



A triterpenoid and Steroids from the Twigs of *Millettia utilis* Dunn. and Their Antioxidant Activities

Thanatip Ruksilp

Department of Chemistry, Faculty of Science and Technology, Loei Rajabhat University, Muang Loei, Loei, 42000, Thailand

Corresponding author. E-mail address: thanatip.ruk@lru.ac.th

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Abstract

A triterpenoid, betulin together with two steroids, β -sitostenone and pregnenolone were isolated from dichloromethane crude extract in the twigs of *Millettia utilis* Dunn. The structures of all isolated compounds were elucidated by basic NMR spectroscopy and compared with previous literature. All compounds were tested for their antioxidant activities. Betulin revealed moderate activity against ABTS assay.

Keywords: *Millettia utilis* Dunn., steroids, triterpenoid, ABTS, antioxidant activities

Introduction

The genus *Millettia*, comprising approximately 200 species, belongs to the Leguminosae family (Huo et al., 2015). The plant *Millettia utilis* Dunn., locally known as “ Satorn ”, is a tree growing in northeastern and northern regions of Thailand. *Millettia* plants revealed the presence of flavonoids (Sritularak & Likhitwitayawuid, 2006; Sritularak, Likhitwitayawuid, Conrad, & Kraus, 2002), isoflavones (Derese et al., 2014), chalcones flavones (Phrutivorapongkul et al., 2003), and rotenoids (Pancharoen, Athipornchai, Panthong, & Taylor, 2008; Ngandeu et al., 2008). Moreover, biological activities of isolated compounds from *Millettia* genus were interesting such as antiviral, anti-inflammatory (Phrutivorapongkul et al., 2003; Pancharoen et al., 2008; Huo et al., 2015), antiplasmodial (Derese et al., 2014), and analgesic activities (Huo et al., 2015). However, there has been no report on the constituents and biological activities from *M. utilis* Dunn., the previous literature revealed for the twigs of *Millettia* plants have many interesting compounds (Ngandeu et al., 2008). In this paper, the isolation and antioxidant activities from dichloromethane crude extract in the twigs of *M. utilis* Dunn. have been reported for the first time.

Methods and Materials

General experiment procedures

NMR spectra were recorded in CDCl_3 on Bruker and Varian 400 MHz spectrometer at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. PHOMO Microplate reader, Autobio model SPR-960 was obtained for DPPH and ABTS assays. Merck's silica gel 60 No.7734 was used as adsorbents for column chromatography. Analytical thin layer chromatography was performed with Merck's Silica gel 60 F_{254} , 0.25 mm precoated TLC aluminium sheets. The detection was visualized under ultraviolet light at the wavelength of 254 and 365 nm. Melting point were obtained on Buchi melting point B-540.

Plant material

The twigs of *M. utilis* Dunn. were collected in June, 2018 from Dansai, Loei, Thailand and identified by Dr. Sawai Mattapha. A voucher specimen was deposited in the Herbarium of Loei Rajabhat University, Faculty of Science and Technology, Loei, Thailand(LRU No. 001).



Extraction and isolation

The air-dried twigs of *M. utilis* Dunn. (1.0 kg) were chopped, ground to powder and macerated with CH₂Cl₂ (4 L x 3, 1 day for each extraction) at room temperature. The crude extract of CH₂Cl₂ (10.5 g) was obtained upon concentration under reduced pressure and separated on a silica gel column chromatography (CC), using *n*-hexane/CH₂Cl₂ (9:1, 4:1, 3:2, 1:1, 3:7, 1:9, 0:10); CH₂Cl₂/EtOAc (9:1 to 0:10) and EtOAc/MeOH (9:1 to 0:10) as the eluent, TLC analysis was used to produce ten fractions. Fraction 3 (500 mg) was chromatographed over silica gel CC and eluted with gradient of *n*-hexane/CH₂Cl₂ (9:1 to 0:10) and CH₂Cl₂/EtOAc (9:1 to 0:10) to give five subfractions (3.1 to 3.5). Subfraction 3.3 (100 mg) was further separated over silica gel CC and eluted with mixtures of *n*-hexane/CH₂Cl₂ (9:1 to 0:10) to afford compound **1** (15 mg) and compound **2** (20 mg). Fraction 4 (300 mg) was further purified over silica gel CC and eluted with mixtures of *n*-hexane/CH₂Cl₂ (9:1 to 0:10) to get three subfractions (4.1 to 4.3). Subfraction 4.3 (50 mg) was further separated over silica gel CC and eluted with mixtures of *n*-hexane/CH₂Cl₂ (1:9) to provide compound **3** (10 mg).

Antioxidant assays

Compounds **1–3** have been tested for their antioxidant activities by DPPH and ABTS assays. PHOMO Microplate reader, Autobio model SPR-960 performed in a 96 well plate was obtained for DPPH and ABTS assays.

The DPPH assay was modified on the method of Kim, Lee, Lee, and Lee (2002). The DPPH reagent was weighed 8 mg, dissolved in EtOH 100 mL for a solution concentration of 80 µg/mL. To determine the scavenging activity, 100 µL DPPH reagent was mixed with 100 µL of sample in a 96-well plate and allowed to stand in the dark at room temperature for 30 min. The absorbance was measured at 515 nm using PHOMO microplate reader. Quercetin was used as standard and 100% ethanol was used as a control.

The ABTS assay was modified on the method of Arnao, Cano, and Acosta (2001). Stock solution of ABTS (2,2'-azobis-(3-ethylbenzothiazoline-6-sulfonic acid)) was prepared by dissolving ABTS 0.0036 g in deionized water 1.0 mL. Potassium persulfate 0.00067 g was mixed to the solution and allowed to stand in the dark for 16 hours to obtain the radical cation ABTS^{•+}. The assay was initiated by the addition of sample 50 µL to ABTS^{•+} solution 100 µL to a final volume of 150 µL in 96 well plate, and allowed to stand for 15 min. The absorbance at 734 nm was monitored, using quercetin as standard compound.

Results and Discussion

Three compounds (**1–3**) were isolated from the twigs of *M utilis* Dunn. They were classified into two groups. Two steroids (compound **1–2**) and one triterpenoid (compound **3**), (Figure 1).

β-sitotenenone (**1**) White powder, m.p. 85–86 °C (m.p. lit. 97–99 °C), ¹H NMR and ¹³C NMR were revealed in Table 1, (Prachasittikul et al., 2009).

Pregnenolone (**2**) White powder, m.p. 187–188 °C (m.p. lit. 192–193 °C), ¹H NMR and ¹³C NMR were revealed in Table 1, (Sheu, Chang, & Duh, 2000).

Betulin (**3**) White powder, m.p. 250–252 °C (m.p. lit. 256–257 °C), ¹H NMR and ¹³C NMR were revealed in Table 1, (Tijjani, Ndukwe, & Ayo, 2012; Aktar, Kaisar, Kabir, Hasari, & Rasid, 2009).

**Table 1** ^1H NMR and ^{13}C NMR spectral data of compound 1-3, β -sitonenone, Pregnenolone and Betulin

Position	Compound 1		β -sitonenone		Compound 2		Pregnenolone		Compound 3		Betulin	
	^1H (ppm)	^{13}C (ppm)	^1H (ppm)	^{13}C (ppm)	^1H (ppm)	^{13}C (ppm)	^1H (ppm)	^{13}C (ppm)	^1H (ppm)	^{13}C (ppm)	^1H (ppm)	^{13}C (ppm)
1		36.61		36.06	1.87	37.3		38.3		38.9		38.6
2		32.04		33.93	2.02	31.8		32.5		27.6		27.2
3		199.71		199.58	3.54, 1.58 (-OH)	71.7	3.42	71.1	3.20 dd, J = 11.5, 4.5 Hz	79.2	3.18 dd, J = 11.2, 5.2 Hz	79.0
4	5.30	123.71	5.74	123.69	2.21	42.3		42.3		39.0		38.8
5		171.78		171.64		140.7		142.4		55.5		55.3
6		32.94		33.86	5.36	121.4	5.33 d, J = 4.5 Hz	121.4		18.5		18.3
7		33.87		32.91	2.05	31.9		32.8		34.4		34.0
8		35.70		35.60	1.88	29.7		32.6		41.1		41.0
9		53.80		53.79	1.02	50.0		52.6		50.6		50.5
10		38.59		39.59		36.5		37.4		37.5		37.2
11		21.01		21.10	1.60	21.1		21.9		21.0		20.9
12		39.61		38.57	2.04	38.8		39.8		25.4		25.2
13		42.37		42.35		44.0		44.4		37.4		37.3
14		56.00		55.85	1.19	56.9		57.7		42.9		42.8
15		24.17		24.14	1.89	24.5		23.5		27.3		27.0
16		28.18		28.13	2.22	22.8		39.6		29.4		29.2
17		55.86		55.99	2.54	63.7	2.61 t, J = 9.0 Hz	64.0		48.0		47.8
18	0.70	12.00	0.71	11.90	0.64	13.3	0.62	13.6		49.0		48.8
19	1.10	19.01	1.18	18.65	1.03	19.4	1.01	19.9	2.40	48.0	2.37	47.8
20		36.10		36.07		209.6		208.5		150.7		150.5
21	0.95 d, J = 6.6 Hz	18.68	1.10	18.98	2.13	31.6	2.06	31.5		29.9		29.7
22		33.96		35.65						34.2		34.2
23		29.13		26.08					0.98	28.2	1.01	28.0
24		45.81		45.81					0.77	15.6	0.76	16.0
25		26.05		29.64					0.83	16.2	0.82	16.1
26	0.82 d, J = 6.8 Hz	17.34	0.80	20.99					1.03	16.3	0.96	15.6
27	0.84 d, J = 6.8 Hz	19.80	0.82	19.75					0.99	15.0	0.97	14.8
28		23.05		23.04					3.34, 3.80 d, J = 11.0 Hz	60.8	3.34, 3.78 d, J = 10.8, 11.2 Hz	60.6
29	0.87 d, J = 7.2 Hz	12.00	0.85	11.14					4.56, 4.68	109.9	4.67, 4.75	109.7
30									1.69	19.3	1.67	19.1

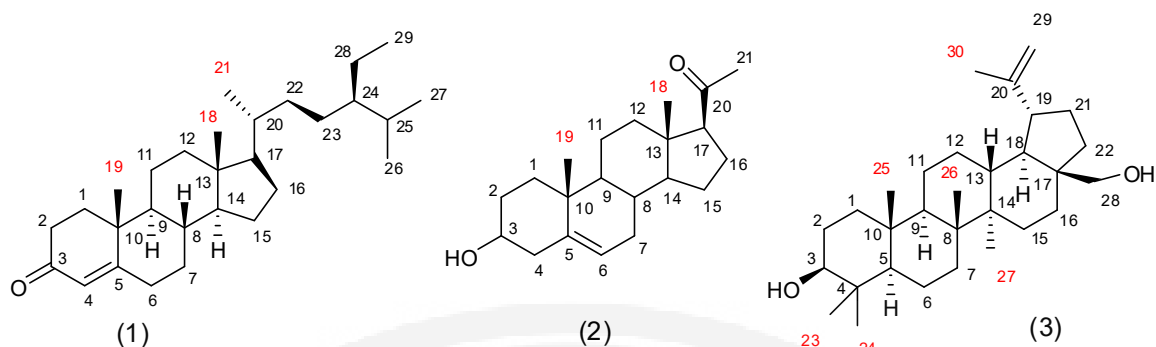


Figure 1 β -sitostenone (1), Pregnenolone (2) and Betulin (3) isolated from the twigs of *M. utilis* Dunn.

The ^1H NMR spectrum of compound **1** displayed an olefinic proton singlet at δ 5.30 (H-4). The spectrum also revealed singlets at δ 1.10 and 0.70 (each 3H, s) assignable to two tertiary methyl groups at C-10 (H-19) and C-13 (H-18), respectively. The three proton doublets at δ 0.95 ($J = 6.6$ Hz), 0.82 ($J = 6.8$ Hz) and 0.84 ($J = 6.8$ Hz) were demonstrative of methyl groups at C-20 (H-21) and C-25 (H-26, H-27), respectively. A three proton doublet ($J = 7.2$ Hz) at δ 0.87 was observed for another methyl group at C-28 (H-29). On this basis, the structure of the compound **1** was determined as β -sitostenone (Ara, Rahman, Rahman, Hasan & Rashid, 2009; Wang, 2014).

The spectrum of ^1H NMR of compound **2** displayed an olefinic proton multiplet at δ 5.36 (H-6), and showed singlets at δ 1.03 and 0.64 (each 3H, s) assignable to two methyl groups at C-10 (H-19) and C-13 (H-18), respectively. The eight methylene groups of H-1, 2, 4, 7, 11, 12, 15, 16 were revealed between δ 1.19–2.22, and one OH group was shown at δ 1.58. The structure of the compound **2** was revealed as pregnenolone (Zahari & Said, 2013).

Compound **3** displayed the ^1H NMR spectrum, a double of doublet ($J = 11.5, 4.5$ Hz) at δ 3.20 which could be assigned for H-3 in the triterpene nucleus. Two broad singlets at δ 4.56 and δ 4.68 revealed the presence of vinylic protons at H-29. The spectrum also showed six three proton singlets at δ 1.69, 0.98, 0.99, 1.03, 0.83 and 0.77 were assigned to methyl protons at C-20 (Me-30), C-4 (Me-23), C-14 (Me-27), C-8 (Me-26), C-10 (Me-25), and C-4 (Me-24), respectively. The multiplet of one proton at δ 2.40 was assigned to H-19. Compound **3** was lupane triterpenoid, betulin (Aktar et al., 2009; Wang, 2014).

Table 2 Antioxidant levels of isolated compounds from the twigs of *M. utilis* Dunn.

Compounds	(IC ₅₀ , $\mu\text{g/mL}$)			
	DPPH	Antioxidant levels	ABTS	Antioxidant levels
β -sitostenone (1)	IC ₅₀ > 50	weak	IC ₅₀ > 50	weak
Pregnenolone (2)	IC ₅₀ > 50	weak	IC ₅₀ > 50	weak
Betulin (3)	IC ₅₀ > 50	weak	10.13 \pm 0.13	moderated
quercetin	9.92 \pm 0.13	high	1.86 \pm 0.03	high

Compounds **1–3** were tested for their antioxidant activities by DPPH and ABTS assays. Compound **3** showed moderate activity by ABTS assay with IC₅₀ value 10.13 \pm 0.13 $\mu\text{g/mL}$. Compound 1 and 2 were weakly active. β -sitostenone was tested *in vitro* against P-388 cell lines and revealed moderate activity with



ED₅₀ value 15.3 µg/mL (Chen, Duh, & Chen, 2005). Pregnenolone was tested for its cytotoxicity toward various cancer cell lines (P-388, KB, A549, and HT-29 cells). Highly active was shown in HT-29 with ED₅₀ value 0.7 µg/mL, and showed moderate activity in P-388 and A549 with ED₅₀ value 7.78, 8.6 µg/mL, respectively (Sheu et al., 2000).

Betulin was tested against Human lung carcinoma (A549), human colorectal adenocarcinoma (DLD-1), human breast adenocarcinoma (MCF7), human prostate adenocarcinoma (PC-3), and human normal skin fibroblasts (WS1) cell lines, highly active were shown by A549, DLD-1 and WS1 with IC₅₀ value 3.8 ± 0.1, 6.6 ± 0.3 and 3.6 ± 0.1 µmol/L, respectively, and showed moderate activity by MCF-7 and PC-3 with IC₅₀ value 23.3 ± 0.5 and 17.9 ± 0.9 µmol/L (Gauthier et al., 2009).

Conclusion and Suggestion

The twigs of *M. utilis* Dunn were ground into powder, extracted with dichloromethane to obtain dichloromethane crude extract. The chromatographic separations and purification of dichloromethane crude extract yielded two steroids, β-sitosterone (1), pregnenolone (2) and one triterpenoid, betulin (3). All compounds were tested for their antioxidant activities by DPPH and ABTS assays. Betulin (3) showed moderate activity with IC₅₀ value 10.13 ± 0.13 µg/mL. by ABTS assay.

The other parts of *M. utilis* Dunn. such as leaves, fruits, roots should be investigated for their constituents, biological activities due to their have no reports.

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