Chemical constituents of *Broussonetia luzonicus*

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ABSTRACT

The leaves of *Broussonetia luzonicus* afforded epitaraxerol (**1**), lupenone (**2**), squalene (**3**), β-carotene (**4**), vitamin K (**5**) and β-sitosterol (**6**), while the flowers yielded **2**, **6**, lupeol (**7**), betulin aldehyde fatty acid ester (**8**) and lupeol fatty acid ester (**9**). The compounds were isolated by silica gel chromatography and identified by NMR spectroscopy. Triterpenes **1**, **2** and **8** were tested for cytotoxicity using the MTT assay. They did not exhibit cytotoxic effect against a human cancer cell line colon carcinoma (HCT 116). Results of the antimicrobial tests on **1**, **2** and **8** indicated moderate antifungal activity against *C. albicans* and low antimicrobial activity against *T. mentagrophytes, A. niger, S. aureus*, *E. coli, P. aeruginosa,* and *B. subtilis*.

Keywords: *Broussonetia luzonicus*, Moraceae, triterpenes, antimicrobial

INTRODUCTION

Broussonetia luzonicus (Moraceae family), is an endemic tree which grows in thickets and forests throughout the Philippines.[1] The flowers and leaves are eaten as vegetables. There are no reported studies on the chemical constituents and biological activities of *B. luzonicus*. However, *B. papyrifera* and *B. kazinoki* which are congeners of the tree have been used as tonic in traditional Chinese medicines. The chemical components of *B. papyrifera* were shown to decrease the effects of Alzheimer's disease and exhibit anti-aging properties, as well as possess antityrosinase and antioxidant activities.[2] On the other hand, *B. kazinoki* was reported to exhibit cytotoxic properties.[3]

We report herein the isolation and identification of epitaraxerol (**1**), lupenone (**2**), squalene (**3**), *β*-carotene (**4**), vitamin K (**5**) and *β*-sitosterol (**6**) from the leaves; and **2**, **6**, lupeol (**7**), betulin aldehyde fatty acid ester (**8**) and lupeol fatty acid ester (**9**) from the flowers of *B. luzonicus* (Fig. 1). We also report the antimicrobial properties of **1**, **2** and **8**.

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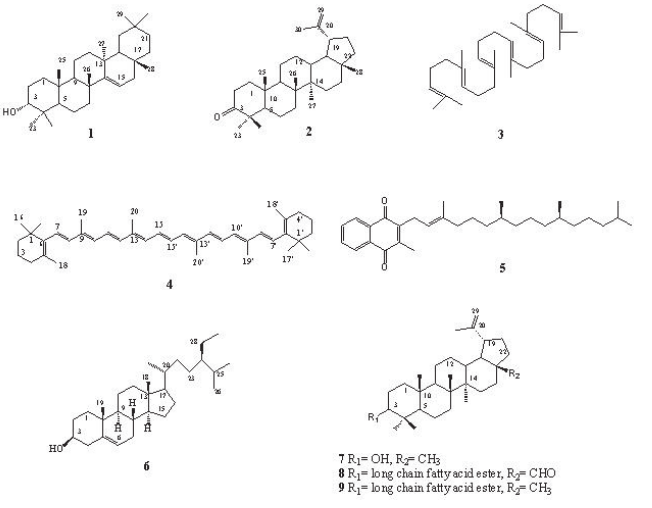

Figure 1. Chemical constituents of Broussonetia luzonicus leaves: epitaraxerol (1), lupenone (2), squalene (3). B-carotene (4), vitamin K (5) and B-sitosterol (6) and flowers: 2, 6, lupeol (7), betulin aldehyde fatty acid ester (8) and lupeol fatty acid ester (9).

MATERIALS AND METHODS

General Experimental Procedures

NMR spectra of **1-4**, **6** and **8** were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR spectra. NMR spectra of **5**, **7** and **9** were recorded on a Varian Unity Inova spectrometer in $CDCl_3$ at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR spectra. Column chromatography was performed with silica gel (230–400 mesh). TLC was performed on aluminium plates coated by silica gel 60 F_{254}. The plates were visualized by spraying with vanillin-sulfuric acid, followed by warming.

Plant Material

The leaves of *Broussonetia luzonicus* were collected from the Bureau of Plant Industry (BPI), Department of Agriculture, Manila, Philippines, while the flowers were collected from Sinait, Ilocos Sur. The samples were authenticated by Josephine T. Garcia of the BPI.

Extraction and Isolation

The air-dried leaves (1.2 kg) of *B. luzonicus* were ground in an osterizer, soaked in dichloromethane (DCM) for three days, and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (50 g) which was chromatographed in increasing proportions of acetone in DCM at 10% increment. The DCM fraction was rechromatographed (9x) in hexane to afford **3** (200 mg), **4** (150 mg) and **6** (80 mg). The 10–20% acetone in DCM fractions were rechromatographed (8x) in 2.5% EtOAc in hexane, followed by 5%, 10% and 15% EtOAc in hexane to afford **1** (3 mg), **2** (7 mg) and **5** (3 mg).

The air-dried flowers (262 g) of *B. luzonicus* were ground in an osterizer, soaked in DCM for three days, and then filtered. The filtrate was concentrated under vacuum to afford a crude extract (10 g) which was chromatographed in increasing proportions of acetone in DCM at 10% increment. The 30% to 50% acetone in DCM fractions were combined and rechromatographed (5x) in 2.5% EtOAc in hexane to afford **8** (6 mg) and **9** (10 mg). The more polar fractions were rechromatographed (2x) by gradient elution technique. The column was first eluted with 10% EtOAc in hexane to afford **2** (12 mg), followed by DCM:diethyl ether:acetonitrile (8:1:1) to afford **6** (25 mg). The 10% acetone fraction was rechromatographed (4x) in DCM to afford **7** (120 mg), after washing with acetone.

Epitaraxerol (**1**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 32.2 (C-1), 25.1 (C-2), 76.2 (C-3), 37.4 (C-4), 49.2 (C-5), 18.7 (C-6), 41.2 (C-7), 39.1 (C-8), 48.9 (C-9), 35.8 (C-10), 17.4 (C-11), 33.7 (C-12), 37.5 (C-13), 158.2 (C-14), 116.7 (C-15), 37.7 (C-16), 38.0 (C-17), 48.7 (C-18), 36.7 (C-19), 28.8 (C-20), 32.2 (C-21), 35.1 (C-22), 28.2 (C-23), 22.2 (C-24), 15.2 (C-25), 26.0 (C-26), 21.2 (C-27), 29.8 (C-28), 33.3 (C-29), 29.9 (C-30).

Lupenone (**2**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 39.6 (C-1), 34.1 (C-2), 218.2 (C-3), 47.3 (C-4), 54.9 (C-5), 19.7 (C-6), 33.5 (C-7), 40.8 (C-8), 49.8 (C-9), 36.9 (C-10), 21.5 (C-11), 25.1 (C-12), 37.5 (C-13), 42.9 (C-14), 27.4 (C-15), 35.5 (C-16), 43.0 (C-17), 48.2 (C-18), 47.9 (C-19), 150.8 (C-20), 29.8 (C-21), 40.6 (C-22), 26.6 (C-23), 21.0 (C-24), 15.8 (C-25), 16.0 (C-26), 14.5 (C-27), 18.0 (C-28), 109.4 (C-29), 19.3 (C-30).

Squalene (**3**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 25.7 (C-1), 131.3 (C-2), 124.3 (C-3), 26.7 (C-4), 39.7 (C-5), 134.9 (C-6), 124.3 (C-7), 26.7 (C-8), 39.7 (C-9), 134.9 (C-10), 124.3 (C-11), 28.3 (C-12), 17.7 (C-2'), 16.0 (C-6'), 16.0 (C-10').

β-Carotene (**4**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 34.3 (C-1, 1'), 39.6 (C-2, 2'), 19.3 (C-3, 3'), 33.1 (C-4, 4'), 129.3 (C-5, 5'), 137.9 (C-6, 6'), 126.6 (C-7, 7'), 137.8 (C-8, 8'), 136.0 (C-9, 9'), 132.4 (C-10, 10'), 130.0 (C-11, 11'), 137.2 (C-12, 12'), 136.5 (C-13, 13'), 130.8 (C-14, 14'), 125.0 (C-15, 15'), 29.7 (C-16, 16'), 29.0 (C-17, 17'), 21.8 (C-18, 18'), 12.82 (C-19, 19'), 12.76 (C-20, 20').

Vitamin K (**5**): ^{13}C NMR (125 MHz, $CDCl_3$): δ 12.7, 16.3, 19.70, 19.71, 22.6, 22.7, 24.4, 24.8, 25.3, 26.0, 28.0, 32.6, 32.8, 36.6, 37.3, 37.4, 39.4, 40.0, 118.8, 126.2, 126.3, 132.15, 132.19, 133.3, 134.0, 138.0, 143.4, 146.2, 184.6, 185.5.

β-Sitosterol (**6**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 37.2 (C-1), 31.6 (C-2), 71.8 (C-3), 42.3 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9 (C-7), 31.9 (C-8), 50.1 (C-9), 36.1 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-13), 56.7 (C-14), 24.3 (C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-19), 36.5 (C-20), 19.0 (C-21), 33.9 (C-22), 29.1 (C-23), 45.8 (C-24), 26.0 (C-25), 18.8 (C-26), 19.8 (C-27), 23.1 (C-28), 12.0 (C-29).

Lupeol (7): ^{13}C NMR (125 MHz, $CDCl_3$): δ 38.9 (C-1), 27.4 (C-2), 79.0 (C-3), 38.7 (C-4), 55.3 (C-5), 18.0 (C-6), 34.3 (C-7), 40.8 (C-8), 50.4 (C-9), 37.2 (C-10), 20.9 (C-11), 25.1 (C-12), 38.1 (C-13), 42.8 (C-14), 27.4 (C-15), 35.6 (C-16), 48.0 (C-17), 48.3 (C-18), 48.0 (C-19), 151.0 (C-20), 29.8 (C-21), 40.0 (C-22), 28.0 (C-23), 15.4 (C-24), 16.1 (C-25), 16.0 (C-26), 14.5 (C-27), 18.3 (C-28), 109.3 (C-29), 19.3 (C-30).

Betulin aldehyde fatty acid ester (**8**): ^{13}C NMR (150 MHz, $CDCl_3$): δ 38.7 (C-1), 26.7 (C-2), 80.5 (C-3), 39.7 (C-4), 55.4 (C-5), 18.1 (C-6), 34.9 (C-7), 40.8 (C-8), 50.4 (C-9),

37.8 (C-10), 20.7 (C-11), 25.5 (C-12), 39.7 (C-13), 42.5 (C-14), 26.7 (C-15), 29.2 (C-16), 59.3 (C-17), 47.5 (C-18), 48.0 (C-19), 149.7 (C-20), 29.5 (C-21), 33.2 (C-22), 28.8 (C-23), 16.0 (C-24), 16.2 (C-25), 16.5 (C-26), 14.2 (C-27), 206.7 (C-28), 110.2 (C-29), 19.0 (C-30), 173.7 (C-1'), 34.9 (C-2'), 31.9 (C-3'), 22.7, 25.2, 29.2-29.8 (CH_2)$_n$, 14.1 (CH_3).

Lupeol fatty acid ester (**9**): ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.77, 0.82, 0.84, 0.86, 0.92, 1.02 and 1.66 (each 3H, s, H-23, 24, 25, 26, 27, 28, and 30), 2.35 (1H, m, H-19), 4.46 (1H, dd, *J* = 4.5, 11.0, H-3), 4.55 (1H, brs, H-29b), 4.73 (1H, brs, H-29a), fatty acid: 1.21-1.35 (br s, CH_2), 2.30 (2H, t, *J* = 7.0 Hz, H-2'), 1.60 (2H, H-3'), 0.86 (CH_3, t, *J* = 6.5 Hz).

BIOASSAYS

Cytotoxicity Tests

Four milligrams each of **1**, **2** and **8** were dissolved in 1 milliliter of DMSO to make 4 mg/mL solutions. Compounds **1**, **2** and **8** were tested for cytotoxic activity against a human cancer cell line colon carcinoma (HCT 116) at the Institute of Biology, University of the Philippines, Diliman, Quezon City. Doxorubicin was used as the positive control, while DMSO was used as the negative control. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay reported in the literature was employed.[4,5]

Antimicrobial Tests

The microorganisms used: *Pseudomonas aeruginosa* (UPCC 1244), *Bacillus subtilis* (UPCC 1149), *Escherichia coli* (UPCC 1195), *Staphylococcus aureus* (UPCC 1143), *Candida albicans* (UPCC 2168), *Trichophyton mentagrophytes* (UPCC 4193) and *Aspergillus niger* (UPCC 3701) were obtained from the University of the Philippines Culture Collection (UPCC). The test compound was dissolved in 95% ethanol. The positive control for the bacteria and fungi, respectively are chloramphenicol and Canesten which contains 1% chlotrimazole. The antimicrobial assay procedure reported in the literature[6] was employed. The clearing zone was measured in millimeters, and the average diameter of the clearing zones was calculated. The activity index was computed by subtracting the diameter of the well from the diameter of the clearing zones divided by the diameter of the well.

RESULTS AND DISCUSSION

The structures of **1**, **2**, **5**, and **7** were elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of their ^{13}C NMR data with those reported in the literature for epitaraxerol,[7] lupenone,[8] vitamin K[9] and lupeol,[10] respectively. The structures of **3**, **4** and **6** were confirmed by comparison of their ^{13}C NMR data with those reported in the literature for squalene,[11] β-carotene[12] and β-sitosterol,[12] respectively. The structure of **8** was elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of its ^{13}C NMR data with those of betulin aldehyde for the triterpene part[13] and fatty acid esters of 16-hydroxycycloartenol for the fatty acid part.[14] The structure of **9** was deduced by comparison of its ^{1}H NMR data with those of lupeol fatty acid ester.[15]

Since a congener of the tree exhibited cytotoxic properties,[3] **1**, **2** and **8** were tested for cytotoxicity against a human cancer cell line colon carcinoma (HCT 116) by the MTT assay. These compounds did not show linear interpolation with HCT 116, thus IC_{50} could not be computed. This implied that **1**, **2** and **8** did not exhibit cytotoxic effect against this cell line.

Compounds **1**, **2** and **8** were further tested for antimicrobial activity against seven microorganisms (Table 1). Results of the study indicated that these compounds exhibited moderate antifungal activity against *C. albicans* with activity index (AI) of 0.3, 0.4, and 0.4, respectively. They showed low antifungal activity against *T. mentagrophytes* (AI = 0.2, 0.3 and 0.4, respectively) and *A. niger* (AI = 0.1, 0.2 and 0.2, respectively). They also exhibited low antibacterial activity against *S. aureus* (AI = 0.2, 0.3, 0.3, respectively), *E. coli* (AI = 0.2, 0.2 and 0.1, respectively), *P. aeruginosa* (AI = 0.2, 0.2 and 0.2, respectively), and partial inhibition of *B. subtilis* (AI = 0.3, 0.5 and 0.5, respectively).

Although there are no known medicinal properties of *B. luzonicus*, results of the study implied that **1**, **2** and **8** exhibit antimicrobial potentials. The compounds isolated from *B. luzonicus* have shown biological activities. Squalene exhibited chemopreventive activity against colon carcinogenesis and possessed antioxidant properties.[16,17] Lupeol showed antiurolithiatic and diuretic activities, prevented the vesical calculi and reduced the stone size in the rats.[18] Long-chain fatty acid esters of lupeol demonstrated an antiplasmodial activity,[15] while β-sitosterol induced apoptosis in human tumors for colon and breast cancers.[19] Lupenone and lupeol exhibited antimicrobial, antiviral, anticancer, and anti-inflammatory activities.[20] Vitamin K is a factor required for normal blood coagulation and it reduced undercarboxylated osteocalcin and improved the bone turnover profile.[21]

Table 1. Antimicrobial Activity of Compounds 1, 2 and 8.

Microorganism	Compounds	Clearing Zone,[c] mm	Activity Index (AI)
Pseudomonas aeruginosa	1	12	0.2
	2	12	0.2
	8	12	0.2
	Chloramphenicol[c]	14	1.3
Staphylococcus aureus	1	12	0.2
	2	13	0.3
	8	13	0.3
	Chloramphenicol[c]	23	2.8
Candida albicans	1	(13)[c]	(0.3)[c]
	2	(15)[c]	(0.5)[c]
	Chloramphenicol[c]		
	1	13	0.3
	2	14	0.4
	8	14	0.4
	Canesten, 0.2 g[c]	18	0.8
T. mentagrophytes	1	12	0.2
	2	813	0.3
	8	14	0.4
	Canesten, 0.2 g[c]	55	4.5
Aspergillus niger	1	11	0.1
	2	12	0.2
	Canesten, 0.2 g[c]	23	1.3

[a]Average of 3 trials, [b]Partial inhibition of growth of test organism, [c]Chloramphenicol disc - 6 mm diameter, [d]Contains 1% Chlotrimazole

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