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Ergosterol Peroxide and Stigmasterol from The Stembark of *Aglaia simplicifolia* (Meliaceae) and Their Cytotoxic against HeLa Cervical Cancer Cell Lines

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Abstract

Two steroid compounds, ergosterol peroxide (**1**) and stigmasterol (**2**) have been isolated from the stembark of *Aglaia simplicifolia* belong to Meliaceae family. The chemical structures of **1** and **2** were identified based on spectroscopic evidence including UV, IR, 1D NMR, 2D NMR as well as mass spectra and by comparison with those previously reported spectra data. Both compounds were evaluated for their cytotoxic effects against cervical cancer HeLa cells in vitro. Compounds **1** and **2** showed cytotoxicity activity against HeLa cervical cancer cells with IC_{50} values of 0.80 and 26.42 µM, respectively.

Keywords : *Ergosterol peroxide, stigmasterol, Aglaia simplicifolia, HeLa cervical cancer, IC_{50}*

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1. INTRODUCTION

Meliaceae is the important plant families that have utilized and generally grow in tropical countries. Meliaceae plant is known for the presence of the various secondary metabolite compounds that exhibit interesting biological activity such as hypoglycemia, anticancer, anti-inflammation, antifeedant, antitumor (Awang *et al*., 2012; Leong *et al*., 2016; Su *et al*., 2006) and insecticidal activity (Nugroho *et al*., 1999).

The *Aglaia* genus is a plant of the tropical rain forest in the Indomalesiana region and mainly distributed in tropical countries including India, Indonesia, Malaysia and parts of the Western Pacific. Aglaia is the largest genus belongs to the Meliaceae family contains more than 150 species (Hidayat *et al*., 2017a; Hidayat *et al*., 2017b; Awang *et al*., 2012) and about 65 species grown in Indonesia (Wood, *et al*., 1970; Heyne 1982). Phytochemical studies on Aglaia species have led to the identification of main compounds such as sesquiterpenoid, diterpenoid, triterpenoid, limonoid, steroid, lignan, and alkaloid groups (Harneti & Supratman, 2021).

Aglaia simplicifolia is found in Sumatra and Kalimantan, Indonesia. So far, reports on the content of secondary metabolite compounds from this plant are the only senecracidiol isolated from the bark of the stem (Kurniasih *et al.*, 2019). Although steroids of other Aglaia species have been investigated previously, the ergosterol peroxide of *A. simplicifolia* is yet to be reported.

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2. MATERIALS AND METHOD

Tools and Materials

Thin layer chromatography (TLC): silica gel plates (GF254, Merck, 0.25 mm); visualized by heating and immersing in 10% H_2SO_4 in EtOH. Column chromatography (CC): commercial SiO_2 (100 – 200 and 200 – 300 mesh; Merck, Darmstadt, Germany), and reversed-phase C18 (RP-C_{18} ; 40 – 63 mm; Fuji Sylisia, Japan); fractions were monitored by TLC. IR Spectra: Perkin-Elmer spectrum-100 FT-IR (Waltwam, MA, USA); KBr disks. ^{1}H- and ^{13}C-NMR spectra: Bruker Topspin spectrometer at 500 and 125 MHz respectively (Bruker BioSpin GmbH, Silberstreifen 4, D-76287 Rheinstetten, Germany); in $CDCl_3$; at room temperature; d in ppm relative to Me_4Si as internal standard, J in Hz. HR-TOF-MS: Synapt G2 mass spectrometer instrument (Waters, Milford, MA, USA); in m/z.

Cervical cancer line HeLa was maintained in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% pen strep (Gibco). Cultures were grown in a humidified incubator at 37 °C and 5% CO_2. The stembark collected from Bogor Botanical Garden and taxonomically identified as A. simplicifolia by Mr. Didik Widyatmoko. A voucher specimen (No. BO-1295311) was deposited in Bogoriense Herbarium, Bogor, West Java Province, Indonesia.

Extraction and Isolation

Air-dried stems (1.10 kg) were extracted three times with MeOH (3x4 L; 3 h, 3 h, and 2 h, respectively) at room temperature. After removal of MeOH under reduced pressure with a rotary evaporator, the viscous residue was suspended in H_2O:MeOH (4:1) and partitioned successively with *n*-hexane (10 L), ethyl acetate (10 L), and *n*-butanol (10 L). Evaporated of these extracts resulted of *n*-hexane (14.5 g), ethyl acetate (28.0 g) and *n*-butanol (14.5 g), respectively.

The *n*-hexane -soluble extract (14.5 g) was fractionated by vacuum liquid chromatography (silica gel G60; aq. *n*-hexane-ethyl acetate-methanol, gradient) to give nine fractions, Frs. A – I, combined according to the TLC results. Fraction D (1.29 g) was further subjected to column chromatography (SiO_2; *n*-hexane-ethyl acetate 100:0 to 40:60, gradient) to give nine subfractions, Frs. D.1 – D.9. Fraction C.6 (142.3 mg) was subjected to CC (SiO_2; methylene chloride: ethyl acetate (49: 1) to yield compound 1 (5.6 mg). Fraction D.7 (109.0 mg) was separated by CC (SiO_2; methylene chloride: ethyl acetate (49: 1) to yield 2 (20.6 mg).

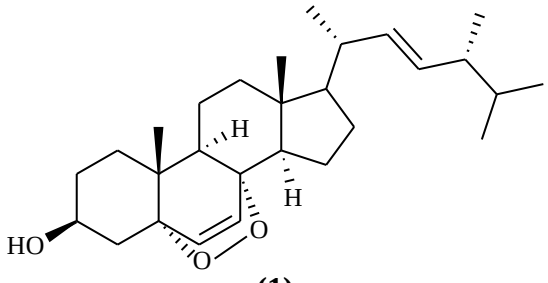
Cytotoxic Activity (Resazurin assay)

Cell viability was assessed by resazurin assay following the previously reported procedures (Sittampalam et al., 2004). Cells were seeded into a 96-well plates at a density of 17,000 cells/well and stabilized at 37 °C in 5% CO_2 for 24 h. Cells were incubated for 24 h with compounds **1** and **2**. Ten cells were treated with 10 µL of Presto Blue™ Cell Viability Reagent for another 1-2 hours. Cell viability assessed by measuring the absorbance at 570 nm with a reference wavelength of 600 nm using an EMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). For the positive control, cells were incubated for 24h with 100 µL of Cisplatin.

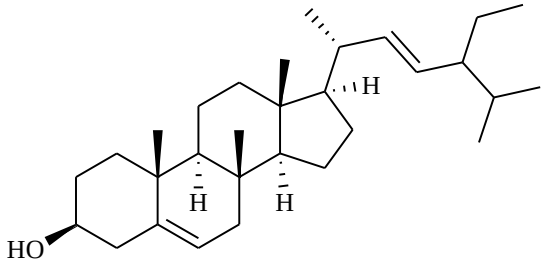
3. RESULT AND DISCUSSION

Structure Elucidation

In our phytochemical research on *Aglaia simplicifolia*, two steroids, ergosterol peroxide (**1**) and stigmasterol (**2**) (**Figure 1**) were isolated from the nonpolar fractions. Their structures were determined by a detailed analysis of their spectroscopic data.



(1)



(2)

Figure 1. Chemical structures of compounds **1** and **2**

Table 1. NMR data for compounds 1 and 2 ($CDCl_3$, 500 MHz for ^1H and 125 MHz for ^{13}C) compared with Ergosterol Peroxide (Nowak *et al*., 2016) and Stigmasterol (Cayme & Ragasa, 2004)

Position	Compound (1)		Ergosterol peroxide*		Compound (2)		Stigmasterol**	
	^1H NMR δ_H (Integral, mult, *J*=Hz)	δ ^{13}C	^1H NMR δ_H (Integral, mult, *J*=Hz)	δ ^{13}C	^1H NMR δ_H (Integral, mult, *J*=Hz)	δ ^{13}C	^1H NMR δ_H (Integral, mult, *J*=Hz)	δ ^{13}C
1	1.70, dd, J = 13.8;3.4	34.71	1.73, dd, J = 13.8; 3.4	34.7	1.08 , m; 1.84, m	37.4 (t)	1.73, dd, J = 13.8; 3.4	34.7
2	-	30.13		30.1	1.49, m; 1.81, m	31.8 (t)		30.1
3	3.95, m	66.47	3.98, m	66.5	3.52, m	72.0 (d)	3.98, m	66.5
4	-	36.98		37.0	2.28, dd, J = 2.0;5.2 2.30, dd, J = 2.0;5.2	42.5 (t)		37.0
5	-	82,15		82.2	-	140.9 (s)		82.2
6	6.22, d, J=8.5	135.41	6.25, d, J = 8.5	135.4	5.35, d, J = 5.2	121.9 (d)	6.25, d, J = 8.5	135.4
7	6.51, d, J=8.6	130.75	6.52, d, J = 8.6	130.8	1.54, m; 1.96, m	32.1 (t)	6.52, d, J = 8.6	130.8
8	-	79.42		79.4	1.46, m	32.0 (d)		79.4
9	-	51.13		51.1	0.94, m	50.3 (d)		51.1
10	-	36.95		36.9	-	36.7 (s)		36.9
11	1.21, m; 1.55, m	20.63	1.23, m; 1.55, m	20.6	1.46, m; 1.49, m	21.3 (t)	1.23, m; 1.55, m	20.6
12	1.25, m; 1.98, m	39.36	1.27, m; 1.98, m	39.4	1.15, m; 1.95, m	39.9 (t)	1.27, m; 1.98, m	39.4
13	-	44.57		44.6	-	42.5 (s)		44.6
14	1.55, m	51.69	1.59, m	51.7	1.03, m	56.9 (d)	1.59, m	51.7
15	1.42, m; 1.66, m	23.40	1.42, m; 1.66, m	23.4	1.07, m; 1.56, m	24.5 (t)	1,42, m; 1,66, m	23.4
16	1.32, m; 1.81, m	28.62	1.33, m; 1.81, m	28.7	1.26, m; 1.67, m	28.4 (t)	1.33, m; 1.81, m	28.7
17	1.25, m	56.23	1.25, m	56.2	1.13, m	56.1 (d)	1.25, m	56.2
18	0.81s	12.87	0.83, s	12.9	0.67, s	12.1 (q)	0.83, s	12.9
19	0.83 s	18.16	0.89, s	18.2	1.00, s	19.5 (q)	0.89, s	18.2
20	2.03, m	39.69	2.05, m	39.7	2.02, m	40.7 (d)	2.05, m	39.7
21	1.17, d, J = 6.7	20.87	1.00, d, J = 6.7	20.9	0.92, d, J = 6.5	21.2 (q)	1.00, d, J = 6.7	20.9
22	5.17, dd, J = 7.5; 15.3	135.19	5.16, dd, J = 7.5; 15.3	135.2	5.16, dd, J = 8.5; 15.0	138.5 (d)	5.16, dd, J = 7.5; 15.3	135.2
23	5.11, dd, J = 8.0; 15.3	132.33	5.14, dd, J = 8.0; 15.3	132.3	5.00, dd, J = 8.5; 15.0	129.5 (d)	5.14, dd, J = 8.0; 15.3	132.3
24	1.88, m	42.78	1.86, m	42.8	1.53, m	51.4 (d)	1.86, m	42.8
25	1.62, m	33.07	1.6, m	33.1	1.45, m	31.8 (d)	1,6, m	33.1
26	0.84, d, J = 6.8	19.63	0.82, d, J = 6.8	19.6	0.84, d, J = 6.4	21.3 (q)	0.82, d, J = 6.8	19.6
27	0.87, d, J = 6.6	19.93	0.83, d, J = 6.6	20.0	0.82, d, J = 6.1	19.1 (q)	0.83, d, J = 6.6	20.0
28	0.93, d, J = 6.8	17.55	0.91, d, J = 6.8		1.15, t, J = 3.2	25.6 (d)	0.91, d, J = 6.8	
29				17.6	0.80, t, J = 6.0	12.2 (q)		17.6

* ($CDCl_3$, 500 MHz for ^1H and 125 MHz for ^{13}C)
** ($CDCl_3$, 400 MHz for ^1H and 100 MHz for ^{13}C)

Compound **1** was obtained as colorless needles with a melting point between 179–182 °C. The HR-TOF-MS result at m/z 451.3748 ([M + Na^+]; calc. 428.3704) indicated that it has a molecular formula of $C_{28}H_{44}O_3$ with seven degrees of unsaturation. The IR spectrum showed the functional group of hydroxyl (3401 cm^{-1}) and ether groups (1052

cm^{-1}). The 13C-NMR spectrum showed 28 carbons signals (**Table 1**), which could be classified with the help of HSQC data as six Me, seven CH_2, and eleven CH groups (two oxygenated), and four Cq -atoms. The presence of two disubstituted olefins (δ 130.78 (C-7), 132.33 (C-23), 135.41 (C-6), 135.19 (C-22)), indicating that the sterol fragment of compound **1** is an ergosterol derivative. Besides, two oxygenated quaternary carbons of δ 82.15 (C-5) and 79.42 (C-8) suggested the presence of a peroxide structure.

The signals at δ_H 6.22 and 6.51 (d, J = 8 Hz, 2H, H-6, H-7) in the ^{1}H-NMR spectrum revealed the presence of a disubstituted double bond which were correlated with carbon signals of 135.41 (C-6) and 130.78 (C-7) in the HMBC spectrum. The ^{1}H-NMR showed also signals for six methyl groups, two singlets at 0.81 and 0.83, and four doublets at 0.84 (J = 6.8 Hz), 0.87 (J = 6.6 Hz), 0.93 (J = 6.8 Hz) and 1.17 (J = 6.7 Hz). Moreover, a multiplet at 3.95, characteristic of a steroid oxymethine signal located at C-3, was observed. The 2D-NMR experiments confirmed that compound **1** is a steroid, containing a peroxy function at C-5/C-8 and two double bonds in the side chain and at C-6/C-7.

In the HMBC correlations (**Figure 2**), these three methylene proton signals were correlated to the methine carbon signal at δ_C 66.84 (C-3), and the methylene carbon signal at δ_C 35.06 (C-1) was correlated with the methyl proton signal at δ_H 0.89 (H-19). The methylene proton signals at δ_H 1.94 and 2.11 (H-4) were clearly correlated to two carbon signals at δ_C 83.10 (C-5) and 135.80 (C-6) in 5α,8α-epidioxy system. The methyl proton signal at δ_H 0.89 (H-19) was long-range-correlated to the methyne carbon signal at δ_C 83.10 (C-5) to which the proton signals at δ_H 1.94 and 2.11 (H-4) were correlated, but not to the signal at δ_C 79.82 (C-8). The methyl proton signal was also correlated to the methine carbon signal at δ_C 51.43 (C-9), but not to the signal at 52.05 (C-14) which correlated with the methyl proton signal at δ_H 0.83 (H-18). Also an olefinic proton signal in 5α,8α-epidioxy system at (5 6.25 (H-7) was correlated to the carbon signals at δ_C 51.43 (C-9) and 52.05 (C- 14), while the other olefinic proton signal at δ_H 6.51 (H- 6) was correlated to carbon signals at δ_C 37.29 (C-4) and 37.33 (C-10). Therefore, the structure of ergosterol peroxide (5,8-epidioxy-5 ,8 -ergosta-6,22E-dien-3 -ol) was thus elucidated to be **1**. In this paper ergosterol peroxide was isolated from *A. simplicifolia* first time, so it is a new compound for this species. The known compounds stigmasterol (**2**), were confirmed by comparison and biogenetic analysis of these compounds with values reported by Cayme & Ragasa (2004).

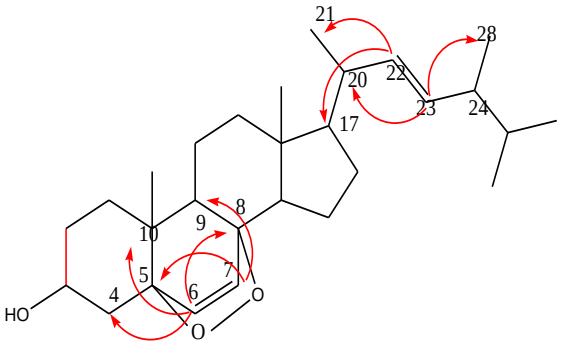


Figure 2. The long-range correlations of ergosterol peroxide through three bond connections observed in HMBC. All proton signals were correlated to carbon signals through two bond connections except H-3, H-16 and H-17. The arrow indicates the correlation from proton to carbon.

Cytotoxic Activity

The cytotoxic effects of compounds **1** and **2** against HeLa cervical cancer cells were conducted according to the resazurin assay method (Sittampalam *et al*., 2004) and were used cisplatin (IC_{50} 0.67 µM) as a positive control. As shown in Figure 3, treatment with both compounds resulted in the dose-dependent inhibition of HeLa cervical cancer cell growth when assessed at 24 hours post-treatment. Higher concentrations of both compounds were required to inhibit cell growth.

Ergosterol peroxide (**1**) has a much stronger activity with an IC_{50} value of 0.80 µM compared to stigmasterol (**2**) which has an IC_{50} value of 26.42 µM. This shows that the value of cytotoxic activity against HeLa cervical cancer cells is influenced by the presence of peroxide groups bound to C-5 and C-8. In fact, the ergosterol peroxide compound from the stem bark of *Aglaia simplicifolia* is much stronger to inhibit the growth of cervical cancer cells HeLa than those isolated from marine fungus Phoma sp with an IC_{50} value of 29.20 µM (Wu *et al.*, 2018).

Ergosterol peroxide was sensitive to cancer cells, while less sensitive or nontoxic to normal cells. Wu *et al*., (2018) isolated

ergosterol peroxide from marine fungus Phoma sp. The bioassay results demonstrated that ergosterol peroxide reduced the viability of various cancer cells. EP induced caspase-dependent apoptosis through mitochondrial damage, induced ROS generation and apoptosis, and reduced the LPS/ATP induced proliferation and migration of A549 cells through attenuated NLRP3 inflammasome activity.

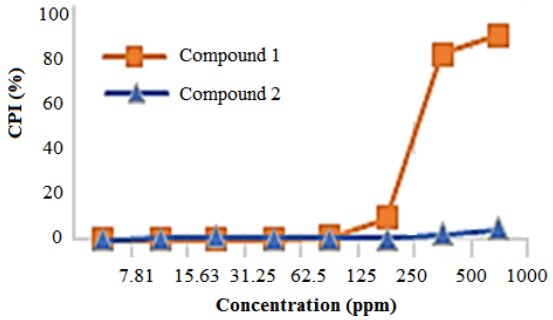


Figure 3. Effects of 24 h treatment various concentrations of compound **1** and **2** to HeLa cervical cancer cell (CPI: cell proliferation inhibition)

4. CONCLUSIONS

Two steroid compounds, ergosterol peroxide (**1**) and stigmasterol (**2**) have been isolated from the stembark of *Aglaia simplicifolia* and were shown for the first time in this species. The presence of peroxide in steroid structure plays an important role in cytotoxic activity against HeLa cervical cancer cells.

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