CYTOTOXIC STEROIDS FROM THE STEM BARK OF *Chisocheton cumingianus* (Meliaceae)

STEROID DENGAN AKTIVITAS SITOTOKSIK DARI KULIT BATANG *Chisocheton cumingianus* (Meliaceae)

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ABSTRACT

Three cytotoxic steroids, stigmasterol (**1**), stigmast-5-en-3β-ol (**2**) and β-sitosterol-3-*O*-acetate (**3**) were isolated from the stem bark of *Chisocheton cumingianus.* The chemical structures of those compounds were identified based on spectroscopic data and by comparison with those data previously reported. All of the compounds isolated were evaluated for their cytotoxic effects against P-388 murine leukemia cells *in vitro*. Compounds **1**-**3** showed cytotoxicity activity against P-388 murine leukemia cells with IC_{50} values of 12.4, 60.8, and > 100 μg/mL, respectively.

Keywords: *C. cumingianus*, *Chisocheton*, cytotoxic activity, Meliaceae, Steroids

ABSTRAK

Tiga senyawa steroid yang beraktivitas sitotoksik, stigmasterol (**1**), stigmast-5-en-3β-ol (**2**) dan β-sitosterol-3-*O*-acetate (**3**) telah diisolasi dari kulit batang *Chisocheton cumingianus.* Struktur kimia senyawa tersebut diidentifikasi berdasarkan data-data spektroskopi dan perbandingan dengan data spektra yang diperoleh sebelumnya. Semua senyawa hasil isolasi dievaluasi sifat sitotoksiknya terhadap sel murine leukimia P-399 secara *in vitro*. Senyawa **1**-**3** menunjukkan aktivitas sitotoksik terhadap sel murine leukimia P-388 dengan nilai IC_{50} beturut-turut 12,4; 60,8 dan > 100 μg/mL.

Kata kunci: *C. cumingianus*, *Chisocheton*, sifat sitotoksik, Meliaceae, Steroids.

INTRODUCTION

The *Chisocheton* genus belongs to the Meliaceae family is a second largest genus in the family of Meliaceae comprising more than 50 plant species and distributed in Nepal, India, Burma, Myanmar, South China, Thailand, Malaysia, Papua New Guinea and Indonesia (Vossen and Umali, 2002). Previous phytochemical studies on *Chisocheton* plants reported the presence of compounds with interesting biological activities such sesquiterpenoids (Phongmaykin, Kumamoto, Ishikawa, Suttisri, & Saifah, 2008), dammarane-type triterpenoids (Inada et al., 1993; Phongmaykin et al., 2008), tirucallane-type triterpenoids (Zhang, Feng, Bin, Sheningg, & Mian, 2012; Yang, Wang, Luo, Wang, & Kong, 2011), apo-tirucallane-type triterpenoids (Zhang et al., 2012), limonoids (Maneerat, Laphoohiero, Koysomboon, & Chantraprомma., 2008; Laphookhieo et al., 2008; Mohamad et al., 2009; Yang, Wang, Luo, Wang, & Kong, 2009; Najmuldeen et al., 2010; Wong et al., 2011; Lim, 2008), steroids and phenolics (Phongmaykin et al., 2008).

As a part of our studies on anticancer candidate compounds from Indonesia *Chisocheton* plants, we already isolated a 7-hydroxy coumarin from the stem bark of *C. celibicus* (Katja et al., 2015), and a 30-nor trijugin-type limonoid, chisotrijugin and lanostan-type triterpenoid, 3β-hydroxy-25-ethyl-lanost-9(11),24(24′)-diene from the stem bark of *C. cumingianus* (Katja et al., 2016a, Katja et al., 2016b). In further search of cytotoxic compounds from Indonesia

Chisocheton species, we found that *n*-hexane and ethyl acetate extracts of the stem bark of *C. cumingianus* exhibited a moderate cytotoxic activity against P-388 murine leukemia cells with IC_{50} value of 16.9 and 19.9 μg/mL, respectively. We report herein the isolation and structural identification of the steroids **1-3**, together with the cytotoxic activity against P-388 murine leukemia cells.

MATERIAL AND METHODS

General

Melting points were measured on an electrothermal melting point apparatus IA9000. The IR spectra were recorded on a Perkin-Elmer 1760X FT-IR in KBr. Mass spectra were obtained with a Water Qtof HR-MS XEV^{otm} mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained with a JEOL JNM A-500 spectrometer using TMS as an internal standard. Chromatographic separations were carried out on silica gel 60 and ODS. TLC plates were precoated with silica gel and Octa desyl silane GF_{254} (ODS), detection was achieved by spraying with 10% H_2SO_4 in ethanol, followed by heating and under ultra-violet light with wavelength at 254 and 367 nm.

Plant material

The stem bark of *C. cumingianus* was collected in Bogor Botanical Garden, Bogor, West Java Province, Indonesia in April 2014. The plant was identified by the staff of the Bogoriense Herbarium, Bogor, Indonesia and a voucher specimen (No. Bo-1305316) was deposited at the herbarium.

Extraction and isolation

Dried ground bark of *C. cumingianus* (2.2 kg) was extracted successively with *n*-hexane, ethyl acetate, and methanol. Evaporation resulted in the crude extracts of *n*-hexane (26.8 g), ethyl acetate (23.6 g), and methanol (30.0 g), respectively. The *n*-hexane extract of *C. cumingianus* (25 g) was subjected to vacuum liquid chromatography over silica gel using a gradient elution mixture of *n*-hexane-ethyl acetate (10:0-0:10) as eluting solvents to afford 15 fractions (A01-A15). Fraction A04 (3.8 g) was subjected to column chromatography over silica gel using a mixture of *n*-hexane:acetone (9:1) as eluting solvents to afford ten fractions (B01-B10). Fraction B04-B07 were combined (130 mg) and fractionated using column chromatography technique over silica gel with a mixture of *n*-hexane-ethyl acetate (10:0-0:10) as eluting solvents to give six fractions (C01-C06). Fractions C04-C05 were combined (25.8 mg) and crystallized with methanol to yield **1** (12.4 mg).

Fraction A06 (450 mg) was column chromatographed over silica gel with a mixture of *n*-hexane:acetone (9:1) as eluting solvents to afford 8 fractions (D01-G08). Fraction D05 (120 mg) was column chromatographed over silica gel with a mixture of *n*-hexane-ethyl acetate (10:0-0:10) as eluting solvents to give seven fractions (E01-E07). Fraction E03-E04 were combined (26.4 mg) and crystallized with methanol to give **2** (16.5 mg). The ethyl acetate extract of *C. cumingianus* (20 g) was subjected to vacuum liquid chromatography over silica gel using a gradient elution mixture of *n*-hexane-ethyl acetate (10:0-0:10) as eluting solvents to afford 12 fractions (F01-F15).

Fraction F04 (3.8 g) was column chromatographed over silica gel with a mixture of *n*-hexane:acetone (9:1) as eluting solvents to afford seven subfractions (G01-10). Subfraction G04-07 were combined (140 mg) and column chromatographed over octadecyl silane with a mixture of water-methanol (10:0-0:5) as eluting solvents to give five subfractions (H01-H06). Subfractions H04-H05 were combined (35.8 mg) and crystallized with methanol to yield **3** (15.4 mg).

Stigmasterol (1)

White needle-like crystals; m.p. 160-171 °C; IR (KBr) ν_{max} 3401, 2860, 1457, 1052 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz), see **Table 1**; ^{13}C NMR ($CDCl_3$, 125 MHz), see **Table 1**; HR-TOFMS *m/z* 413.3748 $[M+H]^+$, (calcd. for $C_{29}H_{48}O$, 412.3704).

Stigmast-5-en-3β-ol (2)

White needle-like crystals; m.p.138-139 ^{o}C; IR (KBr) ν_{max} 3424, 2925, 2850, 1464, 1056 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz), see **Table 1**; ^{13}C NMR ($CDCl_3$, 125 MHz), see **Table 1**; HR-TOFMS *m/z* 413.7211 $[M+H]^+$, (calcd. for $C_{29}H_{50}O$, 414.7204).

β-sitosterol-3-*O*-asetat (3)

White needle-like crystals; m.p.133-136 ^{o}C; IR (KBr) ν_{max} 2920, 2875, 1728, 1280, 1172 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz), see

Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see **Table 1**; HR-TOFMS *m/z* 457.7234 $[M+H]^+$, (calcd. for $C_{31}H_{52}O_2$, 456.7434).

Determination of cytotoxic activities

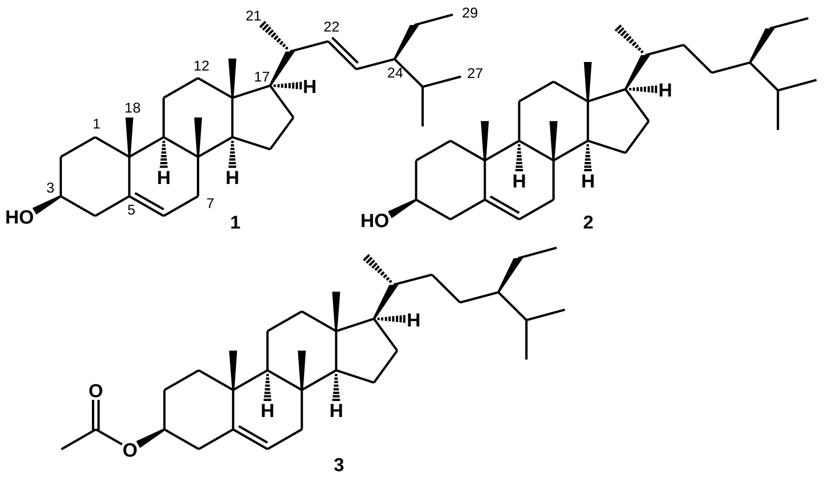
The cytotoxicity assay was conducted according to the method described previously (Sahidin et al., 2005; Alley et al., 1998). P-388 cells were seeded into 96-well plates at an initial cell density of approximately 3 x 10^4 cells cm^{-3}. After 24 h of incubation for cell attachment and growth, varying concentrations of samples were added. 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 a 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 microplate 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 stem bark of *C. cumingianus* was grounded and successively extracted with *n*-hexane, ethyl acetate, and methanol. The *n*-hexane and ethyl acetate extract were chromatographed over a vacuum-liquid chromatographed (VLC) column packed with silica gel 60 by gradient elution. The fractions were repeatedly subjected to normal-phase and reverse-phase column chromatography to afford compounds **1-3** (**Figure 1**).

Stigmasterol (**1**) was obtained as a whiteness needle crystals, with m.p. 160-171 ^{o}C. The molecular formula was established to be $C_{29}H_{48}O$ by HR-TOFMS data (*m/z*413.3748 $[M+H]^+$, calculated for $C_{29}H_{48}O$ *m/z* 412.3704) together with ^{1}H and ^{13}C NMR spectral data (**Table 1**), thus requiring six degrees of unsaturation. Mass spectra of **1** showed molecular ions at *m/z* 369, 351, 327, 301, 300 and 271, suggested the presence of Δ^5 and Δ^{22} sterol-type (Yayli and Baltaci, 1996). The infra red spectrum suggested the presence of a hydroxyl (ν_{max} 3401 cm^{-1}), saturated aliphatic (ν_{max} 2860 cm^{-1}), olefinic (ν_{max} 1457 cm^{-1}) and ether groups (ν_{max} 1178 cm^{-1}). ^{1}H NMR spectrum showed the presence of six methyl signals consist of two tertiary methyls at δ_H 0.67 (3H, s) dan 1.00 (3H, s), three secondary methyl signals at δ_H 0.92 (3H, d, *J*=6.5 Hz), 0.84 (3H, d, *J*=6.4 Hz) dan 0.82 (3H, d, *J*=6.1 Hz), and a primary methyl at δ_H 0.80 (3H, t, *J*=6.0 Hz), suggested the presence of steroid skeleton in compound **1** (Yayli and Baltaci, 1996). The presence of three methine signals at δ_H 5.35 (1H, d, *J*=5.2 Hz, H-6), 5.16 (1H, dd, *J*=8.5, 15.0 Hz, H-22) and 5.00 (1H, dd, *J*=8.5, 15.0 Hz, H-23) and an oxygenated methine signal at δ_H 3.52 (1H, m, H-3), suggested the the characteristic of stigmasterol structure (Cayme & Ragasa, 2004; Yayli & Baltaci, 1996).

Twenty nine carbon resonances were observed in the ^{13}C NMR spectrum. These were assigned by DEPT experiment to six methyls, ten methylenes, eleven methines and two quartenary carbons. The presence of six methyl signals at δ_C 12.1 (C-19), 19.5 (C-19), 21.2 (C-21), 21.3 (C-26), 19.1 (C-27) and 12.2 (C-29), an oxymethine signal at δ_C 72.0 (C-3), three sp^2 methines at δ_C 121.9 (C-6), 138.5 (C-22), 129.5 (C-23), and one quartenary sp^2 carbon at δ_C 140.9 (C-5), suggested that compound **1** to be a stigmasterol (Cayme and Ragasa, 2004; Yayli and Baltaci, 1996). These functionalities accounted for two out of the six degrees of unsaturation. The remaining four degrees of unsaturation were consistent with a tetracyclic stigmastane structure (Cayme and Ragasa, 2004). A detailed comparison of NMR data of **1** to stigmasterol (Cayme and Ragasa, 2004), revealed that **1** was identified as a stigmasterol. It was shown for the first time in this species.



1 2 3

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

Stigmast-5-en-3β-ol (**2**), was obtained as a colorless needle crystals, with m.p. 138-139 °C. The molecular formula was established to be $C_{29}H_{50}O$ by HR-TOFMS *m/z* 413.7211 $[M+H]^+$, calculated for $C_{29}H_{50}O$ *m/z* 414.7204, together with NMR spectral data (**Table 1**), thus requiring five degrees of unsaturation. The IR spectrum of **2** showed the presence of hydroxyl ((ν_{max} 3424 cm^{-1}), saturated aliphatic (ν_{max} 2925 cm^{-1}), olefinic (ν_{max} 1464 cm^{-1}) and ether groups ((ν_{max} 1056 cm^{-1}). The NMR spectra of **2** was similar to those of **1**, except the absence of *trans*-olefinic signals at [δ_H 5.16 (1H, dd, *J*=8.5, 15.0 Hz, H-22), δ_H 5.00 (1H, dd, *J*=8.5, 15.0 Hz, H-23), δ_C 138.5 (C-22) and δ_C 129.5 (C-23)] and appearance the methylene signals at 1.67 (1H, m), 2.03 (1H, m), 1.42 (1H, m), 1.52 (1H, m), 34.1 (t) and 26.2 (t)], suggested that **2** was derivative of **1** with loss of a double bond. A comparison of the NMR data of **2** with those of stigmast-5-en-3β-ol (Chaturvedula & Prakash, 2012), revealed that the compound **2** was identified as a stigmast-5-en-3β-ol.

β-sitosterol-3-*O*-acetate (**3**), was obtained as a colorless needle crystals, with m.p. 133-136 °C. The molecular formula was established to be $C_{31}H_{52}O_2$ by HR-TOFMS *m/z* 457.7234 $[M+H]^+$, calculated for $C_{31}H_{52}O_2$, together with NMR spectral data (**Table 1**), thus requiring six degrees of unsaturation. The IR spectrum of **3** suggested the presence of saturated aliphatics (ν_{max} 2875 cm^{-1}), carbonyl (ν_{max} 1728 cm^{-1}) and ether group (ν_{max} 1172 cm^{-1}). The NMR spectra of **3** was similar to compound **1**, except the absence of hydroxyl group and appearance of acetyl signals at [δ_H 1.60 (3H, s), δ_C 20.0, 173.5], suggested that **3** was 3-*O*-acetyl derivative of **1**. A detailed comparison of the NMR data of **3** to those of β-sitosterol-3-*O*-acetate (Elkader et al., 2013), revealed that the structure of both compounds were similar, therefore compound **3** was identified as a β-sitosterol-3-*O*-acetate (**Figure 1**) and was shown for the first time in this species. The stereochemistry of **3** was determined in line with β-sitosterol-3-*O*-acetate based on the chemical shift in ^{13}C NMR spectrum, proton-proton coupling constant values in ^1H NMR spectrum and biogenetic point of view the occurrences of steroid compounds in *Chisocheton* genus (Harneti et al., 2014; Yang et al., 2009).

The cytotoxic effects of the three isolated compounds **1-3** against P-388 murine leukemia cells were conducted according to the method described previous paper (Harneti et al., 2014; Sahidin et al., 2005; Alley et al., 1988) and were used an artonin E (IC_{50} 0.3 μg/mL) as a positive control (Hakim et al., 2007). Compounds **1-3** showed cytotoxic activity with IC_{50} values of 12.4, 60.8 and > 100 μg/mL, respectively. Among these steroid structures, compound **1** having two olefinic moieties showed the strongest activity, whereas compound **2** lacking one olefinic and compound **3** adding one acetyl groups showed decrease cytotoxic activity. These results suggested that the olefinic and acetyl moieties were important structural components for for cytotoxic activity.

Table 1. NMR data for compounds **1-3** ($CDCl_3$, 500 MHz for ^{1}H and 125 MHz for ^{13}C)

Position Carbon	**1**		**2**		**3**	
	δ_H (Integ. Mult., *J*=Hz)	δ_C (mult.)	δ_H (Integ. Mult., *J*=Hz)	δ_C (mult.)	δ_H (Integ. Mult., *J*=Hz)	δ_C (mult.)
1	1.08 (1H, m); 1.84 (1H, m)	37.4 (t)	1.04 (1H, dd, 5.5, 10.5) 1.11 (1H, dd, 5.5, 10.5)	37.4 (t)	1.73 (1H, dd, 5.6, 10.2) 1.84 (1H, dd, 5.6, 10.1)	37.2 (t)
2	1.49 (1H, m); 1.81 (1H, m)	31.8 (t)	1.69 (1H, dt, 6.0, 9.5) 1.72 (1H, dt, 6.0, 9.5)	29.1 (t)	1.69 (1H, dt, 6.0, 9.5) 1.72 (1H, dt, 6.0, 9.5)	32.1 (t)
3	3.52 (1H, m)	72.0 (d)	3.53 (1H, m)	72.0 (d)	4.16 (1H, m)	73.8 (d)
4	2.28 (1H, dd, 2.0, 5.2) 2.30 (1H, dd, 2.0, 5.2)	42.5 (t)	2.23 (1H, d, 5.3) 2.34 (1H, m)	42.4 (t)	2.20 (1H, m) 1.35 (1H, m)	39.9 (t)
5	-	140.9 (s)	-	140.9 (s)	-	140.0 (s)
6	5.35 (1H, d, 5.2)	121.9 (d)	5.35 (1H, d, 4.9)	121.9 (d)	5.37 (1H, d, 5.2)	122.8 (d)
7	1.54 (1H, m); 1.96 (1H, m)	32,1 (t)	1.53 (1H, m); 1.94 (1H, m)	31.8 (t)	1.54 (1H, m); 2.02 (1H, m)	34.0 (t)
8	1.46 (1H, m)	21.3 (t)	1.44 (1H, m)	32.2 (d)	1.44 (1H, m)	32.0 (d)
9	0.94 (1H, m)	50.3 (d)	0.96 (1H, m)	50.3 (d)	0.95 (1H, m)	50.2 (d)
10	-	36.7 (s)	-	37.7 (s)	-	36.8 (s)
11	1.46 (1H, m); 1.49 (1H, m)	21.3 (t)	1.42 (1H, m); 1.46 (1H, m)	21.2 (t)	1.48 (1H, m); 1.50 (1H, m)	21.2 (t)
12	1.15 (1H, m); 1.95 (1H, t)	39.9 (t)	1.18 (1H, m); 1.92 (1H, m)	39.9 (t)	1.17 (1H, m); 2.31 (1H, m)	38.3 (t)
13	-	42.5 (s)	-	42.5 (s)	-	42.5 (s)
14	1.03 (1H, s)	56.9 (d)	1.09 (1H, m)	56.9 (d)	1.10 (1H, m)	56.8 (d)
15	1.07 (1H, m); 1.56 (1H, m)	24.5 (t)	1.03 (1H, m); 1.62 (1H, m)	24.5 (t)	1.05 (1H, m); 1.60 (1H, m)	25.3 (t)
16	1.26 (1H, m); 1.67 (1H, m)	28.4 (t)	1.19 (1H, m); 1.58 (1H, m)	28.4 (t)	1.18 (1H, m), 1.64 (1H,m)	26.2 (t)
17	1.13 (1H, m)	56.1 (d)	1.15 (1H, m)	56.2 (d)	1.05 (1H, m)	56.2 (d)
18	0.67 (3H, s)	12.1 (q)	0.67 (3H, s)	12.0 (q)	0.67 (3H, s)	12.0 (q)
19	1.00 (3H, s)	19.5 (q)	1.01 (3H, s)	18.9 (q)	1.01 (3H, s)	19.5 (q)
20	2.02 (1H, m)	40.7 (d)	1.33 (1H, m)	36.3 (d)	1.33 (1H, m)	36.3 (d)
21	0.92 (1H, d, 9.5)	21.2 (q)	0.79 (3H, d, 6.2)	21.4 (q)	0.92 (3H, d, 5.8)	19.2 (q)
22	5.16 (1H, dd, 8.5, 15.0)	138.5 (d)	1.67 (1H, m); 2.03 (1H, m)	34.1 (t)	1.70 (1H, m), 1.98 (1H, m)	34.9 (t)
23	5.00 (1H, dd, 8.5, 15.0)	129.5 (d)	1.42 (1H, m); 1.52 (1H, m)	26.2 (t)	1.43 (1H, m); 1.50 (1H, m)	24.5 (t)
24	1.53 (1H, m)	51.4 (d)	0.97 (1H, m)	46.0 (d)	0.93 (1H, m)	46.0 (d)
25	1.45 (1H, m)	31.8 (d)	1.13 (1H, m)	29.3 (d)	1.13 (1H, m)	34.5 (t)
26	0.84 (3H, d, 6.4)	21.3 (q)	0.81 (3H, d, 6.1)	21.4 (q)	0.81 (3H, d, 6.2)	14.0 (q)
27	0.82 (3H, d, 6.1)	19.1 (q)	0.78 (3H, d, 6.1)	20.0 (q)	0.78 (3H, d, 6.2)	19.0 (q)
28	1.15 (1H, t, 3.2)	25.6 (d)	1.27 (1H, m); 1.32 (1H, m)	23.2 (t)	1.27 (1H, m); 1.32 (1H, m)	23.2 (t)
29	0.80 (3H, t, 6.0)	12.2 (q)	0.92 (3H, t, 1.89)	12.2 (q)	0.90 (3H, t, 1.89)	14.3 (q)
H_3C-					1.60 (3H, s)	20.0 (q)
C=O					-	173.5 (s)

CONCLUSION

Three steroid compounds, stigmasterol (**1**), stigmast-5-en-3β-ol (**2**) dan β-sitosterol-3-*O*-acetate (**3**) have been isolated from the stembark of *Chisocheton cumingianus* and was shown for the first time in this species. The presence of olefinic and acetyl moieties in steroid structure play important role for cytotoxic activity against P-388 murine leukemia cells.

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