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Structural Diversity, Anti-Fungal Activity and Chemosystematics of Bornean Liverwort *Bazzania harpago* (De Not.) Schiffner

(Kepelbagaian Struktur, Aktiviti Anti-Kulat dan Kemosistematik Lumut Hati Borneo *Bazzania harpago* (De Not.) Schiffner)

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ABSTRACT

*Two terpenes; cneorubin X (**1**), (-)-gymnomitr-3(15)-en-4β-ol (**2**) as well as one benzoic compound; caffeic acid (**3**) were isolated from* Bazzania harpago *collected from Mt. Trus Madi, Sabah, Malaysia. The structures of these secondary metabolites were elucidated based on spectroscopic data (Infra-Red (IR), 1D and 2D Nuclear Magnetic Resonance (NMR), and Mass Spectrum (MS)). This is the first record that cneorubin X (**1**) was isolated from non-vascular plants and it exhibited active anti-fungal activity against* Haliphthoros sabahensis *and* Haliphthoros milfordensis*. Chemosystematics finding suggests that the Marchantiophyta (liverworts) genus* Bazzania *might have a closer chemical relationship to its sister group Bryophyta (mosses) genus* Brachythecium, Kindbergia *and* Mnium*, as well as Anthocerophyta (hornworts) genus* Anthoceros *in their evolutionary history.*

Keywords: Bazzania harpago*; Borneo; chemosystematics; liverwort; secondary metabolite*

ABSTRAK

*Dua terpena; cneorubin X (**1**), (-)-gymnomitr-3(15)-en-4β-ol (**2**) dan satu sebatian benzoik; asid kaffeik (**3**) telah diasingkan daripada* Bazzania harpago *yang diambil dari Gunung Trus Madi, Sabah, Malaysia. Struktur metabolit sekunder ini telah dijelaskan berdasarkan data spektroskopi (Infra-merah (IR), 1D and 2D Resonans Magnet Nukleus (NMR) dan Spektrum Jisim (MS)). Kajian ini merupakan rekod pertama cneorubin X (**1**) yang diasingkan daripada tumbuhan bukan vaskular serta menunjukkan aktiviti anti-kulat yang aktif terhadap* Haliphthoros sabahensis *dan* Haliphthoros milfordensis*. Hasil kajian mencadangkan bahawa Marchantiophyta (lumut hati) genus* Bazzania *mempunyai hubungan kimia yang lebih rapat dengan kumpulan kembarnya, briofit (lumut hati) genus* Brachythecium, Kindbergia *dan* Mnium*, begitu juga dengan Anthocerophyta (lumut tanduk) genus* Anthoceros *dalam sejarah evolusi mereka.*

Kata kunci: Bazzania harpago*; Borneo; lumut hati; metabolit sekunder; sistematik kimia*

Introduction

Bryophytes can be classified into three divisions, namely Bryophyta (mosses), Marchantiophyta (liverworts) and Anthocerotopyhta (hornworts) (Crandall-Stotler et al. 2009). The number of bryophytes species is approximately 24,000 species whereby 14,000 species are mosses, 6,000 species are liverworts, and 300 species are hornworts (Asakawa et al. 2013a). Tropical rainforest of Borneo is one of the 12 mega-biodiversity hotspots on Earth possessing extremely high species richness and abundance of flora and fauna, including high number of endemic liverworts (Ariyanti et al. 2008). There is a total of 38 families, 122 genera and 758 taxa of liverworts can be found in Sabah (Chuah-Petiot 2011). The characteristics that differentiate liverworts from other bryophytes are the present of cellular oil bodies (Asakawa et al. 2013b). There are various types of oil bodies and the oil bodies often produce secondary metabolites which mostly composed of lipophilic terpenoids and aromatic compounds. Asakawa (2004) and Asakawa et al. (2013a) reported that secondary metabolites from liverworts showed various biological activities such as cytotoxic, insect anti-feedant, insecticidal, muscle relaxing, antimicrobial and anti-inflammation activities.

The genus *Bazzania* consists one of the most diverse number of liverwort species in the family of Lepidoziaceae. Out of the 150 *Bazzania* species worldwide, 24 of them could be found in Sabah; *B. conophylla*, *B. densa*, *B. eurosa*, *B. indica*, *B. intermedia*, *B. friabilis*, *B. grandiretis*, *B. harpago*, *B. horridula*, *B. involutiformis*,

B. kokawana, *B. longicaulis*, *B. lowii*, *B. marginela*, *B. parvitexta*, *B. patentistipa*, *B. praerupta*, *B. psedovittata*, *B. spiralis*, *B. subtilis*, *B. tridens*, *B. uncigera*, *B. vittata,* and *B. serpentina* (Chuah-Petiot 2011; Frahm et al. 1990; Menzel 1988). Globally, the phytochemical analysis of *Bazzania* has been studied extensively in liverwort and they have been shown to produce a wide diversity of sesquiterpenoids and aromatic compounds (Asakawa 2004; Asakawa et al. 2013b). Figure 1 shows the chemical skeleton of the studied *Bazzania* species. However, the phytochemical information of the Bornean *Bazzania* is still not well understood. To date, there are only three species of Bornean *Bazzania* that have been chemically investigated which are *B. harpago*, *B. praerupta,* and *B. spiralis*. Interestingly, although *B. harpago*, *B. praerupta,* and *B. spiralis* are of the same genus, they are not chemically related due to the origin of their major metabolites' chemical skeleton. Barbatanes are characteristic of *B. harpago*, whereas drimanes are characteristics of *B. praerupta* and eudesmanes are characteristics of *B. spiralis* (Ludwiczuk & Asakawa 2010).

As part of our on-going interest in bioactive secondary metabolites from Bornean liverworts, we have obtained two terpenes as well as one benzoic compound from *Bazzania harpago* (De Not.) Schiffner collected at Mt. Trus Madi, Sabah, Malaysia. The isolation of these metabolites, their antifungal activity and chemosystematics are reported in this paper.

Materials and Methods

General

Using deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as an internal standard, the NMR spectra were obtained via 600 MHz FT-NMR (Jeol, Japan). The high-resolution mass spectrum was acquired on Liquid Chromatography-Electrospray Ionization-Ion Trap-Time of Flight-Mass Spectrometry (Shimadzu, Japan). The optical rotation was measured at 25 °C using AUTOPOL IV automatic polarimeter (Rudolph Research Analytical, USA). Infrared spectra were recorded on a FTIR spectroscopy (Thermo Nicolet, USA). For purification and compounds isolation, Silica gel preparative TLC (Kieselgel 60, F_{254}) and column chromatography (Kieselgel 60, 70-230 mesh) were performed (Merck, Germany).

Samples Collection

Specimen of *Bazzania harpago* (M. Suleiman & S.Y. Ng 5947) was collected from Mt. Trus Madi (5° 33' 29.736''N, 116° 29' 59.1''E), Sabah, Malaysia on 20th August 2015. Identification was carried out by the forth author (Universiti Malaysia Sabah) and a voucher specimen (BORB0024) is deposited in the BORNEENSIS Herbarium of Institute for Tropical Biology and Conservation (BORH), Universiti Malaysia Sabah. The sample was brought back to the laboratory under cool conditions (4 °C) and processed according to the procedures described by Ng et al. (2016).

Extraction and Isolation

Specimens of plant material (201 g) were air-dried and extracted with methanol (MeOH) at room temperature (1.0 L × 3 for 3 days). Solvent partition of the crude extract was performed between ethyl acetate (EtOAc) (50 mL × 3) and distilled water (150 mL). The combined organic layers were dried over sodium sulfate (anhydrous) and concentrated *in vacuo* to afford EtOAc extract of 2.1 g in weight. The EtOAc extracts (1 g) was chromatographed using a gravitational Silica gel column using n-hexane (Hex) and EtOAc solvent system as eluent with increasing polarity Hex/EtOAc: 9:1, 8:2, 7:3, 1:1 and 100% EtOAc) to yield five fractions, 1-5. The obtained respective compounds yields were calculated as percentages in the EtOAc crudes.

Fraction 2 (162.6 mg) was subjected to PTLC (Hex/EtOAc: 9:1) to yield **1** (27.1 mg) (2.7% yield). Fraction 4 (227.2 mg) was subjected to PTLC (100% $CHCl_3$) to yield **2** (80.1 mg) (8.0% yield) and **3** (83.8 mg) (8.4% yield).

Anti-fungal Activity

The minimum inhibitory concentration (MIC) of fungistatic effect on hyphae was determined. Different concentrations of the pure compound solutions (12.5, 25, 50, 100 μg/mL) were incorporated onto Peptone Yeast Extract Glucose Sugar (PYGS) agar in petri dish followed by inoculation of the *Lagenidium thermophilum*, *Haliphthoros sabahensis,* and *Haliphthoros milfordensis*. The procedure was adopted from Munchan et al. (2009) with slight modification. After incubated at 25 °C for a week, the lowest concentration of agar which showed no visible hyphal growth was determined as the MIC.

Results and Discussion

Approximately 201 g of *Bazzania harpago* was collected from Mt. Trus Madi (Figure 2). Upon extraction and partition process as described above, 2.1 g of EtOAc extract was obtained from *B. harpago*. The cellular oil bodies of the *B. harpago* specimen is as shown in Figure 3.

The structures of **1-3** were elucidated based on extensive analysis of spectroscopic data and comparison with those published literatures. The compounds cneorubin X (**1**) (Brochini & Roque 2000), (-)-gymnomitr-3(15)-en-4β-ol (**2**) (Adio et al. 2002) and caffeic acid (**3**) (Jocković et al. 2008) were identified. Chemical structures of the compound **1** to **3** are shown in Figure 4.

The minor compound **1** was isolated as colourless oil with [a] $\overset{\mathfrak{Z}}{D}$: -19.0 (*c* 0.30, $CHCl_3$). The molecular formula of **1**, $C_{20}H_{32}O$ (corresponding to five degree of unsaturation), was deduced from HR-ESI-MS measurements. The IR absorption at 3370 cm^{-1} indicated the presence of the hydroxyl group. HSQC and ^{13}C-DEPT experiments coupled with ^{13}C- and ^{1}H-NMR signals of compound **1** showed the presence of an olefinic carbons at δ_C 131.9 (C), δ_C 125.5 (CH), δ_H 5.10 (1H, t), one vinylidene at δ_C 154.2 (C), δ_C 106.9 (CH_2), δ_H 4.68 (2H, d), four methyls at δ_C 26.4 (CH_3), δ_H 1.68 (3H, s), δ_C 26.4 (CH_3), δ_H 1.30 (3H, s), δ_C 18.3 (CH_3), δ_H 1.60 (3H, s), δ_C 14.4 (CH_3), δ_H 1.03 (3H, s), six methylenes, four methines and two quaternary carbons including one bearing hetero atom (Table 1). Hence, degree of unsaturation could be attributed to two double bonds and three rings.

Two spin systems were unveiled by the analysis of ^{1}H–^{1}H COSY experiment. The HMBC correlations of H_3-15 with C-3, C-4 and C-5 allowed us to deduce connectivity of C-15 to C-4. The HMBC correlations of H_3-13 with C-6, C-7, C-11 and C-12 allowed us to attach C-13 to C-11. Besides, the three-bond correlations of H_3-19 and H_3-20 to the opposite carbons C-19 and C-20, and the correlations of both to C-17 and C-18, permitted placement of the *gem*-dimethyl group at C-18. The exomethylene group attached between C-1 and C-9 was confirmed by HMBC correlations of H_2-14 to C-1 and C-9. Evidences from the finding suggested the gross structure of **1** as shown in Figure 4. The NOESY experiment and comparison with published literature concluded the relative stereochemistry of **1** (Brochini & Roque 2000). Thus, compound **1** was identified as cneorubin X (Figure 4). The previously assignment of proton chemical shift at H−8α and H-12α are different from our present results (Brochini & Roque 2000; Hernandez et al. 2018). This could be explained by the usage of lower megahertz NMR machine which gave inaccurate results (200 MHz NMR) compared to our present study (600 MHz NMR).

The anti-fungal activity was carried out using all the isolated metabolites against three selected marine fungi, *Lagenidium thermophilum* IPMB 1401, *Haliphthoros sabahensis* IPMB 1402, *Haliphthoros milfordensis* IPMB 1603. Among the three tested compounds, compound **1** showed the best anti-fungal activity against *H. sabahensis* and *H. milfordensis*, both with MIC value of 25 mg/mL. The anti-fungal results of the isolated metabolites were as shown in Table 2.

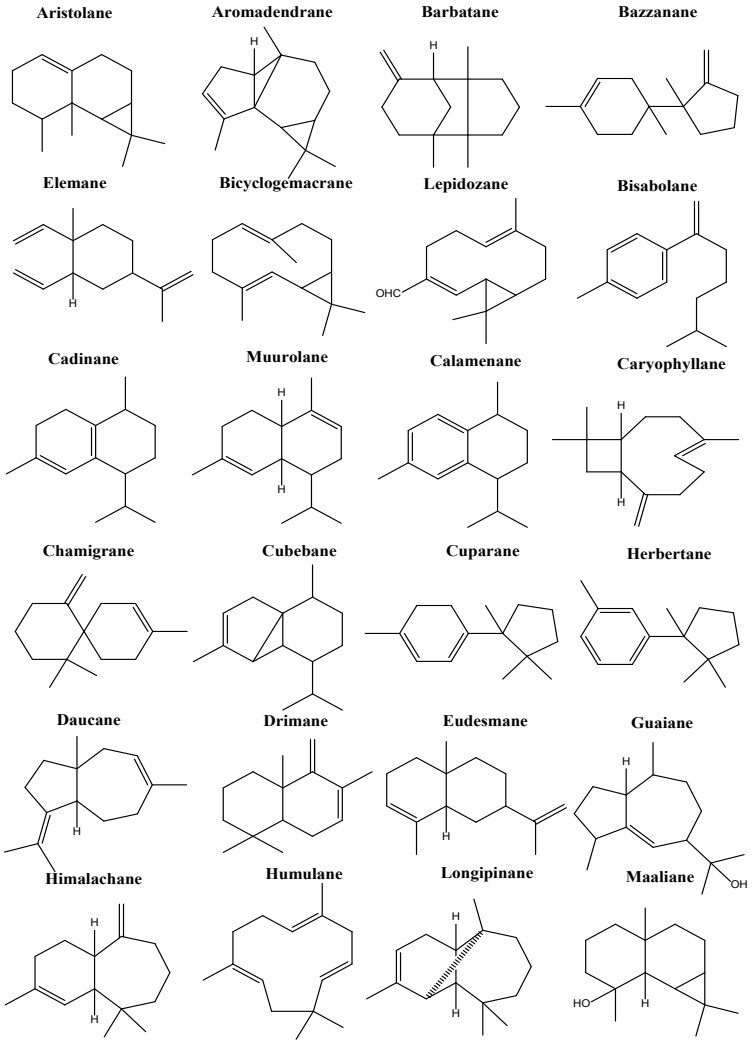

FIGURE 1. Characteristic chemical skeleton isolated from studied *Bazzania* species worldwide

Cneorubin X (**1**) was isolated from sample *Bazzania harpago*. Literature reviews showed that **1** was initially isolated and reported from *Cneorum tricoccon* (Trautmann et al. 1980), *Guarea guidonia* (Brochini & Roque 2000), *Ptaeroxylon obliquum* (Mulholland & Mahomed 2000) and *Trichilia sylvatica* (Soares et al. 2014), that are all vascular plants. To the best of our knowledge, this is the first report of Cneorubin X been reported from a non-vascular plant. This unique diterpenoid was absence in previous phytochemical analysis of *B. harpago* collected from Mount Kinabalu conducted by Ludwiczuk and Asakawa (2010). This might be due to the differences of genetics, environmental variation or microhabitat differences from two mountain areas (Ng et al. 2017). Besides, it is also not surprising that (-)-gymnomitr-3(15)-en-4β-ol (**2**) was found as the major metabolites in this specimen since these Barbatane-type metabolites are known to be the characteristic of *B. harpago* (Asakawa 1982). Interestingly, the caffeic acid (**3**) which only presented from some mosses genera such as *Brachythecium*, *Kindbergia,* and *Mnium*, as well as in hornworts genus *Anthoceros*, was only reported in liverworts genus *Bazzania* (Asakawa 1995; Asakawa et al. 2013b). This finding suggests that the Marchantiophyta (liverworts) genus *Bazzania* might have a closer chemical relationship to its sister group Bryophyta (mosses) genus *Brachythecium*, *Kindbergia,* and *Mnium*, as well as Anthocerophyta (hornworts) genus *Anthoceros* in their evolutionary history.

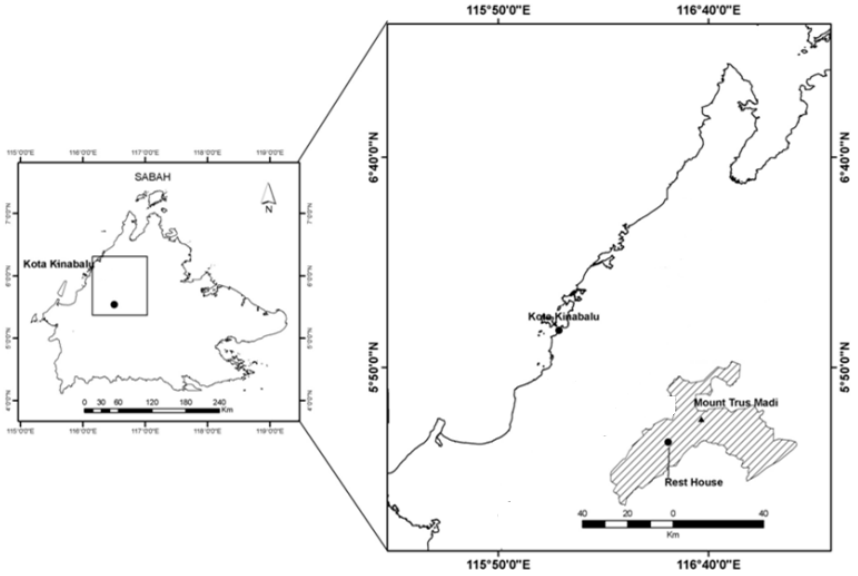

FIGURE 2. Map of sample collection site

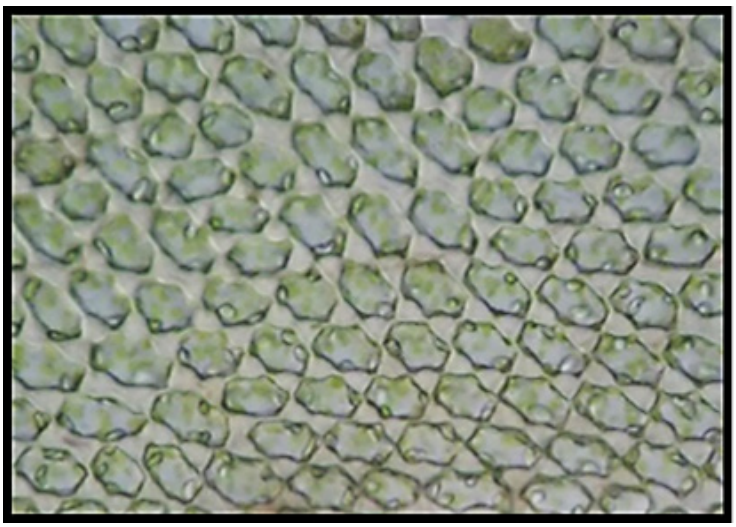

FIGURE 3. Oil bodies of *Bazzania harpago* specimen

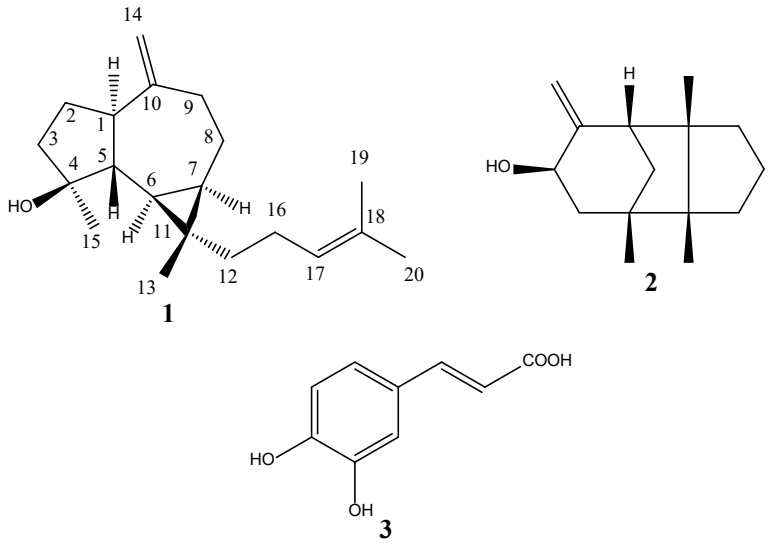

FIGURE 4. Structures of isolated compounds **1-3**

TABLE 1. ^{13}C-NMR (150 MHz) and ^{1}H-NMR (600 MHz) data for **1**

		1		Literature (Brochini & Roque 2000)	
C[b]	^{13}C (δ)	^{1}H (δ)	multiplicity, *J* (Hz)	^{13}C (δ)	^{1}H (δ)
1	52.9	2.22	m	52.9	2.19
2α	26.3	1.62	m	26.3	
2β		1.89	m		
3α	41.6	1.60	m	41.6	
3β		1.76	m		
4	81.1			81.0	
5	53.8	1.34	m	53.8	1.32
6	29.1	0.49	dd, *J* = 9.6, 11.0	29.1	0.48
7	26.8	0.72	m	26.8	0.71
8α	24.8	1.01	m	24.8	0.90
8b		1.97	m		2.01
9α	38.9	2.03	m	38.9	
9β		2.42	dd, *J* = 6.2, 13.8		2.40
10	153.5			153.5	
11	24.3			24.3	
12α	43.3	1.14	m	43.2	1.03
12β		1.34	m		1.37
13	13.7	1.03	s	13.6	1.02
14α	106.2	4.68	d, *J* = 17.2	106.2	4.65
14b		4.68	d, *J* = 17.2		4.68
15	25.9	1.30	s	25.7	1.28
16α	25.2	2.03	m	25.2	2.09
16β		2.06	m		
17	124.8	5.10	t, *J* = 6.9	124.8	5.10
18	131.2			131.1	
19	17.6	1.60	s	17.6	1.59
20	25.7	1.68	s	25.9	1.66

[a] Measured in chloroform-d_1, [b] Assignment was made by the HSQC spectrum

TABLE 2. Minimum inhibitory concentration (MIC) of isolated secondary metabolites against the selected marine fungi

Fungus strain / Compounds	*Lagenidium thermophilum* MIC (mg/mL)	*Haliphthoros sabahensis* MIC (mg/mL)	*Haliphthoros milfordensis* MIC (mg/mL)
1	50	25	25
2	100	100	100
3	100	100	100

CONCLUSION

In conclusion, presented finding is the first report on the isolation of cneorubin X (**1**) from non-vascular plant *B. harpago*. Barbatane-type of secondary metabolites represents the chemotaxonomical marker for Bornean *B. harpago* and these secondary metabolites displayed antifungal activity against three marine fungi. Chemosystematics results showed that liverworts genus *Bazzania* has an evolutionary closer chemical relationship to certain mosses genera (*Brachythecium*, *Kindbergia*, *Mnium*) and hornworts genus (*Anthoceros*).

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REFERENCES

Adio, A.M., Paul, C., König, W.A. & Muhle, H. 2002. Volatile components from European liverworts *Marsupella emarginata*, *M. aquatic* and *M. alpina*. *Phytochemistry* 61(1): 79-91.

Ariyanti, N.S., Bos, M.M., Kartawinata, K., Tjitrosoedirdjo, S.S., Guhardja, E. & Gradstein, S.R. 2008. Bryophytes on tree trunks in natural forest, selectively logged forests and cacao agroforests in Central Sulawesi, Indonesia. *Biological Conservation* 141(10): 2516-2527.

Asakawa, Y. 2004. Chemosystematics of the Hepaticae. *Phytochemistry* 65(6): 623-669.

Asakawa, Y. 1995. Chemical constituents of the Bryophytes. In *Progress in the Chemistry of Organic Natural Products*, edited by Herz, W., Kirby, G.W., Moore, R.E., Steglich, W. & Tamm, Ch. Vienna: Springer. pp. 1-618.

Asakawa, Y. 1982. Chemical constituents of Hepaticae. In *Progress in the Chemistry of Organic Natural Products*, edited by Herz, W., Grisebach, H. & Kirby, G.W. Vienna: Springer. pp. 1-285.

Asakawa, Y., Ludwiczuk, A. & Nagashima, F. 2013a. Phytochemical and biologica studies of bryophytes. *Phytochemistry* 91: 52-80.

Asakawa, Y., Ludwiczuk, A. & Nagashima, F. 2013b. Chemical constituents of bryophytes. In *Progress in the Chemistry of Organic Natural Products,* edited by Kinghorn, A.D., Falk, H. & Kobayashi, J. Vienna: Springer. pp. 1-796.

Brochini, C.B. & Roque, N.F. 2000. Two new cneorubin related diterpenes from the leaves of *Guarea guidonia* (Meliaceae). *Journal of the Brazillian Chemical Society* 11(4): 361-364.

Chuah-Petiot, M.S. 2011. A checklist of Hepaticae and Anthocerotae of Malaysia. *Polish Botanical Journal* 56(1): 1-44.

Crandall-Stotler, B.J., Stotler, R.E. & Long, D.G. 2009. Phylogeny and classification of the Marchantiophyta. *Edinburgh Journal of Botany* 66(1): 1-44.

Frahm, J.P., Frey, W., K**ü**rschner, H. & Menzel, M. 1990. *Mosses and Liverworts of Mt. Kinabalu*. Kota Kinabalu: Sabah Parks Trustees. pp. 12(2): 1-91.

Hernandez, V., De Leo, M., Cotugno, R., Braca, A., De Tommasi, N. & Severino, L. 2018. New tirucallane-type triterpenoids from *Guarea guidonia*. *Planta Medica* 84(9-10): 716-720.

Jocković, N., Andrade, P.B., Valentão, P. & Sabovljević, M. 2008. HPLC-DAD of phenolics in bryophytes *Lunularia cruciata*, *Brachytheciastrum velutinum* and *Kindbergia praelonga*. *Journal of the Serbian Chemical Society* 73(12): 1161-1167.

Ludwiczuk, A. & Asakawa, Y. 2010. Chemosystematics of selected liverworts collected in Borneo. *Tropical Bryology* 31: 33-42.

Menzel, M. 1988. Annotated catalogue of the Hepaticae and Anthocerotae of Borneo. *Journal of Hattori Botanical Laboratory* 65: 145-206.

Mulholland, D.A. & Mahomed, H.A. 2000. Isolation of Cneorubin X, an unusual diterpenoid from *Ptaeroxylon obliquum* (Ptaeroxylaceae). *Biochemical Systematics and Ecology* 28(7): 713-716.

Munchan, C., Hatai, K., Takagi, S. & Yamashita, A. 2009. *In vitro* and *in vivo* effectiveness of itraconazole against *Ochroconis humicola* isolated from fish. *Aquaculture Science* 57(3): 399-404.

Ng, S.Y., Kamada, T. & Vairappan, C.S. 2017. New pimarane-type diterpenoid and *ent*-eudesmane-type sesquiterpenoid from Bornean liverwort *Mastigophora diclados*. *Records of Natural Products* 11(6): 508-513.

Ng, S.Y., Kamada, T., Suleiman, M. & Vairappan, C.S. 2016. A new *Seco*-Clerodane-type diterpenoid from Bornean liverwort *Schistochila acuminata*. *Natural Product Communications* 11(8): 1071-1072.

Soares, A.D.O., Ferreira, A.G.L., Soares, L.R., Corsino, J., Garcez, F.R. & Garcez, W.S. 2014. Chemical study of leaves of *Trichilia silvatica* (Meliaceae). *Química Nova* 37: 1487-1490.

Trautmann, D., Epe, B., Oelberman, U. & Mondon, A. 1980. Diterpene aus Cneoraceen, I. Konstitution und Konfiguration der Cneorubine. *Chemische Berichte* 113(12): 3848-3865.

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