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 IC₅₀ values of 0.80 and 26.42 µM, respectively.

**Keywords**: Ergosterol peroxide, stigmasterol, *Aglaia simplicifolia*, HeLa cervical cancer, IC₅₀

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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., 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.
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% H₂SO₄ in EtOH. Column chromatography (CC): commercial SiO₂ (100 – 200 and 200 – 300 mesh; Merck, Darmstadt, Germany), and reversed-phase C18 (RP-C18; 40 – 63 mm; Fuji Sylisia, Japan); fractions were monitored by TLC. IR Spectra: Perkin-Elmer spectrum-100 FT-IR (Waltwam, MA, USA); KBr disks. ¹H- and ¹³C-NMR spectra: Bruker Topspin spectrometer at 500 and 125 MHz respectively (Bruker BioSpin GmbH, Silberstreifen 4, D-76287 Rheinstetten, Germany); in CDCl₃; at room temperature; d in ppm relative to Me₄Si 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% CO₂. The stembark collected from Bogor Botanical Garden and taxonomically identified as A. simplicifolia by Mr. Didik Widyatmoko. A voucher specimen (No. BO1295311) 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 H₂O: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 (SiO₂; 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 (SiO₂; methylene chloride: ethyl acetate (49: 1) to yield compound 1 (5.6 mg). Fraction D.7 (109.0 mg) was separated by CC (SiO₂; 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% CO₂ 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 24 h with 100 µL of Cisplatin.

3. RESULT AND DISCUSSION

Structure Elucidation

In our phytochemical research on A. 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.
**Table 1.** NMR data for compounds 1 and 2 (CDCl₃, 500 MHz for ¹H and 125 MHz for ¹³C) compared with Ergosterol Peroxide (Nowak et al., 2016) and Stigmasterol (Cayme & Ragasa, 2004)

| Position | Compound (1) | Ergosterol peroxide | Stigmasterol |
|----------|--------------|---------------------|--------------|
|          | δ ¹H (Integral, mult., J=Hz) | δ ¹³C | δ ¹H (Integral, mult., J=Hz) | δ ¹³C | δ ¹H (Integral, mult., J=Hz) | δ ¹³C |
| 1        | 1.70, dd, J = 13.8, 3.4 | 34.7 | 1.73, dd, J = 13.8; 3.4 | 34.7 | 1.08; m; 1.84; m | 37.4 (t) |
| 2        | 30.13 | 30.1 | 30.1 | 31.8 (t) | 1.49; m; 1.81; m | 30.1 |
| 3        | 3.95, m | 66.47 | 3.98, m | 66.5 | 3.52, m | 72.0 (d) |
| 4        | 36.98 | 37.0 | 2.28, dd, J = 2.0; 5.2 | 42.5 (t) | 2.30, dd, J = 2.0; 5.2 | 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) |
| 7        | 130.75 | 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       | 20.63 | 20.6 | 1.46, m; 1.49, m | 21.3 (t) | 1.23, m; 1.55, m | 20.6 |
| 12       | 39.36 | 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       | 51.69 | 51.7 | 1.03, m | 56.9 (d) | 1.59, m | 51.7 |
| 15       | 23.40 | 23.4 | 1.07, m; 1.56, m | 24.5 (t) | 1.42, m; 1.66, m | 23.4 |
| 16       | 28.62 | 28.7 | 1.26, m; 1.67, m | 28.4 (t) | 1.33, m; 1.81, m | 28.7 |
| 17       | 56.23 | 56.2 | 1.13, m | 56.1 (d) | 1.25, m | 56.2 |
| 18       | 0.81s | 0.83, s | 0.67, s | 12.1 (q) | 0.83, s | 12.9 |
| 19       | 0.83 s | 0.72 | 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) |
| 21       | 1.00, d, J = 6.7 | 20.87 | 1.00, d, J = 6.7 | 20.9 | 0.92, d, J = 6.5 | 21.2 (q) |
| 22       | 135.19 | 135.2 | 5.16, dd, J = 5.1; 15.3 | 138.5 (d) | 5.16, dd, J = 7.5; 15.3 | 135.2 |
| 23       | 132.33 | 132.3 | 5.00, dd, J = 8.5; 15.0 | 129.5 (d) | 5.14, dd, J = 8.0; 15.3 | 132.3 |
| 24       | 42.78 | 42.8 | 1.53, m | 51.4 (d) | 1.86, m | 42.8 |
| 25       | 33.07 | 33.1 | 1.45, m | 31.8 (d) | 1.6, m | 33.1 |
| 26       | 19.63 | 19.6 | 0.84, d, J = 6.4 | 21.3 (q) | 0.82, d, J = 6.8 | 19.6 |
| 27       | 19.93 | 20.0 | 0.82, d, J = 6.1 | 19.1 (q) | 0.83, d, J = 6.6 | 20.0 |
| 28       | 17.55 | 17.6 | 1.15, t, J = 3.2 | 25.6 (d) | 0.91, d, J = 6.8 | 17.6 |
| 29       | 0.91, d, J = 6.8 | 18.5 | 0.80, t, J = 6.0 | 12.2 (q) | 0.91, d, J = 6.8 | 17.6 |

*(CDCl₃, 500 MHz for ¹H and 125 MHz for ¹³C)*

© (CDCl₃, 400 MHz for ¹H and 100 MHz for ¹³C)*

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 C₃₀H₄₄O₃ 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 CH₂, and eleven CH groups (two oxygenated), and four C=O- 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 ¹H-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 ¹H-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 oxymethylene 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 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carbon signal 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 methine carb
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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 stem bark 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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