Cytotoxic Steroids from the Bark of *Aglaia argentea* (Meliaceae)

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ABSTRACT

The study aimed to find a potential anticancer agent by isolating and identifying the chemical structure of compounds from Aglaia argentea and testing their cytotoxic effects against P-388 murine leukimia cells. Five steroids – stigmast-5-en-3β-ol (β-sitosterol) (1), stigmast-5-en-3β-ol-3β-oleate (β-sitosterol oleate) (2), stigmast-5-en-3β-ol-3-O-(6'-O-oleoyl)-β-D-glucopyranoside (sitoindoside II) (3), stigmast-5-en-3β-ol-3-O-β-D-glucopyranoside (β-sitosterol glucoside) (4), stigmast-5,22-dien-3β-ol-3-O-β-D-glucopyranoside (stigmasterol glucoside) (5) – were isolated from the bark of Aglaia argentea. The chemical structures of 1-5 were identified with spectroscopic data, including IR, NMR (^{1}H, ^{13}C, DEPT 135°, HMQC, HMBC, ^{1}H-^{1}H COSY) and HRTOFMS, as well as by comparing with previously reported spectral data. All compounds were evaluated for their cytotoxic effects against P-388 murine leukemia cells. Compounds 1-5 showed cytotoxicity against P-388 murine leukemia cell with IC_{50} values of 12.45 ± 0.050, 85.25 ± 0.050, >100, 52.27 ± 0.031 and 62.52 ± 0.076 μg/mL, respectively.

Keywords: *Aglaia argentea*, Cytotoxic activity, Meliaceae, P-388 murine leukemia cells, Sterol

INTRODUCTION

Sterols, a type of steroid, are an important class of bioorganic molecules similar to cholesterol in structure and found widely in plants, animals, and fungi (Saeidnia et al., 2014). They include β-sitosterol, campesterol, stigmasterol, and

cycloartenol (Ostlund et al., 2002). Sterols, especially β-sitosterol, have been reported to have the following activities or effects: anti-inflammatory (Prieto et al., 2006), inducing apoptosis (Ju et al., 2004; Park et al., 2007; Chai et al., 2008), chemoprotective or chemopreventive (Ovesna et al., 2004), hypocholesterolemic (Zak et al., 1990), angiongenic (Moon et al., 1999), anti-diabetic (Jamaluddin et al., 1994; Gupta et al., 2011; Radika et al., 2013), and anti-oxidant (Vivancos and Moreno, 2005; Baskar et al., 2012).

Aglaia is the largest genus of the family Meliaceae; it includes more than 100 species, distributed mainly in India, Indonesia, Malaysia and parts of the Western Pacific (Pannell, 1992; Inada et al., 2001). *Aglaia argentea*, also known as *langsat hutan* in Indonesia, is a higher plant traditionally used to moisturize the lungs, reduce fever, and treat contusions, coughs and skin diseases (Hidayat and Hutapea, 1991; Mabberley et al., 1995; Muellner et al., 2010). Previous phytochemical studies on the genus *Aglaia* have revealed the presence of a variety of compounds with interesting biological activities, including recoglamides (Ishibashi et al., 1993; Wu et al., 1997; Nugroho et al., 1999), triterpenoid bisamides (Brader et al., 1998), dammarane-type triterpenoids (Roux et al., 1998; Khalit et al., 1999; Xie et al., 2007; Zhang et al., 2010; Harneti et al., 2012), and cycloartane-type triterpenoids (Khalit et al., 1999; Awang et al., 2012).

As part of our studies on anticancer candidate compounds from Indonesian *Aglaia* plants, we isolated and described cytotoxic triterpenoids from the bark of *A. smithii* and *A. eximia* (Harneti et al., 2012; 2014), as well as a lignan and bisamides from the bark of *A. eximia* (Sianturi et al., 2015; 2016). In the further screening for cytotoxic compounds from Indonesian *Aglaia species*, we found that *n*-hexane and ethyl acetate extracts of the bark of *A. argentea* exhibited a cytotoxic activity against P-388 murine leukemia cells with IC_{50} of 26.72 ± 0.02 and 15.48 ± 0.03 µg/mL. We report herein the isolation and structure elucidation of five steroids together with their cytotoxic activity against P-388 murine leukimia cells.

MATERIALS AND METHODS

General

Melting points were measured on an electrothermal melting point apparatus and are uncorrected. 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, with TMS as internal standard. Column chromatography was conducted on silica gel 60 (Kanto Chemical Co., Inc., Japan). TLC plates were precoated with silica gel GF_{254} (Merck, 0.25 mm) and detection was achieved by spraying with 10% H_2SO_4 in ethanol, followed by heating.

Plant material

The bark of *A. argentea* was collected in Bogor Botanical Garden, Bogor, West Java Province, Indonesia in June 2015. The plant was identified by the staff

of the Bogoriense Herbarium, Bogor, Indonesia and a voucher specimen (No. Bo-1288718) was deposited at the herbarium.

Extraction

The dried bark (2.5 kg) was extracted with methanol (12 L) at room temperature for 5 days. After removal of the solvent under vacuum, the viscous concentrated extract of MeOH (133.5 g) was first suspended in H_2O and then partitioned with *n*-hexane, EtOAc, and *n*-BuOH, successively. Evaporation resulted in crude extracts of *n*-hexane, EtOAc, and *n*-BuOH. All the extracts were tested for their cytotoxic activity against P-388 murine leukemia cells.

Determination of cytotoxic activities

The P-388 cells were seeded into 96-well plates at an initial cell density of approximately 3 x 10^4 cells cm^{-3}. After 24 h of incubation for cell attachment and growth, varying concentrations of samples were added. Before adding, the compounds were dissolved in DMSO at the required concentration. The subsequent six desired concentrations were prepared using PBS (phosphoric buffer solution, pH = 7.30 - 7.65). Control wells received only DMSO. The assay was terminated after a 48 h incubation period by adding MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; also named thiazol blue] and the incubation was continued for another 4 h during 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

Extraction and isolation

The crude extracts of *n*-hexane (26.3 g), EtOAc (12.4 g), and *n*-BuOH (12.6 g) were tested for their cytotoxic activity against P388 murine leukemia cells and showed cytotoxic activity with IC_{50} values of 26.72 ± 0.02, 15.49 ± 0.03, and 85.67 ± 0.02 µg/mL, respectively. The *n*-hexane soluble fraction (26.3 g) was fractionated by vacuum liquid chromatography on silica gel 60 using a gradient *n*-hexane and EtOAc to give nine fractions (A–I), combined according to TLC results. Fraction A (6 g) was chromatographed on a column of silica gel, eluted successively with a gradient of *n*-hexane–CH_2Cl_2 (10:0–1:1) to give ten subfractions (A01–A10). Subfraction A03 was chromatographed on a column of silica gel, eluted with *n*-hexane:$CHCl_3$ (9:1) to give **2** (74.5 mg). Fraction C (2.68 g) was chromatographed on a column of silica gel, eluted successively with a gradient of *n*-hexane–EtOAc (10:0–4:1) to give nine subfractions (C01-C9). Subfraction C03 was recrystallized in *n*-hexane, to give **1** (188.6 mg). The EtOAc soluble fraction (12.4 g) was fractionated by column chromatography on silica gel using

a gradient *n*-hexane and EtOAc to give eight fractions (J–Q), combined according to TLC results. Fraction K (927.6 mg) was chromatographed on a column of silica gel, eluted successively with a gradient of *n*-hexane–EtOAc (10:0–0:10) to give **3** (106.3 mg). Fraction P (3.86 g) was chromatographed on a column of silica gel, eluted successively with a gradient of $CHCl_3$-Me_2CO (10:0–4:1), to give seven subfractions (P01-P07). Subfraction P05 was chromatographed on a column of silica gel, eluted with *n*-hexane-Me_2CO (10:0-0:10) to give **4** (5 mg) and 5 (3 mg).

Stigmast-5-en-3β-ol (1). White needle-like crystals; m.p. 134-136 °C; IR (KBr) ν_{max} 3424, 2937, 2870, 1464, 1379, 1056 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) see Table 1; ^{13}C-NMR ($CDCl_3$, 150 MHz), see Table 1; TOFMS (negative ion mode) *m/z* 413.0811 $[M-H]^-$, (calcd. $C_{29}H_{49}O^-$, *m/z* 413.3789).

Stigmast-5-en-3β-ol-3β-oleate (2). White waxy solid; IR (KBr) ν_{max} 2937, 2870, 1710, 1620, 1464, 1379, 1056 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz) see Table 1; ^{13}C-NMR ($CDCl_3$, 150 MHz), see Table 1; TOFMS (positive ion mode) *m/z* 679.6013 $[M+H]^+$, (calcd. $C_{47}H_{83}O_2^+$, *m/z* 679.6388).

Stigmast-5-en-3β-ol-3-*O*-(6′-*O*-oleoyl)-β-D-glucopyranoside (3). White waxy solid; IR (KBr) ν_{max} 3425, 3342, 1707, 1566, 1292, 1020 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 600 MHz), see Table 1; ^{13}C-NMR ($CDCl_3$, 150 MHz), see Table 1.

Stigmast-5-en-3β-ol-3-*O*-β-$_D$-glucopyranoside (4). White amorphous powder; m.p. (decomposed); IR (KBr) ν_{max} 3433, 1639, 1461, 1380, 1053 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 600 MHz), see Table 2; ^{13}C-NMR (pyridine-d_5, 150 MHz), see Table 2.

Stigmast-5,22-dien-3β-ol-3-*O*-β-D-glucopyranoside (5). White amorphous powder; m.p. (decomposed); IR (KBr) ν_{max} 3450, 1630, 1445, 1370, 1050 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 600 MHz), see Table 2; ^{13}C-NMR (pyridine-d_5, 150 MHz), see Table 2.

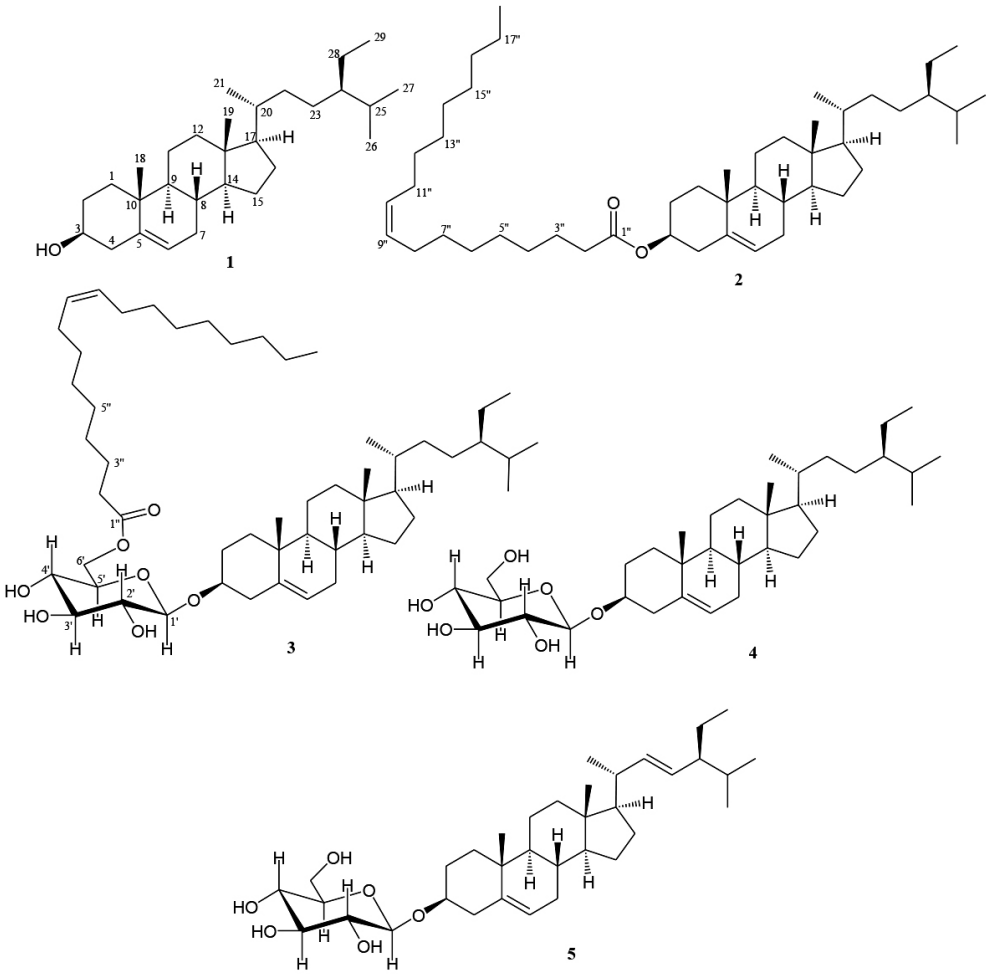

Figure 1. Structures of compounds **1-5**.

Table 1. NMR data (600 MHz for ^{1}H and 150 MHz for ^{13}C, in $CDCl_3$) for **1-3**.

Position	1		2		3	
	^{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)	^{13}C NMR δc (mult.)	^{1}H NMR δ_H (Int., mult, *J*=Hz)
1	37.4 (t)	1.68 (2H, m)	37.1 (t)	1.85 (2H,m)	37.4 (t)	1.82 (2H, dd, 3.6, 9.6)
2	31.8 (t)	1.50 (2H, m)	32.1 (t)	1.97 (2H,m)	32.0 (t)	1.95 (1H, m)
						1.45 (1H, t, 10.3)
3	72.0 (d)	3.52 (1H, m)	73.7 (d)	4.59 (1H, m)	79.8 (d)	3.51 (1H, m)
4	42.3 (t)	2.23 (2H, m)	39.8 (t)	1.99 (2H, m)	39.0 (t)	2.35 (1H, d, 10.3)
						2.24 (1H, dt, 3.2, 10.3)
5	140.9 (s)	-	139.8 (s)	-	140.4 (s)	-
6	121.9 (d)	5.35 (1H, t, 5.2)	122.7 (d)	5.35 (1H, s)	122.2 (d)	5.33 (1H, d, 3.3)
7	32.1 (t)	1.99 (2H, m)	32.0 (t)	1.93 (2H, m)	29.4 (t)	1.60 (1H, m)
						1.92 (1H, m)
8	32.1 (d)	1.43 (1H, m)	31.9 (d)	1.49 (1H, m)	31.9 (d)	1.46 (1H, m)
9	50.3 (d)	0.82 (1H, m)	50.1 (d)	0.93 (1H, m)	50.2 (d)	0.89 (1H, m)
10	36.7 (s)	-	36.7 (s)	-	36.8 (s)	-
11	21.3 (t)	1.46 (2H, m)	21.1 (t)	1.47 (2H, m)	21.2 (t)	1.48 (2H, m)
12	39.9 (t)	1.23 (2H, m)	38.2 (t)	2.29 (2H, m)	39.8 (t)	2.00 (1H, m)
						1.12 (1H, m)
13	42.4 (s)	-	42.4 (s)	-	42.4 (s)	-
14	56.9 (d)	0.95 (1H, m)	56.7 (d)	1.00 (1H, m)	56.8 (d)	0.97 (1H, m)
15	26.2 (t)	1.58 (2H, m)	24.4 (t)	0.98 (2H, m)	24.4 (t)	1.59 (2H, m)
16	28.4 (t)	1.16 (2H, m)	28.3 (t)	1.83 (2H, m)	28.3 (t)	1.81 (2H, m)
17	56.2 (d)	1.10 (1H, m)	56.1 (d)	1.07 (1H, m)	56.2 (d)	1.07 (1H, m)
18	12.0 (q)	1.00 (3H, s)	11.9 (q)	0.65 (3H, s)	11.9 (q)	0.66 (3H, s)
19	19.0 (q)	0.68 (3H, s)	19.4 (q)	1.00 (3H, s)	19.5 (q)	0.98 (3H, s)
20	36.3 (d)	1.86 (1H, m)	36.2 (d)	1.31 (1H, m)	36.3 (d)	1.32 (1H, m)
21	19.2 (q)	0.92 (3H, d, 6.2)	18.9 (q)	0.90 (3H, d, 3.6)	18.9 (q)	0.90 (3H, d, 6.0)
22	34.1 (t)	1.05 (2H, m)	33.9 (t)	1.23 (2H, m)	34.0 (t)	0.99 (2H, m)
23	26.2 (t)	1.07 (2H, m)	26.0 (t)	1.14 (2H, m)	26.2 (t)	1.15 (2H, m)
24	45.9 (d)	1.52 (1H, m)	45.9 (d)	0.91 (1H, m)	45.9 (d)	0.92 (1H, m)
25	29.3 (d)	1.60 (1H, m)	29.2 (d)	1.29 (2H, m)	29.2 (d)	1.60 (1H, m)
26	19.6 (q)	0.83 (3H, d, 6.5)	19.9 (q)	0.85 (3H, d, 6.6)	19.9 (q)	0.81 (3H, d, 5.0)
27	20.0 (q)	0.79 (3H, d, 5.2)	19.1 (q)	0.81 (3H, d, 6.6)	19.1 (q)	0.79 (3H, d, 5.0)
28	23.2 (t)	1.27 (2H, m)	23.1 (t)	1.25 (2H, m)	23.1 (t)	1.29 (2H, m)
29	12.2 (q)	0.84 (3H, t, 5.2)	12.1 (q)	0.83 (3H, t, 1.8)	12.1 (q)	0.83 (3H, t, 2.8)
1′					101.3 (d)	4.36 (1H, d, 8.2)
2′					73.5 (d)	3.33 (1H, dd, 8.2, 9.0)
3′					73.9 (d)	3.45 (1H, dd, 7.1, 9.0)
4′					76.2 (d)	3.53 (1H, dd, 7.1, 7.5)
5′					70.4 (d)	3.35 (1H, dd, 7.5, 7.7)
6′					63.6 (t)	4.28 (2H, ddd, 3.4, 5.0, 7.7)
1″			173.4 (s)	-	174.5 (s)	-
2″			34.8 (t)	2.25 (2H, t, 7.8)	34.4 (t)	2.30 (2H, t, 7.5)
3″			25.5 (t)	1.59 (2H, m)	25.1 (t)	1.58 (2H, m)
4″			29.2 (t)	1.29 (2H, m)	29.3 (t)	1.29 (2H, m)
5″			29.6 (t)	1.23 (2H, m)	29.7 (t)	1.23 (2H, m)
6″			29.4 (t)	1.23 (2H, m)	29.5 (t)	1.23 (2H, m)
7″			29.8 (t)	1.23 (2H, m)	29.9 (t)	1.23 (2H, m)
8″			27.2 (t)	1.98 (2H, m)	27.3 (t)	1.98 (2H, m)
9″			129.9 (d)	5.32 (1H, dd, 3.2, 9.5)	130.1 (d)	5.32 (1H, dd, 3.2, 9.5)
10″			130.1 (d)	5.32 (1H, dd, 3.2, 9.5)	130.2 (d)	5.32 (1H, dd, 3.2, 9.5)
11″			27.3 (t)	1.98 (2H, m)	27.4 (t)	1.98 (2H, m)
12″			29.9 (t)	1.23 (2H, m)	29.9 (t)	1.23 (2H, m)
13″			29.5 (t)	1.23 (2H, m)	29.6 (t)	1.23 (2H, m)
14″			29.7 (t)	1.23 (2H, m)	29.8 (t)	1.23 (2H, m)
15″			29.3 (t)	1.29 (2H, m)	29.4 (t)	1.29 (2H, m)
16″			27.9 (t)	1.85 (2H, m)	27.5 (t)	1.85 (2H, m)
17″			22.8 (t)	1.26 (2H, m)	22.8 (t)	1.26 (2H, m)
18″			14.3 (q)	0.86 (3H, t, 6.6)	14.2 (q)	0.86 (3H, t, 6.0)

Table 2. NMR data (600 MHz for ^{1}H and 150 MHz for ^{13}C, in pyridine-d_5) for **4** and **5**.

Position	4		5	
	^{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	37.3 (t)	1.83 (2H, t, 1.8)	37.3 (t)	1.83 (2H, m)
2	31.9 (t)	1.90 (1H, dd, 3.0, 9.0) 2.42 (1H, t, 10.8)	31.9 (t)	1.90 (1H, dt, 2.4, 11.4) 2.42 (1H, t, 11.4)
3	78.5 (d)	3.90 (1H, m)	78.5 (d)	3.90 (1H, m)
4	39.2 (t)	2.06 (1H, dd, 1.8, 12.8) 2.68 (1H, dd, 1.8, 12.8)	39.2 (t)	2.06 (1H, d, 11.4) 2.67 (1H, dt, 2.4, 11.4)
5	140.7 (s)	-	140.7 (s)	-
6	121.8 (d)	5.28 (1H, d, 4.8)	121.8 (d)	5.28 (1H, d, 3.6)
7	29.2 (t)	1.76 (2H, m)	29.3 (t)	1.80 (2H, m)
8	32.0 (d)	1.69 (1H, m)	32.0 (d)	1.69 (1H, m)
9	50.1 (d)	0.96 (1H, m)	50.2 (d)	1.00 (1H, m)
10	36.7 (s)	-	36.7 (s)	-
11	21.1 (t)	1.47 (2H, m)	21.1 (t)	1.47 (2H, m)
12	39.7 (t)	1.30 (2H, m)	39.8 (t)	1.32 (2H, m)
13	42.3 (s)	-	42.3 (s)	-
14	56.6 (d)	1.04 (1H, m)	56.7 (d)	1.05 (1H, m)
15	24.3 (t)	1.49 (2H, m)	24.4 (t)	1.48 (2H, m)
16	28.4 (t)	1.53 (2H, m)	28.4 (t)	1.66 (2H, m)
17	56.0 (d)	1.20 (1H, m)	56.1 (d)	1.19 (1H, m)
18	11.8 (q)	0.58 (3H, s)	11.8 (q)	0.59 (3H, s)
19	19.8 (q)	0.86 (3H, s)	19.8 (q)	0.86 (3H, s)
20	36.2 (d)	1.36 (1H, m)	36.2 (d)	1.36 (1H, m)
21	19.0 (q)	0.91 (3H, d, 6.0)	19.0 (q)	0.92 (3H, d, 6.6)
22	34.0 (t)	1.17 (2H, m)	138.7 (d)	4.98 (1H, dd, 8.4, 15.0)
23	26.1 (t)	1.29 (2H, m)	129.3 (d)	5.13 (1H, dd, 8.4, 15.0)
24	45.8 (d)	1.01 (1H, m)	45.8 (d)	1.01 (1H, m)
25	30.1 (d)	1.59 (1H, m)	30.1 (d)	1.63 (1H, m)
26	19.3 (q)	0.80 (3H, d, 5.0)	19.3 (q)	0.81 (3H, d, 4.9)
27	18.8 (q)	0.78 (3H, d, 5.0)	18.8 (q)	0.79 (3H, d, 4.9)
28	23.2 (t)	1.24 (2H, m)	23.2 (t)	1.25 (2H, m)
29	12.0 (q)	0.83 (3H, t, 2.5)	12.0 (q)	0.83 (3H, t, 2.4)
1′	102.4 (d)	4.53 (1H, d, 7.5)	102.4 (d)	4.52 (1H, d, 8.5)
2′	75.2 (d)	4.25 (1H, dd, 7.5, 8.3)	75.2 (d)	4.22 (1H, dd, 8.5, 9.3)
3′	77.7 (d)	3.95 (1H, dd, 7.7, 8.3)	77.7 (d)	3.94 (1H, dd, 7.3, 9.3)
4′	78.4 (d)	4.03 (1H, dd, 7.4, 7.7)	78.4 (d)	4.02 (1H, dd, 7.3, 7.5)
5′	71.5 (d)	4.39 (1H, d, 7.4)	71.5 (d)	4.37 (1H, dd, 7.5, 7.7)
6′	62.6 (t)	4.27 (2H, ddd, 3.2, 5.0, 12.0)	62.6 (t)	4.25 (2H, ddd, 3.1, 5.0, 7.7)

The cytotoxicity effects of the five isolated compounds against P-388 murine leukemia cells were conducted according to the method described previously (Harneti et al., 2012; Sahidin et al., 2005; Alley et al., 1988); artonin E (IC_{50} 0.3 μg/mL) was used as the positive control (Hakim et al., 2007). The cytotoxicity activities of the isolated compounds **1-5** are shown in Table 3.

Table 3. Cytotoxicity activity of compounds **1-5** against P-388 murine leukemia cells.

Compounds	IC_{50} (µg/mL)
stigmast-5-en-3β-ol **(1)**	12.45 ± 0.050
stigmast-5-en-3β-ol-3β-oleate **(2)**	85.25 ± 0.050
stigmast-5-en-3β-ol-3-*O*-(6′-O-oleoyl)-β-D-glucopyranoside **(3)**	> 100
stigmast-5-en-3β-ol-3-*O*-β-D-glucopyranoside **(4)**	52.27 ± 0.031
stigmast-5,22-dien-3β-ol-3-*O*-β-D-glucopyranoside **(5)**	62.52 ± 0.076

DISCUSSION

The phytochemical test for the *n*-hexane and EtOAc extract showed the presence of steroids. By using cytotoxic assay to guide separations, the *n*-hexane and EtOAc fraction was separated by column chromatography over silica gel by gradient elution. The fractions were repeatedly subjected to normal-phase column chromatography and preparative TLC on silica gel GF_{254} yielded five cytotoxic steroids **1-5** (Figure 1).

Compound **1** was obtained as a white needle-like crystal. The TOFMS spectrum showed $[M-H]^+$ *m/z* 413.0811 (calcd. *m/z* 413.3789), which coresponded to the molecular formula $C_{29}H_{50}O$ and thus required five degrees of unsaturation, originating from one pair of C sp^2 and the remaining tetracyclic stigmastane-type steroid. The IR spectra showed absorption peaks at 3,424 cm^{-1} (OH), 2,937 and 2,870 cm^{-1} (C-H sp^3), 1,464 cm^{-1} (C=C), 1379 cm^{-1} (*gem*-dimethyl groups), and 1,056 cm^{-1} (C-O). The ^{1}H-NMR ($CDCl_3$ 600 MHz) spectrum showed the presence of six methyl groups: two tertiary methyl groups resonating at δ_H 1.00 (CH_3-18) and 0.68 (CH_3-19); three secondary methyl groups resonating at δ_H 0.92 (3H, d, J = 6.2 Hz, CH_3-21), 0.83 (3H, d, J = 6.5 Hz, CH_3-26), and 0.81 (d, J = 5.2 Hz, CH_3-27); and one primary methyl group resonating at δ_H 0,84 (t, J = 5.2 Hz, CH_3-29); this was indicative of the presence of stigmastane-type steroid skeleton. One olefinic methine group, resonating at δ_H 5.35 (d, J = 5.2 Hz, H-6) and an oxymethine group resonating at δ_H 3.52 (1H, m, H-3) were also observed in the ^{1}H NMR spectra. Proton pairing was also confirmed with the ^{1}H-^{1}H COSY spectrum (Figure 2). ^{1}H-^{1}H COSY cross peak observed at H-2/H-3/H-4 indicated that the hydroxy group was positioned at C-3. The cross peak also observed at H-6/H-7/H-8 indicated the position of a double bond at C-5/C-6. The ^{13}C-NMR ($CDCl_3$ 150 MHz) and DEPT 135° spectra showed the presence of six methyl groups: one olefinic methine, one olefinic quartenary carbon, and an oxygenated methine group, resonating at δ_C 72.0 (C-3); this indicated that had the characteristic of a stigmastane-type steroid (Cayme and Ragasa, 2004). These functionalities accounted for one of five degrees of unsaturation. The remaining four degrees of unsaturation were consistent with the stigmastane-type steroid. A comparison of the NMR data of **1** with the data for β-sitosterol (Chaturvedula and Prakash, 2012), revealed that the structure of the two compounds were very similar; con-

sequently, compound **1** was identified as stigmast-5-en-3β-ol (β-sitosterol); *m/z* 679.6013 $[M+H]^+$, (calcd $C_{47}H_{83}O_2^+$, *m/z* 679.6388).

Compound **2** was obtained as a white waxy solid. The TOFMS spectrum showed [M+H]+ *m/z* 679.6013 (calcd. $C_{47}H_{82}O_2$, *m/z* 678.6388); the fragment ion peaks occurred at *m/z* 415.2132 [M+H-265], indicating the loss of a fatty acid (terminal oleate acid unit), which coresponded to the molecular formula $C_{29}H_{50}O$ and thus required five degrees of unsaturation. The ^1H and ^{13}C-NMR spectrum of **2** resembled that of **1** – the main difference was that compound **2** was substituted with a fatty acid in the ester linkage. The position of the fatty acid was determined by HMBC correlation (Figure 2). The presence of a fatty acid (oleate acid) attached at C-3 in ester form was supported by HMBC correlation from oxymethine proton at H-3 (δ_H 4.59) to C-1″ (δ_C 173.4) in the ester group, and correlation at H-2″ (δ_H 2.25) to C-1″ (δ_C 173.4). The position of the double bond in oleic acid at C-9″/C-10″ was evidenced by HMBC correlation from H-9″ (δ_H 5.32) to C-6″ (δ_C 29.4) and C-7″ (δ_C 29.8). This correlation also supported the appearance of oleate acid in mass spectra, which appeared at molecular ion peak *m/z* 265. The coupling constant of H-9/H-10 was 9.5 Hz; this indicated that each proton was in *cis* position. Compound **2** agreed well with data from the literature (Tesemma et al., 2013; Ragasa et al., 2016), supporting its identification as stigmast-5-en-3β-ol-3β-oleate (β-sitosterol oleate).

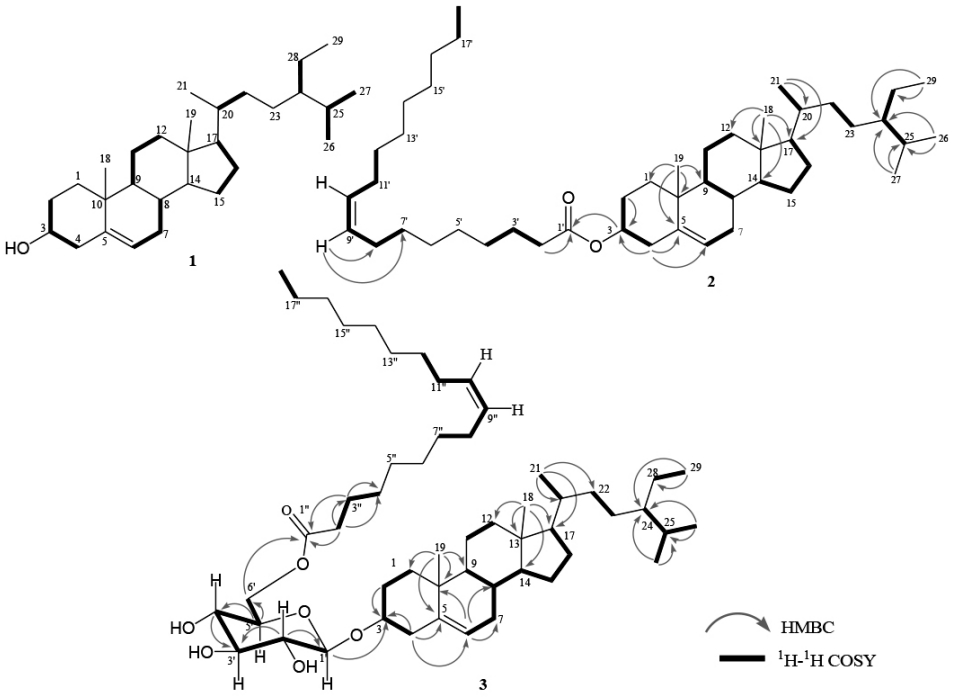

Figure 2. Selected HMBC and ^1H-^1H COSY correlations for **1, 2,** and **3**.

Compound **3** was obtained as a white waxy solid. Its molecular composition $C_{53}H_{92}O_7$, was established from NMR data (Table 1). The IR spectrum suggested the presence of hydroxyl (3,425 cm^{-1}), carbonyl (1,707 cm^{-1}), olefinic (1,566 cm^{-1}) and ether groups (1,292 and 1,020 cm^{-1}). The ^{1}H and ^{13}C-NMR spectrum of **3** resembled that of **2**; the main difference was that compound **3** was substituted with sugar moiety in the ether link. The position of the fatty acid and the sugar unit were determined by HMBC and ^{1}H-^{1}H COSY correlations (Figure 2). The presence of oxygenated methylene at δ_H 4.28 (2H, ddd, J = 3.4, 5, 7, H-6′), together with an anomeric signal proton at δ_H 4.36 (1H, d, J = 8.2, H-1′), as well as four oxygenated methines, resonating at δ_H 3.33 (1H, dd, J = 8.2, 9.0, H-2′), 3.45 (1H, dd, J = 7.1, 9.0, H-3′), 3.53 (1H, dd, J = 7.1, 7.5, H-4′), and 3.35 (1H, dd, J = 7.5, 7.7, H-5′), was typical of a glucose moiety. The ^{13}C NMR of the anomeric carbon resonated at δ_C 101.3 (C-1′), indicating β-glucose. The HMBC spectrum showed correlations between H-1′ (δ_H 4.36) to C-3 (δ_C 79.8), indicating that the sugar unit was linked at the C-3 position. The presence of fatty acid in ester form at C-6′ was established by correlation between H-6′ (δ_H 4.28) and H-2″ (δ_H 2.30) to C-1″ (δ_C 174.5) in an ester group. The fatty acid was identified as oleate acid based on observation of the NMR spectrum and the coupling constant of H-9/H-10, which was 9.5 Hz; this indicated that each proton was in the *cis*-position. Compound **3** agreed well with data from the literature (Tesemma et al., 2013; Ragasa et al., 2016), supporting its identification as stigmast-5-en-3β-*O*-(6′-*O*-oleoyl)-β-D-glucopyranoside (sitoindoside II).

Compound **4** was obtained as a white amorphous powder. Its molecular composition $C_{35}H_{60}O_6$, was established from NMR data (Table 2). The IR spectrum suggested the presence of hydroxyl groups (3,433 cm^{-1}), olefinic carbons (1,639 cm^{-1}), *gem*-dimethyl group (1,461 and 1,380 cm^{-1}), and C-O bond (1,053 cm^{-1}). The ^{1}H and ^{13}C-NMR spectrum of **4** resembled that of **1**; the main difference was that compound **4** was substituted with sugar moiety in the ether link. The presence of oxygenated methylene at δ_H 4.27 (2H, ddd, J = 3.2, 5.0, 12.0 Hz, H-6′), together with an anomeric signal proton at δ_H 4.53 (1H, d, J = 7.5 Hz, H-1′), as well as four oxygenated methines resonating at δ_H 4.25 (1H, dd, J = 7.5, 8.3 Hz, H-2′), 3.95 (1H, dd, J = 7.7, 8.3 Hz, H-3′), 4.03 (1H, dd, J = 7.4, 7.7 Hz, H-4′), and 4.39 (1H, d, J = 7.4 Hz, H-5′), was typical of a glucose moiety. The ^{13}C NMR of anomeric carbon resonated at δ_C 102.4 (C-1′), indicating β-glucose. Compound **4** agreed well with data from the literature (Harneti et al., 2014), supporting its identification as stigmast-5-en-3β-ol-3-*O*-β-D-glucopyranoside (β-sitosterol glucoside).

Compound **5** was obtained as white amorphous powder. Its molecular composition $C_{35}H_{58}O_6$, was established from NMR data (Table 2). The IR spectrum suggested the presence of hydroxyl groups (3,450 cm^{-1}), olefinic carbons (1,630 cm^{-1}), *gem*-dimethyl group (1,445 and 1,370 cm^{-1}), and C-O bond (1,050 cm^{-1}). The ^{1}H and ^{13}C-NMR spectrum of **5** were very similar to **4**; the main difference was that compound **5** had an additional double bond at δ_H 4.98 and 5.13 (each 1H, dd, J = 8.4, 15.0 Hz, H-22, H-23) and δ_C 138.7 (C-22) and 129.3 (C-23). Compound **5** agreed well with data from the literature (Harneti et al., 2014),

supporting its identification as stigmast-5,22-dien-3β-ol-3-*O*-β-D-glucopyranoside (stigmasterol glucoside).

Among the five steroid compounds identified here, stigmast-5-en-3β-ol **(1)**, with a 3-hydroxyl group, showed the strongest cytotoxicity activity, whereas stigmast-5-en-3β-ol-3β-oleate **(2)**, stigmast-5-en-3β-ol-3-*O*-(6′-*O*-oleoyl)-β-D-glucopyranoside **(3)**, stigmast-5-en-3β-ol-3-*O*-β-D-glucopyranoside **(4)**, and stigmast-5,22-dien-3β-ol-3-*O*-β-D-glucopyranoside **(5)**, with 3-glucosides, 3β-oleate, and 3β-*O*-(6′-*O*-oleoyl)-β-D-glucopyranoside, showed weak or no activity. This indicated that the presence of a 3β-hydroxyl group may be an important sructural feature for cytotoxic activity, whereas the presence of sugar and fatty acid moieties may decrease cytotoxic activity.

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