonymous with the genus *Nephthea*) has been found on Borneo Island, Sabah, Malaysia (Ishii et al., 2010a; Ishii et al., 2010b; Ishii et al., 2016; Ishii et al., 2018; Tani et al., 2019), but their identification and detailed information are still dearth. During this investigation, efforts were made to incorporate chemotaxonomic identification features for *Litophyton* species. As an initial step, comparative profiling of structure types of their secondary metabolites in *L. arboreum* were carried out for soft coral populations from Sepanggar Bay. In addition, to their importance as a tool for phytogenic relation, bioactivity potentials of these secondary metabolites were also investigated and reported in this paper.

Materials and Methods

General Experimental Procedures

^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectra were recorded on a JEOL ECA 600 NMR spectrometer using $CDCl_3$ with tetramethylsilane as an internal

standard. The AUTOPOL IV automatic polarimeter (Rudolph Research Analytic) was used to acquire their physical data. Preparative thin layer chromatography (TLC) was performed with silica gel glass plates (Merck, Kieselgel 60 F_{254}), and column chromatography (CC) with silica gel (Merck, Kieselgel 60, 70-230 mesh).

Sample Collection

The specimen of *L. arboreum* was collected from Sepanggar Bay, Sabah, North Borneo (6º4.683'N, 116º4.710'E), in July 2016. The voucher specimen (BORMI0055) was deposited in the BORNEENSIS Collection of the Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Malaysia.

Extraction and Isolation

The fresh soft coral (2.5 kg wet wt) was extracted in methanol (MeOH) at room temperature (24 ℃, 1.0 L × 3 each for five days), subsequently filtered, concentrated *in vacuo* and partitioned between ethyl acetate (EtOAc) / distilled water (H_2O) followed by partitioned with *n*-hexane / 90% MeOH from EtOAc fraction. The resulting crude extracts were subjected to CC eluting with a gradient of *n*-hexane and EtOAc with increasing polarity. The MeOH fraction 1 gave **1** (3.1 mg: 0.6%) after purification by preparative TLC using *n*-hexane. In addition, the MeOH fraction 3 was subjected to preparative TLC in *n*-hexane-EtOAc (3:1 (v/v)) and toluene-EtOAc (3:1 (v/v)) to yield **2** (2.9 mg; 0.6%). Percentages of compounds were the average of the respective compounds in 90% MeOH (2.1 g) crude extracts.

Alismol (1): colourless oil; $C_{15}H_{25}O$: $[\alpha]_D^{25}$ + 3.3 (*c* 0.2, $CHCl_3$): ^{1}H-NMR ($CDCl_3$, 600 MHz) δ_H: 5.56 (1H, s, H-6), 4.79 (2H, s, H-15), 4.70 (2H, s, H-15), 2.50 (2H, m, H-9), 2.27 (1H, s, H-5), 2.25 (1H, m, H-1), 2.22 (1H, m, H-11), 2.20 (2H, m, H-8), 2.05 (2H, m, H-9), 2.03 (2H, m, H-8), 1.90 (2H, m, H-2), 1.75 (2H, m, H-3), 1.75 (2H, m, H-3), 1.71 (2H, m, H-2), 1.23 (3H, s, H-14), 0.99 (3H, d, H-12, *J* = 6.9 Hz), 0.98 (3H, d, H-13, *J* = 6.9 Hz); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ_C: 48.0 (C-1), 25.4 (C-2), 41.0 (C-3), 81.0 (C-4), 55.7 (C-5), 122.0 (C-6), 150.5 (C-7), 30.7 (C-8), 37.8 (C-9), 154.6 (C-10), 38.1 (C-11), 22.2 (C-12), 22.0 (C-13), 24.8 (C-14), 107.2 (C-15).

10α-Methoxy-4ß-hydroxy guaian-6-ene (2): colourless oil; $C_{16}H_{28}O_2$: $[\alpha]_D^{25}$ - 0.5 (*c* 0.2, $CHCl_3$): ^{1}H-NMR ($CDCl_3$, 600 MHz) δ_H: 5.43 (1H, s, H-6), 3.14 (3H, s, 4-OMe), 2.22 (1H, m, H-1), 2.21 (1H, m, H-11), 2.19 (2H, m, H-8), 2.03 (1H, m, H-5), 1.85 (2H, m, H-8), 1.73 (2H, m, H-3), 1.70 (2H, m, H-2), 1.65 (2H, m, H-9), 1.60 (2H, m, H-3), 1.59 (2H, m, H-9), 1.55 (2H, m, H-2), 1.19 (3H, s, H-14), 1.18 (3H, s, H-15), 0.97 (3H, s, H-12), 0.95 (3H, s, H-13); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ_C:

48.7 (C-1), 22.3 (C-2), 41.2 (C-3), 79.9 (C-4), 50.8 (C-5), 121.9 (C-6), 150.4 (C-7), 25.3 (C-8), 36.2 (C-9), 79.9 (C-10), 37.9 (C-11), 22.4 (C-12), 21.9 (C-13), 23.2 (C-14), 18.6 (C-15), 49.4 (10-OMe).

Antifungal Assay

The *in vitro* screening used compounds **1** and **2** to evaluate the antifungal activity (minimum inhibitory concentration (MIC)) against eight marine fungal strains. The eight strains of marine fungi (*Exophiala* sp. NJM 1551, *Fusarium moniliforme* NJM 8995, *F. oxysporum* NJM 0179, *F. solani* NJM 8996, *Haliphthoros milfordensis* IPMB 1603, *H. sabahensis* IPMB 1402, *Lagenidium thermophilum* IPMB 1401, and *Ochroconis humicola* NJM 1503) used for screening were kindly provided by the Borneo Marine Research Institute (BMRI), Universiti Malaysia Sabah. The MIC was determined visually as the lowest concentration showing no hyphal growth when this was incubated at 25 °C for 7 days. Besides this, the assay was performed three times, and sterile seawater with same values of MeOH containing no compound was prepared as a control for this assay (Tani et al., 2019).

Results and Discussion

The MeOH extract was chromatographed repeatedly over preparative TLC to obtain pure secondary metabolites **1** and **2**. Compounds **1** and **2** showed similar NMR spectroscopic data as the previous literature data (Peng et al., 2003; Rao et al., 2000). Thus, **1** and **2** were identified as alismol (**1**) and 10α-methoxy-4ß-hydroxy guaian-6-ene (**2**), respectively (**Figure 1**).

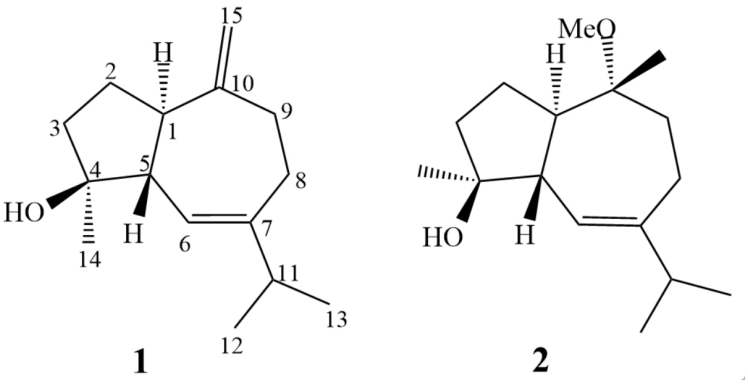
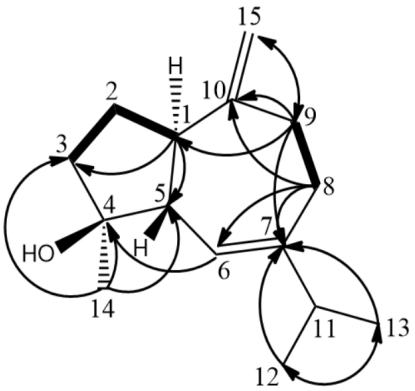


Figure 1. Structures of compounds **1** and **2** from *L. arboreum*.

Compound **1** was isolated as a colourless oil, with $[\alpha]_D^{25}$ + 3.3 (*c* 0.2, $CHCl_3$). The ^{13}C-NMR spectroscopic data showed the presence of 15 carbon signals and four signals attributable to olefinic carbons (δ_C 154.6 (C-10), 150.5 (C-7), 122.0 (C-6), and 107.2 (C-15)). In addition, their multiplicities were confirmed by ^{1}H-NMR signal, DEPT, and HSQC measurements as three methyls at δ_C 24.8 (C-14), 22.2 (C-12), and 22.0 (C-13); δ_H 1.23 (H_3-14), 0.99 (H_3-12), and 0.98 (H_3-13), four sp^3 methylenes at δ_C 41.0 (C-3), 37.8 (C-9), 30.7 (C-8), and 25.4 (C-2); δ_H 2.50 (H_2-9), 2.20 (H_2-8), 2.05 (H_2-9), 2.03 (H_2-8), 1.90 (H_2-2), 1.75 (H_2-3), 1.75 (H_2-3), and 1.71 (H_2-2), three sp^3 methines at δ_C 55.7 (C-5), 48.0 (C-1), and 38.1 (C-11); δ_H 2.27 (H-5), 2.25 (H-1), and 2.22 (H-11), one sp^2 methylene at δ_C 107.2 (C-15); δ_H 4.79 (H_2-15) and 4.70 (H_2-15), one sp^2 methine at δ_C 122.0 (C-6); δ_H 5.56 (H-6), and three quaternary carbons at 154.6 (C-10), 150.5 (C-7), and 81.0 (C-4) (**Table 1**).



1

Figure 2. ^{1}H-^{1}H COSY and key HMBC correlation of compound **1** and **2**.

Construction of compound **1** based on the ^{1}H-^{1}H COSY and key HMBC correlations are shown in **Figure 2.** The cross peaks of ^{1}H-^{1}H COSY determined the partial spin systems H-1/H_2-2/H_2-3. In addition, the HMBC correlations between H-1 to C-3/C-5, H_2-9 to C-1/C-10, H_3-14 to C-3/C-4/C-5 and H_3-15 to C-9 revealed the connectivity for the two structures (5-membered ring and 7-membered ring). Thus, our results also support that the ^{13}C-NMR data for alismol in reference (Peng et al., 2003) at C-1 and C-5 are incorrect, and that a previously reported reference (Thinh et al., 2019) is correct. In addition, the relative configuration of **1** was determined to be identical to previously reported compounds based on

comparison of chemical shifts, *J*-based configurations, and HMBC correlations (Peng et al., 2003).

On the other hand, compound **2** was isolated as colourless oil, with $[\alpha]_D^{25}$ - 0.5 (*c* 0.2, $CHCl_3$). The ^{13}C- NMR spectroscopic data of **2** indicated the presence of 16 carbon signals and two signals attributable to olefinic carbons (δ_C 150.4 (C-7) and 121.9 (C-6)), where their multiplicities were confirmed by ^1H-NMR spectroscopic data, DEPT and HSQC measurements as five methyls (including one methoxy) at δ_C 49.4 (10-OMe), 23.2 (C-14), 22.4 (C-12), 21.9 (C-13), and 18.6 (C-15); δ_H 3.14 (10-OMe), 1.19 (H_3-15), 1.18 (H_3-14), 0.97 (H_3-12), and 0.95 (H_3-13), four sp^3 methylenes at δ_C 41.2 (C-3), 36.2 (C-9), 25.3 (C-8), and 22.3 (C-2); δ_H 2.19 (H_2-8), 1.85 (H_2-8), 1.73 (H_2-9), 1.70 (H_2-2), 1.65 (H_2-3), 1.60 (H_2-9), 1.59 (H_2-3), and 1.55 (H_2-2), three sp^3 methines at δ_C 50.8 (C-5), 48.7 (C-1), and 37.9 (C-11); δ_H 2.22 (H-5), 2.21 (H-11), and 2.03 (H-1), one sp^2 methine at δ_C 121.9 (C-6); δ_H 5.43 (H-6), and three quaternary carbons at 150.4 (C-7), 80.9 (C-4), and 79.9 (C-10) (**Table 1**). Based on the above NMR spectroscopic data and comparison with literature, compound **2** was determined to be 10α-methoxy-4 β -hydroxy guaian-6-ene.

Table 1. ^1H (600 MHz) and ^{13}C NMR (150 MHz) spectra of **1** and **2** in $CDCl_3$, δ in ppm and J in Hz.

	1		**2**	
Position	δ_H **(Mult. *J*)**	δ_C	δ_H **(Mult. *J*)**	δ_C
1	2.25 (m)	48.0	2.03 (m)	48.7
2	1.90 (m) 1.71 (m)	25.4	1.70 (m) 1.55 (m)	22.3
3	1.75 (m) 1.75 (m)	41.0	1.65 (m) 1.59 (m)	41.2
4	-	81.0	-	80.9
5	2.27 (s)	55.7	2.22 (m)	50.8
6	5.56 (s)	122.0	5.43 (s)	121.9
7	-	150.5	-	150.4
8	2.20 (m) 2.03 (m)	30.7	2.19 (m) 1.85 (m)	25.3
9	2.50 (m) 2.05 (m)	37.8	1.73 (m) 1.60 (m)	36.2
10	-	154.6	-	79.9
11	2.22 (m)	38.1	2.21 (m)	37.9
12	0.99 (d, 6.9)	22.2	0.97 (d, 8.3)	22.4
13	0.98 (d, 6.9)	22.0	0.96 (d, 8.3)	21.9
14	1.23 (s)	24.8	1.18 (s)	23.2
15	4.79 (s) 4.70 (s)	107.2	1.19 (s)	18.6
4-OMe	-	-	3.14 (s)	49.4

In addition, the antifungal potential of compounds **1** and **2** were tested against eight fungal strains (*Exophiala* sp., *F. moniliforme*, *F. oxysporum*, *F. solani*, *H. milfordensis*, *H. sabahensis*, *L. thermophilum*, and *O. humicola*). The result of antifungal screening analysis showed that strongest activity was **2** with MIC 12.5 µg/mL against *H. milfordensis* (**Table 2**).

Table 2. Antifungal activities of compounds **1** and **2**.

Strains	MIC (µg/mL)	
	1	**2**
Exophiala sp.	50	50
F. moniliforme	50	50
F. oxysporum	100	100
F. solani	100	100
H. milfordensis	50	12.5
H. sabahensis	100	50
L. thermophilum	100	100
O. humicola	50	50

Note: Positive control; Clotrimazole with MIC 3.1 µg/mL.

The structures (guaiane types) similar to compound **1** and **2** have been reported to isolate from various terrestrial plants (Ma et al., 2019; Pan et al., 2021). Besides that, the guaiane type sesquiterpenes were isolated and reported from *L. arboretum*, among others in the genus *Litophyton* (Ellithey et al., 2013; El-Kassem et al., 2018). In addition, the results of antifungal activity provide important information on the functional quality of secondary metabolites from the genus *Litophyton* and can be used to better characterize the genus soft corals. Hence, secondary metabolites and their bioactivities can be considered a chemical taxonomic marker of the genus level.

Conclusion

In this study, the variety in structure types and bioactivities of *Litophyton arboreum* in Malawali Island, Sabah, Northern Borneo, not fully studied previously was used as an additional tool for their identification. As a result, a total of two secondary metabolites (alismol (**1**) and 10α-methoxy-4ß-hydroxy guaian-6-ene (**2**)) were isolated from Bornean soft corals *L. arboretum*. Moreover, **2** showed relatively better inhibition against *H. milfordensis* at MIC value of 12.5 µg/mL. As far as we know, these results are the first record of sesquiterpenes isolated from the soft coral *L. arboreum* in Northern Borneo and

is a step towards enriching our knowledge of Bornean soft corals. These sesquiterpenes derivatives might be considered as a chemical taxonomic marker for *L. arboreum*.

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Graphical Abstract