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New triterpenoid saponin glycosides from the fruit fibers of *Trichosanthes cucumerina* L.†

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Five new triterpenoid saponin glycosides, trichocucumerisides A–E (1–5), together with eleven known compounds (6–16) were isolated from *Trichosanthes cucumerina* fruit fibers. The structures of the new compounds were elucidated by detailed analysis of NMR and mass spectroscopic data as well as chemical reactions. The anti-inflammatory study against nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells shows that compounds 7 and 9 exhibited stronger NO inhibitory activity, with IC₅₀ values of 3.0 and 2.7 μM, respectively, with comparison to positive references Celecoxib and aminoguanidine (IC₅₀ values 75.7 and 75.0 μM, respectively). Compounds 7 and 9 also possessed a greater selectivity index (SI) of approximately 3–4-fold activity than that of the positive references.

Introduction

Trichosanthes cucumerina L. is a Thai medicinal plant in the Cucurbitaceae family and the fruits are known for their strong bitter taste. *Trichosanthes* is mainly distributed in the tropical zone of Southeast Asia and Australia, and it is the largest genus of the family with over 100 species, 17 species of which are found in Thailand.¹ Previous reports revealed its pharmacological effects including cytotoxic,² anti-inflammatory,³ antidiabetic,^{4,5} hypoglycemic,⁶ hepatoprotective,⁷ gastroprotective,⁸ antifertility⁹ and larvicidal¹⁰ activities. The main chemical constituents present in *T. cucumerina* are triterpenoids, especially cucurbitacin groups,² which make the plant pharmacologically and therapeutically important. Studies on the cytotoxic activities of cucurbitacins from *T. cucumerina* have been reported.^{11–13} However, the phytochemical investigation of the constituents of this plant has not much been undertaken. In the present study, we wish to report five new triterpenoid saponin glycosides 1–5 together with eleven known compounds 6–16^{14–24} from the fruit fibers of *T. cucumerina*. These compounds were elucidated by analysis of varying spectroscopic evidence, as well as comparison with the literature data of

known compounds. All these compounds were evaluated against nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells.

Results and discussion

The crushed fruit fiber of *T. cucumerina* was extracted successively with *n*-hexane, EtOAc and MeOH. The extracts were repeatedly subjected to silica gel, Sephadex LH-20 and reversed phase RP C-18 column chromatography to afford five new triterpenoid saponin glycosides (1–5), two known steroids (6 and 13) and nine known cucurbitacins (7–12 and 14–16). The structures of the new compounds were elucidated by spectroscopic techniques and chemical means to be hederagenin-3-*O*-β-D-glucopyranosyl-28-*O*-β-D-xylopyranosyl-(1→6)-[β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (1), bayogenin-3-*O*-β-D-glucopyranosyl-28-*O*-β-D-xylopyranosyl-(1→6)-[β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (2), oleanolic acid-3-*O*-β-D-glucopyranosyl-28-*O*-β-D-xylopyranosyl-(1→6)-[β-D-xylopyranosyl-(1→2)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (3), oleanolic acid-3-*O*-β-D-glucopyranosyl-28-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-β-D-xylopyranoside (4), and gypsogenin-3-*O*-β-D-glucopyranosyl-28-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)-β-D-xylopyranoside (5) (Fig. 1). The NMR and mass spectra of the new compounds are included in the ESI.† The known compounds were identified as α-spinasterol (6),¹⁴ cucurbitacin B (7),^{15–17} cucurbitacin E (8),^{17–19} isocucurbitacin B (9),²⁰ isocucurbitacin D (10),²¹ cucurbitacin D (11),^{17,18,22} 25-acetyl cucurbitacin F (12),²³ α-spinasterol-3-*O*-β-D-

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Compound 2 was isolated as a white powder, $[\alpha]_D^{26} -22.7^\circ$ (c 0.24 in MeOH). Its ESI-TOF-MS data showed a sodium adduct ion

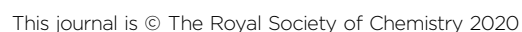


Table 1 ^1H NMR data for aglycone moieties of compounds 1–5 in pyridine- d_5 at 400 MHz (δ in ppm, J in Hz)

Position	1	2	3	4	5
1	0.94 ^a 1.45 m	1.30 m 2.32 dd (12.7, 4.1)	0.91 ^a 1.44 m	0.85 m 1.41 ^a	0.88 m 1.40 ^a
2	1.89 ^a 2.23 m	4.17 m	1.84 m 2.24 m	1.83 ^a 2.43 dd (11.8, 3.2)	1.84 m 2.17 m
3	4.26 m	4.25 m	3.39 dd (11.3, 4.6)	3.31 dd (11.8, 3.9)	4.04 m
5	1.66 m	1.84 ^a	0.84 m	0.74 brd (11.4)	1.66 dd (9.2, 8.1)
6	1.45 m 1.89 ^a	1.49 ^a 1.92 ^a	1.44 m 1.68 m	1.31 ^a 1.51 m	1.06 ^a 1.40 ^a
7	1.76 m 2.02 m	1.77 ^a 2.06 m	1.82 ^a 2.08 m	1.41 m 1.47 m	1.25 ^a 1.43 ^a
9	1.75 ^a	1.84 ^a	1.67 m	1.60 t (8.1)	1.36 m
11	1.89 ^a 1.94 m	1.94 ^a 2.08 m	1.97 m	1.87 m	1.84 m
12	5.40 brt (3.2)	5.40 brt (3.2)	5.46 brs	5.46 brt (3.2)	5.44 brs
15	1.46 m 2.05 m	1.49 m 2.08 ^a	1.57 m 2.12 m	1.27 ^a 2.11 m	1.22 ^a 2.07 m
16	1.92 ^a 2.05 m	1.96 ^a 2.04 ^a	2.00 m 2.16 m	2.03 m 2.11 m	2.02 m 2.10 ^a
18	3.13 dd (14.0, 4.3)	3.14 dd (13.9, 3.6)	3.18 brd (12.1)	3.31 dd (13.7, 3.9)	3.28 brd (11.6)
19	1.14 m 1.68 m	1.19 ^a 1.68 brd (13.6)	1.25 m 1.80 ^a	1.27 ^a 1.81 ^a	1.22 ^a 1.78 m
21	1.11 ^a 1.26 brd (12.0)	1.14 ^a 1.30 m	1.19 m 1.35 ^a	1.18 brd (12.7) 1.41 ^a	1.75 ^a 2.01 m
22	1.75 ^a	1.49 m 1.77 ^a	1.44 ^a 1.75 m	1.75 m 2.11 m	1.17 m 1.40 ^a
23	3.74 d (10.9) 4.29 d (10.9)	3.76 d (10.6) 4.51 d (10.6)	0.99 s	1.12 s	9.95 s
24	0.91 s	0.98 s	1.32 s	1.34 s	1.43 s
25	0.94 s	1.06 s	0.91 s	0.82 s	0.79 s
26	1.11 s	1.14 s	1.20 s	1.07 s	1.02 s
27	1.18 s	1.16 s	1.32 s	1.29 s	1.25 s
29	0.80 s	0.83 s	0.89 s	0.94 s	0.94 s
30	0.90 s	0.93 s	0.96 s	1.01 s	1.00 s

^a Signals overlapped.

$[\text{M} + \text{Na}]^+$ at m/z 1245.5869 (calcd for $\text{C}_{58}\text{H}_{94}\text{NaO}_{27}$, 1245.5875), corresponding to the molecular formula of $\text{C}_{58}\text{H}_{94}\text{O}_{27}$. The IR spectrum indicated the presence of hydroxyl (3442 cm^{-1}), ester (1727 cm^{-1}) and olefinic (1643 cm^{-1}) groups. The ^1H NMR data (Table 1 and Fig. S44–S66†) showed very similar spectral feature to that of compound 1. Comparison of the molecular formula of compound 2 with that of compound 1 indicated that 2 constituted one oxygen atom more than 1, suggesting the presence of one additional hydroxyl group in the aglycone unit. The large down-field shifts at C-2 signals of 2 (δ_{H} 4.17 and δ_{C} 67.6) comparing with those of 1 (δ_{H} 1.89, 2.23 and δ_{C} 26.4) were observed. Considerable down-field shifts of proton and carbon resonances at C-1 and C-3 of 2 (Tables 1 and 3) were also noted. These observations indicated that the hydroxyl group should be located at the 2-position. This was confirmed by the ^1H – ^1H COSY correlation of H-2 and H-3, and HMBC correlation between H-2 and C-3.

The orientation of H-2 was difficult to determine owing to the obscured nature of its ^1H signal. However, bayogenin-3-*O*- β -D-glucopyranoside (2b, Fig. S2†),³⁰ the alkaline hydrolysis product of compound 2 (see Experimental section), exhibited well-resolved ^1H -NMR spectrum (Table S1 and Fig. S13†) and H-2 signal (δ 4.24, J = 11.6, 3.9 Hz) showed

relatively large coupling constant with the neighbouring H-1ax. It was therefore concluded that H-2 was in the β -orientation. From the existing NMR spectra (Fig. S5 and S6†) and comparison with those of glycosides with bayogenin as the aglycone,^{31,32} it could therefore be concluded that the aglycone moiety was 2 β ,3 β ,23-trihydroxyolean-12-en-28-oic acid (bayogenin) (2a).

For the sugar moiety of compound 2, the ^1H NMR data were closely resembled those of compound 1 (Table 2) and the ^{13}C NMR data of the sugar moiety of 2 (Table 4) were almost identical to those of compound 1 (Table 4). Acid hydrolysis of 2 also produced the same sugars, D-glucose, L-rhamnose and D-xylose, together with the aglycone which was identified as bayogenin (2a, Fig. S2†).^{31,32} HMBC correlations of the proton of one sugar unit to the carbon of the next sugar unit were in the same manner to those occurred in the sugar unit of compound 1. The sugar linkages of compounds 1 and 2 were therefore identical. Alkaline hydrolysis of 2 (see Experimental section) gave bayogenin-3-*O*- β -D-glucopyranoside (2b, Fig. S2†),³⁰ the structure of which was confirmed by NMR data (Tables S1 and S2†). In addition, the ^1H – ^1H COSY correlation of the sugar units (Fig. 2 and S51–S53†) and the NOESY correlations of the sugar



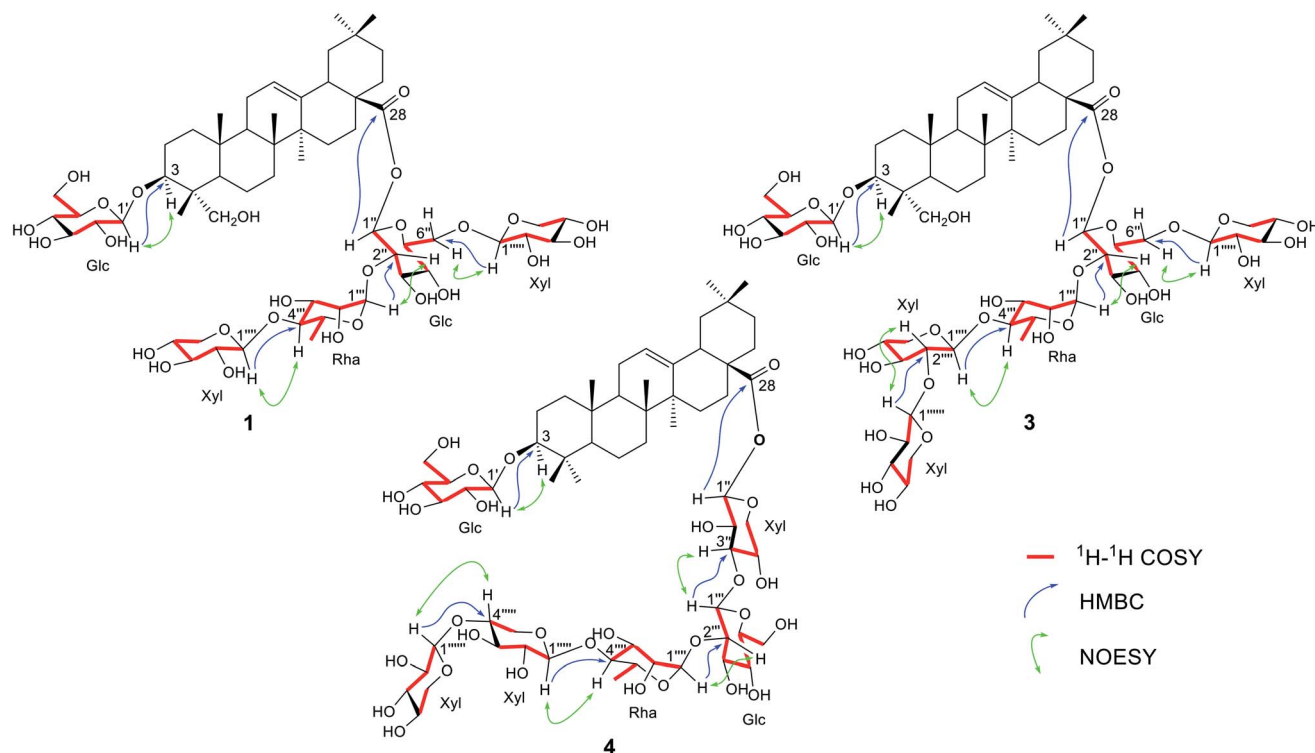


Fig. 2 Key correlations of compounds 1, 3 and 4.

and the aglycone units (Fig. 2 and S64–S66†) confirmed the structure of 2. Compound 2 was therefore identified as bayogenin-3-*O*- β -D-glucopyranosyl-28-*O*- β -D-xylopyranosyl-(1 \rightarrow 6)-[β -D-xylopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 2)]- β -D-glucopyranoside and was named trichocucumeriside B.

Compound 3 was isolated as a white powder with $[\alpha]_{\text{D}}^{26} -39.6^\circ$ (c 0.20 in MeOH). The ESI-TOF-MS gave a sodiated molecular ion at m/z 1345.6331 $[\text{M} + \text{Na}]^+$ compatible with the molecular formula $\text{C}_{63}\text{H}_{102}\text{O}_{29}$. The ^1H and ^{13}C NMR data (Tables 1–4 and Fig. S68–S90†) revealed that the aglycone moiety of 3 was similar to that of 1. The significant difference was the absence of an oxymethylene group at C-23 and this was replaced by a methyl group at δ_{H} 0.99 and δ_{C} 17.6. The aglycone moiety of 3 was suggested to be 3 β -hydroxyolean-12-en-28-oic acid (oleanolic acid, 3a) from its NMR spectra (Fig. S7 and S8†) and by comparison with the NMR data of the glycosides of oleanolic acid.³³ Acid hydrolysis of 3 (see Experimental section) gave D-glucose, L-rhamnose and D-xylose, together with the aglycone oleanolic acid (3a). The NMR data (Tables 2 and 4) of 3 suggested that the sugar chain at C-28 consisted of three β -D-xylose, one β -D-glucose and one α -L-rhamnose and the sugar unit at C-3 was determined as one β -D-glucose in the molecule. The assignments of the sugar moieties at both positions were further confirmed by alkaline hydrolysis (see Experimental section) which yielded oleanolic acid-3-*O*- β -D-glucopyranoside (3b, Fig. S2†),³⁴ the NMR spectral data of which are presented in Fig. S15, S16, Tables S1 and S2.† The anomeric protons appeared at δ_{H} 4.94 (1H, d, J = 7.8 Hz, H-1'), 6.16 (1H, d, J = 8.0 Hz, H-1''), 6.47 (1H, brs, H-1'''), 5.08 (1H, d, J = 7.0 Hz, H-1'''), 4.90 (1H, d, J = 7.4 Hz, H-1''') and 5.23 (1H, d, J = 7.5 Hz, H-1'''). These six anomeric proton signals

showed correlations with six anomeric carbon signals at δ_{C} 107.4 (C-1'), 95.2 (C-1''), 101.9 (C-1'''), 108.2 (C-1'''), 106.1 (C-1''') and 106.4 (C-1'''), respectively, in the HMQC spectrum (Fig. S78–S81†). These observations suggested that 3 contained six sugar units, with one more sugar unit than that of compound 1. Comparison of the molecular formula of 3 with that of 1 also suggested that the extra sugar moiety was compatible with a xylose unit deduced from the NMR data. The HMBC experiments (Fig. 2) showed correlations between δ_{H} 4.94 (Glc-1') and δ_{C} 89.6 (C-3), δ_{H} 6.16 (Glc-1'') and δ_{C} 177.3 (C-28), δ_{H} 6.47 (Rha-1''') and δ_{C} 76.9 (Glc-2''), δ_{H} 5.08 (Xyl-1''') and δ_{C} 85.9 (Rha-4'''), and between δ_{H} 4.90 (Xyl-1''') and δ_{C} 69.5 (Glc-6''). The sequence of connection of the sugar units was therefore the same as those of compounds 1 and 2. The additional sixth sugar moiety, the xylose unit, was determined to reside at the lower xylose unit from the HMBC correlation between δ_{H} 5.23 (Xyl-1''') and δ_{C} 87.8 (Xyl-2'''). These data have been used to establish the positions of the sugar units in the chain. In addition, ^1H - ^1H COSY correlations of the sugar protons were observed (Fig. 2). Furthermore, key NOESY correlations of the sugar moieties (Fig. 2) were also observed. These correlations were key interactions to support the gross structure of 3. Compound 3 was therefore identified as oleanolic acid-3-*O*- β -D-glucopyranosyl-28-*O*- β -D-xylopyranosyl-(1 \rightarrow 6)-[β -D-xylopyranosyl-(1 \rightarrow 2)]- β -D-xylopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 2)]- β -D-glucopyranoside and was named trichocucumeriside C.

Compound 4 was isolated as a white powder with $[\alpha]_{\text{D}}^{26} -31.1^\circ$ (c 0.38 in MeOH). The ESI-TOF-MS gave a sodiated molecular ion at m/z 1345.6340 $[\text{M} + \text{Na}]^+$ which was compatible with the molecular formula $\text{C}_{63}\text{H}_{102}\text{O}_{29}$. The ^1H and ^{13}C NMR data (Tables 1–4 and



Fig. S92–S115†) showed that the aglycone of **4** was almost identical to **3**. Acid hydrolysis of **4** (see Experimental section) yielded D-xylose, D-glucose, L-rhamnose and the triterpenoid oleanolic acid

(**3a**).³³ Alkaline hydrolysis of **4** (see Experimental section) gave oleanolic acid-3-O-β-D-glucopyranoside (**3b**, Fig. S2†)³⁴ which was identical to that obtained from alkaline hydrolysis of compound **3**.

Table 2 ¹H NMR data for sugar moieties of compounds 1–5 in pyridine-*d*₅ at 400 MHz (δ in ppm, *J* in Hz)

	1	2	3	4	5
Position	Glc'	Glc'	Glc'	Glc'	Glc'
1'	5.10 d (7.2)	5.20 ^a	4.94 d (7.8)	4.93 d (7.6)	4.83 d (7.6)
2'	4.04 t (8.3)	4.11 m	4.06 m	4.20 d (8.2)	4.12 m
3'	4.18 d (8.8)	4.13 t (8.4)	4.31 m	4.34 t (8.8)	4.27 t (8.8)
4'	4.26 m	4.27 t (10.1)	4.27 m	4.07 m	4.06 m
5'	3.90 ddd (9.0, 4.6, 2.5)	3.93 ddd (9.4, 5.0, 2.2)	4.02 t (7.7)	3.95 ddd (9.5, 5.0, 1.9)	3.87 m
6'	4.40 brd (10.2) 4.51 m	4.32 dd (10.2, 4.9) 4.52 dd (9.5, 6.5)	4.43 dd (11.8, 5.0) 4.62 brd (11.0)	4.37 dd (8.8, 4.5) 4.57 m	4.34 dd (8.8, 3.8) 4.55 m
^a Signals overlapped.					
	1	2	3	4	5
Position	Glc''	Glc''	Glc''	Xyl''	Xyl''
1''	6.14 d (8.0)	6.16 d (7.8)	6.16 d (8.0)	6.54 d (7.7)	5.24 d (7.1)
2''	4.29 d (8.3)	4.32 m	4.32 m	4.60 t (9.6)	4.59 m
3''	4.21 m	4.24 m	4.26 t (8.9)	4.23 m	4.15 m
4''	4.29 m	4.11 m	4.36 t (8.7)	4.08 m	4.07 t (8.1)
5''	4.04 t (8.3)	4.05 m	4.07 m	4.00 dd (10.4, 4.0) 4.39 m	3.99 dd (7.0, 3.5) 4.45 m
6''	4.28 m 4.64 dd (9.6, 1.6)	4.30 m 4.67 dd (11.3, 1.6)	4.31 m 4.66 brd (10.9)		
	1	2	3	4	5
Position	Rha'''	Rha'''	Rha'''	Glc'''	Glc'''
1'''	6.46 brs	6.47 brs	6.47 brs	5.24 d (7.6)	5.18 d (7.2)
2'''	4.82 brs	4.84 brs	4.84 m	4.64 t (9.2)	4.59 m
3'''	4.70 brd (8.8)	4.72 brd (9.4)	4.72 m	4.74 brs	4.61 m
4'''	4.37 t (9.4)	4.39 t (9.3)	4.49 dd (9.2, 3.4)	4.19 d (8.6)	4.17 m
5'''	4.50 t (6.4)	4.52 t (9.5)	4.53 m	4.07 t (6.6)	4.06 m
6'''	1.82 d (6.1)	1.84 d (6.1)	1.81 d (5.9)	4.64 m 4.42 m	4.17 m 4.56 m
	1	2	3	4	5
Position	Xyl''''	Xyl''''	Xyl''''	Rha''''	Rha''''
1''''	5.05 d (7.5)	5.08 d (7.2)	5.08 d (7.0)	5.78 brs	5.82 brs
2''''	4.09 m	4.08 m	4.09 m	4.60 m	4.61 m
3''''	4.09 m	4.24 m	4.09 m	4.45 m	4.45 m
4''''	4.46 m	4.32 m	4.36 t (8.7)	4.41 m	4.41 m
5''''	3.52 t (10.3) 4.26 m	3.55 t (10.2) 4.27 t (10.1)	3.57 t (10.8) 4.31 t (9.7)	4.41 m	4.40 m
6''''				1.79 d (5.0)	1.76 d (4.7)
	1	2	3	4	5
Position	Xyl'''''	Xyl'''''	Xyl'''''	Xyl'''''	Xyl'''''
1'''''	4.87 d (7.4)	4.90 d (7.4)	4.90 d (7.4)	5.19 ^a	5.15 d (7.5)
2'''''	3.97 t (7.9)	3.99 t (7.8)	4.00 t (8.1)	4.07 m	4.07 m
3'''''	4.10 m	4.11 m	4.17 t (8.7)	4.59 m	4.59 m
4'''''	4.17 t (8.8)	4.19 m	4.21 t (8.2)	4.08 m	4.07 m
5'''''	3.63 t (10.6) 4.29 t (10.8)	3.67 t (10.8) 4.32 t (8.2)	3.66 t (10.6) 4.31 m	3.48 t (9.8) 4.23 t (9.4)	3.51 t (10.1) 4.24 t (9.4)



Table 2 (Contd.)

Position	1	2	3	4	5
			Xyl''''''	Xyl''''''	Xyl''''''
1''''''			5.23 d (7.5)	5.28 d (7.6)	5.23 ^a
2''''''			4.13 t (8.4)	4.07 m	4.06 m
3''''''			4.22 m	4.19 m	4.17 m
4''''''			4.09 m	4.20 m	4.19 m
5''''''			3.53 t (10.0)	3.70 t (10.0)	3.70 t (10.0)
			4.31 t (9.7)	4.31 m	4.29 t (10.4)

The sugar unit at C-3 was therefore determined as one β -D-glucose and the sugar chain was located at C-28. Six anomeric proton signals at δ 4.93 (1H, d, J = 7.6 Hz, H-1'), 6.54 (1H, d, J = 7.7 Hz, H-1''), 5.24 (1H, d, J = 7.6 Hz, H-1'''), 5.78 (1H, brs, H-1'''), 5.19 (1H, overlapping signals, H-1''') and 5.28 (1H, d, J = 7.6 Hz, H-1''') showed correlations with six anomeric carbon signals at δ 105.5 (C-1'), 93.8 (C-1''), 107.7 (C-1'''), 101.4 (C-1'''), 106.9 (C-1''') and 106.5 (C-1'''), respectively, in the HMQC spectrum (Fig. S102–S105†). In the HMBC spectrum (Fig. 2 and S106–S112†), the correlation between δ_{H} 4.93 (Glc-1') and δ_{C} 89.4 (C-3), which was the mono sugar unit of β -D-glucose, was observed. The correlations between δ_{H} 6.54 (Xyl-1'') and δ_{C} 176.8 (C-28), δ_{H} 5.24 (Glc-1'') and δ_{C} 84.9

(Xyl-3''), δ_{H} 5.78 (Rha-1''') and δ_{C} 75.3 (Glc-2'''), δ_{H} 5.19 (Xyl-1''') and δ_{C} 84.3 (Rha-4'''), δ_{H} 5.28 (Xyl-1''') and δ_{C} 87.5 (Xyl-4''') characterized that the sequence of the sugar chain was β -D-Xyl-(1 \rightarrow 4)- β -D-Xyl-(1 \rightarrow 4)- α -L-Rha-(1 \rightarrow 2)- β -D-Glc-(1 \rightarrow 3)- β -D-Xyl and that the glycosidic site was at C-28. In addition, ^1H - ^1H COSY correlations of the sugar protons were observed (Fig. 2) and the NOESY correlations (Fig. 2) were also key interactions to support this gross structure. Compound 3 was therefore assigned as ole-anolic acid-3-O- β -D-glucopyranosyl-28-O- β -D-xylopyranosyl-(1 \rightarrow 4)- β -D-xylopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-glucopyranosyl-(1 \rightarrow 3)- β -D-xylopyranoside and was named trichocucumeriside D.

Table 3 ^{13}C NMR data for aglycone moieties of compounds 1–5 in pyridine- d_5 at 100 MHz (δ in ppm)

Position	1	2	3	4	5
1	39.3	47.9	39.4	39.2	38.6
2	26.4	67.6	27.2	27.1	25.4
3	82.8	89.1	89.6	89.4	83.7
4	43.9	45.2	40.1	40.0	55.6
5	48.3	47.9	56.5	56.3	48.7
6	18.9	18.9	19.2	18.9	21.0
7	32.9	32.9	33.0	33.7	33.0
8	40.5	40.5	40.5	40.3	40.6
9	48.7	48.6	48.6	48.4	48.2
10	37.5	38.6	37.6	37.4	36.7
11	24.4	23.8	24.4	24.2	24.2
12	123.3	123.2	123.5	123.5	123.1
13	144.5	144.5	144.6	144.7	144.8
14	42.7	42.8	42.9	42.6	42.6
15	29.1	29.1	29.0	28.7	28.7
16	23.8	24.4	24.0	23.6	23.6
17	47.7	47.7	47.8	47.8	47.8
18	42.4	42.3	42.5	42.2	42.2
19	46.8	46.8	47.0	46.7	46.7
20	31.2	31.2	31.3	31.4	31.4
21	34.4	34.4	34.5	34.6	33.2
22	33.6	33.4	33.7	33.2	34.6
23	65.2	64.4	17.6	17.3	210.2
24	14.2	15.3	28.8	28.7	11.4
25	16.8	18.0	16.2	16.0	16.2
26	18.1	18.1	18.1	18.0	17.9
27	26.5	26.4	26.6	26.5	26.5
28	177.1	177.1	177.3	176.8	176.8
29	33.6	33.6	33.7	33.7	33.7
30	24.4	24.4	24.4	24.2	24.2

Compound 5 was isolated as a white powder, $[\alpha]_{\text{D}}^{26} -11.6^\circ$ (c 0.30 in MeOH), with the molecular formula of $\text{C}_{63}\text{H}_{100}\text{O}_{30}$ obtained by the ESI-TOF-MS from the $[\text{M} + \text{Na}]^+$ ion at m/z 1359.6145 (calcd for $\text{C}_{63}\text{H}_{100}\text{NaO}_{30}$, 1359.6192). The ^{13}C and ^1H -NMR spectra (Tables 1–4) of the aglycone moiety of 5 was similar to that of 4. The major difference was that the chemical shifts of 23- CH_3 at δ_{H} 1.12 and δ_{C} 17.3 of 4 were replaced by the CHO group at δ_{H} 9.95 (1H, s, H-23) and δ_{C} 210.2 in the ^1H and ^{13}C -NMR spectrum, respectively, indicated an aldehydic group in the aglycone moiety of 5 at C-23. The aglycone moiety of 5 was therefore suggested to be gypsogenin (4a, Fig. S2†) by comparison of its spectroscopic data with those reported in the literature. Acid hydrolysis of 5 yielded D-xylose, D-glucose, L-rhamnose and the triterpenoid 4a (Fig. S2†). Alkaline hydrolysis gave the glycoside gypsogenin-3-O- β -D-glucopyranoside (4b) (Fig. S2, Tables S1 and S2†). The ^1H - ^1H COSY (Fig. 2 and S126–S129†), HMBC (Fig. 2 and S134–S141†) and HMQC data (Fig. S130–S133†) confirmed the same sugar moiety of β -D-Glc at C-3. Analysis of the HMBC correlations (Fig. 2 and S134–S141†) supported by the COSY (Fig. 2 and S126–S129†) and NOESY (Fig. 2 and S142–S144†) correlations revealed the sugar linkage at C-28 as β -D-Xyl-(1 \rightarrow 4)- β -D-Xyl-(1 \rightarrow 4)- α -L-Rha-(1 \rightarrow 2)- β -D-Glc-(1 \rightarrow 3)- β -D-Xyl, which is the same as that of compound 4. All of the above data led to the formulation of 5 as gypsogenin-3-O- β -D-glucopyranosyl-28-O- β -D-xylopyranosyl-(1 \rightarrow 4)- β -D-xylopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-glucopyranosyl-(1 \rightarrow 3)- β -D-xylopyranoside and was named trichocucumeriside E.

The anti-inflammatory activity of all the isolated compounds was evaluated against nitric oxide (NO) production in LPS-stimulated RAW264.7 cells using the Griess assay³⁷ (Table 5). Compared to the positive references, Celecoxib and amino-guanidine (IC_{50} 75.7 and 75.0 μM , respectively), cucurbitacin B (7) and isocucurbitacin B (9) showed more potent inhibitory



Table 4 ^{13}C NMR data for sugar moieties of compounds 1–5 in pyridine- d_5 at 100 MHz (δ in ppm)

Position	1	2	3	4	5
	Glc'	Glc'	Glc'	Glc'	Glc'
1'	106.3	106.3	107.4	105.5	103.4
2'	76.4	76.0	76.4	72.0	71.7
3'	79.3	79.1	79.3	78.9	78.8
4'	72.1	71.8	72.3	75.8	75.5
5'	78.6	78.9	78.9	78.5	78.8
6'	63.2	62.8	63.5	63.3	63.1

Position	1	2	3	4	5
	Glc''	Glc''	Glc''	Xyl''	Xyl''
1''	95.1	95.1	95.2	93.8	93.9
2''	76.7	76.7	76.9	70.0	76.6
3''	79.9	79.9	79.9	84.9	83.3
4''	71.4	76.7	71.2	69.5	69.5
5''	78.1	78.1	78.1	63.3	63.4
6''	69.4	69.5	69.5		

Position	1	2	3	4	5
	Rha'''	Rha'''	Rha'''	Glc'''	Glc'''
1'''	101.9	101.9	101.9	107.7	106.8
2'''	72.3	72.3	72.1	75.3	75.4
3'''	73.1	73.1	73.1	70.0	75.0
4'''	86.0	86.0	85.9	75.5	75.4
5'''	68.8	68.8	68.8	75.5	75.6
6'''	19.2	19.2	19.2	61.7	62.6

Position	1	2	3	4	5
	Xyl''''	Xyl''''	Xyl''''	Rha''''	Rha''''
1''''	108.2	108.2	108.2	101.4	101.5
2''''	79.2	79.3	87.8	73.2	73.2
3''''	76.7	76.7	75.8	66.4	66.7
4''''	71.6	71.4	71.4	84.3	84.4
5''''	68.0	68.0	67.9	69.0	69.0
6''''				18.9	18.9

Position	1	2	3	4	5
	Xyl'''''	Xyl'''''	Xyl'''''	Xyl'''''	Xyl'''''
1'''''	106.1	106.1	106.1	106.9	107.6
2'''''	75.3	75.3	75.4	77.4	76.6
3'''''	78.9	78.6	78.6	72.3	72.3
4'''''	71.4	71.6	71.6	87.5	87.6
5'''''	67.6	67.6	67.6	67.4	67.4

Position	1	2	3	4	5
			Xyl''''''	Xyl''''''	Xyl''''''
1''''''			106.4	106.5	106.5
2''''''			76.6	75.6	75.8
3''''''			79.7	78.7	77.6
4''''''			78.6	71.4	71.4
5''''''			67.5	67.8	67.8

Table 5 Anti-inflammatory activity of compounds 1–16

Compound	(IC ₅₀ , μM)		SI ^c
	NO ^a	MTT ^b	
1	97.7 \pm 12.4	>160	>1.6
2	99.0 \pm 15.6	>160	>1.6
3	115.7 \pm 16.9	>160	>1.4
4	29.2 \pm 5.3	49.0 \pm 6.4	1.7
5	22.5 \pm 3.8	52.0 \pm 3.8	2.3
7	3.0 \pm 1.1	19.4 \pm 1.6	6.5
9	2.7 \pm 0.9	17.2 \pm 4.5	6.4
10	13.3 \pm 2.3	41.7 \pm 9.4	3.1
11	21.7 \pm 8.1	67.0 \pm 16.4	3.1
12	11.3 \pm 3.3	19.0 \pm 2.3	1.7
13	71.7 \pm 3.0	>160	>2.2
14	148.3 \pm 6.4	141.7 \pm 9.3	1.0
15	153.0 \pm 6.5	>160	>1.1
16	110.0 \pm 9.0	>160	>1.5
Celecoxib ^d	75.7 \pm 12.8	102.7 \pm 8.8	1.4
Aminoguanidine ^d	75.0 \pm 15.5	>160	>2.1

^a Nitric oxide (NO) production in LPS-stimulated RAW264.7 cells. ^b Cell viability in LPS-stimulated RAW264.7 cells. ^c Selectivity index. ^d Positive references. Mean \pm SD, $n = 3$.

activity against NO production in LPS-stimulated RAW264.7 cells with IC₅₀ values of 3.0 and 2.7 μM , respectively. More importantly, both natural compounds showed approximately 3–4-fold higher selectivity index (SI) than that of positive references. Compounds 4, 5 and 10–12 exhibited moderate inhibitory activity towards NO production with IC₅₀ range from 11.3 to 29.2 μM , while compounds 1–3 and 13–16 exhibited weaker activity with the IC₅₀ values of 71.7–153.0 μM .

Conclusions

In summary, five new triterpenoid saponin glycosides (1–5) have been isolated from *T. cucumerina* fruit fibers, together with eleven known compounds (6–16) in this work. The anti-inflammatory assays showed that the natural compounds 7 and 9 exhibited more potent NO inhibitory activities than those of positive references Celecoxib and aminoguanidine. In addition, these two compounds possessed approximately 3–4-fold higher selectivity index (SI) than the positive references.

Experimental

General

Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. Optical rotations were acquired using a JASCO P-1020 digital polarimeter. IR spectra were recorded on a PerkinElmer FT-IR 400 spectrophotometer. Spectra of solid sample were recorded in the ATR mode. NMR spectra were recorded on a Bruker AVANCE 400 FT-NMR spectrometer, operating at 400 (^1H) and 100 (^{13}C) MHz. For the spectra taken in CDCl_3 (δ_{H} 7.24 and δ_{C} 77.0) and pyridine- d_5 (δ_{H} 8.74 and δ_{C} 150.3), the residual nondeuterated solvent were used as references for ^1H and ^{13}C NMR spectra. The electrospray ionization mass spectra (ESIMS) were measured with a Finnigan



LC-Q mass spectrometer. High resolution electrospray ionization time-of-flight mass spectra (ESI-TOF-MS) were measured with a Bruker micrOTOF-II mass spectrometer. Column chromatography was carried out using Merck silica gel 60 (<0.063 mm) and reversed phase RP C-18 (40–63 μ m, E. Merck). For TLC, Merck precoated silica gel 60 F₂₅₄ plates and RP-TLC, RP-18 F₂₅₄ precoated on aluminium plate (E. Merck) were used. Spots on TLC were detected under UV light and by spraying with anisaldehyde-H₂SO₄ reagent followed by heating. The standard sugars, D(+)-glucose, L(+)-rhamnose and D(+)-xylose, were purchased from Sigma, St. Louis, USA.

Plant material

The dried fruit fibers of *T. cucumerina* used in this study were collected from Takli district, Nakhon Sawan province, Thailand in 2009 and were identified by Assoc. Prof. Nopporn Damrongsiri, Department of Biology, Faculty of Science, Ramkhamhaeng University. A voucher specimen (Apichart Suksamrarn, No. 058) was deposited at the Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand.

Extraction and isolation

The dried fruit fibers of *T. cucumerina* (18.0 kg), whose fruit peels and seeds were separated, were chilled in liquid N₂ and crushed into small pieces and extracted successively with *n*-hexane (3 \times 50 L), EtOAc (3 \times 50 L) and MeOH (3 \times 50 L). The extracts were evaporated to dryness under reduced pressure at temperature 40–45 °C to afford the hexane (168.0 g), EtOAc (350.0 g) and MeOH (830.0 g) extracts, respectively. The hexane extract (25.0 g) was subjected to column chromatography (CC) (Merck silica gel 60, 0.063–0.200 mm, 300 g) using gradient elution of *n*-hexane : EtOAc (100 : 0 to 0 : 100, v/v) and EtOAc : MeOH (100 : 0 to 0 : 100, v/v) to give 3 fractions (H1 to H3). H1 (8.5 g) was repeatedly CC over silica gel eluted with *n*-hexane : EtOAc (100 : 10, v/v) to afford 4 subfractions (H1-1 to H1-4), then H1-3 (1.2 g) was purified by CC eluted with CH₂Cl₂ : MeOH (100 : 0.4, v/v) to give compound 6 (148.9 mg). H2 (10.8 g) was applied to silica gel using isocratic elution of *n*-hexane : EtOAc (50 : 50, v/v) to obtain 4 subfractions (H2-1 to H2-4). H2-3 was identified to be compound 7 (6.5 g). Subfraction H2-1 (0.3 g) was subjected to silica gel CC eluted with *n*-hexane : EtOAc (50 : 50, v/v) to yield compound 8 (20.0 mg). H2-2 (3.5 g) was purified by CC eluted with *n*-hexane : EtOAc (50 : 50, v/v) to afford compound 9 (2.8 g). The EtOAc extract (140.0 g) was fractionated by CC on silica gel (400 g), eluting with *n*-hexane : EtOAc (100 : 0 to 0 : 100, v/v) and EtOAc : MeOH (100 : 0 to 0 : 100, v/v) to give 4 fractions (E1 to E4). E3 (21.4 g) was applied to silica gel using isocratic elution of CH₂Cl₂ : MeOH (98 : 2, v/v) to yield 4 subfractions (E3-1 to E3-4). E3-2 (1.8 g) was repeated CC with EtOAc : *n*-hexane (50 : 35, v/v) to give compound 10 (137.7 mg). E3-3 (3.2 g) was subjected to silica gel CC eluted with EtOAc : *n*-hexane (50 : 35, v/v) to yield 3 subfractions (E3-3-1 to E3-3-3). E3-3-3 (0.8 g) was repeatedly CC over silica gel eluting with CH₂Cl₂ : MeOH (98 : 2, v/v) to afford compounds 11 (30.4 mg) and 12 (6.4 mg). E4 (67.0 g) was subjected to silica gel CC eluted with CH₂Cl₂ : MeOH (95 : 5, v/v) to

yield 4 subfractions (E4-1 to E4-4). E4-2 (5.4 g) was chromatographed on silica column using gradient elution of CH₂Cl₂ : MeOH (98 : 2 to 95 : 5, v/v) to give 4 subfractions (E4-2-1 to E4-2-4). E4-2-3 (1.5 g) yielded compound 13 (49.0 mg) by recrystallization with MeOH. The filtrate upon evaporation gave a residue (849.0 mg) which was CC on silica gel eluting under gradient condition of CH₂Cl₂ : MeOH (98 : 2 to 96 : 4, v/v) to give compound 14 (217.8 mg). E4-3 (6.9 g) was subjected to silica gel CC eluted with EtOAc : MeOH (97 : 3, v/v) to yield 4 subfractions (E4-3-1 to E4-3-4). E4-3-3 (3.5 g) was purified by CC eluted with CH₂Cl₂ : MeOH (95 : 5, v/v) to afford 2 subfractions (E4-3-3-1 and E4-3-3-2). E4-3-3-2 was identified to be compound 15 (34.9 mg). E4-3-3-1 (0.4 g) was purified by CC eluted with CH₂Cl₂ : MeOH (98 : 2, v/v) to give compound 16 (35.4 mg). The MeOH part (60.0 g) was fractionated by CC on silica gel (300 g), and it was eluted with CH₂Cl₂ : MeOH (100 : 0 to 0 : 100, v/v) to give 6 fractions (M1 to M6). M5 (22.9 g) was subjected to reversed phase RP-18 CC eluted with MeOH : H₂O (50 : 25, v/v) to afford 6 subfractions (M5-1 to M5-6). M5-2 (3.2 g) was repeatedly chromatographed on Sephadex LH-20 eluting with 100% MeOH, followed by reversed phase RP-18 CC using MeOH : H₂O (50 : 25, v/v) to afford compound 2 (105.0 mg). M5-3 (1.6 g) was purified by reversed phase RP-18 CC eluted with MeOH : H₂O (50 : 25, v/v) to give compound 1 (46.4 mg). Similarly, M5-4 (5.6 g) was subjected to reversed phase RP-18 gel CC to afford 5 subfractions (M5-4-1 to M5-4-5). M5-4-5 (2.3 g) was separated by reversed phase RP-18 CC eluted with MeOH : H₂O (50 : 20, v/v) to give 3 subfractions (M5-4-5-1 to M5-4-5-3). Then, M5-4-5-3 (0.5 g) was purified by reversed phase RP-18 CC using H₂O : ACN (50 : 25, v/v) to afford compound 4 (26.4 mg). M5-4-3 (2.5 g) was subjected to reversed phase RP-18 CC eluted with MeOH : H₂O (50 : 20, v/v) to obtain 3 subfractions (M5-4-3-1 to M5-4-3-3). Then, M5-4-3-2 (0.4 g) was repeatedly chromatographed on RP-18 column using H₂O : ACN (50 : 25, v/v) to afford compound 5 (42.8 mg) and M5-4-3-3 (0.9 g) was CC using RP-18 with MeOH : H₂O (50 : 25, v/v) as eluting solvent to yield compound 3 (158.8 mg).

Compound 1. White powder; $[\alpha]_D^{26} -17.2^\circ$ (*c* 0.39 in MeOH); Mp 219–222 °C; IR (ATR) ν_{\max} : 3337, 2932, 1734, 1639, 1455, 1387, 1255, 1073, 894, 815 cm⁻¹; ¹H-NMR (pyridine-*d*₅, 400 MHz) data and ¹³C-NMR (pyridine-*d*₅, 100 MHz) data: see Tables 1–4; ESI-TOF-MS *m/z* 1229.5924 [M + Na]⁺ (calcd for C₅₈H₉₄O₂₆Na, 1229.5926).

Compound 2. White powder; $[\alpha]_D^{26} -22.7^\circ$ (*c* 0.24 in MeOH); Mp 215–217 °C; IR (ATR) ν_{\max} : 3442, 2928, 1727, 1643, 1455, 1374, 1258, 1073, 894, 837 cm⁻¹; ¹H-NMR (pyridine-*d*₅, 400 MHz) data and ¹³C-NMR (pyridine-*d*₅, 100 MHz) data: see Tables 1–4; ESI-TOF-MS *m/z* 1245.5869 [M + Na]⁺ (calcd for C₅₈H₉₄O₂₇Na, 1245.5875).

Compound 3. White powder; $[\alpha]_D^{26} -39.6^\circ$ (*c* 0.20 in MeOH); Mp 204–206 °C; IR (ATR) ν_{\max} : 3332, 2933, 1751, 1639, 1455, 1361, 1258, 1030, 894, 820, 809 cm⁻¹; ¹H-NMR (pyridine-*d*₅, 400 MHz) data and ¹³C-NMR (pyridine-*d*₅, 100 MHz) data: see Tables 1–4; ESI-TOF-MS *m/z* 1345.6331 [M + Na]⁺ (calcd for C₆₃H₁₀₂O₂₉Na, 1345.6399).

Compound 4. White powder; $[\alpha]_D^{26} -31.1^\circ$ (*c* 0.38 in MeOH); Mp 213–215 °C; IR (ATR) ν_{\max} : 3338, 2934, 1734, 1639, 1458,



1390, 1258, 1034, 892, 818, 779 cm^{-1} ; $^1\text{H-NMR}$ (pyridine- d_5 , 400 MHz) data and $^{13}\text{C-NMR}$ (pyridine- d_5 , 100 MHz) data: see Tables 1–4; ESI-TOF-MS m/z 1345.6340 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{63}\text{H}_{102}\text{O}_{29}\text{Na}$, 1345.6399).

Compound 5. White powder; $[\alpha]_{\text{D}}^{26} -11.6^\circ$ (c 0.30 in MeOH); Mp 180–182 $^\circ\text{C}$; IR (ATR) ν_{max} : 3344, 2933, 1719, 1640, 1455, 1387, 1261, 1042, 894, 818, 782 cm^{-1} ; $^1\text{H-NMR}$ (pyridine- d_5 , 400 MHz) data and $^{13}\text{C-NMR}$ (pyridine- d_5 , 100 MHz) data: see Tables 1–4; ESI-TOF-MS m/z 1359.6145 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{63}\text{H}_{100}\text{O}_{30}\text{Na}$, 1359.6192).

Acid hydrolysis

The new compounds 1–5 (5.0 mg each) were individually dissolved in MeOH (0.2 mL) and TFA (2 N, 1 mL each) was added. The mixture was heated at 95 $^\circ\text{C}$ for 24 h and the progress of the reaction was monitored by TLC. The residues were evaporated to dryness by repeatedly adding MeOH to remove the acid. The residue was analyzed by TLC over silica gel together with authentic sugar standards using $\text{CHCl}_3 : \text{MeOH} : \text{H}_2\text{O}$ (3 : 1.5 : 0.2) and the sugars were then separated by silica gel using the same eluting solvent system as the mobile phase.^{38,39} The monosaccharides of compounds 1–5 were identified as D-glucose, L-rhamnose and D-xylose by TLC and NMR comparisons with those of standard sugars. It was detected at 589 nm on a polarimeter that the optical rotation of 1.02% (w/v) of D-glucose ($[\alpha]_{\text{D}}^{26} +18.7^\circ$),^{40–43} 1.12% (w/v) of L-rhamnose ($[\alpha]_{\text{D}}^{26} +5.0^\circ$)⁴⁴ and 0.35% (w/v) of D-xylose ($[\alpha]_{\text{D}}^{26} +11.6^\circ$)⁴⁵ consisting with a homologous standard distilled water solution. Moreover, by comparison of the spectroscopic data (Fig. S3–S10†) with those reported in literature, the aglycones obtained from acid hydrolysis were identified as hederagenin²⁵ (**1a**, Fig. S2†) from compound 1, bayogenin^{31,32} (**2a**, Fig. S2†) from compound 2, oleanolic acid³³ (**3a**, Fig. S2†) from compounds 3 and 4, and gypsogenin³⁵ (**4a**, Fig. S2†) from compound 5.

Alkaline hydrolysis

The new compounds 1–5 (5 mg each) were dissolved in MeOH (0.2 mL each), then saturated solution of NaOH (1 mL each) were added. The mixture was heated at 60 $^\circ\text{C}$ for 24 h, acidified with 1 N HCl and the mixture was then evaporated to dryness *in vacuo*. The triterpenoid glycosides were separated by silica column chromatography using $\text{CHCl}_3 : \text{MeOH} : \text{H}_2\text{O}$ (3 : 1.5 : 0.2) as the mobile phase.⁴⁶ Comparison of these compounds with the corresponding aglycones, the glycosides hederagenin-3-O- β -D-glucopyranoside²⁹ (**1b**, Fig. S2, Tables S1 and S2†), bayogenin-3-O- β -D-glucopyranoside³⁰ (**2b**, Fig. S2, Tables S1 and S2†), oleanolic acid-3-O- β -D-glucopyranoside³⁴ (**3b**, Fig. S2, Tables S1 and S2†) and gypsogenin-3-O- β -D-glucopyranoside³⁶ (**4b**, Fig. S2, Tables S1 and S2†), which was derived from compounds 1–5, respectively.

Anti-inflammatory assay

Cell culture. RAW264.7 murine macrophages (ATCC, Rockville, MD, USA) were cultured in RPMI-1640 medium (Corning, NY, USA) supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin (Gibco, MA, USA). Cells were incubated at 37 $^\circ\text{C}$ with 5% CO_2 in a humidified incubator.

Cell viability test. RAW264.7 cells were grown in a 96-well plate at a density of 1.0×10^5 cells per well for 24 h. Cells were treated with the compounds at various concentrations ranged from 5–160 μM in combination with 10 ng mL^{-1} of LPS. After 24 h, cells were then incubated with 0.5 mg mL^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution for another 3 h. After removal of MTT solution, the formazan crystal products were dissolved in DMSO (200 μL per well). The absorbance was measured using a microplate reader at a wavelength of 560 nm. Three independent experiments were performed in triplicates. IC_{50} of cell viability by MTT was calculated from a dose–response curve using GraphPad Prism 6 (GraphPad Software Inc., CA, USA).

Nitric oxide assay. RAW264.7 cells of 1.0×10^5 cells per well were seeded into 96-well plates and grown for 24 h. Cells were pre-treated with various concentrations of compounds (5–160 μM) followed by 10 ng mL^{-1} LPS stimulation for 24 h. Then, culture supernatants were collected and Griess reagent (1% sulfanilamide and 0.1% naphthylethylene in 2.5% phosphoric acid solution) was added in a 96-well plate according to the described method.³⁷ The absorbance was measured at 540 nm using a microplate reader. Nitrite levels were determined using a NaNO_2 standard curve with $r^2 > 0.999$. Data were obtained from three independent experiments in triplicates. IC_{50} by nitric oxide assay was determined from a dose–response curve using GraphPad Prism 6 (GraphPad Software Inc., CA, USA). The selectivity index (SI) was calculated as the ratio of IC_{50} (MTT) to IC_{50} (NO).

Conflicts of interest

Authors declare no conflict of interest.

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