Secondary Metabolites from Steambarks of *Dysoxylum alliaceum*

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Abstract

Plants from the Dysoxylum genus (Meliaceae) are rich sources of structurally diverse secondary metabolites with interesting biological activities. One of the interesting activities is capability to act as agent of P-gp in MCF-7 breast cancer cells.

In the course of our continuing search of bioactive compounds from Meliaceae plants, the bark of D. alliaceum was extracted with n-hexane, ethyl acetate and methanol successively. The ethyl acetate extract was separated and purified using several chromatographic techniques guided with thin layer chromatografi analysis, and two compounds were obtained, i.e. alliacene (1) and 6,7-dimethoxy-2H-chromane-2-one (2).

The chemical structures of these compounds have been determined based on spectroscopic interpretation and compared against spectral data from previous studies. All compounds were evaluated for their cytotoxic activity against MCF-7 breast cancer cell line. Compounds 1-2 showed weak cytotoxic activities with IC_{50} values >100 mg/mL.

Keywords: *D.alliaceum*, chromane, phenolic, MCF-7.

Introduction

Meliaceae is a group of plant known as a rich source of compounds with various structural and high biological activity. *Dysoxylum* is a member of Meliaceae family containing approximately 80 species and mainly distributed in Asia and Australia, in tropical and subtropical regions[1]. The *Dysoxylum* is known as a source of variety compounds with interesting biological activities such as cytotoxic alkaloids[2-4], cytotoxic biflavonoid[5,6], cytotoxic sesquiterpene[7,8], anticancer diterpene[9], cytotoxic triterpene[10,11], antibacterial triterpene[12], and antibacterial steroid[13].

D. alliaceum, is a member of *Dysoxylum* genus, also known as *kayu bawang* in Indonesia, which is typical of higher plant. There is no chemical constituent having been reported from this species. In our continuous search to investigate cytotoxic constituents from Indonesian *Dysoxylum* plants against MCF-7 breast cancer cell lines, herein we isolated chemical constituents from *D. alliaceum*, determined their chemical structures, and tested the compounds towards MCF-7 breast cancer cell lines.

Material and Methods

General: The IR spectra were recorded on a Perkin-Elmer spectrum-100 FT-IR in KBr. Mass spectra were obtained with a Synapt G2 mass spectrometer instrument. NMR data were recorded on a Jeol ECZ-600 spectrometer at 600 MHz for ^{1}H and 150 MHz for ^{13}C, and TMS as internal standard. Column chromatography was conducted on silica gel 60 (Merck, German). TLC plates were pre-coated with silica gel GF_{254} (Merck, 0.25 mm) and detection was achieved by spraying the TLC plate with 10% H_2SO_4 in water followed by heating.

Plant Material: The stembark of *Dysoxylum alliaceum* were collected in Bogor Botanical Garden, Bogor, West Java, Indonesia in June 2015. The plant was identified by the staff of the Bogoriense Herbarium, Bogor, Indonesia and a voucher specimen (No. BO-1295763) was deposited at the herbarium.

Extraction and isolation: The dried bark (3.5 kg) was extracted with methanol exhaustively (15 L) at room temperature for 7 days. After removal of the solvent under vacuum, the viscous concentrate of MeOH extract (116 g) was first suspended in H_2O and then partitioned with *n*-hexane and EtOAc successively. Evaporation resulted in the crude extracts of *n*-hexane (65 g) and EtOAc (16 g) respectively. All of the extracts were tested for their cytotoxic activity against MCF-7 breast cancer cell lines and showed cytotoxic activity with IC_{50} values of 58.4 and 495.5 µg/mL respectively.

The EtOAc soluble fraction (16 g) was fractionated by vacum liquid chromatography on silica gel 60 using a gradient *n*-hexane, EtOAc, and MeOH to give fractions A–D, all fractions were combined according to TLC results. Fraction B (410 mg) was chromatographed on a column of silica gel, eluted successively with a gradient of *n*-hexane–EtOAc (10:0–1:1) to give six subfractions (B1–B6). Subfraction B3 was chromatographed on a column of silica gel, eluted successively with a gradient of $CHCl_3$–MeOH (9:1), to give 1 (8 mg).

Fraction C (1 g) was chromatographed on a column of silica gel, eluted successively with a gradient of *n*-hexane–EtOAc (10:0–1:1), to give five subfractions (C1–C5). Subfraction

C4 was chromatographed on a column of silica gel, eluted successively with a gradient of *n*-hexane–Me_2CO (10:0–7:3), to give six subfractions (C4A–C4F). Subfraction C4D was chromatographed on a column of silica gel, eluted successively with a gradient of *n*-hexane–EtOAc (2:3), to give 2 (12 mg).

Alliacene (1): White amorphous powder; IR (KBr) ν_{max} 3410, 2924, 1603, 1090, 824 cm^{-1}. ^1H NMR (acetone-d_6, 600 MHz), table 1; ^{13}C NMR (acetone-d_6, 150 MHz), table 1; HR-ESI-TOFMS (negative ion mode) *m/z* 301.6000 $[M-H]^-$, (calcd. for $C_{18}H_{22}O_4$, *m/z* 302.3700).

6,7-dimethoxydihydro coumarin (2): Colourless needle like crystal; IR (KBr) ν_{max} 2924, 1700, 1603, 1090, 824 cm^{-1}. ^1H NMR (acetone-d_6, 600 MHz), table 1; ^{13}C NMR (acetone-d_6, 150 MHz), table 1; ESI-TOFMS (negative ion mode) *m/z* 205.99 $[M-H]^-$, (calcd. for $C_{11}H_{10}O_4$, *m/z* 206.20).

Determination of Cytotoxic Activities: The MCF-7 cells were seeded into 96-well plates at an initial cell density of approximately 3×10^4 cells.cm^{-3}. After 24 h of incubation for cell attachment and growth, varying concentrations of samples were added. The compounds added were first dissolved in DMSO at the required concentration. Subsequent six desirable concentrations were prepared using PBS (phosphoric buffer solution, pH = 7.30 - 7.65). Control wells received only DMSO. The assay was terminated after 48 h incubation period by adding MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; also named as thiazol blue] and the incubation was continued for another 4 h, in which the MTT-stop solution containing SDS (sodium dodecyl sulphate) was added and another 24 h incubation was conducted.

Optical density was read by using a micro plate reader at 550 nm. IC_{50} values were taken from the plotted graph of percentage live cells compared to control (%), receiving only PBS and DMSO, versus the tested concentration of compounds (μg/mL). The IC_{50} value is the concentration required for 50% growth inhibition. Each assay and analysis was run in triplicate and averaged.

Results and Discussion

The phytochemical test for the EtOAc extract showed the presence of phenolic compounds. By using cytotoxic assay to guide separations, the EtOAc fraction was separated by column chromatography over silica gel by gradient and isocratic elution and yielded a new polycyclic geranyl hydroquinone derivative, compound 1 and a coumarin derivative, compound 2 (figure 1).

Compound 1 was obtained as a white amorphous powder. The HR-TOFMS spectrum showed $[M-H]^-$ *m/z* 301.6000 (calcld *m/z* 302.3700) corresponding to the molecular formula of $C_{18}H_{22}O_4$ and thus required eight degrees of unsaturation, originating from four pairs of C sp^2 and the remaining tetracyclic geranyl hydroquinone derivative. The IR spectra showed absorption peaks at 3410 cm^{-1} (OH), 2924 cm^{-1} (C-H sp^2), 1603 cm^{-1} (C=C), 1090 cm^{-1} (C-O stretch), and 824 cm^{-1} (substituted benzene). The ^1H-NMR (acetone-d_6, 600 MHz) spectrum showed the presence of two tertiary methyl groups, resonating at δ_H 1.11 (3H, s, H-13) and 1.08 (3H, s, H-11), one methoxy at δ_H 3.80 (3H, s, H-4-OMe), two aromatic protons at δ_H 7.24, 7.26 (each 1H, d, J = 8.4 Hz, H-2 and H-3), indicating that both protons in *ortho* position, and two olefinic protons at δ_H 6.02, 6.03 (each 1H, d, J = 9.7 Hz, H-6 and H-7), indicating that this protons in *cis* oriented.

All of these signal suggest the presence of geranyl hydroquinone-derived skeleton with additional methyl group[13]. The ^{13}C-NMR spectrum displayed 18 carbon signals (table 1) which were assigned, by the DEPT spectra, into three methyls, two methylenes, seven methines (including two sp^3 oxymethines and four sp^2 methines), and six quaternary carbons (including two sp^3 and two sp^2 oxygenated carbons). The eight sp^2 carbons indicated the presence of four double bonds. Thus, the remaining four degrees of unsaturation indicated a tetracyclic skeleton for compound 1.

A comparison of the NMR data of 1 with those of arnebacene isolated from *Arnebia hispidissima*[14] revealed that the structures of the two compounds are closely related. The main difference was the additional methyl group of 1 instead of hydroxyl group of arnebacene, attached on position 8. This difference was proven by HMBC correlation of H-13 (δ_H 1.11) to C-7 (δ_C 144.4), C-8 (δ_C 73.2), and C-12 (δ_C 69.8).

The HMBC spectrum (figure 2) also showed correlations between H-11 (δ_H 1.08) to C-5 (δ_C 78.1), C-10a (δ_C 78.2), C-10 (δ_C 26.2), and C-8a (δ_C 102.2) and correlation between H-6 (δ_H 6.02) to C-5 (δ_C 78.1) and C-7 (δ_C 144.4), indicating that hydroxy group was linked at C-5 position and olefinic carbons at C-6/C-7 (this correlation was also supported by ^1H-^1H COSY crosspeak of H-6/H-7). The position of ether group (oxygene bridge) at C-12/C-9 was proven by HMBC correlation between H-12 (δ_H 4.43 and 5.02) to C-9 (δ_C 90.9). Methoxy group attached on C-4 evidenced by HMBC correlation of H-4-OMe (δ_H 3.80) to C-4 (δ_C 160.9). Therefore, the structure of 1 was elucidated as a new polycyclic geranyl hydroquinone derivative, named alliacene.

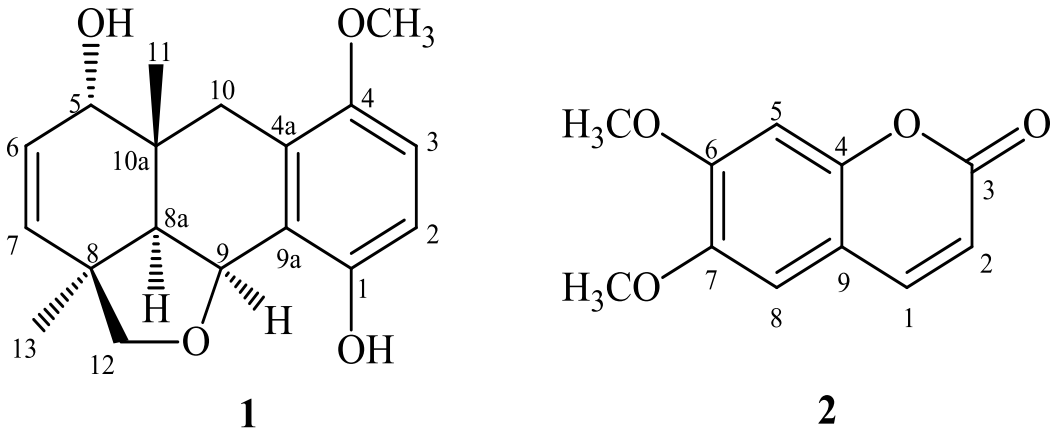
Compound 2 was obtained as a colourless needle-like crystal. The HR-TOFMS spectrum showed $[M-H]^-$ *m/z* 205.99 (calcld. *m/z* 206.20), which coresponded to the molecular formula of $C_{11}H_{10}O_4$ and thus required seven degrees of unsaturation, originating from four pairs of C sp^2, a carbonyl group, and the remaining bicyclic coumarine-derived.

The IR spectra showed absorption peaks at 2924 cm^{-1} (OH), 1700 cm^{-1} (C=O), 1603 cm^{-1} (C=C), 1090 cm^{-1} (C-O

stretch), and 824 cm^{-1} (substituted benzene). The ^{1}H-NMR (acetone-d_6, 600 MHz) spectrum showed the presence of two methoxy groups, resonating at δ_H 3.81 (3H, s, H-6-OMe) and 3.82 (3H, s, H-7-OMe), two aromatic protons at δ_H 6.93, 6.80 (each 1H, s, H-5 and H-8), indicating that both protons in *para* position, and two olefinic protons at δ_H 7.59, 6.27 (each 1H, d, J = 9 Hz, H-1 and H-1), indicating that this protons in *cis* oriented.

All of these signal were suggested the presence of coumarin-derived skeleton[15]. The ^{13}C-NMR spectrum displayed 11 carbon signals (table 1) which were assigned, by the DEPT spectra, into two methoxy groups at δ_C 56.2 (C-6-OMe) and 56.4 (C-7-OMe), four sp^2 methines, four sp^2 quaternary carbons, and one carbonyl groups, which correlated to a lactone at δ_C 180.3 (C-3). These functionalities accounted for five out of the total seven degrees of unsaturation.

The remaining two degrees of unsaturation were consistent with the bicyclic coumarin-derived. Therefore, the ^{13}C NMR data was supported the ^{1}H NMR data. A comparison of the NMR data of 2 with those of 6,7-dimethoxydihydrocoumarin, isolated from *Edgeworthia chrysantha*[15] revealed that the structures of the two compounds are very similar. Consequently, compound 2 was identified as 6,7-dimethoxy dihydrocoumarin.



1 **2**

Figure 1: Structures of Compounds 1 and 2

Table 1
NMR Data (600 MHz for ^{1}H and 150 MHz for ^{13}C, in acetone-d_6) for 1 and 2.

Position	1		2	
	^{13}C NMR δc (mult.)	^{1}H NMR δ_H (Int., mult, J=Hz)	^{13}C NMR δc (mult.)	^{1}H NMR δ_H (Int., mult, J=Hz)
1	160.9 (s)	-	162.8 (d)	7.59 (1H, d, 9)
2	123.0 (d)	7.24 (1H, d, 8.4)	103.3 (d)	6.27 (1H, d, 9)
3	105.4 (d)	6.60 (1H, d, 8.4)	180.3 (s)	-
4	106.0 (s)	-	143.5 (s)	-
4a	160.9 (s)	-		
5	78.1 (d)	3.45 (1H, m)	89.9 (d)	6.93 (1H, s)
6	129.1 (d)	6.02 (1H, d, 9.7)	165.3 (s)	-
7	144.4 (d)	6.83 (1H, d, 9.7)	166.3 (s)	-
8	73.2 (s)	-	107.5 (d)	6.80 (1H, s)
8a	102.2 (d)	5.25 (1H, m)		
9	90.9 (d)	4.86 (1H, s)	113.5 (s)	-
9a	160.8 (s)	-		
10	26.8 (t)	2.68 (1H, m)		
		2.57 (1H, m)		
10a	78.2 (s)	-		
11	24.8 (q)	1.08 (3H, s)		
12	69.8 (t)	4.43 (1H, d, 9.75)		
		5.02 (1H, d, 10.4)		
13	26.3 (q)	1.11 (3H, s)		
4-OMe	56.5 (q)	3.80 (3H, s)		
6-OMe			56.2 (q)	3,81 (3H, s)
7-OMe			56.4 (q)	3,82 (3H, s)

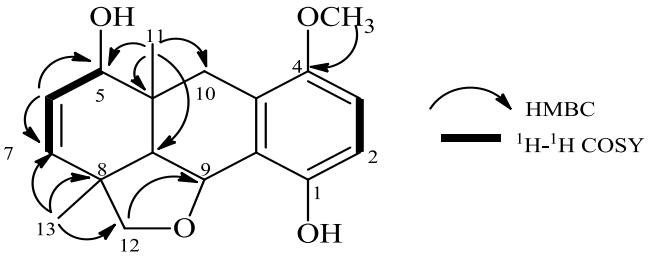


Figure 2: Selected HMBC and ^{1}H-^{1}H COSY correlations for 1.

The cytotoxic effects of the two isolated compounds 1 and 2 against MCF-7 breast cancer cell lines were investigated according to a described method[16]. The cytotoxic activity of isolated compounds 1 and 2 in IC_{50} value were 546.5 and 353.6 µg/mL respectively. Activity of phenolic compound in this species and also in this genus was weak. In fact, the type of compound, which showed high cytotoxic activity in this genus is alkaloid, especially rohitukine[2-4] which was abundant in several species of *Dysoxylum*, for example in *D. binectariferum*[4].

Conclusion

Two phenolic compounds, a new polycyclic geranyl hydroquinone derivative, named alliacene 1, and a coumarin derivative, 6,7-dimethoxydihydro coumarin 2 have been isolated from the stembark of *Dysoxylum alliaceum*. Compounds 1 and 2, were evaluated for their cytotoxic activity against MCF-7 breast cancer cell lines *in vitro* and showed IC_{50} value of 546.5 and 353.6 µg/mL respectively and therefore the compounds considered have weak activity against MCF-7 cell lines.

Acknowledgement

This investigation was financially supported by Directorate General of Higher Education, Ministry of Science, Technology and Higher Education, Indonesia (Hibah Kompetensi, 2016-2017 by TM). We thank Mrs. Tenny Putri Wikayani and Mrs. Nurul Qomarilla at Cells and Tissues Culture Laboratory, Faculty of Medicine, Universitas Padjadjaran for the cytotoxic assay.

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