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Chemical constituents of *Wrightia pubescens* (R.Br.)

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ABSTRACT

*Chemical investigation of the dichloromethane extracts of Wrightia pubescens (R.Br.) led to the isolation of ursolic acid (**1**), oleanolic acid (**2**), squalene (**3**), β-sitosterol (**4**) and chlorophyll a (**5**) from the leaves; and **1**, **2** and α-amyrin acetate (**6**) from the twigs. The structures of **1**-**6** were identified by comparison of their ^{1}H NMR and/or ^{13}C NMR data with those reported in the literature.*

Keywords: *Wrightia pubescens* (R.Br.), Apocynaceae, ursolic acid, oleanolic acid, squalene, β-sitosterol, chlorophyll a, α-amyrin acetate

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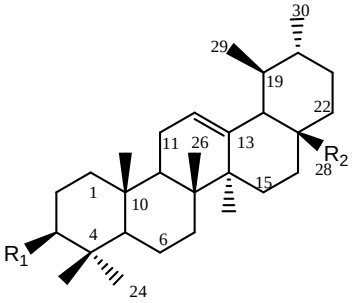
INTRODUCTION

Wrightia pubescens (R.Br.) of the family Apocynaceae is one of the eight recognized species of *Wrightia* in Malesia [1]. This species, which grows to 35 m tall in deciduous lowland thickets and forests, also occurs in mainland China, India and Australia [2, 3]. In the Philippines, it is locally known as “lanete” or “laniti”. In folk medicine, extracts from the roots and bark of the tree are used to treat scrofula and rheumatic arthralgia [3], while the latex is used against severe dysentery [4]. Furthermore, the latex of the plant has been shown to be anti-inflammatory and antinociceptive. The inhibitory activity of the plant’s latex on prostaglandin E2 (PGE_2) production and cyclooxygenase 2 (COX-2) protein expression in RAW 264.7 mouse macrophages were studied and linked to the reduction of inflammation and pain [5]. Chinese medicine preparations containing *W. pubescens* for intractable hiccups [6], osteoarthritis [7, 8], and acute upper respiratory infection of children [9] have been reported.

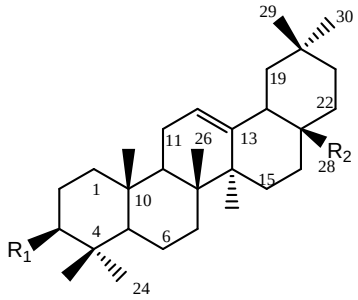
There is no reported study on the chemical constituents of *Wrightia pubescens*. However, a review on the chemical constituents and biological activities of its congener, *Wrightia tinctora* R. Br. has been provided [10]. The chemical constituents reported from this tree include 3,4-seco-lup-20(29)-en-3-oic acid, lupeol, stigmasterol and campetosterol, indigotin, indirubin, tryptanthrin, isatin, anthranillate, triacontanol, wrightial, cycloartenone, cycloeucalenol, β-amyrin, α-amyrin, β-sitosterol, 14α-methylzymosterol, desmosterol, clerosterol, 24-methylene-25-methylcholesterol, and 24-dehydropollinastanol [10].

This study is part of our research on the chemical constituents of trees found at the De La Salle University – Science and Technology Complex (DLSU–STC) riparian forest and reforested area. We recently reported the isolation of squalene, β-sitosterol, polyprenols and triglyceride from the leaves of *Dysoxylum gaudichaudianum* (A. Juss.) Miq. collected from the riparian forest. The dichloromethane extract of the leaves of *D. gaudichaudianum* exhibited IC_{50} values of 7.35 and 13.19 μg/mL against breast cancer (MCF-7) and colon cancer (HT-29) cells, respectively [11]. A tree from the reforested area of DLSU-STC, *Kibatalia gitingensis* (Elm.) Woodson afforded ursolic acid, squalene, α-amyrin acetate and lupeol acetate from the leaves and twigs, while the twigs also yielded isoscopoletin [12].

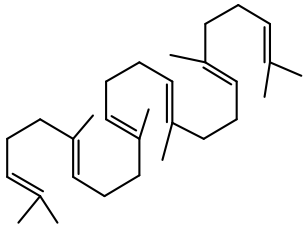
We report herein the isolation and identification of ursolic acid (**1**), oleanolic acid (**2**), squalene (**3**), β-sitosterol (**4**) and chlorophyll a (**5**) from the leaves; and **1**, **2** and α-amyrin acetate (**6**) from the twigs of *W. pubescens* (Fig. 1). To the best of our knowledge, this is the first report on the isolation of **1**-**6** from *W. pubescens.*



1 R_1 = OH, R_2 = COOH
6 R_1 = OAc, R_2 = CH_3



2 R_1 = OH, R_2 = COOH



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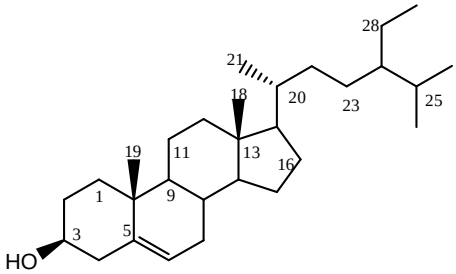

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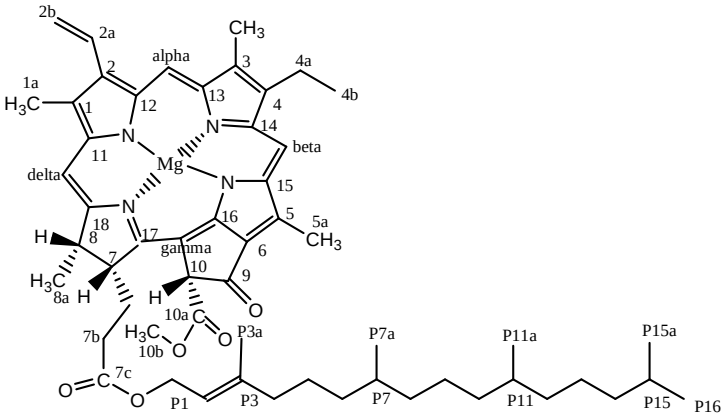

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Fig. 1. Chemical constituents of *Wrightia pubescens*: ursolic acid (1), oleanolic acid (2), squalene (3), β-sitosterol (4), chlorophyll a (5), and α-amyrin acetate (6)

MATERIALS AND METHODS

General Experimental Procedure

NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR spectra. Column chromatography was performed with silica gel 60 (70-230 mesh). Thin layer chromatography was performed with plastic backed plates coated with silica gel F_{254} and the plates were visualized by spraying with vanillin/H_2SO_4 solution followed by warming.

Sample Collection

Samples of leaves and twigs of *Wrightia pubescens* (R.Br.) were collected from the De La Salle University – Science and Technology Complex (DLSU-STC) riparian forest in February 2014. The samples were authenticated by one of the authors (EHM) and deposited at the De La Salle University Herbarium with voucher specimen #915.

General Isolation Procedure

A glass column 20 inches in height and 2.0 inches internal diameter was packed with silica gel. The crude extract from the leaves were fractionated by silica gel chromatography using increasing proportions of acetone in dichloromethane (10% increment) as eluents. One hundred milliliter fractions were collected. All fractions were monitored by thin layer chromatography. Fractions with spots of the same *Rf* values were combined and rechromatographed in appropriate solvent systems until TLC pure isolates were obtained. A glass column 12 inches in height and 0.5 inch internal diameter was used for the rechromatography. Five milliliter fractions were collected. Final purifications were conducted using Pasteur pipettes as columns. One milliliter fractions were collected.

Isolation

The leaves of *W. pubescens* were air-dried for about one week. The air-dried leaves (321 g) were ground in a blender, soaked in CH_2Cl_2 for 3 days and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (23 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The CH_2Cl_2 fraction was rechromatographed (2 ×) in 1% EtOAc in petroleum ether to afford **3** (25 mg). The 30% acetone in CH_2Cl_2 fraction was rechromatographed (4 ×) using 15% EtOAc in petroleum ether to afford **5** (32 mg) after washing with petroleum ether, followed by Et_2O. The 40% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) using CH_3CN:Et_2O:CH_2Cl_2 (0.5:0.5:9, v/v) to afford **4** (6 mg) after washing with petroleum ether. The 90% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) using CH_3CN:Et_2O:CH_2Cl_2 (1:1:8, v/v) to afford a mixture of **1** and **2** (2.08 g) after trituration with petroleum ether.

The twigs of *W. pubescens* were air-dried for about one week. The air-dried twigs (391.4 g) were ground in a blender, soaked in CH_2Cl_2 for 3 days and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (3.8 g) which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The 10% acetone in CH_2Cl_2 fraction was rechromatographed (4 ×) using 2.5% EtOAc in petroleum ether to afford **6** (7 mg) after washing with petroleum ether. The 90% acetone in CH_2Cl_2 fraction was rechromatographed (3 ×) using CH_3CN:Et_2O:CH_2Cl_2 (1.5:1.5:7, v/v) to afford a mixture of **1** and **2** (18 mg) after trituration with petroleum ether.

Ursolic Acid (**1**): colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): δ 36.99 (C-1), 28.12 (C-2), 79.04 (C-3), 38.58 (C-4), 55.20 (C-5), 18.28 (C-6), 32.92 (C-7), 39.46 (C-8), 47.52 (C-9), 38.74 (C-10), 23.27 (C-11), 125.84 (C-12), 137.91 (C-13), 41.95 (C-14), 27.21 (C-15), 24.12 (C-16), 47.90 (C-17), 52.61 (C-18), 39.03 (C-19), 38.81 (C-20), 30.59 (C-21), 36.68 (C-22), 28.00 (C-23), 15.46 (C-24), 15.58 (C-25), 17.08 (C-26), 23.56 (C-27), 181.96 (C-28), 16.97 (C-29), 21.16 (C-30).

Oleanolic acid (**2**): colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): δ 38.39 (C-1), 27.17 (C-2), 79.02 (C-3), 38.74 (C-4), 55.20 (C-5), 18.28 (C-6), 32.62 (C-7), 39.25 (C-8), 47.61 (C-9), 37.06 (C-10), 23.38 (C-11), 122.63 (C-12), 143.55 (C-13), 41.62 (C-14), 27.67 (C-15), 22.94 (C-16), 46.49 (C-17), 41.03 (C-18), 45.86 (C-19), 30.66 (C-20), 33.78 (C-21), 32.42 (C-22), 28.08 (C-23), 15.53 (C-24), 15.31 (C-25), 17.08 (C-26), 25.90 (C-27), 182.25 (C-28), 33.05 (C-29), 23.56 (C-30).

Squalene (**3**): colorless oil. ^{13}C NMR (150 MHz, $CDCl_3$): δ 25.69 (C-1), 131.24 (C-2), 124.30 (C-3), 26.66 (C-4), 39.73 (C-5), 134.88 (C-6), 124.40 (C-7), 26.76 (C-8), 39.75 (C-9), 135.09 (C-10), 124.30 (C-11), 28.27 (C-12), 17.67 (C-13), 16.03 (C-14), 16.00 (C-15).

β-Sitosterol (**4**): colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.51 (m, H-3), 2.26, 2.21 (H_2-4), 5.33 dd (*J* = 3.0, 2.4 Hz, H-6), 0.66 (s, H_3-18), 0.99 (s, H_3-19), 0.90 (d, *J* = 6.6 Hz, H_3-21), 0.79 (d, *J* = 7.2 Hz, H_3-26), 0.82 (d, *J* = 6.6 Hz, H_3-27), 0.85 (t, *J* = 7.8 Hz, H_3-29).

Chlorophyll a (**5**): dark green crystals. ^{13}C NMR (150 MHz, $CDCl_3$): δ 131.78 (C-1), 12.11 (C-1a), 136.45 (C-2), 129.05 (C-2a), 122.99 (C-2b), 136.09 (C-3), 11.25 (C-3a), 145.10 (C-4), 19.49 (C-4a), 17.36 (C-4b), 137.91 (C-5), 12.11 (C-5a), 129.05 (C-6) 51.22 (C-7), 29.69 (C-7a), 31.19 (C-7b), 172.92 (C-7c), 50.16 (C-8), 23.08 (C-8a), 189.58 (C-9), 64.71 (C-10), 169.52 (C-10a), 52.87 (C-10b), 142.85 (C-11), 136.16 (C-12), 155.56 (C-13), 150.92 (C-14), 128.99 (C-15), 149.67 (C-16), 161.25 (C-17), 172.92 (C-18), 97.53 (C-α), 104.47 (C-β), 105.24 (C-γ), 93.09 (C-δ), 61.47 (P-1), 117.76 (P-2), 142.16 (P-3), 16.27 (P-3a), 39.78 (P-4), 24.97 (P-5), 36.62 (P-6), 32.60 (P-7), 19.64 or 19.70 (P-7a), 37.30 (P-8), 24.44 (P-9), 37.39 (P-10), 32.74 (P-11), 19.64 or 19.70 (P-11a), 37.24 (P-12), 24.75 (P-13), 39.33 (P-14), 27.94 (P-15), 22.60 (P-15a), 22.70 (P-16).

α-Amyrin acetate (**6**): colorless solid. ^{13}C NMR (150 MHz, $CDCl_3$): δ 38.45 (C-1), 23.59 (C-2), 80.95 (C-3), 37.74 (C-4), 55.24 (C-5), 18.23 (C-6), 32.85 (C-7), 40.01 (C-8), 47.63 (C-9), 36.78 (C-10), 23.36 (C-11), 124.30 (C-12), 139.62 (C-13), 42.05 (C-14), 26.58 (C-15), 28.08 (C-16), 33.74 (C-17), 59.04 (C-18), 39.64 (C-19), 39.59 (C-20), 31.23 (C-21), 41.52 (C-22), 28.74 (C-23), 16.85 (C-24), 15.73 (C-25), 16.73 (C-26), 23.21 (C-27), 28.75 (C-28), 17.50 (C-29), 21.39 (C-30), 171.03, 21.32 (OAc).

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extracts of *Wrightia pubescens* afforded a mixture of ursolic acid (**1**) [12] and oleanolic acid (**2**) [13] in a 1:1 ratio, squalene (**3**) [14], β-sitosterol (**4**) [13] and chlorophyll a (**5**) **[**15, 16**]** from the leaves; and a mixture of **1** and **2** in a 1:1 ratio, and α-amyrin acetate (**6**) [17] from the twigs. The structures of **1**-**6** were identified by comparison of their ^{1}H NMR and/or ^{13}C NMR data with those reported in the literature [12-17]. The 1:1 ratio of **1** and **2** was deduced from the integrations of the ^{1}H NMR resonances for the olefinic protons of **1** at δ 5.24 (t, *J* = 3.6 Hz) and **2** at δ 5.28 (t, *J* = 3.6 Hz).

Although no biological activity tests were conducted on the isolated compounds (**1**-**6**), literature search revealed that these have diverse bioactivities as follows.

Ursolic acid (**1**) was found to induce apoptosis in tumor cells by activation of caspases and modulation of other pathways involved in cell proliferation and migration. It decreases proliferation of cells and induces apoptosis, thereby inhibiting growth of tumor cells both *in vitro* and *in vivo* [18]. An earlier study reported that **1** exhibited anti-tumor activity against human colon carcinoma cell line HCT15 [19]. Moreover, **1** inhibits the growth of colon cancer-initiating cells by targeting STAT3 [20]. Furthermore, **1** has potential therapeutic use in prostate cancer through its antiproliferative and apoptotic effects [21]. A recent study reported that **1** inhibited cell growth and proliferation of Jurkat leukemic T-cells, as well as suppressed PMA/PHA induced IL-2 and TNF-α production in a concentration and time dependent manner [22]. Another study reported that ursolic acid-activated autophagy induced cytotoxicity and reduced tumor growth of cervical cancer cells TC-1 in a concentration-dependent manner [23].

Oleanolic acid (**2**) exhibited anti-inflammatory effects by inhibiting hyperpermeability, the expression of CAMs, and the adhesion and migration of leukocytes [24]. It showed anti-inflammatory activities through the inhibition of the HMGB1 signaling pathway [25]. It exhibited anti-inflammatory, hepatoprotective, gastroprotective, immunoregulatory and anti-ulcer activities [26], and gastroprotective effect on experimentally induced gastric lesions in rats and mice [27]. Oleanolic acid was also reported to inhibit mouse skin tumor [28], protect against hepatotoxicants and treat hepatitis [29], and showed significant antitumor activity on human colon carcinoma cell line HCT 15 [30].

Squalene (**3**) was reported to significantly suppress colonic ACF formation and crypt multiplicity which strengthened the hypothesis that it possesses chemopreventive activity against colon carcinogenesis [31]. It showed cardioprotective effect which is related to inhibition of lipid accumulation by its hypolipidemic properties and/or its antioxidant properties [32]. A recent study reported that tocotrienols, carotenoids, squalene and coenzyme Q10 have anti-proliferative effects on breast cancer cells [33]. The preventive and therapeutic potential of squalene containing

compounds on tumor promotion and regression have been reported [34]. A recent review on the bioactivities of squalene has been provided [35].

β-Sitosterol (**4**) was observed to have growth inhibitory effects on human breast MCF-7 and MDA-MB-231 adenocarcinoma cells [36]. It was shown to be effective for the treatment of benign prostatic hyperplasia [37]. It was also reported to attenuate β-catenin and PCNA expression, as well as quench radical *in-vitro*, making it a potential anticancer drug for colon carcinogenesis [38]. It can inhibit the expression of NPC1L1 in the enterocytes to reduce intestinal cholesterol uptake [39]. It was reported to induce apoptosis mediated by the activation of ERK and the downregulation of Akt in MCA-102 murine fibrosarcoma cells [40].

Chlorophyll a (**5**) and its various derivatives are used in traditional medicine and for therapeutic purposes [41]. Natural chlorophyll and its derivatives have been studied for wound healing [42], anti-inflammatory properties [43], control of calcium oxalate crystals [44], utilization as effective agents in photodynamic cancer therapy [45-47], and chemopreventive effects in humans [48, 49]. A review on digestion, absorption and cancer preventive activity of dietary chlorophyll has been provided [50].

α-Amyrin acetate (**6**) at 100 mg/kg showed significant ($p < 0.05$) inhibition of egg albumen-induced paw edema with 40 % inhibition at the 5th hour. Triterpene **6** isolated from the *Alstonia boonei* stem bark exhibited profound anti-inflammatory activity [51]. β-Amyrin acetate and **6** were also reported to exhibit sedative, anxiolytic and anticonvulsant properties [52].

CONCLUSION

The dichloromethane extract of the leaves of *Wrightia pubescens* afforded ursolic acid, oleanolic acid, squalene, β-sitosterol and chlorophyll a which were reported to exhibit anticancer and cytotoxic properties. This is the first report on the isolation of these compounds from *W. pubescens* which has no reported anticancer property.

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