Oleanane-type Triterpene Glucuronides from the Roots of *Glycyrrhiza uralensis* Fischer

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Key words
- *Glycyrrhiza uralensis* Fischer
- *Glycyrrhiza*
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- cytotoxic activities

Abstract

Investigation of characteristic constituents of the roots of *Glycyrrhiza uralensis* Fischer led to isolation of four new triterpene glucuronides, namely uralsaponins C–F (**1**–**4**), an artificial product, namely the methyl ester of glycyrrhizin (**5**), as well as six known triterpene glucuronides (**6**–**11**). These new compounds were identified by 1D and 2D NMR spectroscopic analysis. The cytotoxicity of the selected compounds and their aglycones were evaluated against HeLa and MCF-7 cancer cell lines, and the preliminary structure-activity relationship was also elucidated.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Introduction

Glycyrrhiza uralensis Fischer, named "licorice", is one of the largest and most widely distributed plants belonging to the genus *Glycyrrhiza* (Leguminosae). Its dried roots have been extensively used in Oriental countries as a medicine and sweetening agent. Chemical investigations showed that saponins and flavonoids were two main types of ingredients in licorice roots [1,2]. Glycyrrhizin is a characteristic saponin constituent in licorice that has been shown to possess diverse biological activities including cytotoxic [3, 4], antiallergic [5], and antiviral [6] ones. However, there are few reports regarding biological activities of other saponins with similar structures. In this work, we have isolated and purified eleven saponins from the roots of *G. uralensis* including four new oleanane-type triterpene glucuronides named uralsaponins C–F (**1**–**4**), an artificial product named methyl ester of glycyrrhizin (**5**), as well as six known triterpene glucuronides (**6**–**11**), using an ingenious pre-processing method for co-application of polyamide and macroporous resin column chromatography. The cytotoxicity against HeLa and MCF-7 cancer cell lines was evaluated for selected compounds and their aglycones, and preliminary structure-activity relationship was also elucidated.

Materials and Methods

General experimental procedures

IR spectra were recorded in KBr disks on a Thermo Nicolet IR100 spectrophotometer. UV spectra were performed on a Shimadzu UV-2401 spectrophotometer. ^{1}H and ^{13}C NMR spectra were determined on a Bruker ASR-500 NMR spectrometer. Spectra were generally recorded in pyridine-d_5 solutions and the solvent signals used as the internal standard for chemical shifts. HRESIMS and ESI-MS/MS spectra were recorded on an Agilent 1200 HPLC/Q-TOF mass spectrometer instrument (Agilent Corp.) in positive ion mode. Column chromatography was performed on polyamide (30–60 mesh; Sinopharm Chemical Reagent Co., Ltd.) and macroporous (20–40 mesh, D101; Haiguang Chemical Reagent Co., Ltd.) resins. Medium pressure liquid chromatography (MPLC) was carried out on a BUCHI apparatus (BUCHI Chromatography Pump B-688, 6.5 cm × 50 cm, column) with RP-18 silica gel (25–50 µm; Merck). Semi-preparative HPLC was performed on Waters 600 liquid chromatography with an Econosil C_{18}, 2.2 × 25 cm, column. HPLC was performed on an Agilent 1100 HPLC instrument with UV detector.

Plant materials

The aerial parts of *G. uralensis* were collected in September 2008 in Hangjinqi County, Nei Menggu Province, China. Voucher specimens (GCN

20080917) were stored in a dry and dark room, deposited at the Key Laboratory of Modern Chinese Medicines (China Pharmaceutical University) and identified by Prof. Ping Li, a professor in the field of pharmacognosy.

Extraction and isolation

The air dried material of *G. uralensis* (20 kg) was extracted with 50% aqueous ethanol (100 L × 2, each extraction lasted 2 hours). After solvent removal, the combined residues were passed over a polyamide resin column (10 kg, 30–60 mesh, 20 × 200 cm) with flow velocity 150 mL/min, and the effluent was chromatographed on a macroporous resin column (12 kg, 20–40 mesh, 20 × 200 cm) to afford crude saponin fractions using EtOH-H_2O (60 : 40, 40 L, flow rate 120 mL/min) as an eluent. After concentration under vacuum, the residue was preliminarily analyzed by HPLC on a ZORBAX C_{18} column (250 × 4.6 mm × 5.0 µm). The mobile phases consisted of 0.1% trifluoracetic acid water (A) and methanol (B) using a gradient program of 60–65% B at 0–15 min and 65–80% B at 15–30 min at a flow rate of 1.0 mL/min. The detection wavelength was set at 254 nm. The retention times of the target compounds in HPLC were as follows: compound **1** at 9.0 min, **2** at 8.7 min, **3** at 7.8 min, **4** at 9.8 min, **6** at 8.3 min, **7** at 10.9 min, **8** at 12.5, **9** at 18.5 min, **10** at 12.9 min, and **11** at 21.1 min. Based on the preliminary data from HPLC, the residue (285 g) was then separated by MPLC on a RP-18 silica gel column (800 g, 25–50 µm, 6.5 × 50 cm) with MeOH-H_2O-AcOH (40 : 60 : 1; 50 : 50 : 1, 60 : 40 : 1, each 4 L) to yield five fractions: fraction A was eluted with a ratio of 40 : 60 : 1 (2 L), fraction B was eluted with a ratio from 40 : 60 : 1 to 50 : 50 : 1 (3 L), fraction C was eluted with a ratio of 50 : 50 : 1 (2 L), fraction D was eluted with a ratio from 50 : 50 : 1 to 60 : 40 : 1 (2 L), and fraction E was eluted with a ratio of 50 : 50 : 1 (3L). Fraction B (about 65 g) was submitted to repeated chromatography over RP-18 silica gel column (400 g, 25–50 µm, 4.5 × 50 cm) eluted with a CH_3CN-H_2O-AcOH (from 20 : 80 : 1 to 35 : 65 : 1) gradient system to obtain four subfractions: fraction B1 was obtained using 1 L CH_3CN-H_2O-AcOH from 20 : 80 : 1 to 25 : 75 : 1, fraction B2 was obtained using 2 L CH_3CN-H_2O-AcOH from 25 : 75 : 1 to 30 : 70 : 1, fraction B3 was obtained using 2 L CH_3CN-H_2O-AcOH with a ratio of 30 : 70 : 1, and fraction B4 was obtained using 2 L CH_3CN-H_2O-AcOH from 30 : 70 : 1 to 35 : 65 : 1. Compounds **2** (80 mg) and **7** (250 mg) were crystallized from fractions B1 and B4, respectively. Fraction B3 was further fractionated by RP-18 silica gel column chromatography (CH_3CN-H_2O-AcOH, 30 : 70 : 1) to afford **1** (10 mg) and **4** (14 mg). Compounds **3** (15 mg) and **6** (20 mg) were obtained by semipreparative HPLC (CH_3CN-H_2O-AcOH, 35 : 65 : 1) from B2. Separation of fraction C by RP-18 silica gel column chromatography yielded **8** (18 mg) and **10** (15 mg) using CH_3CN-H_2O-AcOH (30 : 70 : 1). Compound **11** (900 mg) was crystallized from fraction E. Compound **5** (15 mg) was obtained by RP-18 silica gel column chromatography using CH_3OH-H_2O-AcOH with a ratio of 65 : 35 : 1 and then by semipreparative HPLC using CH_3OH-H_2O-AcOH with a ratio of 65 : 35 : 1 from fraction E. Isolate **9** (200 mg) was obtained from fraction D by repeated RP-18 silica gel column chromatography (CH_3OH-H_2O-AcOH, 60 : 30 : 1).

Chemical conversion

A 5 mL MeOH solution containing 2 mg of compound **4** was treated with 5 mL 2% NaOH, and then heated at 60 °C for 1 h. After cooling, the reaction mixture was neutralized with 10% hydrochloric acid and then extracted with 5 mL *n*-butanol. Approximately 1 mg of the compound **6** was obtained after the *n*-butanol solvent was removed. This compound was then dissolved in MeOH and injected into HPLC-ESI-MS for analysis. The product in the reaction mixture was unambiguously identified as 3-*O*-[*β*-D-glucuronopyranosyl-(1 → 2)-*β*-D-glucuronopyranosyl]-24-hydroxy-glabrolide by comparing the retention time (t_R) and ESI fragmentation behaviors with those of compound **6**.

Two-phase acid hydrolysis and determination of aglycones of 2, 7, 9, and 11

Two-phase acid hydrolysis of glycyrrhizin (**11**) (30 mg) was carried out in a solution of 10% hydrochloric acid (50 mL) and $CHCl_3$ (25 mL) under reflux for 6 h [7]. After cooling, the $CHCl_3$ layer was washed with H_2O (3 × 2 mL) and concentrated *in vacuo* to afford the aglycone (glycyrrhetic acid, 13 mg). This compound was identified by HRESIMS experiment (pseudomolecular ion $[M + H]^+$ at *m/z* 471.3468, $C_{30}H_{46}O_4$), and its purity was tested to be higher than 95% by HPLC-UV area normalization method (mobile phase: CH_3OH-H_2O-TFA with a ratio of 80 : 20 : 0.05 and detection wavelength 254 nm). The aglycones of **2, 7,** and **9** (12 mg, 13 mg, and 13 mg, respectively) were obtained using the same method (the aglycone of **2**, *m/z* 499.3051 $[M + H]^+$, $C_{30}H_{42}O_6$, purity > 95%; the aglycone of **7**, *m/z* 469.3322 $[M + H]^+$, $C_{30}H_{44}O_4$, purity > 95%; the aglycone of **9**, *m/z* 486.3345 $[M + H]^+$, $C_{30}H_{46}O_5$, purity > 95%).

Uralsaponin C (**1**): white amorphous powder; UV (MeOH) λ_{max} (log ε) 250.2 (4.14) nm; IR (KBr) ν_{max} 3461, 2962, 1724, 1652, 1045 cm^{-1}; ^1H NMR and ^{13}C NMR (C_5D_5N, 500 MHz), see ◉ **Table 1**; HRESIMS (+), *m/z* 825.4261 $[M + H]^+$, (calcd. for $C_{42}H_{65}O_{16}$, 825.4267).

Uralsaponin D (**2**): white amorphous powder; UV (MeOH) λ_{max} (log ε) 249.2 (4.14) nm; IR (KBr) ν_{max} 3462, 2976, 1745, 1655, 1055 cm^{-1}; ^1H NMR and ^{13}C NMR (C_5D_5N, 500 MHz), see ◉ **Table 1**; HRESIMS (+), *m/z* 873.3499 $[M + Na]^+$, (calcd. for $C_{42}H_{58}O_{18}Na$, 873.3515).

Uralsaponin E (**3**): white amorphous powder; UV (MeOH) λ_{max} (log ε) 249.6 (4.21) nm; IR (KBr) ν_{max} 3425, 2946, 1763, 1654, 1053 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) δ5.68 (1H, s, H-12), 5.40 (1H, overlapped, H-1″), 4.99 (1H, d, *J* = 7.0 Hz, H-1′), 4.57 (1H, overlapped, H-4″), 4.55 (1H, overlapped, H-5″), 4.50 (1H, overlapped, H-5′), 4.48 (1H, overlapped, H-4′), 4.36 (1H, m, H-3″), 4.27 (1H, overlapped, H-3′), 4.25 (1H, overlapped, H-2′), 4.20 (1H, overlapped, H-2″), 4.23 (1H, overlapped, H-22), 4.16, 3.84 (1H each, d, *J* = 11.0 Hz, H-29), 3.32 (1H, m, H-3), 2.94, 0.98 (1H each, m, H-1), 2.52, 2.37 (1H each, m, H-19), 2.36 (1H, overlapped, H-9), 2.32 (1H, overlapped, H-18), 2.29, 2.02 (1H each, overlapped, H-2), 1.95, 1.54 (1H each, m, H-21), 1.89, 1.10 (1H each, overlapped, H-16), 1.63, 1.06 (1H each, overlapped, H-15), 1.47, 1.19 (1H each, overlapped, H-7), 1.45, 1.22 (1H each, overlapped, H-6), 1.35 (3H, s, H-23), 1.31 (3H, s, H-27), 1.19 (3H, s, H-24), 1.14 (3H, s, H-25), 0.95 (3H, s, H-26), 0.92 (3H, s, H-24), 0.69 (1H, br d, *J* = 11.5, H-5); ^{13}C NMR (C_5D_5N, 500 MHz), see ◉ **Table 2**; HRESIMS (+), *m/z* 859.3719 $[M + Na]^+$, (calcd. for $C_{42}H_{60}O_{17}Na$, 859.3723).

Uralsaponin F (**4**): white amorphous powder; UV (MeOH) λ_{max} (log ε) 250.2 (4.12) nm; IR (KBr) ν_{max} 3437, 2975, 1724, 1656, 1049 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) δ5.89 (1H, s, H-12), 5.60 (1H, d, *J* = 7.5 Hz, H-1″), 4.94 (1H, d, *J* = 7.5 Hz, H-1′), 3.39 (1H, dd, *J* = 4.5, 12.0 Hz, H-3), 2.34 (1H, s, H-9), 1.94 (3H, s, $OOCCH_3$), 0.79, 0.94, 1.07, 1.21, 1.36, 1.37 (3H each, s, 6 × CH_3); ^{13}C NMR (C_5D_5N, 500 MHz), see ◉ **Table 2**; HRESIMS (+), *m/z* 897.4113 $[M + H]^+$, (calcd. for $C_{44}H_{65}O_{19}$, 897.4115).

Table 1 ^{13}C NMR and ^{1}H NMR chemical shifts of compounds **1–2** (C_5D_5N, 500 MHz).

Position	1		2		Position	1		2	
	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)		δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)
1	39.5 CH_2	1.02*, 3.01 (m)	39.4 CH_2	1.06*, 2.98 (m)	23	28.1 CH_3	1.39 (s)	28.1 CH_3	1.37 (s)
2	26.7 CH_2	2.05 (m), 2.29 (m)	26.7 CH_2	2.06 (m), 2.31 (m)	24	16.9 CH_3	1.22 (s)	16.9 CH_3	1.21 (s)
3	89.2 CH	3.33 (dd, 4.0, 11.5)	89.1 CH	3.34 (dd, 4.0, 11.5)	25	16.8 CH_3	1.19 (s)	17.6 CH_3	1.17 (s)
4	39.9 qC		39.9 qC		26	18.9 CH_3	1.06 (s)	18.8 CH_3	0.96 (s)
5	55.5 CH	0.72 (br d, *J* = 11.5)	55.4 CH	0.70 (br d, *J* = 12)	27	23.1 CH_3	1.40 (s)	22.3 CH_3	1.30 (s)
6	17.6 CH_2	1.29*, 1.46 (m)	17.6 CH_2	1.24*, 1.44 (m)	28	21.7 CH_3	1.12 (s)	23.7 CH_3	0.94 (s)
7	33.1 CH_2	1.24*, 1.52 (m)	33.1 CH_2	1.19*, 1.47 (m)	29	28.2 CH_3	1.11 (s)	172.1 qC	
8	45.4 qC		45.1 qC		30	69.9 CH_2	3.83 (m)	175.1 qC	
9	62.0 CH	2.42 (s)	62.0 CH	2.36 (s)					
10	37.2 qC		37.3 qC		1′	105.1 CH	5.03 (d, *J* = 7.5)	105.0 CH	5.01(d, *J* = 7.5)
11	199.4 qC		198.9 qC		2′	84.5 CH	4.26*	84.5 CH	4.27*
12	128.7 CH	5.79 (s)	130.3 CH	5.75 (s)	3′	77.7 CH	4.30*	77.6 CH	4.30*
13	169.4 qC		163.9 qC		4′	73.0 CH	4.53*	73.0 CH	4.52*
14	44.0 qC		44.4 qC		5′	77.4 CH	4.56*	77.7 CH	4.56*
15	26.6 CH_2	1.08*, 1.73*	25.3 CH_2	1.08*, 1.64 (m)	6′	172.3 qC		172.3 qC	
16	28.0 CH_2	1.35*, 1.91 (m)	25.9 CH_2	1.14*, 1.89 (m)					
17	37.8 qC		53.1 qC		1″	106.9 CH	5.41(d, *J* = 7.5)	106.9 CH	5.41(d, *J* = 7.5)
18	45.4 CH	2.64 (br d, 12.0)	44.6 CH	2.43 (m)	2″	76.8 CH	4.23*	76.8 CH	4.24*
19	40.6 CH_2	1.49*, 1.76 *	35.9 CH_2	2.28*, 2.56 (m)	3″	77.6 CH	4.38 (t, 7.0)	77.4 CH	4.38 (t, 7.5)
20	35.8 qC		36.2 qC		4″	73.3 CH	4.60*	73.3 CH	4.60*
21	38.6 CH_2	1.70*, 1.98 (dd, 6.5, 11.5)	36.1 CH_2	2.66 (d, *J* = 12), 3.02(m)	5″	78.4 CH	4.62*	78.4 CH	4.62*
22	74.6 CH	3.73 (m)	85.4 CH	4.42 (d, *J* = 5.5)	6″	172.0 qC		172.0 qC	

* Where ^{1}H NMR signals were overlapped, chemical shift data were obtained from 2D correlations

Table 2 ^{13}C NMR chemical shifts of compounds **3–6** (C_5D_5N, 500 MHz).

Pos.	3	4	5	6	Pos.	3	4	5	6
	δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C, mult.		δ_C, mult.	δ_C, mult.	δ_C, mult.	δ_C, mult.
1	39.5 CH_2	39.7 CH_2	39.5 CH_2	39.5 CH_2	24	16.9 CH_3	63.0 CH_2	16.5 CH_2	63.4 CH_2
2	26.4 CH_2	26.3 CH_2	26.7 CH_2	26.0 CH_2	25	16.8 CH_3	16.3 CH_3	16.8 CH_3	16.5 CH_3
3	89.2 CH	89.3 CH	89.2 CH	89.6 CH	26	18.7 CH_3	18.2 CH_3	18.8 CH_3	20.3 CH_3
4	39.9 qC	44.3 qC	38.4 qC	44.4 qC	27	22.3 CH_3	23.8 CH_3	23.6 CH_3	22.3 CH_3
5	55.4 CH	55.6 CH	55.5 CH	56.0 CH	28	24.0 CH_3	21.5 CH_3	27.9 CH_3	23.9 CH_3
6	17.6 CH_2	18.0 CH_2	17.6 CH_2	18.5 CH_2	29	63.2 CH_2	29.1 CH_3	28.7 CH_3	21.3 CH_3
7	33.1 CH_2	32.7 CH_2	32.1 CH_2	33.5 CH_2	30	178.5 qC	178.9 qC	179.2 qC	179.7 qC
8	45.1 qC	43.3 qC	45.6 qC	44.9 qC					
9	62.0 CH	61.7 CH	62.1 CH	61.9 CH	1′	105.1 CH	104.2 CH	105.0 CH	104.7 CH
10	37.3 qC	36.6 qC	37.3 qC	37.1 qC	2′	84.3 CH	80.6 CH	84.7 CH	81.7 CH
11	199.1 qC	199.3 qC	199.5 qC	198.9 qC	3′	77.6 CH	77.4 CH	76.6 CH	77.7 CH
12	130.0 CH	128.5 CH	128.7 CH	130.0 CH	4′	73.1 CH	72.8 CH	73.0 CH	73.0 CH
13	164.6 qC	168.5 qC	169.6 qC	164.4 qC	5′	77.8 CH	77.2 CH	77.4 CH	77.9 CH
14	44.4 qC	44.1 qC	43.5 qC	45.0 qC	6′	172.5 qC	172.2 qC	172.6 qC	172.2 qC
15	25.3 CH_2	25.3 CH_2	26.6 CH_2	25.2 CH_2					
16	26.0 CH_2	26.2 CH_2	26.8 CH_2	25.9 CH_2	1′′	106.6 CH	104.5 CH	106.9 CH	105.4 CH
17	48.9 qC	34.8 qC	48.7 qC	35.0 qC	2′′	76.7 CH	75.4 CH	77.3 CH	75.8 CH
18	44.6 CH	45.4 CH	44.1 CH	44.4 CH	3′′	77.2 CH	77.3 CH	77.7 CH	77.7 CH
19	33.6 CH_2	40.3 CH_2	41.7 CH_2	40.8 CH_2	4′′	73.4 CH	72.9 CH	73.0 CH	73.1 CH
20	36.4 qC	39.1 qC	39.9 qC	42.1 qC	5′′	79.1 CH	77.5 CH	77.8 CH	77.8 CH
21	36.6 CH_2	35.7 CH_2	31.6 CH_2	38.1 CH_2	6′′	172.8 qC	172.1 qC	170.2 qC	172.2 qC
22	84.1 CH	77.1 CH	33.0 CH	84.0 CH	22-Acetyls		170.2 qC, 20.7 CH_3		
23	28.1 CH_3	22.7 CH_3	28.7 CH_3	23.0 CH_3	6′′-OCH_3			51.9 CH_3	

Methyl ester of glycyrrhizin (**5**): white amorphous powder; UV (MeOH) λ_{max} (log ε) 249.2 (4.14) nm; IR (KBr) ν_{max} 3401, 2945, 1732, 1651, 1055 cm^{-1}; ^1H NMR(C_5D_5N, 500 MHz) δ5.94 (1H, s, H-12), 5.35 (1H, d, J = 8.0 Hz, H-1′′), 4.98 (1H, d, J = 7.5 Hz, H-1′), 3.31 (1H, dd, J = 4.0, 11.5 Hz, H-3), 3.81 (3H, s, $COOCH_3$), 2.45 (1H, s, H-9), 0.77, 1.08, 1.15, 1.25, 1.32, 1.33, 1.43 (3H each, s, 6 × CH_3); ^{13}C NMR (C_5D_5N, 500 MHz), see **Table 2**; HRESIMS (+), *m/z* 859.4082 $[M + Na]^+$, (calcd. for $C_{43}H_{64}O_{16}Na$, 859.4087).

Cellular proliferation assay

The cytotoxicity of compounds was evaluated by MTT assay according to a method reported previously [8]. Briefly, the HeLa (cervical cancer cells) and the MCF-7 (breast cancer cells) were plated in 96-well plates at 5 or 8×10^3/well in the complete medium, 100 µL/well. After incubation for 24 h, the medium was replaced with the medium containing various amounts of compounds (10, 40, 80, 100 µM). Finally, 10 µL of 5 µg/mL MTT was directly added to each well. Cells were then incubated at 37 °C for 4 h. Formazan was solubilized by 100 µL of DMSO and measured at 570 nm on the Model E1310 Autoplate reader (Bio-Tek Instruments). Each experiment was performed in triplicate. Results of three independent experiments were used for statistical analysis. Half-maximal inhibitory concentration (IC_{50} value) was calculated by the Logit method. 5-Fluorouracil (Shanghai Xudong Haipu Pharmaceutical Company, Ltd., purity > 98%) was used as the positive control.

Supporting information

HPLC charts of preprocessing samples from the roots of *G. uralensis*, 1D, 2D NMR, and MS spectra of **1**–**3** and 1D NMR and MS spectra of **4**–**5** are available as Supporting Information.

Results and Discussion

Due to the different adsorption capability of polyamide and macroporous resin for licorice-flavonoids and licorice-saponins, a method for co-application of polyamide and macroporous resin column chromatography was carried out to prepare total saponins, which facilitates the subsequent separation of licorice saponins. The HPLC of preprocessing samples is shown as Supporting Information. The resulting extract was separated by MPLC on a RP-18 silica gel column [MeOH–H_2O–AcOH, 40 : 60 : 1, 50 : 50 : 1, 60 : 40 : 1]. Fractions containing the desired compounds were combined and further purified using MPLC and semipreparative HPLC with CH_3CN–H_2O–AcOH (from 20 : 80 : 1 to 35 : 65 : 1). Consequently, we obtained four new oleanane-type triterpene glucuronides named uralsaponins C–F (**1**–**4**), and an artificial product named the methyl ester of glycyrrhizin (**5**). Besides, six known constituents were also obtained, namely 3-*O*-[*β*-D-glucuronopyranosyl-(1 → 2)-*β*-D-glucuronopyranosyl]-24-hydroxyglabrolide (**6**) [9], licorice-saponin E2 (**7**) [10], 22*β*-acetoxy-glycyrrhizin (**8**) [11], licorice-saponin G2 (**9**) [10], licorice-saponin A3 (**10**) [1] and glycyrrhizin (**11**) [12]. Their structures are shown in **Fig. 1**.

Uralsaponin C (**1**), a white and amorphous powder, produced a protonated ion at *m/z* 825.4261 (calcd. 825.4267) in the HRESIMS, corresponding to the molecular formula of $C_{42}H_{64}O_{16}$. The IR spectrum showed absorption bands at 3461 (OH), 1724 (C = O), and 1652 (C = C) cm^{-1}. The carbohydrate chain of **1** consisted of two monosaccharide residues representing the signals of two anomeric carbons at δ_C 105.1 and δ_C 106.9 as deduced from the ^{13}C NMR spectra. This was correlated by the HSQC spectrum with the corresponding signals of anomeric protons at δ_H 5.03 (1H, d, J = 7.5 Hz) and δ_H 5.41 (1H, d, J = 7.5 Hz). The coupling constants of the anomeric protons indicated the glycosidic bonds had a *β*-con-

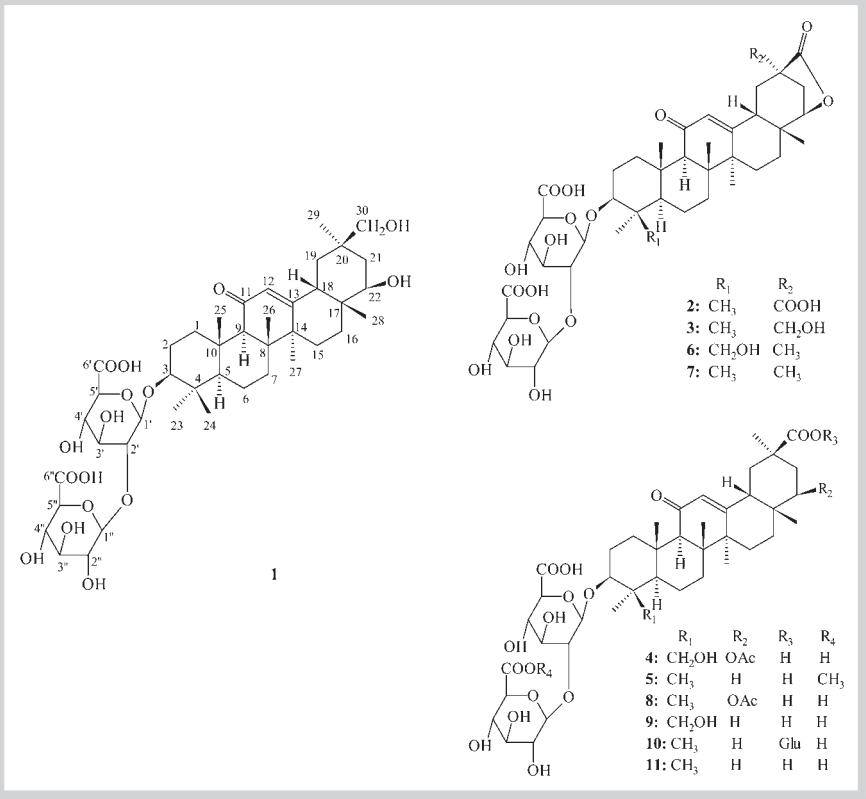

Fig. 1 Chemical structures of compounds **1**–**11**.

figuration [13]. The positive ESIMS of **1** produced a protonated ion peak at *m*/*z* 825.4261 $[M + H]^+$, as well as two fragment ion peaks at *m*/*z* 649.3940 $[M - C_6H_8O_6 + H]^+$ and *m*/*z* 473.3623 $[M - 2C_6H_8O_6 + H]^+$, demonstrating that the carbohydrate chain consists of two glucuronopyranoses, which was confirmed by the signals of two carboxyl carbons at 172.0 and 172.3 ppm, two anomeric carbons at 105.1 and 106.6 ppm, and ten tertiary carbons at 73.1–84.3 ppm. The determination of the linkage sites was obtained from the HMBC correlations between the proton signals at δ_H 5.03 (H-1′) and the carbon resonance at δ_C 89.2 (C-3), and the proton signal at δ_H 5.45 (H-1″) and the carbon resonance at δ_C 84.5 (C-2′). Thus, the carbohydrate sequence of **1** was established as 3-*O*-*β*-D-glucuronopyranosyl-(1 → 2)-*β*-D-glucuronopyranosyl. Complete assignments of the ^1H and ^{13}C NMR signals of the carbohydrate portion were accomplished by HSQC, H-H COSY, and HMBC.

The ^{13}C NMR spectrum of **1** exhibited 30 carbon resonances assigned to the aglycone moiety consisting of seven methyls, nine methines (of which one was oxygenated), five methylenes (including two oxygenated methylenes and one unsaturated methylene), and nine quaternary carbons (including one carbonyl and one unsaturated quaternary carbon). Additionally, the ^1H NMR spectrum showed for the aglycon moiety signals due to a double-bond proton at δ_H 5.79 (1H, s) and seven tertiary methyl groups at δ_H 1.06, 1.12, 1.12, 1.19, 1.22, 1.39, 1.39 (each 3H, s). Thus, compound **1** was considered to be an oleanane-type triterpene glucuronide and indicated the presence of a 12(13)-double bond and a keto group at C-11 in the aglycone moiety. Indeed, the HMBC correlations between the δ_H 5.79 (H-12) vinyl protons and δ_C 199.4 (C-11), δC 62.0 (C-9), δ_C 44.0 (C-14) clearly confirmed the 12(13)-position of the double bond and the presence of a 11-keto group. The presence of an *α*, *β*-unsaturated ketone fragment was confirmed by the UV spectrum of **1** (λ_{max} = 250.2 nm). In addition, the correlations in the HMBC spectrum from δ_H 1.12 (H-28) to δ_C 74.6 (C-22), and δ_H 3.73 (H-22) to δ_C 35.8 (C-20), from δ_H 1.49 (H-19), δ_H 1.11 (H-29) to δ_C 69.9 (C-30), and from δ_H 5.03 (H-1′), δ_H 1.39 (H-23), δ_H 1.22 (H-24) to δ_C 89.2 (C-3) helped in assigning one oxygenated methine at C-30, one oxygenated methylene at C-22, and one oxygenated methylene at C-3, respectively. These correlations and other key H-H COSY and HMBC correlations are shown in ▶ **Fig. 2**.

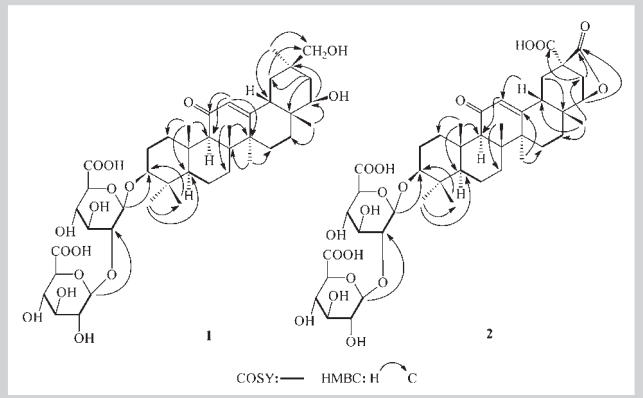

Fig. 2 Key COSY and HMBC correlations of compounds **1**–**2**.

The relative configuration of **1** was established on the basis of NOESY correlations of H-3 with H-1′*α* and H-5, as well as H-16*α* with H-27*α* and H-22, which revealed that both the substituent groups of C-3 and C-22 were *β*-oriented. Consequently, compound **1** was assigned as 3*β*-O-[*β*-D-glucuronopyranosyl-(1 → 2)-*β*-D-glucuronopyranosyl]- oleanane-11-oxo-12(13)-en-

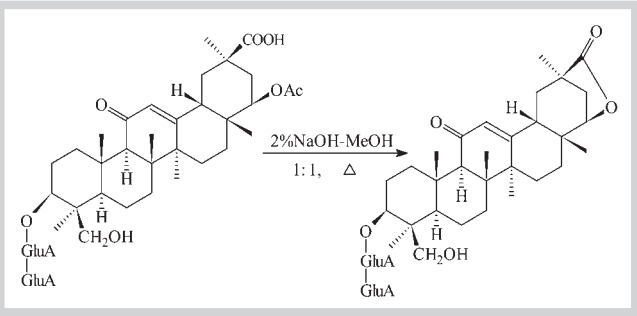

Fig. 3 Chemical conversion of compound **4** to compound **6**.

Table 3 Cytotoxicity data for the aglycones of isolated compounds from *G. uralensis* in selected human cell lines.

Compound	HeLa	MCF-7
Aglycone of **2**	35.3 ± 3.6	38.5 ± 2.8
Aglycone of **7**	86.3 ± 10.0	83.3 ± 8.2
Aglycone of **9**	19.9 ± 2.5	20.5 ± 3.6
Aglycone of **11**	15.5 ± 1.4	11.4 ± 3.0
5-Fluorouracil	11.0 ± 2.1	8.7 ± 1.6

* Results are expressed as IC_{50} values in μM. Cell lines: HeLa human cervical cancer; MCF-7 human breast adenocarcinoma

22*β*,30-diol. This is the first finding of a hydroxyl group at C-30 in oleanane-type glucuronides from *G. uralensis*.

Uralsaponin D (**2**), as a white and amorphous powder, generated a $[M + Na]^+$ ion at *m/z* 873.3499 and a $[M + H]^+$ at *m/z* 851.3610 corresponding to the molecular formula of $C_{42}H_{58}O_{18}$ and fourteen degrees of unsaturation. Two fragment ion peaks at *m/z* 675.3438 $[M - C_6H_8O_6 + H]^+$ and *m/z* 499.3066 $[M - 2C_6H_8O_6 + H]^+$ suggested the presence of two glucuronopyranosyl moieties. The ^1H and ^{13}C NMR spectra of **2** were similar to those of **1**, and further spectroscopic analysis revealed that the main difference was in the ring-E region, C-17 to C-22, and C-28 to C-30 (**Table 1**). Besides five carbonyls and one carbon-carbon double bond of **2**, the presence of eight rings was necessary to meet the number of unsaturation degrees. Since two rings of carbohydrate unit and five rings of oleanane-type triterpene accounted for seven degrees of unsaturation, another ring must have been present. Moreover, signals at δ_C 172.1 (C-30), δ_C 85.4 (C-22), and δ_H 4.42 (1H, d, J = 5.5, H-22) suggested the presence of a 22 (30)-lactone ring [7] which was confirmed by the HMBC correlations from δ_H 2.66 (H-21α) and δ_H 4.42 (H-22) to δ_C 175.1 (C-30), together with H-H COSY correlation between δ_H 3.02 (H-21β) and δ_H 4.42 (H-22). In addition, the HMBC correlations between δ_H 3.02 (H-21β) and δ_C 172.1 (C-29) indicated that a carboxyl group (δ_C 172.1) was present at C-29 (**Fig. 2**). Considering the fact that the NOESY correlations of 2 were consistent with those of **1**, compound **2** was assigned as 3*β*-*O*-[*β*-D-glucuronopyranosyl-(1 → 2)-*β*-D-glucuronopyranosyl]-29- carboxyl-glabrolide.

Uralsaponin E (**3**) exhibited a pseudomolecular ion at *m/z* 859.3719 ($[M + Na]^+$, calcd. 859.3723) in the HRESIMS, in agreement with the molecular formula of $C_{42}H_{60}O_{17}$, the same as 3-*O*-[*β*-D-glucuronopyranosyl-(1 → 2)-*β*-D-glucuronopyranosyl]-24-hydroxy-glabrolide (**6**). Detailed comparison of the NMR data of **3** with those of **6** suggested that the hydroxyl and methyl groups at C-24 and C-29, respectively, in **6** were transposed in **3**. The HMBC correlations from δ_H 3.83 (H-29) and δ_H 4.27 (H-22) to δ_C 178.5 (C-30) confirmed this deduction. Accordingly, **3** was identified as 3*β*-*O*-[*β*-D-glucuronopyranosyl-(1 → 2)-*β*-D-glucuronopyranosyl]-29- hydroxy-glabrolide.

Uralsaponin F (**4**) produced a protonated ion at *m/z* 897.4113 by HRESIMS. The ^{13}C NMR spectra of **4** were similar to those of 3-*O*-[*β*-D-glucuronopyranosyl-(1 → 2)-*β*-D-glucuronopyranosyl]-24-hydroxy-glabrolide (**6**) [9], and further spectroscopic analysis revealed that the only difference was in the region of C-22. The signals of a CH_3COO (δ_C 20.7, δ_C 170.2) in **4** rather than a lactone ring at C-22 in **6** were observed, and confirmed by the ESI-MS/MS experiment, in which the elimination of 60.0210 Da was assigned to a neutral fragment of the CH_3COOH from ion *m/z* 527.3344 to ion *m/z* 467.3134. Based on this evidence, compound **4** was presumed to be 24-hydroxy-22-acetoxyglycyrrhizin. To further verify the presumption, a chemical conversion of **4** to **6** was undertaken (**Fig. 3**). It was found that treatment of **4** with a 1 : 1 mixture of 2% aqueous sodium hydroxide and methanol at 60°C yielded **6**, which is in agreement with some previous studies [10].

The molecular formula of **5** was determined as $C_{43}H_{64}O_{16}$ by HRESIMS with a pseudomolecular ion $[M + Na]^+$ at *m/z* 859.4082 (calcd. 859.4087). The signals of **5** in the ^{13}C NMR spectrum were very similar to those of glycyrrhizin (**11**), and detailed spectroscopic analysis showed that the only difference was in the carbohydrate unit. Signals assigned to COOMe at δ_C 170.2, δ_C 51.9, and δ_H 3.77 (3H, s) showed one of the glucuronopyranosyl was methyl esterified [14] and the fragment ion at *m/z* 647.3787 $[M - C_7H_{10}O_6 - Na + H]^+$ in positive ion ESIMS analysis revealed that the terminal glucuronopyranose was esterified. Therefore, compound **5** was characterized as 3*β*-*O*-[*β*-D-(6-methyl)-glucuronopyranosyl-(1 → 2)-*β*-D-glucuronopyranosyl]- glycyrrhetic acid. Considering that compound **5** is absent in the HPLC chromatogram of the 50% aqueous ethanol extract of the roots of *G. uralensis*, it might be an artifact from glycyrrhizin due to the use of methanol in the column chromatography.

Previous studies demonstrated that glycyrrhizin and its aglycone had inhibitive effects on the growth of tumor cells [3,4]. Triterpene glucuronides **2, 7, 9, 11**, and their corresponding aglycones obtained by two-phase acid hydrolysis [7] were evaluated for cytotoxic activities against the HeLa (human cervical cancer) and MCF-7 (human breast carcinoma) cell lines, using the MTT colorimetric assays with 5-fluorouracil as the positive control [8]. Other isolated compounds were not tested for cytotoxic activities due to limited amounts. Results demonstrated that triterpene glucuronides showed no cytotoxic activity on tested cancer cell lines with IC_{50} > 100 μM. As shown in **Table 3**, the aglycones of **2, 9,** and **11** showed potently cytotoxic activities against HeLa with IC_{50} values at 35.3 ± 3.6, 19.9 ± 2.5, and 15.5 ± 1.4 μM, as well as cytotoxic activities against MCF-7 at 38.5 ± 2.8, 20.5 ± 3.6, and 11.4 ± 3.0 μM, respectively.

In the cytotoxic assay, compounds **2, 7, 9,** and **11** with the carbohydrate unit displayed no inhibitive effects on the growth of tumor cells, whereas their corresponding aglycones exhibited potent anticancer activity. Comparing the activity of the aglycone of glycyrrhizin (**11**) with that of licorice-saponin E2 (**7**), it seemed that the presence of a 22(30)-lactone ring significantly decreased the cytotoxic activity. Similarly, the aglycone of uralsaponin D (**2**) was shown to have weaker anticancer activity than the aglycone of licorice-saponin G2 (**9**) owing to the presence of a lactone ring at position 22(30). Interestingly, the aglycon of **11** showed ap-

proximately 2-fold stronger anticancer activity than the aglycon of **9**. That might be because an additional CH_2OH group is likely to reduce the lipophilicity of the compound, thus resulting in a decrease in the cancer cell membrane permeability to it.

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