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Triterpenoidal saponins from *Hydrocotyle sibthorpioides*

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Abstract

Oleanane-type triterpenoidal saponins, hydrocosisaponins A–F (**1**–**6**), along with a known saponin, hydrocotyloside VII (**7**), were isolated from *Hydrocotyle sibthorpioides*. Their structures were established on the basis of spectroscopic analyses including NMR spectroscopic techniques (^{13}C, ^{1}H, COSY, HMQC, HMBC, TOCSY and NOESY). Biological evaluation established that saponins possessing four sugar units (three D-glucoses and one L-arabinose) (**4**–**7**) exhibited moderate cytotoxicity against KB, Daoy and WiDr human tumor cell lines.

Keywords: *Hydrocotyle sibthorpioides*; Umbelliferae; Oleanane-type triterpenoidal saponin; Cytotoxicity; Chemotaxonomy

1. Introduction

Hydrocotyle sibthorpioides (family Umbelliferae) is a perennial herb widely distributed in parts of China and Taiwan (Kao, 2000). It has folkloric uses in treatment of illnesses such as fever, edema, detoxication and throat pain (Gan, 1991). It has also been shown to exert an anti-diuretic activity and has proven effective as an external application for skin tumors (Yu et al., 2007). Although several triterpenoid glycosides have been isolated from *H. sibthorpioides* and other *Hydrocotyle* species (Matsushita et al., 2004), nothing was reported concerning the actual cytotoxic constituents in the mentioned plants. In our continuing search for bioactive constituents from Taiwanese plants, it was found that the EtOH extract of *H. sibthorpioides* possessed marginal cytotoxicity against several human tumor cell lines. We report herein bioassay-directed fractionations that led to isolation and characterization of seven oleanane-type saponins including six new designated as hydrocosisaponins A–F (**1**–**6**) and one known saponin, hydrocotyloside VII (**7**), from the title plant. The structural elucidation of **1**–**6** was based on chemical methods and spectroscopic analyses, including 1D and 2D NMR spectroscopic techniques. Biological evaluation of the isolated saponins (**1**–**7**) against a panel of cancer cell lines is also described.

2. Results and discussion

The EtOH extract of *H. sibthorpioides* was partitioned successively with *n*-hexane, EtOAc and *n*-BuOH. After evaporation of the solvent, the *n*-BuOH soluble residue was subjected to column chromatography using Diaion HP-20, Sephadex LH-20 and silica gel, respectively, and then separated by HPLC to afford **1**–**7**.

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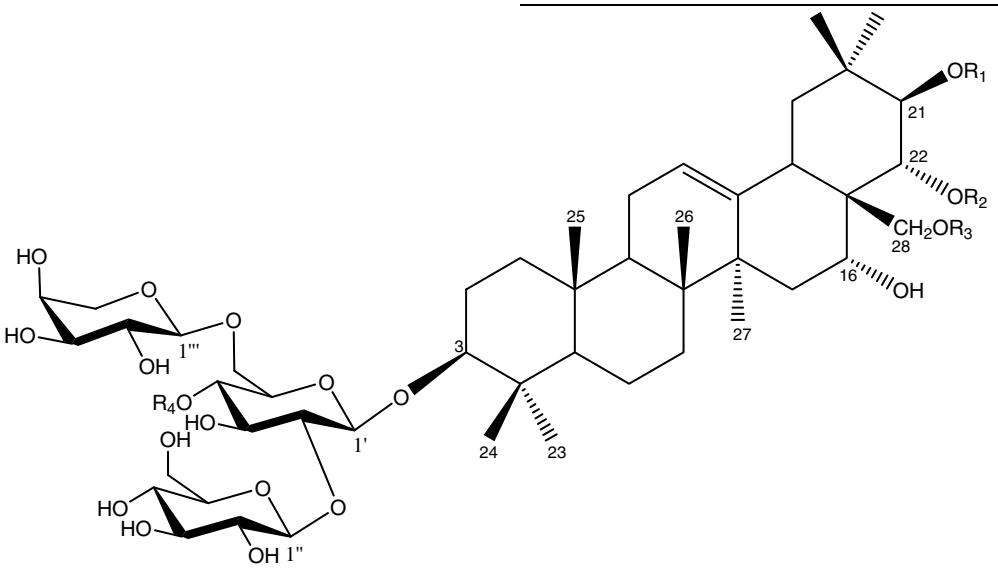
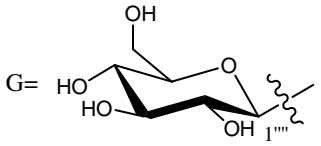


	R_1	R_2	R_3	R_4
1	Ac	Ac	H	H
2	H	Ac	Ac	H
3	Ac	H	Ac	H
4	Ac	H	Ac	G
5	Ac	H	H	G
6	H	H	Ac	G
7	Ac	Ac	H	G



Compound **1** was obtained as a light yellowish glass and its molecular formula was determined to be $C_{51}H_{82}O_{21}$ by HR-FAB-MS molecular ion at *m/z* 1053.5240 $[M+Na]^+$ and ^{13}C NMR spectroscopic analyses. The IR spectrum showed the presence of hydroxyl (3380 cm^{-1}) and olefinic (weak 1640 and 1036 cm^{-1}) groups. Acid hydrolysis of **1** with 2 N methanolic HCl afforded D-glucose and L-arabinose in a ratio of 2:1, both of which were identified by chiral GC. The ^1H NMR spectrum of **1** (Table 1) showed signals of an olean-12-ene-type triterpene [seven singlet methyl resonances at δ_H 0.88, 0.89, 0.99, 1.03, 1.05, 1.12, 1.51 and a trisubstituted olefinic proton signal at δ_H 5.35 (br s)], three anomeric proton resonances (δ_H 4.38, 4.45, 4.68), and two methyl signals [δ_H 2.02 (s) × 2] for two acetyl moieties. Based on HMBC correlations (H-3 with C-2, 4, 5, 23, 24; H-16 with C-15, 17; H-21 with C-17, 20, 22, 29, 30; H-22 with C-18, 20, 21; H-28 with C-16, 17, 22), four oxygenated carbons were assigned at C-3, C-16, C-21, and C-22, respectively, and one primary alcohol was located at C-28. Also, due to the long range correlations, the locations of two acetyl groups were deduced to be at C-21 and C-22, and an olefinic proton was assigned at C-12. The ^1H and ^{13}C NMR spectroscopic data of the triterpene moiety in **1** were found similar to those reported for hydrocotyloside VII (**7**) (Matsushita et al., 2004) and was thus identified as 3,21,22-trisubstituted-olean-12-en-3,16,21,22,28-pentaol. Characteristic signals for H-16 (δ_H 4.00, br s) and CH_3-27 (δ_H 1.51, s) found at relative low-field shifts, suggested the α-configuration for an OH-16 at the aglycone (Herlt et al., 2002; Kitagawa et al., 1974). Predictably, the large coupling constant (10 Hz) for H-21 and H-22 found in **1** indicated the diaxial orientation for these two protons (Gupta and Singh, 1989). Moreover, the correlations between H-21 and CH_3-27α, and between H-22 and H_2-28, observed in the 2D NOE spectrum, were in agreement with the configurations of H-21α and H-22β (Lu et al., 2000). The ^1H NMR signals of the sugar moieties indicated that they consist of two D-glucopyranosyl and one L-arabinopyranosyl units, this being supported by 2D TOCSY data (Fig. 1). Based on the anomeric proton coupling constants ($^3J_{H1,H2}$), glucopyranosyl (7.2–7.6 Hz) and arabinopyranosyl (6.4 Hz) residues were depicted to have β- and α-configurations, respectively. In the ^{13}C NMR spectrum of **1** (Table 2), the C-2′ and C-6′ positions of the central Glc were downfield-shifted to (δ_C 80.73) and 69.60, respectively, directly helping to establish the locations of glycosylation (Orsini et al., 1991; Pal et al., 1995). Furthermore, the interglycosidic linkages in **1** were also established by the HMBC studies (Fig. 1). Thus, cross-peaks between H-1″ of Glc (δ_H 4.68) and C-2′ of Glc (δ_C 80.73), and between H-1‴ of Ara (δ_H 4.38) and C-6′ of Glc (δ_C 69.60) indicated that the β-D-glucopyranosyl and α-L-arabinopyranosyl moieties were linked to C-2′ and C-6′ of Glc, respectively. Cross-peaks between H-1′ of 3-glucopyranosyl residue (δ_H 4.45) and C-3 of the triterpene (δ_C 91.15) indicated that the trisaccharide chain was attached to C-3 of the aglycone. On the basis of these results, the structure of **1** was deduced to be

Table 1
^{1}H NMR spectroscopic data (δ in CD_3OD) for **1**–**7**[a,b]

^{1}H	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Aglycone moiety							
1	1.66 *m*, 1.05 *m*	1.62 *m*, 1.08 *m*	1.65 *m*, 1.07 *m*	1.69 *m*, 1.08 *m*	1.66 *m*, 1.03 *m*	1.62 *m*, 1.07 *m*	1.68 *m*, 1.05 *m*
2	1.74 *m*, 2.02 *m*	1.75 *m*, 2.03 *m*	1.75 *m*, 2.02 *m*	1.73 *m*, 2.02 *m*	1.79 *m*, 2.05 *m*	1.72 *m*, 2.02 *m*	1.75 *m*, 2.00 *m*
3	3.23 *m*	3.24 *m*	3.23 *m*	3.25 *m*	3.24 *m*	3.25 *m*	3.24 *m*
5	0.82 *d* (7.2)	0.84 *d* (7.2)	0.81 *d* (7.2)	0.79 *d* (7.2)	0.85 *d* (7.2)	0.87 *d* (7.2)	0.83 *d* (7.2)
6	1.59 *m*, 1.44 *m*	1.63 *m*, 1.46 *m*	1.67 *m*, 1.45 *m*	1.65 *m*, 1.45 *m*	1.62 *m*, 1.47 *m*	1.59 *m*, 1.46 *m*	1.58 *m*, 1.45 *m*
7	1.75 *m*, 1.43 *m*	1.71 *m*, 1.46 *m*	1.73 *m*, 1.44 *m*	1.71 *m*, 1.42 *m*	1.77 *m*, 1.45 *m*	1.76 *m*, 1.43 *m*	1.75 *m*, 1.42 *m*
9	1.72 *m*	1.72 *m*	1.65 *m*	1.65 *m*	1.68 *m*	1.76 *m*	1.75 *m*
11	1.95 *m*	1.94 *m*	1.91 *m*	1.91 *m*	1.95 *m*	1.96 *m*	1.98 *m*
12	5.35 *br s*	5.33 *br s*	5.33 *br s*	5.30 *br s*	5.34 *br s*	5.35 *br s*	5.40 *br s*
15	1.65 *m*, 1.37 *m*	1.66 *m*, 1.40 *m*	1.66 *m*, 1.39 *m*	1.66 *m*, 1.39 *m*	1.67 *m*, 1.36 *m*	1.63 *m*, 1.38 *m*	1.65 *m*, 1.38 *m*
16	4.00 *br s*	4.08 *br s*	4.08 *br s*	4.09 *br s*	4.09 *br s*	4.04 *br s*	4.03 *br s*
18	2.58 *dd* (14.0, 3.2)	2.59 *dd* (14.0, 3.2)	2.56 *dd* (14.0, 3.2)	2.56 *dd* (14.0, 3.6)	2.54 *dd* (14.0, 3.2)	2.63 *dd* (14.0, 3.2)	2.63 *m*
19	2.65 *dd* (14.0, 14.0)	2.66 *dd* (14.0, 14.0)	2.63 *dd* (14.0, 14.0)	2.64 *dd* (14.0, 14.0)	2.63 *dd* (14.0, 14.0)	2.67 *dd* (14.0, 14.0)	2.68 *m*
20	1.18 *m*	1.18 *dd* (14.0, 3.2)	1.19 *dd* (14.0, 3.2)	1.17 *dd* (14.0, 3.6)	1.19 *dd* (14.0, 3.2)	1.22 *dd* (14.0, 3.2)	1.20 *m*
21	5.80 *d* (10.0)	4.12 *d* (10.0)	5.51 *d* (10.0)	5.51 *d* (10.0)	5.50 *d* (10.0)	3.99 *d* (10.0)	5.79 *d* (10.0)
22	5.50 *d* (10.0)	5.20 *d* (10.0)	3.87 *d* (10.0)	3.87 *d* (10.0)	3.95 *d* (10.0)	3.92 *d* (10.0)	5.46 *d* (10.0)
23	1.12 *s*	1.12 *s*	1.08 *s*	1.09 *s*	1.10 *s*	1.12 *s*	1.13 *s*
24	0.89 *s*	0.88 *s*	0.85 *s*	0.84 *s*	0.87 *s*	0.88 *s*	0.89 *s*
25	1.03 *s*	1.03 *s*	0.96 *s*	0.96 *s*	0.99 *s*	1.03 *s*	1.02 *s*
26	0.99 *s*	0.95 *s*	0.93 *s*	0.92 *s*	0.95 *s*	0.95 *s*	0.97 *s*
27	1.51 *s*	1.49 *s*	1.45 *s*	1.44 *s*	1.48 *s*	1.51 *s*	1.50 *s*
28	2.95 *d* (11.2)	3.78 *d* (11.2)	3.75 *d* (11.2)	3.73 *d* (11.2)	2.85 *d* (11.2)	3.78 *d* (11.2)	3.02 *d* (11.2)
	3.23 *d* (11.2)	3.90 *d* (11.2)	3.89 *d* (11.2)	3.94 *d* (11.2)	3.20 *d* (11.2)	3.97 *d* (11.2)	3.27 *d* (11.2)
29	0.88 *s*	0.88 *s*	0.84 *s*	0.82 *s*	0.86 *s*	0.88 *s*	0.89 *s*
30	1.05 *s*	1.05 *s*	1.01 *s*	1.00 *s*	1.04 *s*	1.05 *s*	1.08 *s*
21-OAc	2.02 *s*		2.08 *s*	2.08 *s*	2.06 *s*		2.04 *s*
22-OAc	2.02 *s*	2.01 *s*					2.03 *s*
28-OAc		2.08 *s*	2.05 *s*	2.05 *s*		2.08 *s*	
Sugar moieties							
Glc-1′	4.45 *d* (7.2)	4.45 *d* (7.2)	4.45 *d* (7.2)	4.46 *d* (7.2)	4.49 *d* (7.2)	4.52 *d* (7.2)	4.50 *d* (7.2)
Glc-2′	3.72 *dd* (8.8, 7.2)	3.74 *dd* (8.8, 7.2)	3.72 *dd* (8.8, 7.2)	3.76 *m*	3.73 *m*	3.74 *m*	3.74 *m*
Glc-3′	3.44 *m*	3.44 *m*	3.45[c]	3.31[c]	3.31[c]	3.32[c]	3.30[c]
Glc-4′	3.37 *t* (9.0)	3.40 *t* (9.2)	3.38 *t* (9.6)	3.78[c]	3.74 *m*	3.75 *m*	3.75 *m*
Glc-5′	3.41 *m*	3.43 *m*	3.42 *m*	3.30[c]	3.30[c]	3.31 *m*	3.30[c]
Glc-6′	4.18 *br d* (11.2), 3.94 *m*	4.16 *br d* (11.2), 3.98 *m*	4.24 *br d* (11.2), 3.95 *m*	4.24 *br d* (11.2), 3.95 *m*	4.14 *br d* (11.2), 3.95 *m*	4.15 *br d* (11.2), 3.96 *m*	4.18 *br d* (11.2), 3.94 *m*
Glc-1″	4.68 *d* (7.6)	4.63 *d* (7.6)	4.66 *d* (7.2)	4.54 *d* (8.0)	4.59 *d* (8.0)	4.62 *d* (8.0)	4.58 *d* (8.0)
Glc-2″	3.74 *dd* (8.8, 7.6)	3.76 *dd* (8.8, 7.6)	3.76 *dd* (8.8, 7.2)	3.78[c]	3.75 *m*	3.80 *dd* (8.8, 8.0)	3.79 *dd* (8.8, 8.0)
Glc-3″	3.43 *m*	3.45 *m*	3.40 *t* (8.8)	3.44 *m*	3.42 *m*	3.42 *m*	3.42[c]
Glc-4″	3.33 *t* (8.8)	3.34 *t* (9.2)	3.32 *t* (9.2)	3.28 *t* (8.8)	3.26 *t* (8.8)	3.28 *t* (9.2)	3.27 *t* (9.6)
Glc-5″	3.45 *m*	3.46 *m*	3.45[c]	3.43 *m*	3.43 *m*	3.44 *m*	3.42[c]
Glc-6″	3.87 *m*, 3.68 *m*	3.86 *m*, 3.71 *m*	3.85 *m*, 3.69 *m*	3.84 *m*, 3.67 *m*	3.81 *m*, 3.68 *m*	3.87 *m*, 3.67 *m*	3.84 *m*, 3.68 *m*
Ara-1‴	4.38 *d* (6.4)	4.35 *d* (6.4)	4.33 *d* (6.4)	4.33 *d* (6.8)	4.37 *d* (6.8)	4.40 *d* (6.8)	4.38 *d* (6.8)
Ara-2‴	3.53 *dd* (8.8, 6.4)	3.52 *dd* (8.8, 6.4)	3.53 *dd* (8.8, 6.4)	3.49 *dd* (8.8, 6.8)	3.52 *dd* (8.8, 6.4)	3.51 *dd* (8.8, 6.4)	3.50 *dd* (8.8, 6.8)
Ara-3‴	3.68 *dd* (8.8, 3.0)	3.66 *dd* (8.8, 3.0)	3.64 *dd* (8.8, 2.4)	3.60 *dd* (8.8, 2.4)	3.57 *dd* (8.8, 3.0)	3.55 *dd* (8.8, 2.4)	3.57 *dd* (8.8, 3.0)
Ara-4‴	3.83 *m*	3.80 *m*	3.82 *m*	3.86 *m*	3.84 *m*	3.86 *m*	3.85 *m*
Ara-5‴	3.91 *m*, 3.50 *m*	3.91 *m*, 3.52 *m*	3.91 *m*, 3.52 *m*	3.90 *m*, 3.48 *m*	3.91 *m*, 3.50 *m*	3.92 *m*, 3.51 *m*	3.91 *m*, 3.50 *m*
Glc-1⁗				4.71 *d* (7.6)	4.77 *d* (7.6)	4.74 *d* (7.6)	4.75 *d* (7.6)
Glc-2⁗				3.30[c]	3.31[c]	3.32 *m*	3.30[c]
Glc-3⁗				3.32 *m*	3.30[c]	3.35 *m*	3.33 *m*
Glc-4⁗				3.34 *t* (9.2)	3.21 *t* (9.2)	3.25 *t* (8.8)	3.23 *t* (9.2)
Glc-5⁗				3.43 *m*	3.45 *m*	3.44 *m*	3.42[c]
Glc-6⁗				3.89 *dd* (11.6, 2.4), 3.60 *m*	3.93 *dd* (11.2, 3.0), 3.71 *m*	3.91 *dd* (11.6, 3.0), 3.71 *m*	3.90 *dd* (11.2, 2.4), 3.71 *m*

[a] Assignments confirmed by TOCSY, HMBC and HMQC.
[b] Compounds **1**–**7** were measured at 400 MHz.
[c] Overlapped with other signals.

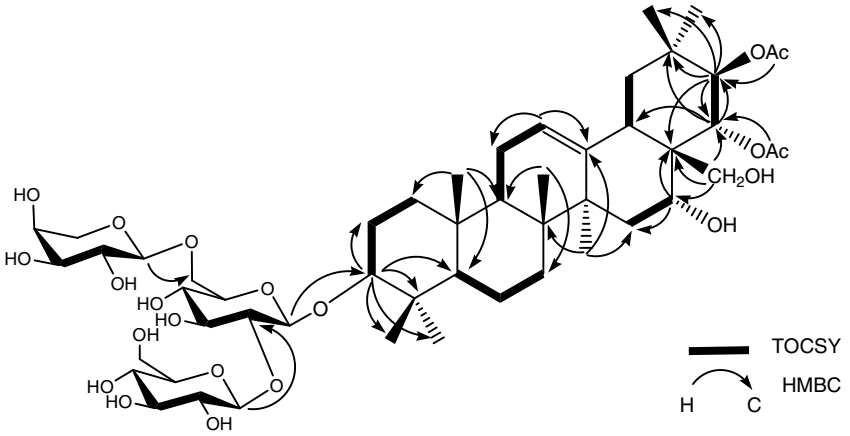


Fig. 1. Key TOCSY and HMBC correlations of **1**.

3-*O*-[α-L-araglucopyranosyl-21,22-*O*-diacetyl-3β,16α,21β,22α,28-pentahydroxy-olean-12-ene, and named as hydrocosisaponin A.

Both **2** (hydrocosisaponin B) and **3** (hydrocosisaponin C) were given a molecular formula of $C_{51}H_{82}O_{21}$, as determined from the HR-FAB-MS data which gave a quasi-molecular ion peak at *m/z* 1053.5256 $[M+Na]^+$ and 1053.5249 $[M+Na]^+$, respectively. The ^1H and ^{13}C NMR spectra of **2**and **3** suggested that each possessed an oleanane-type triterpene, two β-D-glucose [δ_H 4.45 ($J=7.2$ Hz), 4.63 ($J=7.6$ Hz)/δ_C 105.42, 104.49 in **2**; δ_H 4.45 ($J=7.2$ Hz), 4.66 ($J=7.6$ Hz)/δ_C 105.36, 104.47 in **3**] units and one α-L-arabinose [δ_H 4.35 ($J=6.4$ Hz)/δ_C 104.98 in **2**; δ_H 4.33 ($J=6.4$ Hz)/δ_C 105.6 in **3**] unit, as well as two acetate [δ_H 2.01 (s), 2.08 (s)/δ_C 21.05, 21.09, 173.41, 173.88 in **2**; δ_H 2.05 (s), 2.08 (s)/δ_C 20.78, 21.23, 172.61, 173.74 in **3**] units, like **1**. Cross comparison of the ^1H NMR spectra of **2** with that of **1** indicated the obvious changes in chemical shifts of H-21 (δ_H 5.80 in **1**; δ_H 4.12 in **2**) and H-28 (δ_H 2.95, 3.23 in **1**; δ_H 3.78, 3.90 in **2**). Additionally, after comparison with the ^1H NMR spectra of **1** and **3**, high field shifts for H-22 ($\Delta-\delta_H$ 1.63) and low field shifts for H-28 ($\Delta+\delta_H$ 0.80, 0.66) in **3** were apparently observed. This indicated that acetate groups were located at C-22 and C-28 in **2**, respectively, rather than at C-21 and C-28 in **3**. The HMBC spectra displayed cross-peaks for **2** [δ_C 173.88 (acetyl C=O)/δ_H 5.20 (H-22) and δ_C 173.41 (acetyl C=O)/δ_H 3.78, 3.90 (H-28)], and **3** [δ_C 173.74 (acetyl C=O)/δ_H 5.51 (H-21) and δ_C 172.61 (acetyl C=O)/δ_H 3.75, 3.89 (H-28)]. Moreover, locations of sugar moieties in **2** and **3** were also assigned by long-range correlations in their respective HMBC spectra. From the above discussion, structures of **2** and **3** were elucidated as 3-*O*-[α-L-arabinopyranosyl-(1 → 6)]-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-22,28-*O*-diacetyl-3β,16α,21β,22α,28-pentahydroxy-olean-12-ene (**2**) and 3-*O*-[α-L-arabinopyranosyl-(1 → 6)]-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-21,28-*O*-diacetyl-3β,16α,21β,22α,28-pentahydroxyolean-12-ene (**3**), respectively.

The HR-FAB-MS of **4** (hydrocosisaponin D) exhibited a quasi-molecular ion peak at *m/z* 1215.5781 $[M+Na]^+$, consistent with molecular formula, $C_{57}H_{92}O_{26}$. The NMR spectroscopic data of **4** were similar to those of **3**, except for appearance of signals for an additional β-D-glucose [anomeric signal at δ_H 4.71 ($J=7.6$ Hz)/δ_C 104.42]. After acid hydrolysis, the four sugars were identified as one L-arabinose and three D-glucose units by GC. Inspection of the HMBC spectrum of **4**, the correlations: δ_H 4.46 (H-1′ of Glc)/δ_C 91.46 (C-3 of the aglycone); δ_H 4.33 (H-1‴ of Ara)/δ_C 68.52 (C-6′ of Glc); δ_H 4.54 (H-1″ of Glc)/δ_C 80.40 (C-2′ of Glc); δ_H 4.71 (H-1⁗ of 4′-Glc)/δ_C 80.24 (C-4′ of Glc) were conspicuously observed; thus the interglycosidic linkages in **4** were completely established. On the basis of these results, **4** was assigned as 3-*O*-[α-L-arabinopyranosyl-(1 → 6)]-[β-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-21,28-*O*-diacetyl-3β,16α,21β,22α,28-pentahydroxyolean-12-ene.

By HR-FAB-MS {*m/z* 1173.5676 $[M+Na]^+$ for **5**; *m/z* 1173.5679 $[M+Na]^+$ for **6**}, **5** (hydrocosisaponin E) and **6** (hydrocosisaponin F) were assigned the same molecular formula, $C_{55}H_{90}O_{25}$. The IR and ^1H and ^{13}C NMR spectroscopic data indicated that both **5** and **6** possessed an oleanane-type triterpene, an acetate group, as well as sugar moieties comprising three D-glucose and one L-arabinose units. These findings established that **5** and **6** have almost similar structures as for **4**, except for the absence of an acetate moiety in both **5** and **6**. Comparing the ^1H NMR spectra of **5** and **6** with those of **4**, the chemical shifts of H-28 for **5** (δ_H 2.85, 3.20 in **5**; δ_H 3.73, 3.94 in **4**) and H-21 for **6** (δ_H 3.99 in **6**; δ_H 5.51 in **4**) were found shifted to higher field. These results suggested that **5** and **6** lacked an acetate group at C-28 and C-21 of the aglycone, respectively. Furthermore, correlations between δ_C 173.88 (acetyl C=O) and δ_H 5.50 (C-21) in **5** and between δ_C 172.81 (acetyl C=O) and δ_H 3.97, 3.78 (C-28) in **6** were observed in the HMBC spectra, which confirmed the locations of the acetate group at C-21 and C-28 for **5** and **6**, respectively. Accordingly, compounds **5** and **6** were established as

Table 2
^{13}C NMR spectroscopic data for **1**–**7** (CD_3OD)[a,b]

^{13}C	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Aglycone moiety							
1	39.42	39.33	39.95	39.96	39.83	39.80	39.40
2	26.90	26.78	27.20	27.20	27.15	27.16	26.97
3	91.15	91.20	91.36	91.46	91.36	91.35	91.21
4	40.21	40.22	40.43	40.43	40.37	40.31	40.20
5	56.65	56.75	56.93	56.99	56.82	56.80	56.75
6	19.17	19.27	19.26	19.27	19.24	19.20	19.07
7	33.72	33.75	34.00	34.00	34.01	34.04	33.69
8	40.66	40.65	40.89	40.97	40.87	40.89	40.65
9	47.65	47.62	48.00	48.00	47.89	47.83	47.66
10	37.55	37.65	37.74	37.76	37.66	37.63	37.52
11	24.46	24.43	24.65	24.65	24.60	24.59	24.43
12	124.95	124.92	125.28	125.26	125.21	125.20	124.94
13	142.80	142.84	142.97	143.01	142.96	142.98	142.78
14	42.10	42.15	42.39	42.41	42.39	42.45	42.10
15	34.52	34.52	34.88	34.82	34.80	34.72	34.50
16	68.96	68.96	68.74	68.75	68.70	68.81	68.97
17	47.71	47.72	47.43	47.45	47.46	47.56	47.74
18	40.75	40.71	41.18	41.19	40.98	41.02	40.73
19	47.18	47.23	47.95	47.91	47.90	47.89	47.21
20	36.50	36.31	36.48	36.48	36.45	35.56	36.46
21	80.19	71.82	82.45	82.48	82.33	77.60	80.14
22	74.82	79.89	72.14	72.13	72.05	77.01	74.81
23	29.45	29.65	28.40	28.43	28.41	28.48	29.38
24	16.79	16.86	16.91	16.90	16.87	16.87	16.71
25	16.08	16.11	16.17	16.16	16.14	16.22	15.99
26	17.07	17.15	17.40	17.42	17.39	17.19	17.08
27	27.54	27.44	27.54	27.55	27.51	27.42	27.50
28	64.55	66.94	66.56	66.90	64.78	66.84	64.53
29	30.51	30.31	29.76	29.76	29.74	29.96	30.52
30	19.80	19.85	19.90	19.90	19.88	19.97	19.79
21-OAc	173.97		173.74	173.71	173.88		174.02
	21.11		20.78	20.77	21.13		21.03
22-OAc	173.67	173.88					173.77
	21.15	21.09					21.03
28-OAc		173.41	172.61	172.57		172.81	
		21.15	21.23	21.22		21.09	
Sugar moieties							
Glc-1′	105.45	105.42	105.36	105.41	105.24	104.98	105.19
Glc-2′	80.73	80.79	80.87	80.40	80.31	80.28	80.24
Glc-3′	78.32	78.30	78.36	76.38	76.21	76.13	76.14
Glc-4′	71.76	71.86	71.90	80.24	80.16	80.23	80.13
Glc-5′	76.41	76.45	76.29	75.01	74.75	74.70	74.73
Glc-6′	69.60	69.44	69.54	68.52	68.40	68.39	68.28
Glc-1″	104.45	104.49	104.47	104.42	104.31	104.15	104.20
Glc-2″	76.72	76.78	76.70	76.89	76.66	76.71	76.60
Glc-3″	77.91	77.95	77.84	77.82	77.58	77.53	77.55
Glc-4″	71.57	71.71	71.51	71.49	71.70	71.65	71.67
Glc-5″	77.86	77.68	77.84	77.90	77.63	77.63	77.55
Glc-6″	63.21	63.23	63.09	63.12	62.95	62.89	62.88
Ara-1‴	105.16	104.98	105.06	105.26	105.09	104.86	105.01
Ara-2‴	74.82	74.89	74.97	74.86	74.85	74.86	74.81
Ara-3‴	77.98	77.93	77.96	77.93	77.92	77.82	77.88
Ara-4‴	70.78	70.88	70.86	70.74	70.58	70.56	70.51
Ara-5‴	66.79	66.66	66.89	66.99	66.85	66.89	66.81
Glc-1⁗				104.42	104.29	104.13	104.20
Glc-2⁗				75.08	74.99	74.89	74.94
Glc-3⁗				77.82	77.65	77.68	77.63
Glc-4⁗				71.94	71.27	71.35	71.24
Glc-5⁗				78.32	78.11	77.89	78.05
Glc-6⁗				62.41	62.25	62.08	62.19

[a] Assignments confirmed by ^1H–^1H TOCSY, HMBC and HMQC.
[b] Compounds **1**–**7** were measured at 100 MHz.

Table 3
Cytotoxic activity of compounds **1**–**7**

Compound	KB	ED_{50} (μM)	
		Daoy	WiDr
1	–[a]	–[a]	–[a]
2	–[a]	–[a]	–[a]
3	–[a]	–[a]	–[a]
4	–[a]	7.86	7.55
5	8.54	5.21	5.06
6	–[a]	9.41	5.38
7	14.03	4.89	4.24
Mitomycin-C	0.04	0.07	0.05

[a] (–): Inactive, ED_{50} 20 μM.

3-*O*-[α-L-arabinopyranosyl-(1 → 6)]-[β-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-21-*O*-acetyl-3β,16α,21β,22α,28-pentahydroxyolean-12-ene (**5**) and 3-*O*-[α-L-arabinopyranosyl-(1 → 6)]-[β-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-28-*O*-acetyl-3β,16α,21β,22α,28-pentahydroxyolean-12-ene (**6**), respectively.

Compound **7** was isolated and identified as hydrocotyloside VII, 3-*O*-[α-L-arabinopyranosyl-(1 → 6)]-[β-D-glucopyranosyl-(1 → 4)]-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-21,22-*O*-diacetyl-3β,16α,21β,22α,28-pentahydroxyolean-12-ene, by direct comparison with the authentic sample and reported data (Matsushita et al., 2004).

Compounds **1**–**7** were tested against human oral epithelium carcinoma (KB), human medullocarcinoma (Daoy), and human colon adenocarcinoma (WiDr) cells. Although activities were moderate, the bioassay data obtained (Table 3) showed that the aglycone with four sugar units (three D-glucoses, and one L-arabinose) as **4**–**6** exhibited greater available cytotoxicity [Daoy and WiD (ED_{50}) *ca* 4–10 μM], while that of **1**–**3**, which possessed three sugars (2 Glc, 1 Ara) were much less active [Daoy and WiDr (ED_{50})> 20 μM]. The results suggest that the presence of an additional glucose in the sugar moiety of these oleanane-type saponins plays a role in mediating cytotoxicity; this topic is under investigation.

3. Concluding remarks

Saponins **1**–**7** isolated from title plant was consistent with other reports that the aglycone of saponins in *Hydrocotyle* species were comprised of oleanane-type triterpene (Bontems, 1941; Greca et al., 1994a,b; Hiller et al., 1971; Matsushita et al., 2004). Thus, the finding of oleanane-type triterpene saponin may provide a significant chemotaxanomic proof for the genus *Hydrocotyle*.

4. Experimental

4.1. General

Infrared (IR) spectra were measured on a Mattson Genesis II spectrophotometer using a KBr matrix, whereas

optical rotations were acquired on a JASCO P-1020 polarimeter equipped with a sodium lamp (589 nm). Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. NMR spectra were performed on a Bruker NMR spectrometer (Avance 400 MHz) using CD_3OD as solvent for measurement, whereas JEOL FAB-MS data were obtained on a SX-102A instrument. High-resolution FABMS were measured on a Finnigan/Thermo Quest MAT mass spectrometer. GC was carried out using a Agilent Technologies 6890N Network GC System using a Chirasil-L-Val column (25 m × 0.22 mm i.d.) with He as carrier gas. HPLC separations were carried out using a Shimadzu LC-6AD series apparatus with a RID-10 A Refractive Index, equipped with a preparative Cosmosil 5C18-AR II column (25 cm × 20 mm i.d.). Diaion HP-20, Sephadex LH-20, and silica gel (Merck 70–230 mesh and 230–400 mesh) were used for CC, and pre-coated silica gel (Merck 60 F-254) plates were used for TLC. TLC detection was carried out by spraying with 10 % H_2SO_4 and then heating on a hot plate.

4.2. Plant material

Whole *Hydrocotyle sibthorpioides* Lam. plants were collected in August 2003 in the northern mountains of Taiwan, Taipei County, Taiwan, and identified by Professor Muh-Tsuen Kao, National Institute of Chinese Medicine. A voucher specimen (No. NRICM200308B5) has been deposited in the National Research Institute of Chinese Medicine, Taipei, Taiwan.

4.3. Extraction and isolation

Dried *H. sibthorpioides* Lam. (5.4 kg) whole plant tissues were extracted with EtOH (10 L) for 12 h three times at room temperature. The combined EtOH solubles were concentrated in vacuo to give a residue (980 g), which was suspended in H_2O (1 L), and then partitioned successively with *n*-hexane, EtOAc and *n*-BuOH (each 1 L). The condensed *n*-BuOH layer (175 g, dry weight) was applied to a column of Diaion HP-20 (6.0 × 75 cm) and eluted with MeOH–H_2O (1:4, v/v, 2 L); (2:3, v/v, 2 L); (1:1, v/v, 2 L); (3:2, v/v, 2 L); (4:1, v/v, 2 L); (9:1, v/v, 2 L) and MeOH (1.5 L), and finally acetone (1.5 L). Fraction 5 (24 g, dry weight) which was eluted with MeOH–H_2O (4:1), showed cytotoxicity against the WiDr cancer cell line and was repeatedly subjected to Sephadex LH-20 (3.5 × 95 cm; CH_2Cl_2/MeOH, 1:1) CC to afford six subfractions (fraction 5.1–5.6). Fraction 5.3 (4.2 g) was separated by reversed-phase HPLC on a Cosmosil 5C18-AR II column (25 cm × 20 mm, flow rate 5 ml min^{-1}) with CH_3CN/H_2O (35/65) to give **3** (17.5 mg, t_R 72.0 min), **4** (37.4 mg, t_R 24.0 min), **5** (5.6 mg, t_R 67.5 min), **6** (6.2 mg, t_R 82.0 min), **7** (76.3 mg, t_R 62.0 min), respectively; fraction 5.3.5. (82.3 mg) was repeatedly purified as fraction 5.3 with CH_3CN/H_2O (30/70) to yield **1** (7.1 mg, t_R 73.0 min) and **2** (6.1 mg, t_R 76.0 min).

4.4. Acid hydrolysis of saponins

Compounds **1**–**6** (4.0 mg) were each treated with 2 N methanolic HCl (2 mL) under conditions of reflux at 90 °C for 1 h, respectively. The mixture obtained was extracted with CH_2Cl_2two times. The aqueous hydrolysate was neutralized with Na_2CO_3 and filtered. After cooling, the solution was evaporated with a stream of N_2. Then, 1-(trimethylsilyl)imidazole and pyridine (0.2 ml) were added, and the mixture was stirred at 60 °C for 5 min. The reaction mixture was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was analyzed by GC using a Chirasil-L-Val column, with an initial temperature of 100 °C for 1 min, followed by temperature programming to 180 °C at a rate of 4 °C/min. Peaks of hydrolysates derived from **1**–**6** were detected by comparison with retention times of authentic samples (L-arabinose and D-glucose) treated with 1-(trimethylsilyl)imidazole.

4.5. Hydrocosisaponin A (*1*)

Light yellowish glass; $[\alpha]_D^{29}$ −1.6 (MeOH; *c* 0.01); IR: ν_{max} cm^{-1} (KBr) 3380, 2928, 1725, 1630, 1370, 1260, 1072, 1036; for ^{13}C and ^{1}H NMR spectra, see Tables 1 and 2; FAB-MS: *m/z* 1053 $[M+Na]^+$; HR-FAB-MS: *m/z* 1053.5240 $[M+Na]^+$ (calcd. 1053.5246 for $C_{51}H_{82}O_{21}Na$ $[M+Na]^+$).

4.6. Hydrocosisaponin B (*2*)

Light yellowish glass; $[\alpha]_D^{24}$ −2.7 (MeOH; *c* 0.05); IR ν_{max} cm^{-1} (KBr) 3378, 2928, 1726, 1628, 1373, 1259, 1072, 1036; for ^{13}C and ^{1}H NMR spectra, see Tables 1 and 2; FAB-MS *m/z* 1053 $[M+Na]^+$; HR-FAB-MS: *m/z* 1053.5256 $[M+Na]^+$ (calcd. 1053.5246 for $C_{51}H_{82}O_{21}Na$ $[M+Na]^+$).

4.7. Hydrocosisaponin C (*3*)

Light yellowish glass; $[\alpha]_D^{29}$ −6.8 (MeOH; *c* 0.5); IR ν_{max} cm^{-1} (KBr) 3380, 2928, 1722, 1638, 1370, 1260, 1072, 1036; for ^{13}C and ^{1}H NMR spectra, see Tables 1 and 2; FABMS *m/z* 1053 $[M+Na]^+$; HR-FAB-MS *m/z* 1053.5249 $[M+Na]^+$ (calcd. 1053.5246 for $C_{51}H_{82}O_{21}Na$ $[M+Na]^+$).

4.8. Hydrocosisaponin D (*4*)

Light yellowish glass; $[\alpha]_D^{29}$ −2.8 (MeOH; *c* 0.1); IR ν_{max} cm^{-1} (KBr) 3374, 2929, 1720, 1630, 1368, 1259, 1072, 1037; for ^{13}C and ^{1}H NMR spectra, see Tables 1 and 2; FABMS *m/z* 1215 $[M+Na]^+$; HR-FAB-MS *m/z* 1215.5781 $[M+Na]^+$ (calcd. 1215.5775 for $C_{57}H_{92}O_{26}Na$ $[M+Na]^+$).

4.9. Hydrocosisaponin E (*5*)

Light yellowish glass; $[\alpha]_D^{29}$ −1.9 (MeOH; *c* 0.05); IR ν_{max} cm^{-1} (KBr) 3385, 2928, 1726, 1631, 1371, 1262, 1072, 1037; for ^{13}C and ^{1}H NMR spectra, see Tables 1 and 2; FABMS

m/z 1173 $[M+Na]^+$; HR-FAB-MS *m/z* 1173.5676 $[M+Na]^+$ (calcd. 1173.5669 for $C_{55}H_{90}O_{25}Na$ $[M+Na]^+$).

4.10. Hydrocosisaponin F (6)

Light yellowish glass; $[\alpha]_D^{29}$ −4.1° (MeOH; *c* 0.05); IR ν_{max} cm^{-1} (KBr) 3380, 2928, 1725, 1630, 1372, 1260, 1072, 1037; for ^{13}C and ^1H NMR spectra, see Tables 1 and 2; FAB-MS *m/z* 1173 $[M+Na]^+$; HR-FAB-MS *m/z* 1173.5679 $[M+Na]^+$ (calcd. 1173.5669 for $C_{55}H_{90}O_{25}Na$ $[M+Na]^+$).

4.11. Hydrocotyloside VII (7)

Light yellowish glass; $[\alpha]_D^{29}$ −3.3 (MeOH; *c* 0.1); IR ν_{max} cm^{-1} (KBr) 3386, 2929, 1721, 1640, 1371, 1261, 1071, 1036; for ^{13}C and ^1H NMR spectra, see Tables 1 and 2; FAB-MS *m/z* 1215 $[M+Na]^+$; HR-FAB-MS *m/z* 1215.5783 $[M+Na]^+$ (calcd. 1215.5775 for $C_{57}H_{92}O_{26}Na$ $[M + Na]^+$).

4.12. Cytotoxicity assays

The cytocoxicity assay was carried out according to the MTT assay described previously (Kuo et al., 2001). Three human cancer cell lines, human oral epithelium carcinoma (KB), human medullocarcinoma (Daoy), and human colon adenocarcinoma (WiDr) were examined. Mitomycin-C was used as the positive control. The $ED_{50}$was defined by comparison with the untreated cells as the concentration of test sample resulting in 50% reduction of absorbance.

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