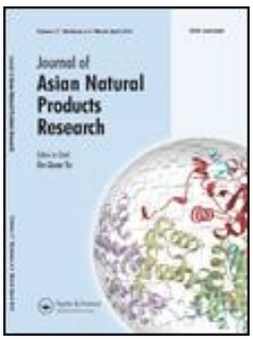

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New norterpenoids and a sphingolipid from *Carissa opaca*

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Chemical investigations on the aerial parts of *Carissa opaca* resulted in the isolation and characterization of two new *nor*-triterpenoids (compounds **1** and **2**) and a new sphingolipid (compound **3**) together with six known compounds. The structures of all the isolates were established using spectral data. All the isolated compounds showed DPPH radical scavenging and enzyme inhibitory activities against enzymes acetylcholinesterase, butyrylcholinesterase, and lipoxygenase.

Keywords: *Carissa opaca*; *nor*-triterpenoid; sphingolipid; acetylcholinesterase inhibition; butyrylcholinesterase inhibition; lipoxygenase inhibition

1. Introduction

Carissa opaca, an important member of the genus *Carissa*, is an evergreen thorny shrub commonly found in the semi-arid coastal areas of the tropical regions. In Pakistan, it is wildly growing in Punjab, Himalayas, and up to the height of 6000 ft in Murree [1]. *C. opaca* is used in folk medicine with hepatoprotective, aphrodisiac, purgative [2], and wound-healing properties [3]. Fruits are effective to cough, fever, eye disorder [4], diarrhea, snakebite [5], and asthma [6]. In Pakistan, its leaves are used as a substitute in cardiac disorders [6,7]. The root's paste is used for the treatment of horn injuries and maggot wounds in animals [3]. The bark is effective for chicken pox and skin diseases [8]. In Thailand, its stem is used as healing agent and as a stimulant [9]. The decoction of fresh leaves of *C. opaca* is effective in the treatment of jaundice, various neuromuscular diseases, and hepatitis [1]. These remarkable properties of *C. opaca* motivated us to carry out the phytochemical investigations on this plant. Herein, we report the isolation and characterization of two new *nor*-triterpenoids 30-*nor*-2α,3β-dihydoxyurs-12-ene (**1**), 30-*nor*-2α,3β,23-trihydroxyurs-12-ene (**2**), a new sphingolipid (**3**) (Figure 1) together with 3β,27-dihydroxylup-12-ene, lupeol-β-hydroxyoctadecanoate, pinoresinol, (−)-carinol, (−)-carissanol, and arjunolic acid).

2. Results and discussion

Compound **1** was isolated as a colorless amorphous solid. Its HR-EI-MS showed the molecular ion peak $[M]^+$ at *m/z* 428.3660 consistent to the molecular formula $C_{29}H_{48}O_2$ with six double bond equivalences (DBE). The Infrared (IR) spectrum showed the absorption bands due to hydroxyl groups (3415 cm^{-1}) and olefinic system (1630 cm^{-1}). The ^1H NMR spectrum of compound **1** (Table 1) showed signals for six tertiary methyl groups at δ 1.11, 1.01, 1.01, 0.96, 0.84, and

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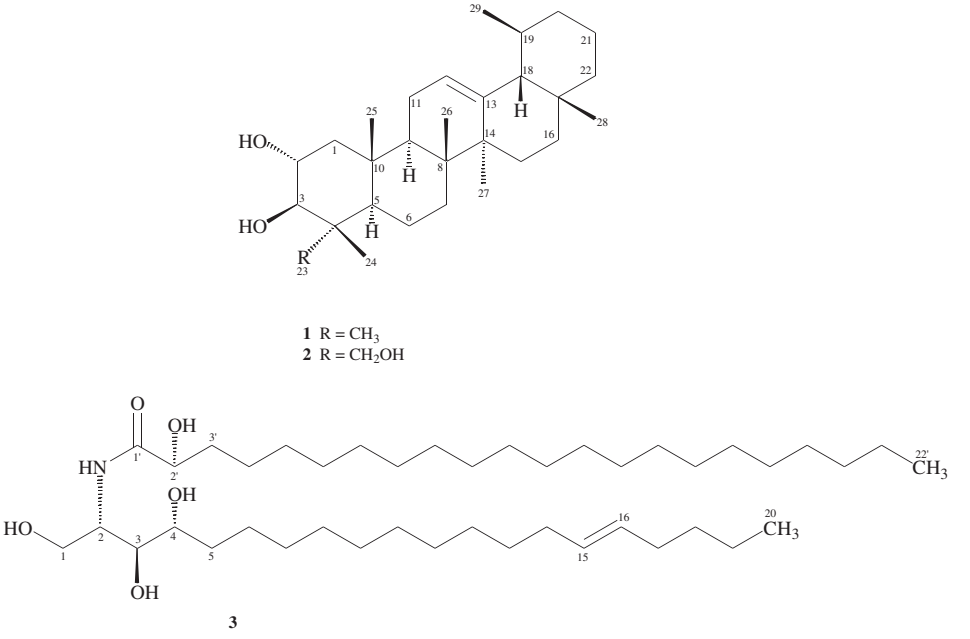

Figure 1. Compounds **1–3** isolated from *Carissa opaca*.

0.80 (3H each, s) corresponding to the signals at δ 24.0, 17.2, 17.5, 17.8, 17.6, and 29.3, respectively, in ^{13}C NMR spectrum and a secondary methyl group at δ 0.89 (3H, d, $J = 6.5$ Hz) corresponding to the carbon signal at δ 21.5. The signal resonating at δ 5.23 (1H, dd, $J = 4.0$, 3.5 Hz) was attested for an olefinic proton. The ^{1}H NMR spectrum showed the presence of two oxymethines at δ 2.91 (1H, d, $J = 9.8$ Hz) and 3.61 (1H, ddd, $J = 9.8$, 9.5, 3.5 Hz), which showed COSY correlations to each other, suggesting their adjacent positions. A doublet methine at δ 2.21 (1H, d, $J = 11.5$ Hz, H-18) suggested that compound **1** could be the ursane-type triterpenoids and the absence of C-30 was confirmed through COSY correlation in which H-29 (δ 0.89) showed correlation with H-19 (δ 1.36–1.34) with further extension to H-18 (δ 2.21) and CH_2-20 (δ 1.64–1.61, 1.49–1.46). This information was further supported by HMBC correlations (Figures 2) in which H-29 showed correlations with the carbons C-20 (δ 30.7), C-19 (δ 40.4), and C-18 (δ 54.3). Twenty-nine carbon signals in the ^{13}C NMR spectrum of compound **1** (Table 1) revealed presence of seven methyl, nine methylene, seven methine, and six quaternary carbon atoms. All the assignments were accomplished through COSY, HSQC, and HMBC information and comparing the data with salvins A and B [10]. The stereochemistry both at C-2 and C-3 was determined by NOESY correlations (Figure 3), molecular modeling, and coupling constants. The larger coupling constant of H-3 ($J = 9.8$ Hz) and absence of NOESY correlation with H-25 confirmed its axial and α orientation, whereas the multiplicity of H-2 (ddd, $J = 9.8$, 9.5, 3.5 Hz) and the NOESY interaction between H-2 with H-25 confirmed it β and axial orientation. The above discussion confirmed compound **1** to be 30-*nor*-2α,3β-dihydoxyurs-12-ene, which is a new natural product and is named carisursane A.

Compound **2** was obtained as colorless amorphous powder whose HR-EI-MS

Table 1. ^{1}H and ^{13}C NMR spectral data of compound **1** in CD_3OD (^{1}H: 500 MHz; ^{13}C: 125 MHz).

	Compound **1**		Compound **2**	
Position	δ_H (J = Hz)	δ_C	δ_H (J = Hz)	δ_C
1	1.95 (1H, dd, 9.5, 7.5)			
0.89 (1H, dd, 7.5, 3.0)	48.5	1.61–1.58, 1.33–1.30 (2H, m)	42.3	
2	3.61 (1H, ddd, 9.8, 9.5, 3.5)	69.5	3.88 (1H, ddd, 10.2, 9.0, 2.0)	67.2
3	2.91 (1H, d, 9.8)	84.4	3.59 (1H, d, 10.2)	78.7
4	–	40.5	–	42.5
5	0.82–0.80 (1H, m)	56.6	1.29–1.26 (1H, m)	44.1
6	1.58–1.55, 1.43–1.40 (2H, m)	19.5	1.61–1.58, 1.43–1.40 (2H, m)	18.6
7	1.62–1.59, 1.42–1.39 (2H, m)	34.2	1.68–1.65,1.40–1.37 (2H, m)	33.8
8	–	40.8	–	40.8
9	1.57–1.54 (1H, m)	49.8	1.76–1.72 (1H, m)	48.4
10	–	39.2	–	39.1
11	1.94–1.91 (2H, m)	24.4	1.97–1.94 (2H, m)	24.4
12	5.23 (1H, dd, 4.0, 3.5)	126.7	5.23 (1H, dd, 4.0, 3.5)	126.6
13	–	139.7	–	139.2
14	–	43.3	–	43.2
15	1.92–1.89, 1.63–1.60 (2H, m)	29.1	1.91–1.87, 1.63–1.60 (2H, m)	29.1
16	1.98–1.96, 1.80–1.77 (2H, m)	25.3	1.99–1.96, 1.82.1.79 (2H, m)	25.3
17	–	40.8	–	40.8
18	2.21 (1H, d, 11.5)	54.3	2.21 (1H, d, 11.0)	54.4
19	1.36–1.34 (1H, m)	40.4	1.39–1.36 (1H, m)	40.4
20	1.64–1.61, 1.49–1.46 (2H, m)	30.7	1.63–1.60, 1.49 (2H, m)	30.7
21	1.44–1.42 (2H, m)	31.7	1.56–1.53 (2H, m)	31.8
22	2.05–2.01 (1H, m)			
1.92–1.89 (1H, m)	38.1	2.06–2.03 (2H, m)	38.4	
23	0.80 (3H, s)	29.3	3.53, 3.39 (1H each, d, 11.0)	71.3
24	1.01 (3H, s)	17.2	0.77 (3H, s)	17.5
25	1.01 (3H, s)	17.5	1.02 (3H, s)	17.6
26	0.84 (3H, s)	17.6	0.84 (3H, s)	17.7
27	1.11 (3H, s)	24.0	1.13 (3H, s)	24.1
28	0.96 (3H, s)	17.8	0.96 (3H, s)	17.8
29	0.89 (3H, d, 6.5)	21.5	0.89 (3H, d, 6.5)	21.5

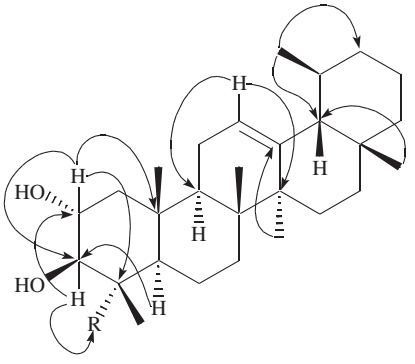

Figure 2. Important HMBC correlations of compounds **1** and **2**.

showed the molecular ion peak $[M]^+$ at *m/z* 444.3610 corresponding to the molecular formula $C_{29}H_{48}O_3$ with six DBE. The IR and NMR spectra showed close resemblance to those for compound **1** except for the absence of a tertiary methyl group and the presence of an oxymethylene group at δ 3.53 (1H, d, $J = 11.0$ Hz) and 3.39 (1H, d, $J = 11.0$ Hz) corresponding to the carbon at δ 71.3. That indicated that one of the tertiary methyl groups has been oxidized to secondary alcohol in compound **2** and its position was confirmed at C-23 by HMBC correlation (Figure 2) in which H-24 (δ 0.77) correlated with C-23 (δ 71.3), H-23 (δ 3.53, 3.39) with C-24 (δ 17.5), and H-3 (δ 3.59) with C-23 (δ 71.3), confirming the oxidation of C-23. The above discussion confirmed compound **2** as 30-*nor*-2α,3β, 23-trihydroxyurs-12-ene, which is a new natural product and named carisursane B.

Compound **3** was obtained as a colorless amorphous solid. The molecular formula $C_{42}H_{83}NO_5$ was determined by the data of HR-EI-MS showing the molecular ion peak at *m/z* 681.6280 with two DBE. The IR absorption bands at 3335, 1644, and 1623 cm^{-1} indicated the presence of hydroxyl, amide, and olefinic groups. The ^{1}H NMR spectrum of compound **3** displayed signals for a secondary amide at δ 7.40 (1H, d, $J = 8.5$ Hz), two oxygenated methylene groups at δ 3.71 (1H, dd, $J = 12.0$, 4.5 Hz) and 3.63 (1H, dd, $J = 12.0$, 2.5 Hz), and three oxymethine groups at δ 3.94 (1H, dd, $J = 8.0$, 3.5 Hz), 3.42 (1H, dt, $J = 6.3$, 4.2 Hz), and 3.39 (1H, dd, $J = 4.5$, 3.9 Hz). A signal resonating at δ 3.99–3.96 (1H, m) corresponded to a carbon atom in ^{13}C NMR spectrum at δ 51.4, which was assigned to the azomethine group. These data indicated compound **3** to be a sphingolipid [11]. The signal appeared at δ 5.32 (1H, dt, $J = 16.5$, 5.0 Hz) and 5.28 (1H, dt, $J = 16.5$, 5.5 Hz), indicating the presence of double bond in hydrocarbon chain. The presence of aliphatic chain was due the signals at δ 1.35–1.22 and terminal methyl groups at δ 0.77 (6H, t, $J = 7.0$ Hz). The ^{13}C NMR spectra (BB and DEPT) of compound **3** displayed the signal for amide carbonyl group at δ 175.6, and δ 75.5, 72.1, 71.8, 60.9 and 51.4 were

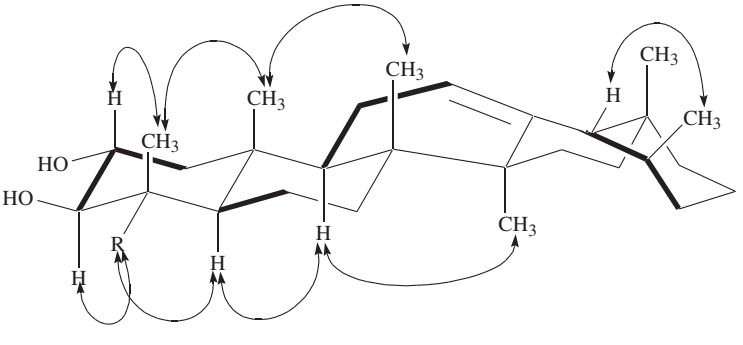

Figure 3. Important COSY and NOESY correlations of compounds **1** and **2**.

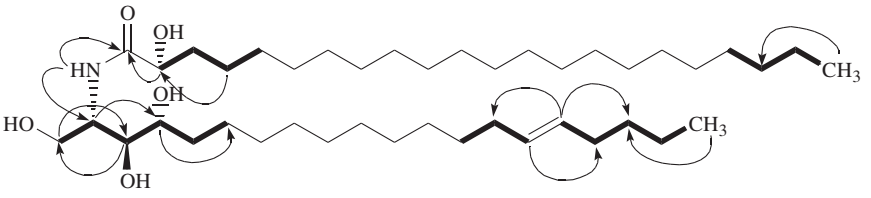

Figure 4. Important HMBC and COSY correlations of compound **3**.

attributed to three oxymethine groups, an oxymethylene group, and an azomethine group, respectively. The presence of double bond was confirmed due to the presence of carbon atoms at δ 130.6, 129.6. The coupling constants and chemical shifts C-14 and C-17 (δ 32.4) next to the double bond indicated its *trans* geometry [12,13]. The entire sequence of the molecule was fixed by HSQC, COSY, and long-range HMBC correlations (Figure 4). The length of the fatty acid chain was fixed by the characteristic fragments at *m/z* 339 and 280 and the amide chain containing a double bond at *m/z* 308 $[M - 2H_2O]^+$ and 225 (Figure 5). Methanolysis [14] of compound **3** with methanolic HCl provided aliphatic oxygenated amine and methyl ester of the fatty acid. These fragments were acetylated [15] and analyzed by GC–MS and were identified as methyl-2-acetoxydocosanoate at *m/z* 412 and 2-acetamino-1,3,4-triacetoxyeicosene at *m/z* 512. The position of the double bond was fixed between C-15 and C-16 by permanganate/periodate oxidative cleavage [16] of 2-acetamino-1,3,4-triacetoxyeicosene yielding a mixture of carboxylic acids, which on methylation and GC–MS analysis provided peaks for 2-acetamino-1,3,4-triacetoxypentadecanoic acid at *m/z* 473 and pentanoic acid at *m/z* 102. The configuration at the stereogenic centers could be determined by the optical rotation of compound **3** ($[\alpha]_D + 33.1$) and its methanolysis products ($[\alpha]_D - 7.2$ and $+17.2$), which were comparable to those of the sphingolipids with (2*S*,3*S*,4*R*,2′*R*) configurations [17,18]. Based on the above evidence, the structure of compound **3** could be assigned as (2*S*,3*S*,4*R*,15*E*)-2-{[(2*R*)-2-hydroxydocosanoyl]amino}eicos-15-ene-1,3,4-triol and named carisnin.

These compounds (**1**–**9**) were evaluated for their DPPH radical scavenging

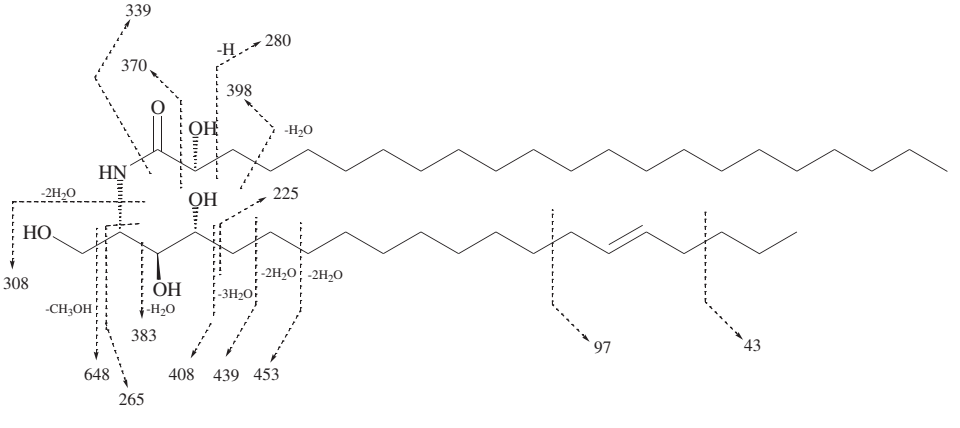

Figure 5. EI-MS mass fragmentations of compound **3**.

and enzyme inhibitory activities against enzymes acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and lipoxygenase (LOX) and results are shown in Table 2.

3. Experimental

3.1 General experimental procedures

IR spectra were recorded on Shimadzu 460 spectrometer (Duisburg, Germany). ^{1}H (400, 500 MHz), ^{13}C NMR (100, 125 MHz), and 2D NMR (HMQC, HMBC, and COSY; 400, 500 MHz) spectra were recorded on Bruker spectrometer (Zurich, Switzerland). The chemical shift values (δ) are reported in ppm and the coupling constants (J) are in Hz. EI-MS, HR-EI-MS, FAB-MS, and HR-FAB-MS were recorded on Finnigan (Varian MAT, Waldbronn, Germany) JMS H × 110 with a data system and JMSA 500 mass spectrometers, respectively. The gas chromatography (GC) was performed on a Shimadzu gas chromatograph (GC-9A, Noisiel, France) (3% OV-1 silanized chromosorb W, column temperature 180°C, injection port and detector temperature 275–300°C, flow rate 35 ml/min, flame-ionization detector). Chromatographic separations were carried out using aluminum sheets pre-coated with silica gel 60 F_{254} (20 cm × 20 cm, 0.2 mm thick; E. Merck; Darmstadt, Germany) for thin-layer chromatography (TLC) and silica gel (230–400 mesh, Darmstadt, Germany) for column chromatography. TLC plates were visualized under UV at 254 and 366 nm and by spraying with ceric sulfate reagent solution (by heating).

3.2 Plant material

The whole plants of *Carissa opaca* Stapf ex Haines were collected from Quaid-i-Azam University, Islamabad, Pakistan, in June 2008 and were identified by Mr. Farrukh Nisar, Plant Taxonomist, Department of Botany, University of Gujrat, Gujrat, Pakistan, where a voucher specimen is deposited (CO-46/08-BUG).

3.3 Extraction and isolation

The shade-dried plant material of *Carissa opaca* (10 kg) was ground and extracted thrice with methanol (35 L). The methanolic extract was concentrated under reduced pressure to get a dark greenish mass (455 g), which was subjected to silica gel column chromatography eluting with *n*-hexane, *n*-hexane:dichloromethane (DCM), DCM, DCM:methanol, and methanol in increasing order of polarity to get seven fractions (1–7). Fraction 1 (2 g) obtained from *n*-hexane:DCM (5.5:4.5) on silica gel column chromatography using isocratic (50% *n*-hexane/DCM) mobile phase yielded 3β,27-dihydroxylup-12-ene (25 mg). Fraction 2 (2.5 g) obtained from the main column using *n*-hexane:DCM (0.5:9.5) on elution with pure DCM yielded lupeol-β-hydroxyoctadecanoate (27 mg). Fraction 3 (3 g) subjected to silica gel eluting with DCM further yielded two sub-fractions. Sub-fraction 1 (1.9 g) was washed out with ethyl acetate to obtain arjunolic acid (17 mg), whereas sub-fraction 2 (3.8 g) when subjected to column chromatography eluted with DCM:MeOH: (9.8:0.2) yielded pinoresinol (26 mg) from the head fractions and (−)-carinol (37 mg) from the tail fractions. Fraction 4 (4.8 g) obtained from DCM: MeOH: (9.7:0.3) was subjected to silica gel column chromatography eluted with DCM: MeOH: (9.6:0.4) and divided into two sub-fractions. Sub-fraction 1 (1.7 g) on purification using silica gel eluting with the same mobile phase yielded (−)-carissanol (19 mg), whereas sub-fraction 2 (2.9 g) on repeated silica gel column chromatography eluting with DCM:MeOH (9.6:0.4) yielded 30-*nor*-2α,3β-dihydoxyurs-12-ene (**1**, 21 mg) from the head fractions and 30-*nor*-2α,3β,23-trihydroxyurs-12-ene (**2**, 23 mg). Fraction 5 (2.5 g) obtained at 6% MeOH in DCM was subjected to repeated silica gel column chromatography using the same solvent system to obtain carisnin (**3**, 25 mg). The remaining fractions contained salts and sugars.

Table 2. DPPH scavenging, AChE, BChE, and LOX inhibitory activities of compounds **1–9**.

Compound	DPPH		AChE		BChE		LOX	
	(%) at 0.5 (mM)	(IC_{50}) (μM)	(%) at 0.5 mM	(IC_{50}) (μM)	(%) at 0.5 mM	(IC_{50}) (μM)	(%) at 0.5 mM	(IC_{50}) (μM)
1	25.14 ± 0.63	<500	43.21 ± 0.82	<700	25.31 ± 0.33	<700	47.51 ± 0.18	<500
2	19.76 ± 0.58	<500	42.17 ± 0.66	<700	51.21 ± 0.61	<400	51.21 ± 0.82	<300
3	12.41 ± 0.75	<500	39.61 ± 0.16	<700	62.18 ± 0.27	336.21 ± 0.14	55.31 ± 0.17	<300
3β,27-Dihydroxylup-12-ene	17.98 ± 0.55	<500	38.52 ± 0.52	<700	58.61 ± 0.18	<400	20.11 ± 0.58	<800
Lupeol-β-hydroxyoctadecanoate	21.19 ± 0.13	<500	25.31 ± 0.18	<700	23.61 ± 0.22	<700	55.21 ± 0.63	<300
Pinoresinol	22.58 ± 0.22	<500	38.56 ± 0.21	<700	53.21 ± 0.17	<400	49.61 ± 0.82	<500
(−)-Carinol	88.24 ± 0.16	84.91 ± 0.07	32.11 ± 0.16	<700	48.91 ± 0.17	<700	48.21 ± 0.68	<500
(−)-Carissanol	90.11 ± 0.18	83.41 ± 0.17	53.61 ± 0.14	<400	88.51 ± 0.12	101.81 ± 0.24	44.58 ± 0.46	<500
Arjunolic acid	15.24 ± 0.86	<500	53.81 ± 0.52	<400	53.21 ± 0.11	<400	52.37 ± 0.57	<300
Quercetin	93.21 ± 0.97	16.96 ± 0.14		-	-	-	-	-
Eserine	–	–	91.29 ± 1.17	0.04 ± 0.0001	82.82 ± 1.09	0.85 ± 0.001	–	–
Baicalein	–	–	–	–	–	–	93.79 ± 1.2	22.4 ± 1.3

3.3.1 Carisursane A (30-nor-2α,3β-dihydroxyurs-12-ene; **1**)

Colorless amorphous solid; $[\alpha]_D^{24}$ + 75.2 (c = 0.0026, MeOH); IR (KBr) ν_{max} cm^{-1}: 3415, 2935, 1630, 830; ^1H and ^{13}C NMR spectral data; see Table 1; HR-EI-MS: *m/z* 428.3660 $[M]^+$ (calcd for $C_{29}H_{48}O_2$, 428.3654).

3.3.2 Carisursane B (30-nor-2α,3β,23-trihydroxyurs-12-ene; **2**)

Colorless amorphous solid; $[\alpha]_D^{24}$ + 58.5 (c = 0.002, MeOH); IR (KBr) ν_{max} cm^{-1}: 3520, 2935, 1655, 830; ^1H and ^{13}C NMR spectral data; see Table 1; HR-EI-MS: *m/z* 444.3610 $[M]^+$ (calcd for $C_{29}H_{48}O_3$, 444.3603).

3.3.3 Carisnin (2S,3S,4R,15E)-2-{[(2R)-2-hydroxydocosanoyl]amino}eicos-15-ene-1,3,4-triol; **3**)

White amorphous solid; $[\alpha]_D^{24}$ + 33.1 (c = 0.0021, MeOH); IR (KBr): ν_{max} 3335, 3215, 2920, 1644, 1623, 1602 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.32 (2H, dt, J = 16.5, 5.0 Hz, H-15), 5.28 (2H, dt, J = 16.5, 5.5 Hz, H-16), 7.40 (1H, d, J = 8.5 Hz, NH), 3.99–3.96 (1H, m, H-2), 3.94 (1H, dd, J = 8.0, 3.5 Hz, H-2′), 3.71 (1H, dd, J = 12.0, 4.5 Hz, H-1a), 3.63 (1H, dd, J = 12.0, 2.5 Hz, H-1b), 3.42 (1H, dt, J = 6.3, 4.2 Hz, H-4), 3.39 (1H, dd, J = 4.5, 3.9 Hz, H-3), 1.94–1.89 (4H, m, H-14,17), 1.70–1.67 (1H, m, H-3′a), 1.61–1.58 (1H, m, H-5a), 1.49–1.46 (1H, m, H-3′b), 1.31–1.28 (1H, m, H-5b), 1.33–1.22 (56H, br s, H-6–13,18–19,4′–21′), 0.77 (6H, t, J = 7.0 Hz, H-20,22′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 175.6 (C-1′), 130.6 (C-16), 129.6 (C-15), 75.5 (C-3), 72.1 (C-4), 71.8 (C-2′), 60.9 (C-1), 51.4 (C-2), 34.2 (C-3′), 32.4 (C-14,17), 31.8 (C-5), 22.5–31.7 (6–13,18–19,4′-21′), 13.8 (C-20,22′); HR-EI-MS: *m/z* 681.6280 $[M]^+$ (calcd for $C_{42}H_{83}NO_5$, 681.6271).

3.4 Methanolysis

Compound **3** (12 mg) was refluxed with 6 ml of 1N HCl and 25 ml of MeOH for 15 h. The reaction mixture was then extracted with *n*-hexane to obtain the corresponding fatty acid methyl ester, which was analyzed by GC–MS after acetylation with Ac_2O-Py. The aqueous layer was evaporated, and the residue was acetylated. Purification over Sephadex LH-20 and elution with CH_2Cl_2/MeOH 1:1 gave the corresponding acetylated sphingosine, which was analyzed by GC–MS.

3.4.1 Methyl ester derived from compound **3**

$[\alpha]_D$ − 7.2; ^1H NMR ($CDCl_3$, 300 MHz): δ 4.09 (1H, t, J = 6.3, H-2′), 3.51 (3H, s, MeO), 1.99 (3H, s, MeCO), 1.32–1.19 (38H, br s, CH_2-3′–21′), 0.84 (3H, t, J = 6.6, CH_3–22′); GC–MS: *m/z* 412.

3.4.2 Acetylsphingamine derived from compound **3**

$[\alpha]_D$ + 17.2; ^1H NMR ($CDCl_3$, 300 MHz): δ 7.51 (1H, d, J = 7.6, NH), 5.31 (2H, dt, J = 16.2, 5.1, H-15,16), 4.68–4.65 (1H, m, H-4), 4.22–4.20 (1H, m, H-2), 4.36 (1H, dd, J = 11.3, 4.5, H-1), 4.21 (1H, dd, J = 5.6, 3.1, H-3), 2.00 (12H, 4 × MeCO), 1.26–1.15 (26H, br s, CH_2-5–14,17–19), 0.85 (t, J = 6.5, CH_3–20); GC–MS: *m/z* 511.

3.5 Oxidative cleavage of the double bond in compound 3

To the solution of methyl ester of compound **3** (4 mg) in acetone, added 1 ml of 0.04 M solution of K_2CO_3, 6 ml of an aqueous solution 0.025 M $KMnO_4$ and 0.09 M $NaIO_4$ in a 100 ml round-bottom flask. The reaction was allowed to proceed at 37°C for 18 h. After acidification with 5 N H_2SO_4, the solution was decolorized with a 1M solution of oxalic acid and

extracted with Et_2O (3–10 ml). The combined organic extract was dried over Na_2SO_4, filtered, and concentrated. The resulting carboxylic acids were methylated with ethereal solution of diazomethane and analyzed by GC–MS.

3.6 Biological activity assays

3.6.1 DPPH radical scavenging activity

The DPPH radical scavenging activity was examined by comparison with that of a known antioxidant, quercetin, using the method developed by Lee and Shibamoto [19] with slight modification of decoloration of the samples and was calculated according to the formula:

$$\text{Antiradicalactivity} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100.$$

Each sample was assayed in triplicate and the mean values were calculated.

3.6.2 Acetylcholinesterase assay

The AChE inhibition activity was performed according to the method used by Ellman [20] with slight modifications. The percent inhibition was calculated using the following equation:

$$\text{Inhibition}(\%) = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

3.6.3 Butyrylcholinesterase assay

The BChE inhibition activity was performed according to the method used by Ellman [20] with slight modifications. The percent inhibition was calculated using the following equation:

$$\text{Inhibition}(\%) = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

IC_{50} values (a concentration at which there is 50% enzyme inhibition) of compounds were calculated using the EZ-Fit enzyme kinetics software (Perella Scientific Inc., Amherst, USA).

3.6.4 Lipoxygenase assay

LOX activity was assayed according to the reported method [21] but with slight modifications. The percentage inhibition was calculated by the formula given below:

$$\text{Inhibition}(\%) = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

Disclosure statement

No potential conflict of interest was reported by the authors.

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