New Triterpenes from *Barringtonia asiatica*

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The leaves of *Barringtonia asiatica* afforded two new triterpenes, germanicol caffeoyl ester (1) and camelliagenone (2). Their structures were elucidated by extensive 1D- and 2D-NMR spectroscopy. It also afforded germanicol *trans*-coumaroyl ester (3), germanicol *cis*-coumaroyl ester (4), germanicol (5), camelliagenin A (6), spinasterol, sitosterol, squalene, lutein and trilinolein. Compounds 3, spinasterol and trilinolein were isolated from the fruits, while the seeds yielded spinasterol, squalene, linoleic acid and trilinolein. Compounds 1—5 exhibited antifungal activity against *Candida albicans*, 1—3 and 5 showed antibacterial activity against *Staphylococcus aureus*, while 5 is active against *Pseudomonas aeruginosa*.

Key words *Barringtonia asiatica*; Lecythidaceae; germanicol caffeoyl ester; camelliagenone; antimicrobial

Barringtonia asiatica, commonly known as botong is found along the seashore throughout the Philippines. The fruits and seeds of *B. asiatica* are used as fish poison, fruit juice for scabies, leaves are applied to stomachache and rheumatism, seeds as vermifuge, and bark for tuberculosis.[1] The aqueous crude extract of *B. asiatica* seeds exhibited high biological activity in the brine shrimp hatchability and lethality assay.[2] The crude methanolic extract of *B. asiatica* (leaves, fruits, seeds, stem and root barks) and the fractions (petrol, dichloromethane, ethyl acetate, butanol) exhibited a broad spectrum antibacterial activity, while a number of fractions demonstrated antifungal activity.[3] Two major saponins from the seeds of *B. asiatica* are putative antifeedants toward *Epilachna* sp. larvae.[4] The seeds of *B. asiatica* were reported to exhibit piscicidal activity. The major compound responsible for this activity is ranuncoside, an oleanane glycoside.[5] An earlier study also reported the isolation of a mixture of saponins (A_1-barrinin) from *B. asiatica* which was hydrolyzed to afford A_1-barrigenol and A_2-barrigenol. The latter was identical to camelliagenin A[6] which is of relevance to our present report.

We report here the isolation, structure elucidation and antimicrobial assay of two new triterpenes, germanicol caffeoyl ester (**1**) and camelliagenone (**2**) from the dichloromethane extracts of the leaves of *B. asiatica*. The leaves also afforded germanicol *trans*-coumaroyl ester (**3**), germanicol *cis*-coumaroyl ester (**4**), germanicol (**5**), camelliagenin A (**6**) (Fig. 1), sitosterol, lutein, spinasterol, squalene and trilinolein. The fruits yielded **3**, spinasterol, squalene and trilinolein, while the seeds afforded linoleic acid, spinasterol,

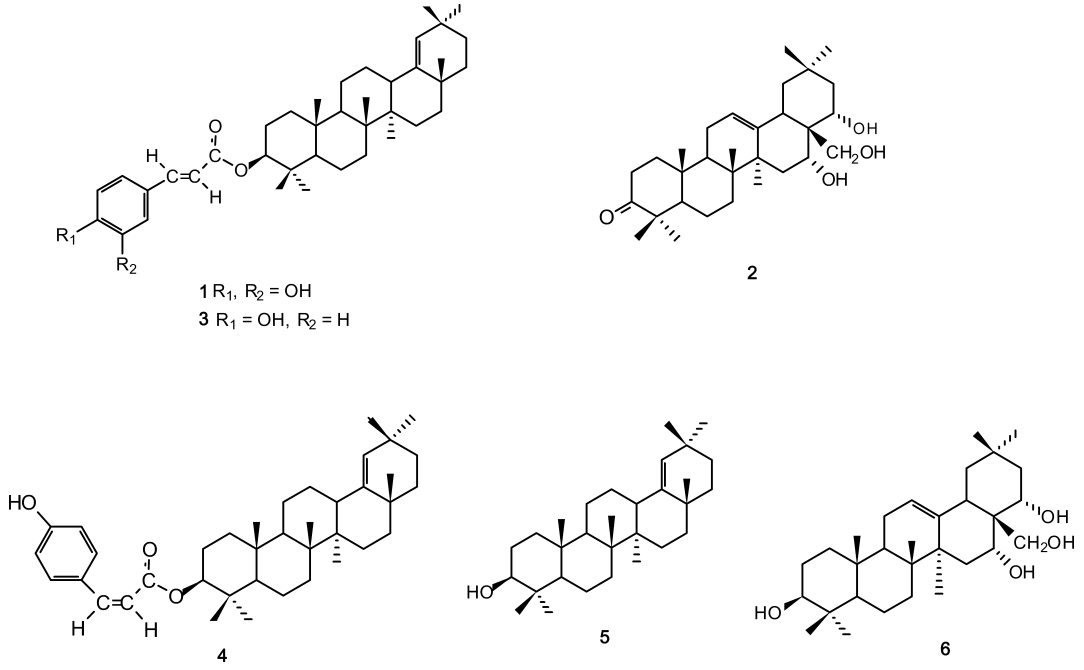

Fig. 1. Chemical Structures of **1**—**6**

This study was conducted in commemoration of the De La Salle University centennial.

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squalene and trilinolein. To the best of our knowledge this is the first report on the isolation of these compounds from *B. asiatica*.

Results and Discussion

The dichloromethane extract of the air-dried leaves of *Barringtonia asiatica* afforded the new triterpenes **1** and **2**. Their structures were elucidated by extensive 1D- and 2D-NMR spectroscopy as follows.

The ^{1}H-NMR spectrum of **1** (Table 1) gave resonances for conjugated olefinic protons at δ 6.25 (d, J=15.6 Hz), 7.54 (d, J=15.6 Hz) and aromatic protons at δ 7.09 (d, J=1.8 Hz), 6.85 (d, J=7.8 Hz) and 6.99 (dd, J=1.8, 7.8 Hz). The large coupling constant (J=15.6 Hz) for δ 6.25 and 7.54 indicated they were *trans*, while the coupling constants for the aromatic protons suggested a 1,3,4-trisubstituted benzene. These data suggested a caffeoyl moiety. An olefinic proton singlet was found at δ 4.85, while an oxymethine proton was suggested by the resonance at δ 4.59 (dd, J=7.2, 9.6 Hz). Eight methyl singlets were deduced from the resonances at δ 0.73, 0.87, 0.90, 0.91, 0.93, 0.94, 1.00 and 1.07.

The ^{13}C-NMR spectrum of **1** (Table 1) gave resonances for thirty-nine carbons with the following functionalities: a carbonyl carbon of a conjugated ester at δ 167.5, an oxymethine at δ 81.2, ten olefinic and aromatic carbons, eight methyl, ten methylene, three methine and six quaternary carbons. These resonances indicated a triterpene with an olefin and a caffeoyl ester substituent.

The high resolution-electron ionization-mass spectra (HR-EI-MS) of **1** gave a molecular ion of m/z 588.4182 [M^+] which corresponded to a molecular formula of $C_{39}H_{56}O_4$. The molecular formula indicated an index of hydrogen deficiency of twelve. With six double bonds deduced from the carbonyl and five olefins, the compound is hexacyclic.

Six isolated spin systems (Fig. 2) were deduced from the correlation spectroscopy (COSY) spectrum of **1** as follows: isolated olefinic protons (H-2′/H-3′); aromatic protons (H-8′/H-9′); coupled methylene protons (H_2-1/H_2-2); a methine proton coupled to methylene protons (H-5/H_2-6/H_2-7); a methine proton coupled to methylene protons which were in turn coupled to a methine proton (H_9/H_2-11/H_2-12/H-13); and two sets of coupled methylene protons (H_2-15/H_2-16) and (H_2-21/H_2-22).

Protons attached to carbons were assigned (Table 1) from ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) 2D-NMR data and the structure of **1** was elucidated by analysis of the heteronuclear multiple bond coherence (HMBC) 2D-NMR data: key HMBC correlations are shown in Fig. 2. The caffeoyl substituent was attached to C-3 based on correlations between the oxymethine proton (H-3) and the carbonyl carbon of the ester C-1′. The olefin was assigned to C-18 on the basis of long-range correlations between the methyl singlets at δ 0.93 (H_3-29) and 0.94 (H_3-30) and the olefinic carbon (C-19), and the correlation between the methyl singlet at δ 1.00 (H_3-28) and the olefinic carbon (C-18). It was earlier suggested that based on coupling constants **1** is a 1,3,4-trisubstituted benzene. This was confirmed by the HMBC spectrum which indicated long-range correlations between H-5′, H-9′ and C-3′; H-5′, H-8′ and C-6′; and H-5′, H-8′, H-9′ and C-7′. All long-range correlations were consistent with the structure of **1**.

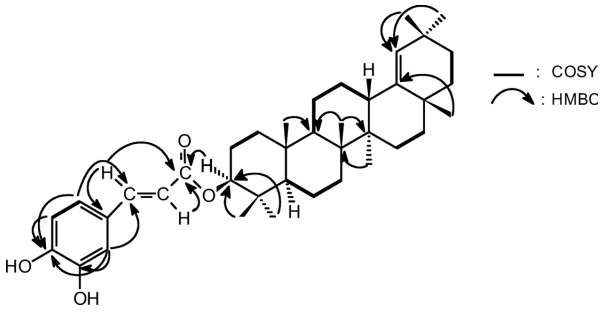

Fig. 2. ^{1}H–^{1}H COSY and ^{1}H–^{13}C Long-Range Correlations for **1**

Table 1. 600 MHz ^{1}H- and 150 MHz ^{13}C-NMR Data of **1** and **2** in $CDCl_3$

No.	**1**		**2**	
	δ_C	δ_H mult. (J in Hz)[a]	δ_C	δ_H mult. (J in Hz)[a]
1	38.4	1.05, 1.78	39.3	1.43, 1.88
2	23.8	1.68, 1.72	34.1	1.66, 2.52
3	81.2	4.59 dd (7.2, 9.6)	217.8	
4	38.1		47.4	
5	55.6	0.82	55.1	1.34
6	18.2	1.40, 1.52	19.6	1.50
7	34.5	1.36, 1.48	32.3	1.35, 1.55
8	40.8		39.7	
9	51.1	1.30	45.8	1.68
10	37.4		36.6	
11	21.1	1.30, 1.56	23.5	1.90
12	26.2	1.20, 1.48	122.9	5.28 t (3.6)
13	38.6	2.26	142.4	
14	43.3		41.7	
15	27.5	1.08, 1.80	33.6	1.42, 2.02
16	37.7	1.32, 1.38	68.2	4.63 br s
17	34.3		43.9	
18	142.7		42.4	1.92
19	129.8	4.85 s	47.3	1.06, 2.26
20	32.3		31.3	
21	33.3	1.32, 1.44	45.8	1.57, 1.76
22	37.2	1.40, 1.45	76.6	4.02 dd (6.6, 12.0)
23	28.0	0.87 s	24.8	1.09 s
24	16.8	0.90 s	21.4	1.042 s
25	16.7	0.91 s	15.4	1.036 s
26	16.1	1.07 s	16.7	0.94 s
27	14.6	0.73 s	26.8	1.40 s
28	25.3	1.00 s	73.0	3.30 d (10.8)
				3.60 dd (4.2, 10.8)
29	31.3	0.93 s	33.0	0.92 s
30	29.2	0.94 s	24.8	0.93 s
1′	167.5			
2′	116.4	6.25 d (15.6)		
3′	144.3	7.54 d (15.6)		
4′	127.7			
5′	114.3	7.09 d (1.8)		
6′	143.8			
7′	146.2			
8′	115.4	6.85 d (7.8)		
9′	122.4	6.99 dd (1.8, 7.8)		
6′-OH, 7′-OH		5.80 br s, 6.00 br s		

a) Multiplet unless otherwise indicated.

The relative configuration of **1** (Fig. 3) was deduced from nuclear Overhauser effect spectroscopy (NOESY). The methyl singlet at δ 1.00 (H_3-28) was close in space to the methine proton at δ 2.26 (H-13), which was in turn close to the methyl singlet at δ 1.07 (H_3-26), which was finally close to the methyl singlet at δ 0.91 (H_3-25). These correlations indicate that they are on the same face of the molecule. On the

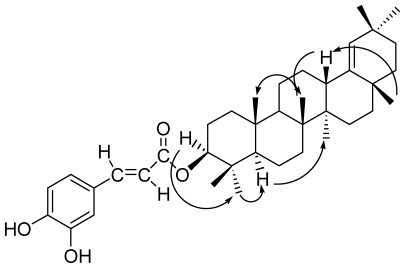

Fig. 3. NOESY Correlations of **1**

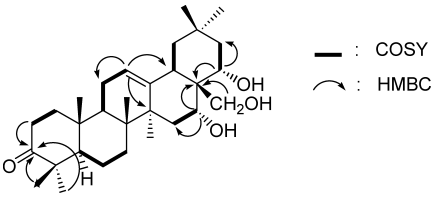

Fig. 4. ^{1}H–^{1}H COSY and Key ^{1}H–^{13}C Long-Range Correlations for **2**

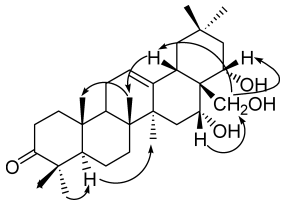

Fig. 5. NOESY Correlations of **2**

opposite face of **1**, the following correlations were observed. The oxymethine proton at δ 4.59 (H-3) was close in space to the methyl singlet at δ 0.87 (H_3-23), which was in turn close to the methine proton at δ 0.82 (H-5), which was finally close to the methyl singlet at δ 0.73 (H_3-27). All NOESY correlations were consistent with the relative configuration of **1**. The trivial name, germanicol caffeoyl ester is proposed for **1**.

The ^{1}H-NMR spectrum of **2** (Table 1) gave resonances for oxymethylene protons at δ 3.30 (d, J=10.8 Hz), 3.60 (dd, J=4.2, 10.8 Hz), oxymethine protons at δ 4.02 (dd, J=6.6, 12.0 Hz) and 4.63 (br s), an olefinic proton at δ 5.28 (t, J=3.6 Hz) and seven methyl singlets at δ 0.92, 0.93, 0.94, 1.036, 1.042, 1.09 and 1.40. The ^{13}C-NMR spectrum (Table 1) indicated resonances for thirty carbons with the following functionalities: a carbonyl carbon of a ketone at δ 217.8, an oxymethylene at δ 73.0 and two oxymethine carbons at δ 68.2 and 76.6, olefinic carbons at δ 122.9 and 142.4, eight methylene, three methine, seven methyl and six quaternary carbons. These resonances indicated a triterpene with an olefin, a ketone, a methylene hydroxy and two methine hydroxys.

The HR-EI-MS of **2** gave a molecular ion of *m*/*z* 472.3560 [M^+], which corresponded to a molecular formula of $C_{30}H_{48}O_4$. The molecular formula indicated an index of hydrogen deficiency of seven. With two double bonds deduced from the carbonyl and an olefin, the compound is pentacyclic.

The COSY spectrum of **2** indicated seven isolated spin systems (Fig. 4) as follows: α-methylene protons coupled to another set of methylene protons (H_2-2/H_2-1); a methine proton coupled to methylene protons (H-5/H_2-6/H_2-7); an olefinic proton coupled to methylene protons, which was further coupled to a methine proton (H-12/H_2-11/H-9); two sets of oxymethine proton coupled to methylene protons (H-16/H_2-15) and (H-22/H_2-21); a methine proton coupled to methylene protons (H-18/H_2-19); and oxymethylene protons (H_2-28) coupled to each other.

Protons attached to carbons were assigned (Table 1) from HMQC 2D-NMR data and the structure of **2** was elucidated by analysis of the HMBC 2D-NMR data: key HMBC correlations are shown in Fig. 4. The carbonyl was located at C-3 on the basis of long-range correlations between the methyl singlets at δ 1.042 (H_3-24) and 1.09 (H_3-23) and this carbon (δ 217.8). The olefin was assigned to C-12 due to long-range correlations between the olefinic proton at δ 5.28 (H-12) and C-11, C-14 and C-18. The oxymethines (δ 4.02, 4.63) and oxymethylene (δ 3.30, 3.60) were located at C-16, C-22 and C-28, respectively based on long-range correlations between these protons and the quaternary carbon at δ 43.9 (C-17). Futhermore, the oxymethine protons (H-16, H-22) were correlated to C-15 and C-21, respectively. On the other hand, the oxymethylene protons (H_2-28) were also correlated to C-17. All long-range correlations were consistent with the structure of **2**.

The relative configuration of **2** (Fig. 5) was deduced from NOESY. The oxymethine proton at δ 4.63 (H-16) was close in space to one of the oxymethylene protons at δ 3.60 (H-28), while the other oxymethylene proton at δ 3.30 (H-28) was close to the oxymethine proton at δ 4.02 (H-22) and the methine proton at δ 1.92 (H-18). The latter proton was also close to the methyl singlet at δ 0.94 (H_3-26), which was in turn close to another methyl singlet at δ 1.036 (H_3-25), indicating that they are in the same face of the molecule. On the opposite face of the molecule was the methyl singlet at δ 1.09 (H_3-23) which was close to the methine proton at δ 1.34 (H-5), which was finally close to the methyl singlet at δ 1.40 (H_3-27). All NOESY correlations were consistent with the relative configuration of **2**. The trivial name camelliagenone is proposed for **2**.

The structures of germanicol *trans*-coumaroyl ester (**3**),[7] germanicol *cis*-coumaroyl ester (**4**),[7] germanicol (**5**),[8] and camelliagenin A (**6**)[9] were elucidated by extensive 1D- and 2D-NMR spectroscopy. The structures of spinasterol,[10] sitosterol,[11] squalene,[12] lutein,[13] linoleic acid[14] and trilinolein[15] were identified by comparison of their ^{13}C-NMR data with those found in the literature.

Antimicrobial Assay As part of our continuing search for antimicrobial compounds from Philippine medicinal plants and since *B. asiatica* has reported antimicrobial properties, triterpenes **1** to **5** were tested for possible antimicrobial activities by the agar well method. Results of the study (Table 2) indicated that **1**—**5** are active against the fungus, *Candida albicans*, with **1** slightly more active than **2**—**5**. Triterpene **1** is also more active against the bacterium: *Staphylococcus aureus* than **2**, **4** and **5**, while **3** is inactive. On the other hand, **4** is the only compound which is slightly active against *Pseudomonas aeruginosa*. All triterpenes were found inactive against *Escherichia coli*, *Bacillus subtilis*, *Trichophyton mentagrophytes* and *Aspergillus niger*.

Table 2. Antimicrobial Activity of Compounds (**1**—**5**)

Microorganism	Compound[a] (30 μg)	Clearing zone (mm)[b]	Activity index (AI)
Escherichia coli	**1**	—	—
	2	—	—
	3	—	—
	4	—	—
	5	—	—
	Chloramphenicol[c]	23	2.8
Pseudomonas aeruginosa	**1**	—	—
	2	—	—
	3	—	—
	4	12	0.2
	5	—	—
	Chloramphenicol[c]	14	1.3
Staphylococcus aureus	**1**	13	0.3
	2	12	0.2
	3	—	—
	4	12	0.2
	5	12	0.2
	Chloramphenicol[c]	25	3.2
Bacillus subtilis	**1**	—	—
	2	—	—
	3	—	—
	4	—	—
	5	—	—
	Chloramphenicol[c]	20	2.3
Candida albicans	**1**	13	0.3
	2	12	0.2
	3	12	0.2
	4	11	0.1
	5	12	0.2
	Canesten, 0.2 g[d]	18	0.8
Trichophyton mentagrophytes	**1**	—	—
	2	—	—
	3	—	—
	4	—	—
	5	—	—
	Canesten, 0.2 g[d]	55	4.5
Aspergillus niger	**1**	—	—
	2	—	—
	3	—	—
	4	—	—
	5	—	—
	Canesten, 0.2 g[d]	23	1.3

a) Sample −10 mm well diameter. *b*) Average of 3 trials. *c*) Chloramphenicol disk −6 mm diameter. *d*) Contains 1% chlotrimazole.

Experimental

General Experimental Procedures Optical rotations were taken with a Jasco DIP-370 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 1600 Fourier Transform IR spectrometer. UV spectra were recorded on a U-2000 Hitachi UV–vis spectrometer. HR-EI-MS was obtained on a Finnigan/Thermo Quest MAT 95 XL spectrometer. NMR spectra 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. Column chromatography was performed with silica gel 60 (70—230 mesh), while the TLC was performed with plastic backed plates coated with silica gel F_{254}. The plates were visualized with vanillin-H_2SO_4 and warming.

Plant Material Fresh leaves (2.2 kg) and fruits (880 g) of *Barringtonia asiatica* were collected from the De La Salle University-Manila campus. The sample was authenticated at De La Salle University-Manila. Voucher specimen # 175 (leaves), 176 (fruit) and 177 (seeds) were deposited at the Chemistry Department, De La Salle University, Manila. The fruits were collected in November 2009, while the leaves were collected in February 2010.

Extraction and Isolation The leaves were air-dried, then ground in an osterizer. The seeds were separated from the flesh of the fruit, chopped into small pieces, then freeze-dried. The air-dried leaves (428.4 g) and the freeze-dried fruits (145.5 g) and seeds (196.3 g) were soaked in dichloromethane for three days, then filtered. The filtrates were concentrated under vacuum to afford the crude extracts: leaves (32.2 g), fruit (3.6 g) and seeds (5.0 g). 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 18 inches in height and 1.0 inch internal diameter was used for the crude extracts from the fruits and seeds. Five milliliter fractions were collected. A glass column 12 inches in height and 0.5 inch internal diameter was used for the rechromatography. Two milliliter fractions were collected. Final purifications were conducted using Pasteur pipettes as columns.

Isolation of Constituents from the Leaves of *B. asiatica* The crude dichloromethane extract of the leaves of *B. asiatica* was chromatographed in increasing proportions of acetone in dichloromethane as eluents. The DCM fraction from the crude extract was rechromatographed in petroleum ether, followed by 2.5% ethyl acetate in petroleum ether, then 5% ethyl acetate in petroleum ether. The fractions eluted with petroleum ether were rechromatographed (3×) in petroleum ether. The less polar fractions afforded squalene (11.5 mg), while the more polar fractions yielded trilinolein (25 mg). The fractions eluted with 2.5% ethyl acetate in petroleum ether were rechromatographed (5×) in 5% ethyl acetate in petroleum ether to afford **5** (20.8 mg). The fractions eluted with 5% ethyl acetate in petroleum ether were rechromatographed (3×) in 5% ethyl acetate in petroleum ether to afford **4** (14.9 mg). The 10% acetone in dichloromethane fraction was rechromatographed (5×) in 2.5% ethyl acetate in petroleum ether to afford **3** (3 mg). The 20—30% acetone in dichloromethane fractions from the crude extract were combined and rechromatographed in 15% ethyl acetate in petroleum ether, followed by 20% ethyl acetate in petroleum ether. The fractions eluted with 15% ethyl acetate in petroleum ether were rechromatographed (3×) in 15% ethyl acetate in petroleum ether to afford a mixture of spinasterol and sitosterol (4.5 mg) after washing with petroleum ether. The fractions eluted with 20% ethyl acetate in petroleum ether were rechromatographed (4×) in dicloromethane to afford **1** (3.4 mg). The 40 to 60% acetone in dichloromethane fractions from the crude extract were combined and rechromatographed (6×) in acetonitrile : diethyl ether : dichloromethane (1 : 1 : 8). The less polar fractions afforded lutein (12 mg) after washing with petroleum ether, followed by diethyl ether. The more polar fractions afforded **2** (1.0 mg). The 70—80% acetone in dichloromethane fractions were rechromatographed (7×) in acetonitrile : diethyl ether : dichloromethane (1 : 1 : 8) to afford **6** (0.4 mg).

Isolation of Constituents from the Fruits of *B. asiatica* The crude dichloromethane extract of the fruits of *B. asiatica* was chromatographed in increasing proportions of acetone in dichloromethane as eluents. The DCM fraction was rechromatographed (5×) in petroleum ether to afford the trilinolein (8.5 mg). The 10% acetone in dichloromethane fractions were combined and rechromatographed (5×) in 2.5% ethyl acetate in petroleum ether to afford **3** (4.0 mg). The 20% acetone in dichloromethane fraction was rechromatographed in 15% ethyl acetate in petroleum ether to afford spinasterol (6.1 mg).

Isolation of Constituents from the Seeds of *B. asiatica* The crude dichloromethane extract of the seeds of *B. asiatica* was chromatographed in increasing proportions of acetone in dichloromethane as eluents. The DCM fraction was rechromatographed (6×) in petroleum ether. The less polar fractions afforded squalene (6.4 mg), while the more polar fractions afforded trilinolein (61.2 mg). The 20% acetone in dichloromethane fraction was rechromatographed (3×) in 15% ethyl acetate in petroleum ether. The less polar fractions afforded spinasterol (25.8 mg), while the more polar fractions yielded linoleic acid (30.2 mg).

Germanicol Caffeoyl Ester (**1**): Colorless solid; mp 171 °C; $[\alpha]_D^{25}$ +11.7 (c=0.99, $CHCl_3$); IR (neat) ν_{max} (cm^{-1}) 3352 (OH), 1674 (C=O), 1274, 1187 (C–O), 1603, 1519 (C=C), 2947, 2860, 1448, 1372, 1112, 1014, 979, 739; UV (EtOH) λ_{max} 205 (ε 4896), 220 (ε 3878), 244 (ε 2424), 300 (ε 2806); ^{1}H- and ^{13}C-NMR data, see Table 1; HR-EI-MS m/z 588.4182 [M^+] ($C_{39}H_{56}O_4$).

Camelliagenone (**2**): Colorless oil; $[\alpha]_D^{25}$ +6.87 (c=0.60, $CHCl_3$); IR (neat) ν_{max} (cm^{-1}) 3436 (OH), 1699 (C=O), 1266, 1107, 1076 (C–O), 1513 (C=C), 2930, 2867, 1459, 1383; ^{1}H- and ^{13}C-NMR data, see Table 1; HR-EI-MS m/z 472.3560 [M^+] ($C_{30}H_{48}O_4$).

Antimicrobial Tests The microorganisms used in these tests were obtained from the University of the Philippines Culture Collection (UPCC). These are *P. aeruginosa* (UPCC 1244), *B. subtilis* (UPCC 1149), *E. coli* (UPCC 1195), *S. aureus* (UPCC 1143), *C. albicans* (UPCC 2168), *T. menta-*

grophytes (UPCC 4193) and *A. niger* (UPCC 3701). The test compound (30 mg) was dissolved in 95% ethanol. The positive control for the bacteria is chloramphenicol disc (HiMedia Laboratories, Ltd.) which contains 30 mg chloramphenicol in a 6 mm disc. The positive control for the fungi is Canesten (Bayer) which contains 1% chlotrimazole. The antimicrobial assay procedure reported in the literature[16] was employed. The clearing zone was measured in millimeters, and the average diameter of the clearing zones was calculated. The diameter of the well for the test compounds was 10 mm. 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, *e.g.* activity index (AI)=(diameter of clearing zone−diameter of well)/diameter of well.

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