Antioxidant Activities of Triterpenoids and Steroid from the Roots of *Millettia utilis* Dunn

Thanatip Ruksilp[1]*, Napatsorn Wongpriaw[1], Thitinan Thammasom[1], Niramol Srichana[1] and Wira Isaro[2]

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

[2]General Education, Loei Rajabhat University, Muang Loei, Loei, 42000, Thailand

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

Received: 26 April 2021; Revised: 2 August 2021; Accepted: 9 August 2021; Available online: 28 October 2021

Abstract

Two triterpenoids, oleanolic acid acetate (**1**), corosolic acid (**2**) and one steroid, stigmasterol (**3**) were isolated from dichloromethane crude extract in the roots of *Millettia utilis* Dunn. The structures of all isolated compounds were elucidated by spectroscopic methods and compared with previous literatures. The antioxidant activities using DPPH and ABTS assays of three isolated compounds were tested. Oleanolic acid acetate (**1**), corosolic acid (**2**) were revealed moderate and stigmasterol (**3**) was weak activity against DPPH and ABTS assays.

Keywords: *Millettia utilis* Dunn., steroids, triterpenoids, DPPH, ABTS

Introduction

The genus *Millettia* belongs to Leguminosae (Fabaceae) family. It has about 260 species widely distributed over the tropical regions of Africa, Australia, Asia and America (Buyinza, Chalo, Derese, Ndakala, & Yenesew, 2020). The plant *Millettia utilis* Dunn., locally known as "Satorn", is a tree growing in northeastern and northern regions of Thailand. *Millettia* is a traditional medicine used in the treatment of gynecological diseases, dysentery, cardiovascular diseases, intestinal pain, rheumatic arthritis, skin diseases, bruises, and haematonic (Yan et al., 2019; Ma et al., 2020; Pailee, Mahidol, Ruchirawat, & Prachyawarakorn, 2019). It revealed the presence of flavonoids (Yan et al., 2019; Ma et al., 2020; Pailee et al., 2019), isoflavonoids (Xue et al., 2020; Raksat, Maneerat, Andersen, Pyne, & Laphookhieo, 2018), chalcones(Deyou et al, 2015), and rotenoids (Deyou et al, 2015; Deyou et al, 2017; Perez et al., 2014). Moreover, biological activities of isolated compounds from genus of *Millettia* were interesting such as anticancer (Yan et al., 2019; Pailee et al., 2019; Deyou et al., 2015), antibacterial (Raksat et al., 2018), antiplasmodial (Deyou et al, 2015) and anti-inflammatory (Xue et al., 2020). In the previously study, a triterpenoid, steroids, fatty acids and an ester were isolated from the twigs and the leaves of *M. utilis* Dunn and to exhibited antioxidant activities (Ruksilp, 2020a, 2020b).

In this paper, the isolation and antioxidant activities from dichloromethane crude extract in the roots of *M. utilis* Dunn have been reported.

Methods and Materials

General experiment procedures

NMR spectra were recorded in $CDCl_3$, Pyridine-d_5 on Bruker 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 wavelength 254 and 365 nm of ultraviolet light were used for detection. Melting point were rercorded on Buchi melting point B-540. All isolated compounds were identified by comparison of their spectroscopic data in the literatures.

Plant material

The roots of *M. utilis* Dunn. were collected in August, 2019 from Dansai, Loei, Thailand . Voucher specimen(LRU No. 001) was identified and deposited in the Herbarium of Loei Rajabhat University, Faculty of Science and Technology, Loei, Thailand.

Extraction and isolation

The air-dried roots of *M. utilis* Dunn. (1.0 kg) were cut, mashed to powder and macerated with CH_2Cl_2(4 L x 3, 1 day for each extraction) at room temperature. The crude extract of CH_2Cl_2 (16.5 g) was obtained upon concentration under reduced pressure and separated on a silica gel column chromatography (CC), using *n*-hexane/CH_2Cl_2(9:1, 4:1, 3:2, 1:1, 3:7, 1:9, 0:10); CH_2Cl_2/EtOAc (9:1 to 0: 10) and EtOAc/MeOH (9:1 to 0: 10) as the eluent , TLC analysis was used to produce eleven fractions. Fraction 4 (400 mg) was chromatographed over silica gel CC and eluted with gradient of *n*-hexane/CH_2Cl_2 (9:1 to 0: 10) and CH_2Cl_2/EtOAc (9:1 to 0: 10) to give five subfractions 4.1 to 4.5). Subfraction 4.3 (100 mg) was further seperated over silica gel CC and eluted with mixtures of *n*-hexane/CH_2Cl_2 (9:1 to 0: 10) to afford compound **1** (25 mg) . Fraction 5 (350 mg) was further purified over silica gel CC and eluted with mixtures of *n*-hexane/CH_2Cl_2 (9:1 to 0: 10) to get five subfractions (5.1 to 5.5). Subfraction 5.3 (100 mg) was further seperated over silica gel CC and eluted with mixtures of *n*-hexane/ CH_2Cl_2 (9:1 to 0: 10) to provide compound **2** (15 mg) and compound **3** (20 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 used for DPPH and ABTS assays.

The DPPH assay was modified on the method of Sridhar and Charles (Sridhar & Charles, 2019). The DPPH (2,2 diphenyl-1-picrylhydrazyl) 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. Ascorbic acid was used as standard and 100% ethanol was used as a control.

The ABTS assay was modified on the method of Sridhar and Charles (Sridhar & Charles, 2019). The ABTS (2,2′-azobis-(3-ethylbenzothiazoline-6-sulfonic acid)) stock solution was prepared by adding ABTS 0.0036 g in deionized water 1.0 mL. Potassium persulfate 0.00067 g was added to the solution and allowed to stand in the dark for 16 hours to get the radical cation $ABTS^{\bullet+}$. The assay was initiated by the addition of sample 50 μL to $ABTS^{\bullet+}$ 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 ascorbic acid as standard compound.

Results and Discussion

Three compounds (**1-3**) were isolated from the roots of *M utilis* Dunn. They were classified into two groups. Two triterpenoids (compound **1-2**) and one steroid (compound **3**), (Figure 1).

Table 1 ^{1}H NMR and ^{13}C NMR spectral data of compound **1**(oleanolic acid acetate) and 2 (corosolic acid)

Position	Compound 1		Oleanolic acid acetate		Compound 2		Corosolic acid	
	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
1		38.0		38.1	2.23, d (*J*=8.0 Hz)	46.8	2.13, ddd (*J*= 13.0, 12.5, 3.5 Hz)	47.7
2		27.7		23.5	4.08, d (*J*=4.0 Hz)	67.1	4.05, ddd (*J* = 11.0, 9.5,4.0 Hz)	68.3
3	4.60, m	80.9	4.49, m	80.9	3.56, d (*J*=8.0 Hz)	82.2	3.34, d (*J*=9.0 Hz)	83.5
4		39.0		37.7		38.8		39.6
5	0.85, m	55.3	0.83-0.88, m	55.3		54.7		55.6
6		18.1		18.2		18.0		18.6
7		32.8		32.6		32.6		33.2
8		39.2		39.3		39.0		39.7
9		48.0		47.6		47.0		47.8
10		37.7		37.0		37.5		38.2
11		24.0		23.4		22.9		23.4
12	5.30, t (*J*= 4.0 Hz)	122.5	5.28, t (*J*= 3.5 Hz)	122.6	5.26, m	124.4	5.40	125.3
13		143.6		143.6		138.2		139.0
14		41.5		41.6		41.6		42.3
15		28.0		27.7		27.4		28.4
16		23.4		22.9	2.25, d (*J*=8.0 Hz)	23.7	2.31, ddd (*J*= 13.5, 13.0, 4.5 Hz)	24.6
17		46.6		46.5		46.9		47.8
18	2.70, t (*J*= 4.0 Hz)	41.0	2.82, dd (*J*=13.6, 4.1 Hz)	41.0	2.50	52.3	2.57, d (*J*= 10.5 Hz)	53.2
19		45.9		45.9		38.4		39.1
20		30.7		30.7		38.4		39.2
21		33.8		33.8		30.1		30.8
22		32.4		32.4		36.2		37.2
23	0.85	16.7	0.85, s	16.7	1.22, s	28.8	1.21, s	29.1
24	0.87	29.7	0.87, s	28.0	0.98, s	17.1	0.98, s	17.4
25	0.97	15.5	0.94, s	15.4	0.95, s	16.4	0.92, s	16.7
26	0.75	17.2	0.76, s	17.1	1.00, s	16.9	1.02, s	17.2

Table 1 (Cont.)

Position	Compound 1		Oleanolic acid acetate		Compound 2		Corosolic acid	
	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
27	1.18, s	25.9	1.13, s	25.9	1.17, s	23.2	1.15, s	23.6
28		178.6		182.7		178.2		179.1
29	0.95	23.6	0.93, s	23.6	0.80, d (J=8.0 Hz)	21.0	0.85, d (J= 6.5 Hz)	21.1
30	0.90	33.1	0.91, s	33.1	0.90	16.9	0.91, d (J= 6.0 Hz)	17.2
$\underline{CH_3}COO$	1.95	22.8	2.05, s	21.3				
$CH_3\underline{COO}$		171.0		171.0				

Oleanolic acid acetate (**1**) White powder, m.p. 243-244 ^{0}C (m.p. lit. 220-222 ^{0}C), IR(cm^{-1}) 2924, 2860, 1734, 1711 and 1242, EIMS, *m/z* 498 $[M]^{+}$ (calcd. for $C_{32}H_{50}O_4$, 498.3709), ^{1}H NMR and ^{13}C NMR ($CDCl_3$) were revealed in Table 1 (Endo, Shigetomi, Mitsuhashi, Igarashi, & Ubukata, 2019).

Corosolic acid (**2**) White powder, m.p. 250-252 ^{0}C (m.p. lit.254-256 ^{0}C), IR(cm^{-1}) 3415, 2973, 2927 and 2872, EIMS, *m/z* 454 $[M-18]^{+}$ (calcd. for $C_{30}H_{46}O_3$, 454.3447), ^{1}H NMR and ^{13}C NMR (Pyridine-d_5) were revealed in Table 1 (Woo, Sang, Choi, Kim, & Lee, 2014).

Stigmasterol (**3**) White powder, m.p. 169-170 ^{0}C (m.p. lit. 135-136 ^{0}C), IR(cm^{-1}) 3428, 2937, 2852, 1642 and 1465, EIMS, *m/z* 412 $[M]^{+}$ (calcd. for $C_{29}H_{48}O$, 412.3705), ^{1}H NMR (400 MHz, CD_3Cl_3): δ_H 5.35 (1H, m, H-6), 0.70 (3H, s, Me-18), 0.93 (3H, s, Me-19), 1.01 (3H, d, J = 7 Hz, Me-21), 5.05 (1H, m, H-22), 5.15 (1H, m, H-23), 0.85 (3H, d, J = 7 Hz, Me-26), 0.84(3H, d, J = 7 Hz, Me-27), 0.88 (3H, d, J = 7 Hz, Me-29). ^{13}C NMR ($CDCl_3$) : δ_C 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 39.7 (C-4),140.7 (C-5), 121.7 (C-6), 31.9 (C-7), 31.6 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12),40.4(C-13), 56.8 (C-14), 24.3 (C-15), 28.8 (C-16), 56.0 (C-17), 11.8 (C-18), 19.3 (C-19), 33.8 (C-20), 19.8(C-21), 138.2 (C-22), 129.3(C-23), 45.9 (C-24), 31.8 (C-25), 19.0 (C-26), 29.0 (C-27), 25.3(C-28), 12.0 (C-29) (Forgo & Kover, 2004) were compared with previous literatures.

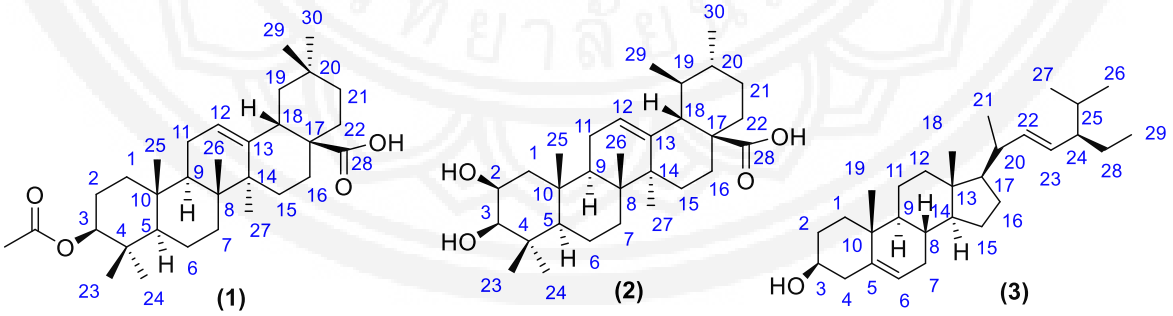

Figure 1 Oleanolic acid acetate (**1**), Corosolic acid (**2**) and Stgmasterol (**3**) isolated from the roots of *M. utilis* Dunn

The ^{1}H NMR spectrum of compound **1** displayed an olefinic proton triplet at δ 5.30 (J = 4.0 Hz, H-12). The spectrum also revealed multiplets at δ 4.60 (H-3) and the three proton singlets at δ 1.18 was demonstrative of methyl groups at C-27 (H-27). A three proton at δ 1.95 was observed for acetyl group at

C-3 (CH_3COO). The ^{13}C NMR spectrum (**Table 1**) displayed signals for 32 carbons, stands for five methine carbon, ten methylene carbon, eight methyl carbon and nine quaternary carbon, the two carbonyl carbon at δ 178.6, 171.0 were assigned to carboxyl (C-28) and acetyl group. On this basis, the structure of the compound **1** was determined as oleanolic acid acetate (Endo et al., 2019).

The spectrum of ^1H NMR of compound **2** displayed an olefinic proton multiplet at δ 5.26 (H-12), and showed doublets at δ 4.08 (J = 4.0 Hz) and 3.56 (J = 8.0 Hz) assignable to two methine groups at C-2 (H-2) and C-3 (H-3), respectively. The ^{13}C NMR spectrum were revealed signals for 30 carbons, eight methine carbon, eight methylene carbon, seven methyl carbon and seven quaternary carbon, the carbonyl carbon at δ 178.2 was assigned to carboxyl group(C-28). The structure of the compound **2** was revealed as corosolic acid (Woo et al., 2014).

Compound **3** displayed the ^1H NMR spectrum of two methyl groups as singlet at δ 0.70, 0.93 were assigned to H-18, H-19, respectively. The three methyl groups doublet at δ 1.01, 0.85, 0.84 were assigned to H-21,H-26, H-27. The signal of three olefinic proton multiplet at δ 5.35, 5.05 and 5.15 were assigned to H-6,H-22 and H-23. The ^{13}C NMR spectrum were shown signals for 29 carbons, eleven methine carbon, nine methylene carbon, six methyl carbon and three quaternary carbon, compared to previously reported (Forgo & Kover, 2004), compound **3** was identified as stigmasterol.

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

Compounds	(IC_{50}, μg/mL)			
	DPPH	Antioxidant levels	ABTS	Antioxidant levels*
Oleanolic acid cetate(1)	19.18± 0.61	moderate	13.12± 0.23	moderate
Corosolic acid (2)	15.07± 0.57	moderate	15.48± 0.58	moderate
Stigmasterol (3)	$IC_{50} > 50$	weak	$IC_{50} > 50$	weak
Ascorbic acid	1.21 ± 0.13	high	0.74 ± 0.03	high

* $IC_{50} \leq 10$ μg/mL high, IC_{50} >10- 50 μg/mL moderate, $IC_{50} > 50$-200 μg/mL weak, $IC_{50} > 200$ μg/mL inactive (Qusti, et al., 2010)

The antioxidant activities of compounds **1-3** were done by DPPH and ABTS assays. Compound **1** and **2** showed moderate activity by DPPH assay with IC_{50} value 19.18 ± 0.61, 15.07± 0.57 and 13.12± 0.23, 15.48± 0.58 μg/mL were revealed moderate by ABTS assay. Compound **3** was weak by DPPH and ABTS assays.

Oleanolic acid acetate was tested for cytotoxicity toward ovarian cancer SKOV3 cells and endometrial cancer HEC-1A cells. The results showed that oleanolic acid acetate effectively suppressed the growth of SKOV3 cell tumor xenografts in immunocompromised mice, and induced apoptosis in SKOV3 and HEC-1A cells. It could be used as a potent anticancer supplementary agent against ovarian and endometrial cancer (Jo, Oh, Park, Lee, & Min, 2020).

Corosolic acid extracted from *Eriobotrya japonica* leaves could strongly improve impaired glucose, hyperlipidemia and insulin resistance in Type 2 diabetes (T2D) models,which were relied on declining the expression of Phosphoenolpyruvate carboxykinase (PEPCK) and other genes involved in carbon metabolism, T2D related oxidative stress and inflammation (Xu et al., 2019).

Conclusion and Suggestions

The roots of *M. utilis* Dunn were ground into powder, extracted with dichloromethane to obtain dichloromethane crude extract. The chromatographic seperations and purification of dichloromethane crude extract yielded two triterpenoids, oleanolic acid acetate (**1**), corosolic acid (**2**) and one steroid, stigmasterol (**3**). The three compounds were tested for their antioxidant activities by DPPH and ABTS assays. Oleanolic acid acetate (**1**) and corosolic acid (**2**) displayed moderate activity by DPPH and ABTS assays.

Acknowledgement

The authors thank Loei Rajabhat University for financial support (year 2021) and Faculty of Science and Technology, Loei Rajabhat University for the provision of laboratory facilities.

References

Buyinza, D., Chalo, D. M., Derese, S., Ndakala, A., & Yenesew, A. (2020). Flavonoids and isoflavonoids of *Millettia dura* and *Millettia ferruginea* : Phytochemical review and chemotaxonomic values. *Biochemical Systematics and Ecology*, *91,* 104053.

Deyou, T., Gumula, I., Pang, F., Gruhonjic, A., Mumo, M., Holleran, J., ... Yenesew, A. (2015). Rotenoids, flavonoids, and chalcones from the root bark of *Millettia usaramensis. Journal of Natural Products*, *78,* 2932-2939.

Deyou, T., Marco, M., Heydenreich, M., Pan, F., Gruhonjic, A., Fitzpatrick, P. A., ... Erdélyi, M. (2017). Isoflavones and rotenoids from the leaves of *Millettia oblata* ssp. teitensis. *Journal of Natural Products*, *80,* 2060-2066.

Endo, M., Shigetomi, K., Mitsuhashi, S., Igarashi, M., & Ubukata, M. (2019). Isolation, structure determination and structure activity relationship of anti toxoplasma triterpenoids from *Quercus crispula* Blume outer bark. *Journal of Wood Science, 65,* 1-11.

Forgo, P., & Kover. K. E. (2004). Gradient enhanced selective experiments in the 1H NMR chemicalshift assignment of the skeleton and side-chain resonances of stigmasterol, a phytosterol derivative. *Steroids, 69,* 43-50.

Jo, H., Oh, J. H., Park, D. W., Lee, C., & Min, C. K. (2020). Oleanolic acid 3-acetate, a minor element of ginsenosides, induces apoptotic cell death in ovarian carcinoma and endometrial carcinoma cells via the involvement of a reactive oxygen specieseindependent mitochondrial pathway. *Journal of Ginseng Research, 44,* 96-104.

Ma, X., Zhao, M., Tang, M. H., Xue, L. L., Zhang, R. J., Liu, L., ... Chen, L. J. (2020). Flavonoids with inhibitory effects on NLRP3 inflammasome activation from *Millettia velutina. Journal of Natural Products*, *83,* 2950-2959.

Pailee, P., Mahidol, C., Ruchirawat, S., & Prachyawarakorn, V. (2019). Diverse flavonoids from the roots of *Millettia brandisiana. Phytochemistry, 162,* 157-164.

Pérez, L. B., Pan, L., Acuña, U. M., Li, J., Chai, H. B., Gallucci, J. C., … Kinghorn, A. D. (2014). Caeruleanone A, a rotenoid with a new arrangement of the D-ring from the fruits of *Millettia caerulea*. *Organic Lett*ers, *16,* 1462-1465.

Qusti, Y. S., Abo-khatwa, N. A. and Bin-Lahwa, A. M. (2010). Screening of antioxidant activity and phenolic content of selected food items cited in the holly ouran. *European Journal of Biological Science*. 2(1). 40-51.

Raksat, A., Maneerat, W., Andersen, R. J., Pyne, S. G., & S Laphookhieo, S. (2018). Antibacterial prenylated isoflavonoids from the stems of *Millettia extensa*. *Journal of Natural Products*, *81,* 1835-1840.

Ruksilp, T. (2020a). A triterpenoid and steroids from the twigs of *Millettia utilis* Dunn. and their antioxidant activities. *Naresuan University Journal : Science and Technology*, *28*(1), 49-54.

Ruksilp, T. (2020b). Fatty acids and an ester from the leaves of *Millettia utilis* Dunn. *Naresuan University Journal : Science and Technology*, *28*(3), 63-68.

Sridhar, K., & Charles, A. L. (2019). *In vitro* antioxidant activity of Kyoho grape extracts in DPPH· and ABTS· assays: Estimation methods for EC_{50} using advanced statistical programs. *Food Chemistry*, *275,* 41-49.

Woo, K. W., Sang, J. Y., Choi, S. U., Kim, K. H., & Lee, K. R. (2014). Triterpenes from *Perilla frutescens* var. *acuta* and their cytotoxic activity. *Natural Product Sciences*, *20*(2), 71-75.

Xu, S., Wang, G., Peng, W., Xu, Y., Zhang, Y., Ge, Y., … Gong, Z. (2019). Corosolic acid isolated from *Eriobotrya japonica* leaves reduces glucose level in human hepatocellular carcinoma cells, zebrafish and rats. Scientific Reports, *9,* 4388.

Xue, L. L., Wub, W. S., Maa, X., Peia, H. Y., Tanga, M. H., Kuanga, S., … & Chena, L. J. (2020). Modulation of LPS-induced inflammation in RAW264.7 murine cells by novel isoflavonoids from *Millettia pulchra*. *Bioorganic Chemistry*, *97,* 103693.

Yan, W., Yang, J., Tang, H., Xue, L., Chen, K., Wang, L., … Chen, L. (2019). Flavonoids from the stems of *Millettia pachyloba* Drake mediate cytotoxic activity through apoptosis and autophagy in cancer cells. *Journal of Advanced Research*, *20,* 117-127.