MOLEKUL
eISSN: 2503-0310

Articles
https://doi.org/10.20884/1.jm.2023.18.3.7824

Steroids Produced by Endophytic Fungus *Lasiodiplodia Theobromae* from *Aglaia argentea* Blume and Their Cytotoxic Activity Against Hela Cervical Cancer Cell Lines

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Received January 19, 2023; **Accepted** June 07, 2023; **Available online** November 20, 2023

ABSTRACT. Endophytes are micro-organism recognised that living beneficial inside the host plant produced secondary metabolites with biological activity such as anticancer, antitumor, antifungal and antibacterial. In this study, we investigated the endophytic fungus *Lasiodiplodia theobromae* from the root of *Aglaia argentea* Blume. *A. argentea* have been phytochemically investigated previously with unique biological activity such as cytotoxic rocaglate derivatives and dammarane-type triterpenoids. In a continuing project on the bioactive compounds from *A. argantea*, we have explored endophytic fungi isolated from this plant. Three steroids, ergosterol **(1)**, ergosterol peroxide **(2)** and stigmasterol **(3)** have been isolated from endophytic fungus, *Lasiodiplodia theobromae* derived from the root of *Aglaia argentea* Blume. The steroids were isolated by vacuum chromatography and column chromatography, the chemical structure was established following the analysis of 1D-NMR, 2D-NMR, IR, MS and by comparison with previously reported spectra data. Ergosterol peroxide **(2)** and stigmasterol **(3)** were reported for the first time isolated from *Lasiodiplodia theobromae* endophytic fungus. Cytotoxic activities of the compounds were tested with resazurin assay against HeLa cervical cancer cells, compound **2** displayed strongest cytotoxic activities against HeLa cervical cancer cells with IC_{50} values of 0.28 µM, while compounds **1** and **3** showed IC_{50} values of 0.34 µM and 27.32 µM, respectively.

Keyword: *Aglaia argentea,* cytotoxic activity, HeLa, *Lasiodiplodia theobromae*, steroid

INTRODUCTION

Endophytes are micro-organism recognised in last century that living mutually beneficial inside the host plant (Arora & Ramawat, 2017). Interaction of endophytic fungi with plants is a symbiosis in the intracellular space of plants without causing damage to their host plants, have received quite a lot attention (Shiono et al., 2013). Endophytic fungi has enormous potential to produce chemical compounds resulting from secondary metabolites that have unique and interesting activities. Many endophytes fungal produced secondary metabolites with biological activity such as anticancer, antitumor, antifungal and antibacterial which strongly inhibit the growth of other microorganisms (Kharwar et al., 2011; Gunatilaka, 2006). In addition, endophytes can produce secondary metabolites that are the same or similar to their host plants (Supratman, et al., 2021). Fungi produced various kind of secondary metabolite, fungi ability to formed new compound can be done by activating silent gene (Brakhage, 2013). One strain many compounds (OSMAC) method is strategy activated silent gene that are not expressed using conventional fermentation, such as controlled temperature, pH, oxygen condition, salinity, addition of enzyme or tryptophan to rice medium (Gao et al., 2020). Using cultivated on fruit and vegetable juice-supplemented solid rice media is one of OSMAC method for increase diversity of secondary metabolite that not produced when the fungi was grown on rice medium without either fruit or vegetable (Hemphill et al., 2017; Supratman, et al., 2021).

Cyclopenta[*b*]benzofurans are mainly accumulated in the roots and stem bark from *Aglaia* species (Brader et al., 1998). In this study, we investigated the endophytic fungus *Lasiodiplodia theobromae* derived from the root of *Aglaia argentea* Blume, belong to Meliaceae family. We isolated three steroid compounds, ergosterol **(1)**, ergosterol peroxide **(2)** and stigmasterol **(3)** using OSMAC method, added

apple juice-supplemented to brown rice medium for the fermentation process. This study also provides isolation, structural elucidation and cytotoxic activity against HeLa cervical cancer cells using resazurin assay.

EXPERIMENTAL SECTION

General Experiment Procedure

NMR spectra were measured by JEOL JNM-ECZ-500R at 500 MHz for ^{1}H and 125 MHz for ^{13}C, chemical shift is given on a d (ppm) scale with TMS as an internal standard and ^{1}H, ^{13}C, DEPT, COSY, HMQC and HMBC spectra were recorded using standard JEOL pulse sequences (Tokyo, Japan). IR spectra were measured on a Perkin–Elmer spectrum-100 FT-IR (Waltham, Massachusetts, USA). Mass Spectra were measured by Waters QTOF-HRTOFMS-XEV^{otm} mass spectrometer (Waters, Milford, MA, USA). Column chromatography were conducted on silica gel 60 (Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on Merck 60 F_{254} silica gel plates, spot detection was achieved by spraying with 10% H_2SO_4 in ethanol followed by heating or by UV irradiation.

Fungal Material and Fermentation

The fungal strain of Arg-8 was isolated from the root of *A. argentea*, collected in August 2019, from Bogor Botanical Garden, Bogor, West Java, Indonesia. Fungal strains Arg-8 was identified as *Lasiodiplodia theobromae* by the using a DNA analysis of the 18S rDNA regions. They have been deposited at Central Laboratory of Universitas Padjadjaran, Indonesia. Arg-8 strain was cultured on slants with Potato Dextrose Agar (PDA) at room temperature for 7 days. Fermentation was carried out in jar (300 mL) containing 50 g of rice and apple juice (70 mL per flask, total of 5000 g). After autoclaving, each flask was inoculated with hyphae inoculum and incubated at 25°C for 30 days. The mycelia and solid rice medium were extracted with ethanol and then was partition with *n*-hexane. The *n*-hexane extract was concentrated under rotary evaporator to give a crude extract (9.0 g).

Extraction and Isolation

The mycelia and solid rice medium (5 kg) were extracted with ethanol for 3 days, then the extract was concentrated under reduced pressure to give a crude extract and the suspended in distilled water. The resulting aqueous concentrated was partitioned into *n*-hexane layer and then concentrated under reduced pressure to give a crude extract of *n*-hexane (9.0 g). The extract was separated by silica gel column chromatography with a stepwise elution of *n*-hexane/ethyl acetate (100:0–0:100, v/v; each 500 mL) to give 11 fractions (Frs. 1 to 11). Frs. 3 (90–10% *n*-hexane / ethyl acetate, 317 mg) were combined and further separated on a silica gel column with *n*-hexane / di isopropyl ether to provide 11 fractions (Frs. 3-1 to 11). Fr. 3-5 was separated by column chromatography with an elution using *n*-hexane containing 20% di isopropyl ether to give 11 fractions (Frs. 3-1 to 11). Fr. 4-6 (26.4 mg) was separated on a silica gel column with *n*-hexane / di isopropyl ether to afford compound **1** (4.9 mg). Fr. 4-7 (46.2 mg) was separated on a silica gel column with *n*-hexane / di isopropyl ether to afford compound **3** (5.4 mg). Fr. 4-7 (46.2 mg) was purified by column chromatography with an elution using *n*-hexane containing 30% di isopropyl ether to afford compound **2** (15.4 mg).

Ergosterol (**1**). White needles crystal; melting point: 158-159 °C; IR (KBr plate) ν_{max} cm^{-1}: 3384, 2952, 2868, 1689, 1605, 1365 and 1025; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 0.61 (3H, s), 0.81 (3H, d, *J*= 7.5 Hz), 0.83 (3H, d, *J*= 7.5 Hz), 0.91 (3H, d, *J*= 7.0 Hz), 0.93 (3H, s), 1.25 (1H, m), 1.28 (2H, m), 1.29 (2H, m), 1.46 (2H, m), 1.58 (1H, m), 1.67 (2H, m), 1.70 (2H, m), 1.84 (1H, m), 1.86 (2H, m), 1.88 (1H, m), 1.96 (1H, m), 2.04 (1H, m), 2.26 (1H, m), 2.45 (1H, m), 3.62 (1H, m), 5.16 (1H, m), 5.20 (1H, m), 5.37 (1H, dd, *J*= 5.4, 2.5 Hz), 5.55 (1H, dd, *J*= 5.5, 3.0 Hz); ^{13}C-NMR ($CDCl_3$, 125 MHz), see Table 1. HR-TOFMS (positive ion mode) *m/z* 397.3500 $[M+H]^+$ (calcd. for $C_{28}H_{45}O^+$, *m/z* 397.3470).

Ergosterol peroxide (**2**). White needle crystals; melting point: 178–179°C; IR (KBr plate) ν_{max} cm^{-1}: 3299, 2954, 2920, 2853, 1722, 1458, 1377 and 1082; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 0.79 (3H, d, *J*= 6.8 Hz), 0.79 (3H, d, *J*= 6.8 Hz), 0.80 (3H, s), 0.82 (3H, d, *J*= 6.4 Hz), 0.86 (3H, s), 0.92 (3H, d, *J*= 6.8 Hz), 0.98 (3H, d, *J*= 7.0 Hz), 1.22 (1H, m), 1.23 (1H, m), 1.35 (1H, m), 1.41 (1H, m), 1.46 (1H, m), 1.5 (1H, m), 1.53 (1H, m), 1.54 (1H, m), 1.56 (1H, m), 1.60 (1H, m), 1.70 (1H, m), 1.75 (1H, m), 1.84 (1H, m), 1.91 (1H, m), 1.95 (1H, m), 1.96 (1H, m), 2.03 (1H, ddd, *J*= 2.0, 5.2, 14.0 Hz), 2.28 (1H, m), 3.95 (1H, m), 5.11 (1H, dd, *J*= 8.4, 15.2 Hz), 5.21 (1H, dd, *J*= 7.2, 15.2 Hz), 6.22 (1H, d, *J*= 8.4 Hz), 6.43 (1H, d, *J*= 8.8 Hz), ^{13}C-NMR ($CDCl_3$, 125 MHz), see Table 2. HR-TOFMS (positive ion mode) *m/z* 451.3179 $[M+Na]^+$ (calcd. for $C_{28}H_{44}O_3\,Na^+$, *m/z* 451.3188).

Stigmasterol (**3**) White needle crystals; melting point: 128-130 °C; IR (KBr plate) ν_{max} cm^{-1}: 3401, 2937, 1457 and 1052; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ_H 0.67 (3H, s), 0.8 (3H, t, *J*= 6.0 Hz), 0.82 (3H, d, *J*= 6.1 Hz), 0.84 (3H, d, *J*= 6.4 Hz), 0.92 (3H, d, *J*= 6.5 Hz), 0.94 (1H, m), 1.00 (3H, s), 1.03 (1H, m), 1.07 (1H, m), 1.08 (1H, m), 1.13 (1H, m), 1.15 (1H, m), 1.15 (1H, t, *J*= 3.2 Hz), 1.26 (1H, m), 1.45 (1H, m), 1.46 (1H, m), 1.49 (1H, m), 1.53 (1H, m), 1.54 (1H, m), 1.56 (1H, m), 1.67 (1H, m), 1.81 (1H, m), 1.84 (1H, m), 1.95 (1H, m), 1.96 (1H, m), 2.02 (1H, m), 2.28 (1H, dd, *J*= 2.0, 5.2 Hz), 2.3 (1H, dd, *J*= 2.0, 5.2 Hz), 5.00 (1H, dd, *J*= 8.5, 15.0 Hz), 5.16 (1H, dd, *J*= 8.5, 15.0 Hz), 5.35 (1H, d, *J*= 5.2 Hz),^{13}C-NMR ($CDCl_3$, 125 MHz), see Table 3; HR-TOF MS *m/z* 413.3748 $[M+H]^+$ (calcd. *m/z* 412.3704 for $C_{29}H_{48}O^+$).

Determination of Cytotoxic Activity

The method used to determine cytotoxic activity of compound **1**-**3** was measured with a cell viability with resazurin base by PrestoBlue® assay. The cells were maintained in RPMI (Rosewell Park Memorial Institute) medium, 10% (*v/v*) FBS (Fetal Bovine Serum), as well as 1 μL/mL antibiotic. The cells were cultured into 96 well plates at 37 ºC in a humidified atmosphere of 5% CO_2 incubation for 24 h or until the cells reach a density of 1.7×10^4 cell/well. The medium was discarded, then added the fresh medium containing sample with different concentration (1,000.00, 500.00, 250.00, 125.00, 62.50, 31.25, 15.63, and 7.81 μg/mL) and also control Cisplatin. The cells with sample then incubated for 48 h, after that PrestoBlue® reagent was added and the result was read using a multimode reader at 570 nm and converted the absorption into cell viability so that the IC_{50} of each compound were obtained.

RESULTS AND DISCUSSION

The HRTOFMS spectrum of compound **1** showed ion peak $[M+H]^+$ *m/z* 397.3500 (calcd. *m/z* 397.3470) which corresponded to the molecular formula as $C_{28}H_{45}O^+$ showed the steroid core moiety (Permatasari et al., 2022). The IR spectra showed absorption peaks at 3384 cm^{-1} (-OH), 2952 cm^{-1} and 2868 cm^{-1} (C-H sp^3), 1605 cm^{-1} and 1689 cm^{-1} (C=C), 1365 cm^{-1} (*gem*-dimethyl groups), and 1025 cm^{-1} (C-O), which corresponding to steroid skeleton (Farabi et al., 2017). ^{1}H NMR spectrum (Table 1) revealed the presence of six sp^3 methyl signal consisting of four methyl doublet 0.81 (3H, d, *J*=7.5 Hz), 0.83 (3H, d, *J*=7.5 Hz), 0.91 (3H, d, *J*=7.0 Hz), 1.03 (3H, d, *J*=7.0 Hz) ppm, two methyl singlet at δ_H 0.63 (3H, s) and 0.95 (3H, s) ppm, there also one oxygenated methine at δ_H 3.64 (1H, m) ppm which indicates the characteristic of steroid (Martinez et al., 2015). A characteristic feature an ergostane-type of steroid is the presence of four olefinic methine resonating at δ_H 5.57 (1H, dd, *J*=5.5, 3.0 Hz), 5.38 (1H, dd, *J*=5.4, 2.5 Hz), 5.19 (1H, m), and 5.18 (1H, m) ppm. The ^{13}C NMR spectrum shows the presence 28 carbon signals, each signal having a distinctive shift. The chemical shift of carbon (δ_C) 12.0-70.0 ppm shows a signal from C sp^3 that supports the ^{1}H NMR data six methyl at δ_C 12.1, 16.3, 17.6, 19.7, 20.0 and 21.1, seven methylene at δ_C 38.4, 32.0, 40.8, 21.1, 39.1, 23.0 and 28.3 ppm, six methine at δ_C 46.3, 54.6, 55.7, 40.5, 42.8 and 33.1 ppm, one oxygenated methine at δ_C 70.5 (C-3) ppm, four olefinic methine at δ_C 119.6, 116.3, 135.6, 132.0 ppm, and two olefinic quaternary carbon at δ_C 139.8 (C-5) and 141.4 (C-8) ppm which is similar to chemical shift of olefinic quaternary carbon of ergostane-type steroid. The NMR data of compound **1** is very similar with reported compound, ergosterol that was isolated from *Laetiporus* sp. (Martinez et al., 2015), therefore compound **1** was identified as an ergosterol. Ergosterol is steroid isolated from endophytic fungi, the most abundant sterol in fungal cell membranes, ergosterol is an immunologically active lipid that almost all steps of its biosynthetic process are potential drugs targets (Rodrigues, 2018). The comparison data between compound **1** and ergosterol is served in **Table 1** while the chemical structure of **1** is described in **Figure 1**. Based on literature for chiral carbon were confirmed by comparison analysis these compounds with the values reported by Martinez et al (2015).

The HRTOFMS spectrum of compound **2** showed ion molecule $[M+Na]^+$ at *m/z* 451.3179 (calcd. *m/z* 451.3188), which corresponded to the molecular formula $C_{28}H_{44}O_3Na^+$ and indicated the steroid core moiety (Farabi et al., 2017; Sari et al., 2022). The IR spectra showed absorption peaks at 3299 cm^{-1} (-OH), 2954 cm^{-1}, 2920 cm^{-1} and 2853 cm^{-1} (C-H sp^3), 1722 cm^{-1} (C=C), 1458 cm^{-1} and 1377 cm^{-1} (*gem*-dimethyl groups), and 1082 cm^{-1} (C-O), which showed the characteristic of steroid skeleton (Farabi et al., 2017). The ^{1}H NMR spectrum (Table 2) revealed the presence of six methyl groups, two singlets at δ_H 0.84 and 0.88 ppm and four doublets at δ_H 0.99 (3H, d, *J*=7.0 Hz), 0.77 (3H, d, *J*=6.8 Hz), 0.82 (3H, d, *J*=6.4 Hz), 0.90 (3H, d, *J*=6.8 Hz) ppm, there also one oxygenated methine at δ_H 3.95 (1H, m, H-3) ppm supports the characteristic of a steroid (Kurniasih et al., 2021). Typical for olefin protons from ergostane-type ergosterol derivatives is the presence of four methine olefins at δ_H 6.24 (1H, d, *J*=8.4 Hz, H-6), 6.50 (1H, d, *J*=8.8 Hz, H-7), 5.21 (1H, dd, *J*=15.2, 7.2, Hz, H-22), and 5.13 (1H, dd, *J*=15.2, 8.4, Hz, H-23) ppm. The ^{13}C NMR spectra data showed a total of 28 carbon signals (**Table 2**), supports the finding of ^{1}H NMR spectrum showing a steroid skeleton in the presence of six methyl groups at δ_C 12.8, 18.2, 20.9, 19.6, 20.6 and 17.5 ppm and one oxygenated methine at δ_C 66.4 ppm. The presence of two oxygenated quaternary carbons at δ_C 82.2 and 79.5 ppm which are thought to originate from the oxygenated cyclic form at B ring with the similar core structure as compound **1**, an ergosterol derivatives. The chemical shift of olefinic methine at δ_C 135.4 and 130.7, suggested the double bond broken of compound **2** yield an oxygenated quaternary carbon at C-5 and C-8. Meanwhile, the olefinic methine of its side chain at δ_C 135.2 and 132.3 is similar to chemical shift of olefinic methine of ergostane-type steroid side chain (Nowak et al., 2016). These functionalities accounted for two out of the seven degrees of unsaturation, the remaining five degrees of unsaturation corresponded to tetracyclic of steroid skeleton and peroxide ring attach through C-5 and C-8. The NMR data of compound **2** comparing with reported compound ergosterol peroxide that was isolated from fresh fruit bodies *Hygrophoropsis aurantiaca* (Nowak et al., 2016) showed very similar data (**Table 2**). In conclusion, the structure of compound **2** was identified

as an ergosterol peroxide. Based on literature for chiral carbon were confirmed by comparison analysis these compounds with the values reported by Nowak et al (2016).

The HRTOFMS spectrum of compound **3** showed ion molecule $[M+H]^+$ *m/z* 413.3748 (calcd. *m/z* 412.3704) which corresponded to the molecular formula $C_{29}H_{48}O^+$ showed the steroid core moiety with eight degree of unsaturations. (Farabi et al., 2017; Sari et al., 2022). The IR spectra showed absorption peaks at 3401 cm^{-1} (hydroxyl), 2937 cm^{-1} (sp^3 aliphatics), 1457 cm^{-1} (*gem*-dimethyl groups), and 1052 cm^{-1} (ether group) (Farabi et al., 2017). The ^1H NMR spectrum of compound **3** (**Table 3**) shown steroid characteristics, there is a proton sp^3 signal from 0.65 to 2.3 ppm, six methyl in the chemical shift 0.68 (3H, s), 0.79 (3H, m), 0.81 (3H, m), 0.83 (3H, m), 1.00 (3H, s), 1.03 (3H, m) ppm, nine methylene groups at 1.44 (2H, m), 1.50 (2H, m), 1.56 (2H, m), 1.72 (2H, m), 1.84 (2H, m), 1.85 (2H, m), 1.97 (2H, m), 2.00 (2H, m), 2.30 (2H, m) ppm, eleven methine in the chemical shift 3.52 (1H, m), 1.46 (1H, m), 0.92 (1H, m), 1.01 (1H, m), 1.15 (1H, m), 2.06 (1H, m), 1.54 (1H, m) and 1.55 (1H, m) ppm, there also an alkene in the chemical shift of 5.35 (1H, m), 5.04 (1H, dd, *J* = 15.2, 8.6, Hz), 5.1 (1H, dd, *J* = 15.1, 8.6, Hz) ppm (Marliyana et al., 2021). The ^{13}C-NMR spectrum showed 29 carbon signals consisting of six methyl, 9 methylene, 11 methine and two quaternary carbon signal who suggested steroid groups compound (Yayli & Baltaci, 1996). The presence of four olefinic carbons (121.8, 129.3, 138.4 and 140.8 ppm), an oxygenated carbon (71.9 ppm), seven methine carbons [31.9 (2 x), 40.6, 50.2, 51.3, 56.0 and 56.8 ppm], two quaternary carbons (36.5 and 42.2ppm), nine methylene carbons (21.1, 24.4, 25.5, 29.0, 31.7, 31.9, 37.3, 39.7 and 42.2 ppm), and six methyl carbons (12.1, 12.3, 19.0, 19.5, 21.1 and 21.3 ppm) (Cayme & Ragasa, 2004). Comparison of the spectroscopic data of the compound **3** and stigmasterol (Cayme & Ragasa, 2004), shows that the two compounds have a very high level of compatibility, thus the compound **3** is identified as a stigmasterol (**Figure 1**). Carbon chiral were confirmed by comparison analysis these compounds with the values reported by Cayme & Ragasa (2004).

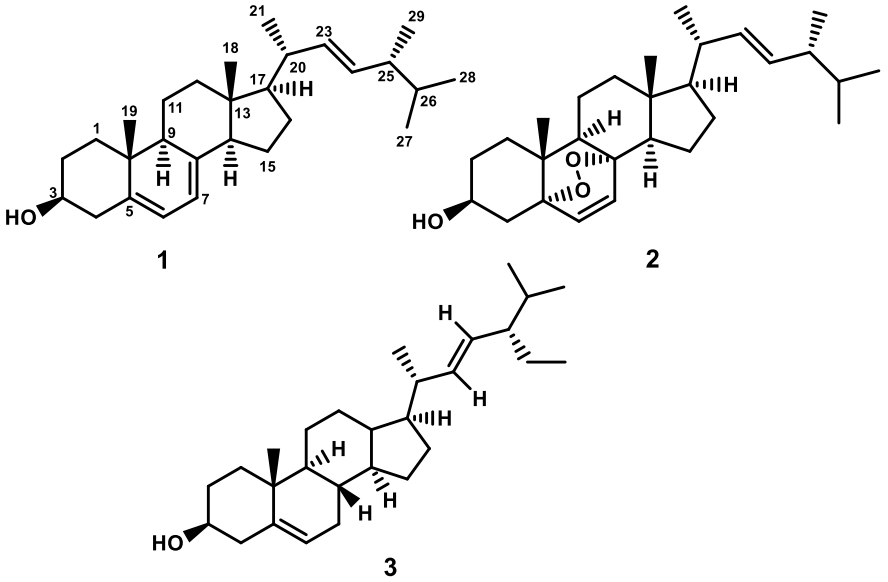

Figure 1. Chemical structure of compounds **1**-**3**

Table. 1. NMR data comparison between compound **1** and ergosterol (Martinez et al., 2015)

Positions	Compound **1**		Ergosterol	
	^{13}C NMR δc ppm	^1H NMR δ_H (Int, mult., *J*=Hz) ppm	^{13}C NMR δc ppm	^1H NMR δ_H (Integral, mult., *J*=Hz) ppm
1	38.4	1.29 (2H, m)	38.4	
2	32.0	1.86 (2H, m)	32.0	
3	70.5	3.62 (1H, m)	70.4	3.64 (1H, m)
4	40.8	2.26 (1H, m) 2.45 (1H, m)	40.8	
5	139.8	-	139.8	
6	119.6	5.55 (1H, dd, 5.5, 3.0)	119.6	5.58 (1H, dd, 5.5, 3.0)
7	116.3	5.37 (1H, dd, 5.4, 2.5)	116.3	5.38 (1H, dd, 5.4, 2.9)
8	141.4	-	141.3	

9	46.3	1.96 (1H, m)	46.2	
10	37.1	-	37.0	
11	21.1	1.67 (2H, m)	21.1	
12	39.1	1.46 (2H, m)	39.1	
13	42.8	-	42.8	-
14	54.6	1.88 (1H, m)	54.6	
15	23.0	1.70 (2H, m)	23.0	
16	28.3	1.28 (2H, m)	28.3	
17	55.7	1.25 (1H, m)	55.7	
18	12.1	0.95 (3H, s)	12.0	0.95 (3H, s)
19	16.3	0.63 (3H, s)	17.6	0.65 (3H, s)
20	40.5	2.04 (1H, m)	40.4	
21	21.1	1.02 (3H, d, 7.0)	21.1	1.04 (3H, d, 6.6)
22	135.6	5.20 (1H, m)	135.6	5.20 (1H, m)
23	132.0	5.16 (1H, m)	132.0	5.21 (1H, m)
24	42.8	1.84 (1H, m)	42.8	
25	33.1	1.58 (1H, m)	33.1	
26	19.7	0.83 (3H, d, 7.5)	19.6	0.84 (3H, d, 6.4)
27	20.0	0.81 (3H, d, 7.5)	19.9	0.82 (3H, d, 6.7)
28	17.6	0.91 (3H, d, 7.0)	16.2	0.92 (3H, d, 6.6)

($CDCl_3$; ^{1}H-NMR 500 MHz; ^{13}C-NMR 125 MHz)

Tabel. 2 NMR data comparison between compound **2** and ergosterol peroxide (Nowak et al., 2016)

Positions	Compound **2**		Ergosterol Peroxide	
	^{13}C NMR δc (125 MHz) ppm	δH (ΣH, mult., *J* (Hz)) 500 Hz ppm	^{13}C NMR δc (125 MHz) ppm	δH (ΣH, mult., *J* (Hz)) 500 Hz ppm
1	34.7	1.70 (1H, m) 1.95 (1H, m)	34.7	1.7 (1H, ddd, 3.5, 3.4, 3.4) 1.90 (1H, m)
2	30.1	1.54 (1H, m) 1.84 (1H, m)	30.1	1.54 (1H, m) 1.84 (1H, m)
3	66.5	3.95 (1H, m)	66.5	3.97 (1H, m)
4	37.0	1.91 (1H, m) 2.28 (1H, m)	37.0	1.91 (1H, dd, 13.7, 11.8) 2.11 (1H, ddd, 13.7, 5.0, 2.0)
5	82.2	-	82.2	-
6	135.4	6.24 (1H, d, 8.4)	135.4	6.25 (1H, d, 8.5)
7	130.8	6.50 (1H, d, 8.4)	130.8	6.52 (1H, d, 8.5)
8	79.5	-	79.4	-
9	51.1	1.5 (1H, m)	51.1	1.5 (1H, m)
10	36.9	-	36.9	
11	23.4	1.23 (1H, m) 1.53 (1H, m)	23.4	1.23 (1H, m) 1.53 (1H, m)
12	39.3	1.23 (1H, m) 1.96 (1H ,m)	39.4	1.23 (1H, m) 1.96 (1H, m)
13	44.6	-	44.6	-
14	51.7	1.56 (1H, m)	51.7	1.56 (1H, m)
15	20.7	1.41 (2H, m)	20,6	1.41(2H, m)
16	28.7	1.60 (1H, m) 1.35 (1H, m)	28.3	
17	56.2	1.75 (1H,m) 1.22 (1H, m)	56.2	1.75 (1H, m) 1.22 (1H, m)
18	12.9	0.84 (3H, s)	12.9	0.82 (3H, s)
19	18.2	0.88 (3H, s)	18.2	0.89 (3H, s)
20	39.8	2.03 (1H, ddd, 14.0, 5.2, 2.0)	39.7	2.02 (1H, ddq, 9.9, 8.6, 6.6)
21	20.9	0.99 (3H, d, 7)	20.9	1.00 (3H, dd, 6.6)
22	135.2	5.21 (1H, dd, 15.2, 7.2)	135.2	5.16 (1H, dd, 15.3, 7.5)

23	132.3	5.13 (1H, dd, 15.2, 8.4)	132.3	5.14 (1H, dd, 15.3, 8.0)
24	42.8	1.84 (1H, m)	42.8	1.85 (1H, ddq, 13.5, 7.8, 6.8)
25	33.1	1.46 (1H, m)	33.1	1.47 (1H, dq, 13.5, 6.8)
26	19.7	0.77 (3H, d, 6.8)	19.6	0.82 (3H, d, 6.8)
27	20.0	0.82 (3H, d, 6.4)	20.0	0.83 (d, 6.8)
28	17.6	0.90 (3H, d, 6.8)	17.6	0.91 (d, 6.8)

(measured in $CDCl_3$; ^{1}H-NMR 500 MHz; ^{13}C-NMR 125MHz)

Table. 3. NMR data comparison between compound **3** and stigmasterol (Cayme & Ragasa, 2004)

Posisi	Compound **3**		Stigmasterol	
	^{13}C NMR δc (mult.)ppm	^{1}H NMR δ_H (Integral, mult., *J*=Hz) ppm	^{13}C NMR δc (mult.) ppm	^{1}H NMR δ_H (Integral, mult, *J*=Hz) ppm
1	37.4	1.08 (1H, m) 1.84 (1H, m)	37.2 (t)	1.08 (1H, m) 1.84 (1H, m)
2	31,8	1.49 (1H, m) 1.81 (1H, m)	31.6 (t)	1.49 (1H, m) 1.81 (1H, m)
3	72.0	3.52 (1H, m)	71.8 (d)	3.53 (1H, m)
4	42.5	2.28 (1H, dd, 5.2, 2.0) 2.30 (1H, dd, 5.2, 2.0)	42.3 (t)	2.24 (1H, dd, 4.7, 1.9) 2.27 (1H, dd, 4.7, 1.9)
5	140.9	-	140.7 (s)	-
6	121.9	5.35 (1H, m)	121.7 (d)	5.35 (1H, d, 4.7)
7	32.1	1.54 (1H, m) 1.96 (1H, m)	31.9 (t)	1.53 (1H, m) 1.98 (1H, m)
8	32.0	1.46 (1H, m)	31.9 (d)	1.46 (1H, m)
9	50.3	0.94 (1H, m)	50.1 (d)	0.94 (1H, m)
10	36.7	-	36.5 (s)	-
11	21.3	1.46 (1H, m) 1.49 (1H, m)	21.6 (t)	1.45 (1H, m) 1.48 (1H, m)
12	39.9	1.15 (1H, m) 1.95 (1H, m)	39.7 (t)	1.15 (1H, m) 1.97 (1H, m)
13	42.5	-	42.2 (s)	-
14	56.9	1.03 (1H, m)	56.8 (d)	1.00 (1H, m)
15	24.5	1.07 (1H, m) 1.56 (1H, m)	24.4 (t)	1.06 (1H, m) 1.55 (1H, m)
16	28.4	1.26 (1H, m) 1.67 (1H, m)	28.9 (t)	1.27 (1H, m) 1.71 (1H, m)
17	56.1	1.13 (1H, m)	55.9 (d)	1.13 (1H, m)
18	12.1	0.67 (3H, s)	12.0 (q)	0.70 (3H, s)
19	19.5	1.00 (3H, s)	19.4 (q)	1.01 (3H, s)
20	40.7	2.02 (1H, m)	40.5 (d)	2.04 (1H, m)
21	21.2	0.92 (3H, d, 6.85)	21.1 (q)	1.02 (3H, d, 6.8)
22	138.5	5.16 (1H, dd, 15.0, 8.5)	138.3 (d)	5.15 (1H, dd, 15.1, 8.4)
23	129.5	5.00 (1H, dd, 15.0, 8.5)	129.2 (d)	5.02 (1H, dd, 15.1, 8.4)
24	51.4	1.53 (1H, m)	51.2 (d)	1.53 (1H, m)
25	31.8	1.45 (1H, m)	31.9 (d)	1.44 (1H, m)
26	21.3	0.84 (3H, d, 6.4)	21.2 (q)	0.84 (3H, d, 6.4)
27	19.1	0.82 (3H, d, 6.1)	19.0 (q)	0.83 (3H, d, 6.1)
28	25.6	1.15 (1H, t, 3.2)	25.4 (d)	1.15 (1H, t, 3.1)
29	12.2	0.80 (3H, t, 6.0)	12.3 (q)	0.80 (3H, t, 6.0)

($CDCl_3$; ^{1}H-NMR 500 MHz; ^{13}C-NMR 125 MHz)

Cytotoxic Activity

The cytotoxic effects of compounds **1**, **2** and **3** against HeLa cervical cancer cells were conducted according to the resazurin assay method and were used cisplatin with IC_{50} 9.05 μM as a positive control. Treatment with compounds **1**, **2** and **3** resulted in the dose dependent inhibition of HeLa cervical cancer cell growth when assessed at 24 hours post-treatment. Ergosterol peroxide (**2**) has a strongest activity with an IC_{50} 0.28 μM compared to ergosterol which has an

IC_{50} 0.34 µM and stigmasterol which has an IC_{50} 27.32 µM. This shows that the peroxide groups bonded to C-5 and C-8 are important structural element to cytotoxic activity against cervical HeLa cancer cell as well as induce apoptosis of cancer cells (Jeong & Park, 2020), whereas ergosterol peroxide nontoxic to normal cells but it was sensitive to cancer cells (Wu et al., 2018).

CONCLUSIONS

Three steroid compounds, ergosterol (**1**), ergosterol peroxide (**2**) and stigmasterol **(3)** have been isolated from endophytic fungus, *L. theobromae* derived from the root of *A. argentea* Blume. Ergosterol peroxide (**2**) and stigmasterol **(3)** were shown for the first time in produced from endophytic fungus, *L. theobromae*. The peroxide groups play an important role for cytotoxic activity against HeLa cervical cancer cells.

ACKNOWLEDGMENTS

The authors are grateful to Mr. Kansi Haikal at the Center Laboratory of Universitas Padjadjaran for performing the HR-ESITOFMS measurement. The authors are grateful to Universitas Padjadjaran, Indonesia for supporting this investigation financially through Academic Leadership Grant (No: 1549/UN6.3.1/PT.00/2023 by Unang Supratman.

REFERENCES

Arora, J., & Ramawat, K. G. (2017). An introduction to endophytes. *Endophytes: Biology and Biotechnology*. *Sustainable Development and Biodiversity*, *15*, 1–23. https://doi.org/10.1007/978-3-319-66541-2_1

Brader, G., Vajrodaya, S., Greger, H., Bacher, M., Kalchhauser, H., Hofer, O., (1998). Bisamides, lignans, triterpenes, and insecticidal cyclopenta[b]benzofurans from *Aglaia* species. *Journal of Natural Product*, *61*, 1482-1490. https://doi.org/10.1021/np9801965

Brakhage, A. A. (2013). Regulation of fungal secondary metabolism. In *Nature Reviews Microbiology*. *11*(1), 21–32. https://doi.org/10.1038/nrmicro2916

Cayme, J.-M. C., & Ragasa, C. Y. (2004). Structure elucidation of β-stigmasterol and β-sitosterol from *Sesbania grandifora* [Linn.] Pers. and β-carotene from *Heliotropium indicum* Linn. by NMR spectroscopy. *KIMIKA*. *20*(1), 5-12. https://doi.org/10.26534/kimika.v20i1.5-12

Farabi, K., Harneti, D., Nurlelasari, Maharani, R., Hidayat, A. T., Supratman, U., Awang, K., & Shiono, Y. (2017). Cytotoxic steroids from the bark of *Aglaia argentea* (Meliaceae). *Chiang Mai University Journal of Natural Sciences*. *16*(4), 293–306. https://doi.org/10.12982/CMUJNS.2017.0024

Gao, Y., Stuhldreier, F., Schmitt, L., Wesselborg, S., Guo, Z., Zou, K., Mándi, A., Kurtán, T., Liu, Z., & Proksch, P. (2020). Induction of new lactam derivatives from the endophytic fungus *Aplosporella javeedii* through an OSMAC approach. *Frontiers in Microbiology*. *11*, 1-13. https://doi.org/10.3389/fmicb.2020.600983

Gunatilaka, A. A. L. (2006). Natural products from plant-associated microorganisms: Distribution, structural diversity, bioactivity, and implications of their occurrence. In *Journal of Natural Products*. *69*(3), 509–526. https://doi.org/10.1021/np058128n

Hemphill, C. F. P., Sureechatchaiyan, P., Kassack, M. U., Orfali, R. S., Lin, W., Daletos, G., & Proksch, P. (2017). OSMAC approach leads to new fusarielin metabolites from *Fusarium tricinctum*. *Journal of Antibiotics*. *70*(6), 726–732. https://doi.org/10.1038/ja.2017.21

Jeong, Y. U., & Park, Y. J. (2020). Ergosterol peroxide from the medicinal mushroom ganoderma lucidum inhibits differentiation and lipid accumulation of 3T3-L1 adipocytes. *International Journal of Molecular Sciences*. *21*(460), 1-13 https://doi.org/10.3390/ijms21020460

Kharwar, R. N., Mishra, A., Gond, S. K., Stierle, A., & Stierle, D. (2011). Anticancer compounds derived from fungal endophytes: Their importance and future challenges. In *Natural Product Reports* . *28*(7), 1208–1228. https://doi.org/10.1039/c1np00008j

Kurniasih, N., Supriadin, A., Harneti, D., Abdulah, R., Taib, M. N. A. bin M., & Supratman, U. (2021). Ergosterol peroxide and stigmasterol from the stembark of *Aglaia simplicifolia* (Meliaceae) and their cytotoxic against HeLa cervical cancer cell Lines. *Jurnal Kimia Valensi*. *7*(1), 46–51. https://doi.org/10.15408/jkv.v7i1.20068

Martinez, A., Martinez, M., Torrez Alvarez, S., Campi, M. G., Bravo, J. A., Vila, J. L., & Bello, A. (2015). Ergosterol from the mushroom Laetiporus sp.; Isolation and structural characerization. In *Bolivian Journal of Chemistry*. *32*(4), 90-94. http://www.bolivianchemistryjournal.org,http://www.scribd.com/bolivianjournalofchemistry

Nowak, R., Drozd, M., Mendyk, E., Lemieszek, M., Krakowiak, O., Kisiel, W., Rzeski, W., & Szewczyk, K. (2016). A new method for the isolation of ergosterol and peroxyergosterol as active compounds of hygrophoropsis aurantiaca and in vitro antiproliferative activity of isolated ergosterol peroxide. *Molecules*. *21*(7), 946. https://doi.org/10.3390/molecules21070946

Rodrigues, M. L. (2018). The multifunctional fungal ergosterol. *mBio*, *9*(5), e01755-18, 1-5.

American Society for Microbiology. https://doi.org/10.1128/mBio.01755-18

Sari, A. P., Nurlelasari, Azhari, A., Harneti, D., Maharani, R., Mayanti, T., Farabi, K., Darwati, Supratman, U., Fajriah, S., Azmi, M. N., & Shiono, Y. (2022). New ergostane-type sterol produced by an endophytic fungus *Fusarium phaseoli* isolated from *Chisocheton macrophyllus* (Meliaceae). *Records of Natural Products*, *16*(6), 614–621. https://doi.org/10.25135/rnp.334.2203.2387

Shiono, Y., Sasaki, T., Shibuya, F., Yasuda, Y., Koseki, T., & Supratman, U. (2013). Isolation of a phomoxanthone a derivative, a new metabolite of tetrahydroxanthone, from a Phomopsis sp. isolated from the mangrove, *Rhizhopora mucronata*. In *NPC Natural Product Communications*. *8*(12), 1735-1737. https://doi.org/10.1177/1934578X1300801220

Supratman, U., Hirai, N., Sato, S., Watanabe, K., Malik, A., Annas, S., Harneti, D., Maharani, R., Koseki, T., & Shiono, Y. (2021). New naphthoquinone derivatives from *Fusarium napiforme* of a mangrove plant. *Natural Product Research*, *35*(9), 1406–1412. https://doi.org/10.1080/14786419.2019.1650358

Supratman, U., Suzuki, T., Nakamura, T., Yokoyama, Y., Harneti, D., Maharani, R., Salam, S., Abdullah, F. F., Koseki, T., & Shiono, Y. (2021). New metabolites produced by endophyte *Clonostachys rosea* B5 – 2. *Natural Product Research*, *35*(9), 1525–1531. https://doi.org/10.1080/14786419.2019.1656629

Wu, H. Y., Yang, F. L., Li, L. H., Rao, Y. K., Ju, T. C., Wong, W. T., Hsieh, C. Y., Pivkin, M. V., Hua, K. F., & Wu, S. H. (2018). Ergosterol peroxide from marine fungus *Phoma* sp. induces ROS-dependent apoptosis and autophagy in human lung adenocarcinoma cells. *Scientific Reports*. *8*(1), 1-14. https://doi.org/10.1038/s41598-018-36411-2