Triterpene Saponins from *Clematis mandshurica* and Their Antiproliferative Activity

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Key words
- *Clematis mandshurica*
- Ranunculaceae
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- antiproliferative activity
- prostate cancer PC-3

Abstract

Six new triterpene saponins, clematomandshurica saponins F–K (**1**–**6**), together with a known compound (**7**), were isolated from the roots and rhizomes of *Clematis mandshurica*. Their structures were elucidated on the basis of spectroscopic evidence and hydrolysis. Compounds **5**–**7** exhibited antiproliferative effects against PC-3 human prostate cancer cells with GI_{50} values of 1.29, 1.50, and 0.71 µM, respectively.

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Introduction

The roots and rhizomes of *Clematis mandshurica* Rupr. (Ranunculaceae) are the main sources of the traditional Chinese medicine "Weilingxian", which is extensively used for the treatment of rheumatism and arthritis [1]. Previous investigations of *C. mandshurica* have demonstrated that it is rich in triterpene saponins, some of which exhibit anti-inflammatory and antitumor activity [2–4]. In addition, *C. mandshurica* contains several other types of chemical components, such as alkaloids [5], phenolic glycosides [6], macrocyclic glucosides [7], and lignans [8]. In our study, six new triterpene saponins, clematomandshurica saponins F–K (**1**–**6**) (**Fig. 1**), together with a known compound (**7**), were isolated from the roots and rhizomes of *C. mandshurica*. Herein, we report the isolation process and structural characterization of the seven triterpene saponins, as well as their antiproliferative activity in the human prostate cancer PC-3 cell line.

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Materials and Methods

General experimental procedures

Melting points were determined on a YanacoMP-S3 apparatus and are uncorrected. Optical rotations were obtained by using a JASCO DIP-370 digital polarimeter. IR spectra were taken on an IR-FT Bruker model IFS-55 spectrometer. HR-ESI-MS spectra were performed on a Bruker micrOTOF-Q mass spectrometer. ^{1}H-NMR, ^{13}C-NMR, HMBC, HSQC, and HSQC-TOCSY spectra were recorded on Bruker-AC(E)-600 spectrometers in C_5D_5N with TMS as an internal standard. HPLC was performed on a JAI LC9103 Recycling preparative HPLC (Japan Analytical Industries Co., Ltd.) equipped with a JAIGEL-ODS-AP-P column (2 × 50 cm, 15 µm) and a JAIGEL-GS310 column (2 × 50 cm, 15 µm), using a JAI refractive index detector and a JAI UV-3702 detector with a MultiChro 2000 workstation. GC analyses were performed on an Agilent GC 6890 instrument with an HP-5 column (320 µm × 30 m, 0.25 µm). TLC was performed on precoated GF_{254} plates (Merck) and detected by spraying with 10% H_2SO_4 in EtOH followed by heating.

Plant material

The roots and rhizomes of *C. mandshurica* were collected from Fushun, Liaoning Province, China, in October 2008, and identified by Professor Jincai Lu (Shenyang Pharmaceutical University). A voucher specimen (CM20081008) was deposited in the Department of Natural Products Chemistry, Shenyang Pharmaceutical University, Shenyang, People's Republic of China.

Extraction and isolation

The air-dried and pulverized roots and rhizomes of *C. mandshurica* (5 kg) were extracted with 60% EtOH (3 × 20 L and 4 h each time) under reflux.

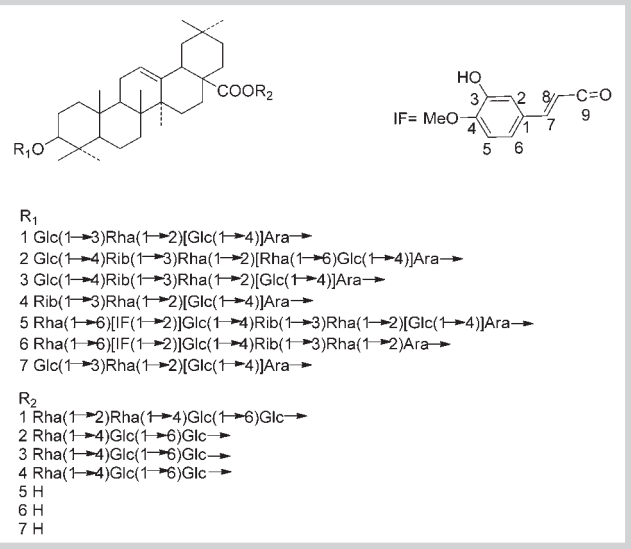

Fig. 1 Chemical structures of compounds **1**–**7** isolated from *Clamatis mandshurica*.

After removing the solvent under reduced pressure at 60 °C, the residue (550 g) was further suspended in water (1.5 L) and partitioned with petroleum ether (PE, 60–90 °C, 3 × 1.5 L), CH_2Cl_2 (3 × 1.5 L), EtOAc (3 × 2 L), and *n*-BuOH (3 × 2 L), successively. The *n*-BuOH extract (180 g) was subjected to D101 porous polymer resin column chromatography and eluted with H_2O and 30%, 50%, 70%, and 95% EtOH, successively. The fraction eluted with 70% EtOH (85 g) was subjected to silica gel column chromatography (CC) (200–300 mesh, 850 g, 8 × 80 cm), eluting with a gradient of CH_2Cl_2-MeOH-H_2O (10 : 1 : 0, 8 : 2 : 0.1, 7 : 3 : 0.1, 3 : 3 : 0.1, 1 : 2 : 0.5, v : v : v, 4 L each) to afford fractions 1–16. Fraction 12 (1.94 g) was performed on HPLC (ODS column) with MeOH-H_2O (62 : 38, 5.0 mL/min) to afford subfractions 12a-12c. Subfraction 12b (0.87 g) was isolated on recycling preparative HPLC (GS column) with MeOH-*n*-hexane (7 : 3, 5.0 mL/min) using the recycling function to yield compounds **3** (28 mg, t_R = 39 min), **1** (87 mg, t_R = 42 min), and **2** (59 mg, t_R = 45 min). Subfraction 12a (0.21 g) was purified on recycling preparative HPLC (ODS column) with CH_3CN-H_2O (30 : 70, 3.0 mL/min) and yielded compound **4** (32 mg, t_R = 32 min). Fraction 9 (0.93 g) was performed on HPLC with (ODS column) MeOH-H_2O (65 : 35) to afford subfractions 9a-9e. Subfraction 9c (0.32 g) was isolated on recycling preparative HPLC (GS column) with MeOH-*n*-hexane (7 : 3, 3.0 mL/min) to yield **5** (29 mg, t_R = 43 min), **6** (28 mg, t_R = 40 min), and **7** (42 mg, t_R = 36 min). The purity (%) of the isolated compounds exceeds 95%.

Isolates

Clematomandshurica saponin F (**1**): white amorphous powder; m. p. 244–245 °C; $[\alpha]_D^{25}$ −96 (*c* 0.032, MeOH); IR (KBr): ν_{max} = 3428, 2926, 1740, 1062 cm^{-1}; ^{1}H-NMR (C_5D_5 N, 600 MHz): δ 6.22 (1H, d, *J* = 7.8 Hz, Glc′-H-1), 6.14 (1H, br s, Rha-H-1), 5.84 (1H, br s, Rha′-H-1), 5.83 (1H, br s, Rha″-H-1), 5.44 (1H, d, *J* = 7.8 Hz, GlcI-H-1), 5.11 (1H, d, *J* = 7.8 Hz, GlcII-H-1), 4.97 (1H, d, *J* = 7.8 Hz, Glc″-H-1), 4.80 (1H, d, *J* = 6.0 Hz, Ara-H-1), 1.67 (3H, d, *J* = 6.0 Hz, Rha′-H-6), 1.56 (3H, d, *J* = 6.0 Hz, Rha″-H-6), 1.53 (3H, d, *J* = 6.0 Hz, Rha-H-6), data of aglycone see **Table 1**; ^{13}C-NMR

Table 1 ^{1}H-NMR data of compounds **1** to **4** (aglycones, C_5D_5 N, δ, 600 MHz).

No.	1	2	3	4
1	0.89 (1H, m)	0.90 (1H, m)	0.89 (1H, m)	0.89 (1H, m)
	1.45 (1H, m)	1.45 (1H, m)	1.44 (1H, m)	1.45 (1H, m)
2	1.85 (1H, m)	1.84 (1H, m)	1.84 (1H, m)	1.85 (1H, m)
	2.05 (1H, m)	2.06 (1H, m)	2.04 (1H, m)	2.06 (1H, m)
3	3.25 (1H, dd, 12.0, 4.8)	3.27 (1H, dd, 12.0, 4.8)	3.27 (1H, dd, 12.0, 4.8)	3.27 (1H, dd, 12.0, 4.8)
5	0.72 (1H, d, 12.0)	0.72 (1H, d, 12.0)	0.72 (1H, d, 12.0)	0.73 (1H, d, 12.0)
6	1.60 (2H, m)	1.61 (2H, m)	1.59 (2H, m)	1.60 (2H, m)
7	1.29 (1H, m)	1.29 (1H, m)	1.28 (1H, m)	1.28 (1H, m)
	1.40 (1H, m)	1.42 (1H, m)	1.39 (1H, m)	1.40 (1H, m)
9	1.61 (1H, m)	1.61 (1H, m)	1.59 (1H, m)	1.58 (1H, m)
11	1.88 (2H, m)	1.90 (2H, m)	1.89 (2H, m)	1.89 (2H, m)
12	5.36 (1H, br s)	5.36 (1H, br s)	5.36 (1H, br s)	5.36 (1H, br s)
15	1.16 (1H, m)	1.17 (1H, m)	1.18 (1H, m)	1.18 (1H, m)
	2.27 (1H, m)	2.25 (1H, m)	2.25 (1H, m)	2.26 (1H, m)
16	1.90 (1H, m)	1.91 (1H, m)	1.90 (1H, m)	1.90 (1H, m)
	2.05 (1H, m)	2.06 (1H, m)	2.04 (1H, m)	2.06 (1H, m)
18	3.13 (1H, d, 12.0)	3.13 (1H, d, 12.0)	3.13 (1H, d, 12.0)	3.13 (1H, d, 12.0)
19	1.21 (1H, m)	1.23 (1H, m)	1.21 (1H, m)	1.22 (1H, m)
	1.73 (1H, m)	1.74 (1H, m)	1.71 (1H, m)	1.73 (1H, m)
21	0.90 (1H, m)	0.93 (1H, m)	0.91 (1H, m)	0.90 (1H, m)
	1.09 (1H, m)	1.09 (1H, m)	1.06 (1H, m)	1.08 (1H, m)
22	1.75 (1H, m)	1.74 (1H, m)	1.79 (1H, m)	1.73 (1H, m)
	1.85 (1H, m)	1.87 (1H, m)	1.84 (1H, m)	1.83 (1H, m)
23	1.29 (3H, s)	1.27 (3H, s)	1.26 (3H, s)	1.25 (3H, s)
24	1.10 (3H, s)	1.13 (3H, s)	1.12 (3H, s)	1.11 (3H, s)
25	0.86 (3H, s)	0.86 (3H, s)	0.88 (3H, s)	0.87 (3H, s)
26	1.05 (3H, s)	1.03 (3H, s)	1.05 (3H, s)	1.05 (3H, s)
27	1.23 (3H, s)	1.21 (3H, s)	1.23 (3H, s)	1.23 (3H, s)
29	0.86 (3H, s)	0.86 (3H, s)	0.88 (3H, s)	0.86 (3H, s)
30	0.84 (3H, s)	0.83 (3H, s)	0.85 (3H, s)	0.84 (3H, s)

Table 2 ^{13}C-NMR data of compounds **1** to **6**.

No.	1	2	2a	3	4	5	6
1	39.1	38.7	38.6	38.7	38.9	38.8	38.8
2	26.8	26.4	26.4	26.6	26.5	26.6	26.5
3	88.7	88.5	88.5	88.7	88.6	88.7	88.5
4	39.6	39.4	39.3	39.5	39.4	39.5	39.4
5	56.0	55.8	55.7	55.9	55.8	55.9	55.7
6	18.6	18.3	18.2	18.3	18.3	18.6	18.3
7	33.2	32.9	32.9	33.0	33.0	33.2	33.2
8	39.9	39.6	39.5	39.8	39.7	39.7	39.6
9	48.1	47.8	47.8	48.0	47.9	48.0	47.9
10	37.1	36.8	36.8	36.9	36.9	37.0	36.9
11	23.7	23.4	23.5	23.6	23.6	23.6	23.5
12	122.6	122.5	122.5	122.7	122.5	122.4	122.4
13	144.9	144.6	144.6	144.8	144.8	144.6	144.6
14	42.2	41.9	41.9	42.0	41.9	42.1	41.9
15	28.3	28.0	28.0	28.2	28.0	28.2	28.1
16	23.8	23.5	23.5	23.7	23.7	23.7	23.6
17	47.0	46.8	46.4	46.9	46.9	46.6	46.5
18	41.7	41.4	41.7	42.0	41.5	41.9	41.8
19	46.3	46.0	46.2	46.2	46.0	46.4	46.4
20	30.8	30.5	30.7	30.6	30.6	30.9	30.8
21	34.0	33.8	33.9	33.9	33.8	34.2	34.0
22	32.6	32.3	32.8	32.4	32.3	33.0	33.0
23	28.2	27.9	27.9	28.2	28.0	28.1	28.0
24	17.2	17.2	16.9	17.0	17.0	17.0	17.0
25	15.7	15.4	15.3	15.6	15.5	15.5	15.4
26	17.5	17.4	17.1	17.4	17.3	17.3	17.2
27	26.1	25.8	25.9	26.0	25.9	26.1	26.1
28	176.4	176.3	180.2	176.5	176.3	180.0	180.0
29	33.2	32.8	32.9	33.0	32.9	33.2	33.2
30	23.7	23.4	23.4	23.6	23.5	23.7	23.7

(C_5D_5N, 150 MHz), see **Tables 2** and **3**; HR-ESI-MS: *m/z* = 1697.7767 $[M + Na]^+$ (calcd. for $C_{77}H_{126}O_{39}Na$: 1697.7774).

Clematomandshurica saponin G (**2**): white amorphous powder; m. p. 254–255 °C; $[\alpha]_D^{25}$ −78 (*c* 0.030, MeOH); IR (KBr): ν_{max} = 3428, 2926, 1740, 1062 cm^{-1}; ^{1}H-NMR (C_5D_5N, 600 MHz): *δ* 6.24 (1H, br s, Rha-H-1), 6.22 (1H, d, *J* = 7.8 Hz, Glc′-H-1), 5.85 (1H, br s, Rha′-H-1), 5.83 (1H, d, *J* = 5.4 Hz, Rib-H-1), 5.42 (1H, br s, RhaI-H-1), 5.08 (1H, d, *J* = 7.8 Hz, GlcII-H-1), 5.05 (1H, d, *J* = 7.8 Hz, GlcI-H-1), 4.94 (1H, d, *J* = 7.8 Hz, Glc″-H-1), 4.84 (1H, d, *J* = 6.0 Hz, Ara-H-1), 1.68 (3H, d, *J* = 6.0 Hz, RhaI-H-6), 1.56 (3H, d, *J* = 6.0 Hz, Rha′-H-6), 1.53 (3H, d, *J* = 6.0 Hz, Rha-H-6), data of aglycone see **Table 1**; ^{13}C-NMR (C_5D_5N, 150 MHz), see **Tables 2** and **3**; HR-ESI-MS: *m/z* = 926.4092 $[M + 2Na]^{2+}$ (calcd. for $C_{82}H_{134}O_{43}Na_2$: 926.4042).

Clematomandshurica saponin H (**3**): white amorphous powder; m. p. 238–239 °C; $[\alpha]_D^{25}$ −62 (*c* 0.026, MeOH); ^{1}H-NMR (C_5D_5N, 600 MHz): *δ* 6.22 (1H, br s, Rha-H-1), 6.21 (1H, d, *J* = 7.8 Hz, Glc′-H-1), 5.82 (1H, br s, Rha′-H-1), 5.80 (1H, d, *J* = 5.4 Hz, Rib-H-1), 5.02 (1H, d, *J* = 7.8 Hz, GlcII-H-1), 4.95 (1H, d, *J* = 7.8 Hz, GlcI-H-1), 4.85 (1H, d, *J* = 7.8 Hz, Glc″-H-1), 4.82 (1H, d, *J* = 6.0 Hz, Ara-H-1), 1.68 (3H, d, *J* = 6.0 Hz, Rha′-H-6), 1.51 (3H, d, *J* = 6.0 Hz, Rha-H-6), data of aglycone see **Table 1**; ^{13}C-NMR (C_5D_5N, 150 MHz), see **Tables 2** and **3**; HR-ESI-MS: *m/z* = 1683.7637 $[M + Na]^+$ (calcd. for $C_{76}H_{124}O_{39}Na$: 1683.7617).

Clematomandshurica saponin I (**4**); white amorphous powder; m. p. 236–237 °C; $[\alpha]_D^{25}$ −58 (*c* 0.028, MeOH); ^{1}H-NMR (C_5D_5N, 600 MHz): *δ* 6.22 (1H, br s, Rha-H-1), 6.21 (1H, d, *J* = 7.8 Hz, Glc′-H-1), 5.82 (1H, br s, Rha′-H-1), 5.80 (1H, d, *J* = 5.4 Hz, Rib-H-1), 4.98 (1H, d, *J* = 7.8 Hz, 4.97 (1H, d, *J* = 7.8 Hz, Glc″-H-1), 4.81 (1H, d, *J* = 6.0 Hz, Ara-H-1), 1.67 (3H, d, *J* = 6.0 Hz, Rha′-H-6), 1.51 (3H, d, *J* = 6.0 Hz, Rha-H-6), data of aglycone see **Table 1**; ^{13}C-NMR (C_5D_5N, 150 MHz), see **Tables 2** and **3**; HR-ESI-MS: *m/z* = 1499.7257 $[M + H]^+$ (calcd. for $C_{70}H_{115}O_{34}$: 1499.7264).

Clematomandshurica saponin J (**5**); white amorphous powder; m. p. 243–244 °C; $[\alpha]_D^{25}$ −95 (*c* 0.031, MeOH); ^{1}H-NMR (C_5D_5N, 600 MHz): *δ* 6.19 (1H, br s, Rha-H-1), 5.80 (1H, d, *J* = 5.4 Hz, Rib-H-1), 5.41 (1H, br s, RhaI-H-1), 5.27 (1H, d, *J* = 7.8 Hz, GlcII-H-1), 4.86 (1H, d, *J* = 7.8 Hz, GlcI-H-1), 4.81 (1H, d, *J* = 6.0 Hz, Ara-H-1), 1.53 (3H, d, *J* = 6.0 Hz, Rha-H-6), 1.52 (3H, d, *J* = 6.0 Hz, RhaI-H-6), data of aglycone see **Table 4**; ^{13}C-NMR (C_5D_5N, 150 MHz), see **Tables 2**, **5**, and **6**; HR-ESI-MS: *m/z* = 1535.7026 $[M + Na]^+$ (calcd. for $C_{74}H_{112}O_{32}Na$: 1535.7029).

Clematomandshurica saponin K (**6**); white amorphous powder; m. p. 246–247 °C; $[\alpha]_D^{25}$ −76 (*c* 0.030, MeOH); ^{1}H-NMR (C_5D_5N, 600 MHz): *δ* 6.20 (1H, br s, Rha-H-1), 5.80 (1H, d, *J* = 5.4 Hz, Rib-H-1), 5.43 (1H, br s, RhaI-H-1), 4.86 (1H, d, *J* = 7.8 Hz, GlcI-H-1), 4.83 (1H, d, *J* = 6.0 Hz, Ara-H-1), 1.59 (3H, d, *J* = 6.0 Hz, RhaI-H-6), 1.52 (3H, d, *J* = 6.0 Hz, Rha-H-6), data of aglycone see **Table 4**; ^{13}C-NMR (C_5D_5N, 150 MHz), see **Tables 2**, **5**, and **6**; HR-ESI-MS: *m/z* = 1389.6263 $[M + K]^+$ (calcd. for $C_{68}H_{102}O_{27}K$: 1389.6240).

Acid hydrolysis of compounds 1–6

Each compound (4 mg) was treated with 1 M HCl (4 mL) at 90 °C for 2 h. Then the reaction mixture was extracted with $CHCl_3$ (3 × 5 mL). The aqueous layer was collected and the water was evaporated under vacuum with the repeated addition of MeOH to remove the solvent completely. The residue was redissolved in anhydrous pyridine (2 mL) and mixed with a pyridine solution

Table 3 ^{13}C-NMR data of compounds **1** to **4** (sugar moieties, C_5D_5N, δ, 150 MHz).

No.	1	2	2a	3	4	No.	1	2	3	4
3-*O*						28-*O*				
Ara						Glc′				
1	105.5	105.2	105.0	105.0	105.0	1	95.5	95.4	95.5	95.5
2	76.5	75.1	75.4	75.2	75.0	2	73.9	73.7	73.8	73.8
3	74.1	74.6	74.6	74.6	74.5	3	78.8	78.5	78.6	78.4
4	80.3	81.7	81.6	81.9	81.7	4	70.9	70.6	70.8	70.6
5	65.4	65.5	65.4	65.5	65.5	5	78.1	77.9	77.9	77.8
Rha						6	69.2	68.9	69.6	68.9
1	101.8	101.2	101.3	101.4	101.2	Glc′′				
2	71.6	71.7	71.7	71.8	71.8	1	104.7	104.6	104.7	104.6
3	83.3	81.8	81.8	82.0	81.8	2	75.4	75.4	75.4	75.4
4	72.8	73.6	73.8	73.8	73.7	3	76.1	76.4	76.4	76.3
5	69.9	69.6	69.6	69.7	69.6	4	78.6	78.0	78.2	78.1
6	18.5	18.4	18.4	18.4	18.4	5	77.2	76.9	77.0	76.9
Rib						6	61.3	61.0	61.2	61.0
1		104.5	104.5	104.6	104.5	Rha′				
2		72.3	72.3	72.4	72.4	1	102.7	102.5	102.6	102.5
3		69.1	69.1	69.1	69.1	2	78.3	72.5	72.7	72.5
4		76.4	76.3	76.4	70.2	3	72.5	72.4	72.4	72.4
5		61.5	61.6	61.5	65.2	4	73.2	73.9	73.9	73.7
GlcI						5	70.3	70.1	70.2	70.1
1	106.9	102.9	102.7	102.0		6	18.6	18.4	18.4	18.4
2	75.9	76.1	76.1	75.4		Rha′′				
3	78.6	78.5	77.9	78.3		1	102.6			
4	71.3	71.6	71.4	71.4		2	72.5			
5	78.5	76.5	76.5	78.5		3	72.7			
6	62.5	61.6	61.5	61.4		4	73.9			
GlcII						5	70.3			
1	106.6	104.7	104.7	103.4	103.3	6	18.6			
2	75.5	73.7	73.8	75.2	75.2					
3	78.6	77.8	77.9	78.5	78.1					
4	71.3	71.4	71.7	71.4	71.3					
5	78.5	76.4	76.3	78.6	78.5					
6	62.6	69.6	69.6	62.4	62.4					
RhaI										
1		102.5	102.5							
2		72.5	72.5							
3		72.4	72.4							
4		73.9	73.9							
5		68.3	68.3							
6		18.4	18.2							

of L-cysteine methyl ester hydrochloride (2 mL). After the mixed solution was heated at 60 °C for 1 h, trimethylchlorosilane (0.5 mL) was added and the resulting mixture was stirred at 60 °C for another 30 min. Then the solution was concentrated to dryness and taken up in water (1 mL × 3), followed by extraction with *n*-hexane (1 mL × 3). The supernatant was analyzed by GC. Separations were carried out on HP-5 columns (320 µm × 30 m, 0.25 µm). Highly pure N_2 was employed as a carrier gas (1.0 mL/min), and the FID detector operated at 280 °C (column temperature 160–200 °C). The retention times of the monosaccharide derivatives were as follows: L-arabinose (12.69 min, compounds **1**–**6**), D-ribose (12.70 min, compounds **2**–**6**), L-rhamnose (12.80 min, compounds **1**–**6**), and D-glucose (14.43 min, compounds **1**–**6**).

Alkaline hydrolysis of compounds 1 and 2

Pure compounds **1** and **2** (each 15 mg) were refluxed in 5% KOH solution (pH 1213) at 90 °C for 1 h, respectively. The reaction mixtures were neutralized with 5% HCl solution and then concentrated to dryness. The residues were extracted with *n*-BuOH and the resulting organic layers were concentrated. After purification using HPLC, two prosapogenins were obtained, and their structures were analyzed by NMR spectroscopy.

Cell growth inhibition assay

The cell growth inhibitory effects of the isolated compounds were investigated using the human prostate cancer PC-3 cell line. The human prostate cancer PC-3 cell line was obtained from American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium (Gibco) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (Gibco). Cell growth inhibition was performed as reported previously [9]. Cells were seeded at a density of 2×10^4 cells/mL and incubated with various concentrations of the tested compounds for 4 days. The compounds were dissolved in dimethyl sulfoxide (DMSO), and the final DMSO concentration in the samples did not exceed 0.1%. The optical density was read at 570 nm using a microplate reader (Tecan), and the cell viability was determined us-

Table 4 ^{1}H-NMR data of compounds **5** and **6** (aglycones, C_5D_5N, δ, 600 MHz).

No.	5	6	No.	5	6
1	0.89 (1H, m) 1.45 (1H, m)	0.89 (1H, m) 1.45 (1H, m)	18	3.23 (1H, d, 12.0)	3.23 (1H, d, 12.0)
2	1.86 (1H, m) 2.06 (1H, m)	1.85 (1H, m) 2.07 (1H, m)	19	1.22 (1H, m) 1.75 (1H, m)	1.21 (1H, m) 1.73 (1H, m)
3	3.27 (1H, dd, 12.0, 4.8)	3.27 (1H, dd, 12.0, 4.8)	21	0.91 (1H, m) 1.14 (1H, m)	0.90 (1H, m) 1.14 (1H, m)
5	0.75 (1H, d, 12.0)	0.74 (1H, d, 12.0)	22	1.78 (1H, m) 1.86 (1H, m)	1.78 (1H, m) 1.85 (1H, m)
6	1.61 (2H, m)	1.61 (2H, m)	23	1.28 (3H, s)	1.28 (3H, s)
7	1.29 (1H, m) 1.43 (1H, m)	1.29 (1H, m) 1.43 (1H, m)	24	1.09 (3H, s)	1.09 (3H, s)
9	1.61 (1H, m)	1.61 (1H, m)	25	0.95 (3H, s)	0.95 (3H, s)
11	1.91 (2H, m)	1.91 (2H, m)	26	0.99 (3H, s)	0.99 (3H, s)
12	5.36 (1H, br s)	5.36 (1H, br s)	27	1.26 (3H, s)	1.26 (3H, s)
15	1.17 (1H, m) 2.10 (1H, m)	1.16 (1H, m) 2.10 (1H, m)	29	0.94 (3H, s)	0.94 (3H, s)
16	1.87 (1H, m) 2.01 (1H, m)	1.86 (1H, m) 2.01 (1H, m)	30	0.80 (3H, s)	0.80 (3H, s)

Table 5 ^{13}C-NMR data of compounds **5** and **6** (sugar moieties, C_5D_5N, δ, 150 MHz).

No.		5	6			5	6
3-*O*-							
Ara				GlcI			
	1	105.1	105.1		1	102.3	102.2
	2	75.5	75.5		2	74.5	74.4
	3	74.5	74.9		3	76.2	76.1
	4	81.3	69.2		4	71.9	71.7
	5	65.3	65.5		5	76.8	76.7
					6	68.1	68.2
Rha				GlcII			
	1	101.6	101.6		1	102.4	
	2	71.8	71.7		2	73.8	
	3	81.7	80.9		3	78.0	
	4	72.7	72.6		4	74.0	
	5	69.8	69.8		5	76.6	
	6	18.4	18.4		6	61.3	
Rib				RhaI			
	1	104.7	104.6		1	102.7	102.6
	2	72.5	72.4		2	71.5	71.5
	3	69.9	69.8		3	72.5	72.5
	4	76.6	76.7		4	74.0	74.0
	5	61.5	61.3		5	69.9	69.9
					6	18.4	18.4

ing MTT. The growth inhibitory ability of these compounds was calculated and expressed as the ratio of the cell number in the treated group to that of the untreated group. The concentration (GI_{50}) which inhibited half of the cell growth was calculated. Podophyllotoxin (98%; prepared by our group) was used as a positive control, and 0.1% of DMSO was used as a negative control.

Supporting information

Spectra of compounds **1**–**6** are available as Supporting Information.

Results and Discussion

The *n*-BuOH layers of the EtOH extract of the roots and rhizomes of *C. mandshurica* were fractionated and purified by repeated chromatography allowing for the isolation of seven triterpene saponins **1**–**7**. The structures of the new compounds were characterized as follows: Compound **1** was obtained as white amorphous powder and its molecular formula $C_{77}H_{126}O_{39}$ was determined by HR-ESI-MS (m/z = 1697.7767 $[M + Na]^+$ calcd. for $C_{77}H_{126}O_{39}Na$: 1697.7774), which was supported by the ^{13}C-NMR data. The IR spectrum of **1** showed a broad absorption band for hydroxyl groups at 3428 cm^{-1}, as well as absorption due to a carbonyl group at 1740 cm^{-1}. The ^{1}H-NMR (**Table 1**) spectrum showed seven tertiary methyl resonances at δ 0.84,

Table 6 NMR data for the isoferuloyl moieties of compounds **5** and **6** (C_5D_5N, δ, 600 MHz).

No.	5		6	
	^{1}H	^{13}C	^{1}H	^{13}C
1		128.4		128.4
2	7.52 (1H, d, *J* = 1.8 Hz)	115.4	7.53 (1H, d, *J* = 1.8 Hz)	115.2
3		148.4		148.4
4		150.9		150.9
5	6.90 (1H, d, *J* = 8.4 Hz)	112.1	6.89 (1H, d, *J* = 8.4 Hz)	112.0
6	7.10 (1H, dd, *J* = 8.4, 1.8 Hz)	121.4	7.09 (1H, dd, *J* = 8.4, 1.8 Hz)	121.4
7	8.06 (1H, d, *J* = 15.6 Hz)	145.9	8.07 (1H, d, *J* = 15.6 Hz)	145.9
8	6.90 (1H, d, *J* = 15.6 Hz)	116.1	6.72 (1H, d, *J* = 15.6 Hz)	116.0
9		166.7		166.9
MeO	3.73 (3H, s)	55.7	3.72 (3H, s)	55.8

0.86, 0.86, 1.05, 1.10, 1.23, and 1.29, and an olefinic proton at δ 5.36, which were typical of the oleanene skeleton. The resonances at δ 122.6, 144.9, and 176.4 in the ^{13}C-NMR (**Table 2**) spectrum also suggested that **1** possessed an oleanolic acid aglycone. The presence of eight monosaccharide units was indicated by eight anomeric protons at δ 6.22 (1H, d, *J* = 7.8 Hz), 6.14 (1H, br s), 5.84 (1H, br s), 5.83 (1H, br s), 5.44 (1H, d, *J* = 7.8 Hz), 5.11 (1H, d, *J* = 7.8 Hz), 4.97 (1H, d, *J* = 7.8 Hz), and 4.80 (1H, d, *J* = 6.0 Hz), and eight anomeric carbons (**Table 3**). The three-proton doublet at δ 1.67 (3H, d, *J* = 6.0 Hz), 1.56 (3H, d, *J* = 6.0 Hz), 1.53 (3H, d, *J* = 6.0 Hz) indicated the presence of three 6-deoxyhexopyranosyl units in **1**. Acid hydrolysis with 1 M HCl gave oleanolic acid, together with L-arabinose, L-rhamnose, and D-glucose. The relatively large coupling constants (5.5–8.0 Hz) of the anomeric protons suggested that the arabinopyranosyl moiety was α-configured and the glucopyranosyl moiety was β-configured [10]. And the α-anomeric configuration of L-rhamanose was judged by its C-5 chemical shift (δ_C 69–70) [11]. In the ^{13}C-NMR spectrum of **1**, the C-3 and C-28 carbon signals were observed at δ 88.7 and 176.4, respectively, implying that **1** was a bisdesmosidic triterpene saponin. Alkaline hydrolysis of **1** afforded a prosapogenin, and its NMR data were identical to those of the known compound **7**, which was identified as oleanolic acid 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)[β-D-glucopyranosyl-(1 → 4)]-α-L-arabinopyranoside by comparison with the literature [12]. After having excluded a signal due to prosapogenin, the remaining signals revealed that the 28-*O*-sugar moiety in **1** contained two β-D-glucopyranosyl units and two α-L-rhamnopyranosyl units, and the linkage of the sugars with the aglycone was established by HMBC correlations (**Fig. 2**) and comparison with literature values [13]. From the above evidence, the structure of **1** was established as oleanolic acid 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)[β-D-glucopyranosyl-(1 → 4)]-α-L-arabinopyranosyl-28-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl ester. In keeping with the nomenclature previously established [2,3], we propose the trivial name clematomandshurica saponin F.

Compound **2** was obtained as white amorphous powder and its molecular formula $C_{82}H_{134}O_{43}$ was determined by HR-ESI-MS (*m/z* = 926.4092, $[M + 2Na]^{2+}$ calcd. for $C_{82}H_{134}O_{43}Na_2$: 926.4042), which was supported by the ^{13}C-NMR data. The ^{1}H-and ^{13}C-NMR spectra indicated the aglycone of **2** was identical to oleanolic acid (**Tables 1** and **2**). The presence of monosaccharide units was due to nine anomeric protons at δ 6.24 (1H, br s), 6.22 (1H, d, *J* = 7.8 Hz), 5.85 (1H, br s), 5.83 (1H, d, *J* = 5.4 Hz),

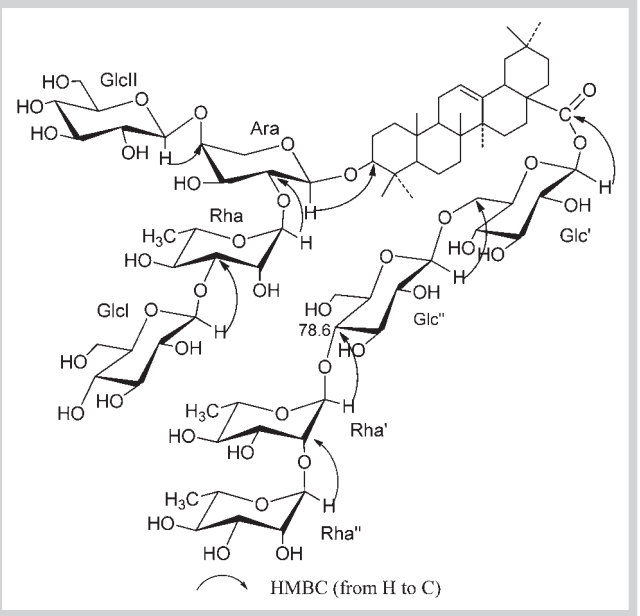

Fig. 2 The key HMBC correlations of **1**.

5.42 (1H, br s), 5.08 (1H, d, *J* = 7.8 Hz), 5.05 (1H, d, *J* = 7.8 Hz), 4.94 (1H, d, *J* = 7.8 Hz), and 4.84 (1H, d, *J* = 6.0 Hz), and nine anomeric carbons (**Table 3**). The three-proton doublet at δ 1.68 (3H, d, *J* = 6.0 Hz), 1.56 (3H, d, *J* = 6.0 Hz), 1.53 (3H, d, *J* = 6.0 Hz) indicated the presence of three 6-deoxyhexopyranosyl units in **2**. Acid hydrolysis with 1 M HCl gave oleanolic acid, together with L-arabinose, L-rhamnose, D-ribose, and D-glucose. The coupling constants (5.5–8.0 Hz) of the anomeric protons suggested the presence of α-arabinopyranosyl, β-glucopyranosyl, and β-ribopyranosyl moieties. Alkaline hydrolysis of **2** afforded **2a** which was identified as oleanolic acid 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-ribopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)[α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 4)]-α-L-arabinopyranoside based on the data of ^{1}H-, ^{13}C-NMR, and HMBC. The 28-*O*-sugar moiety in **2** was determined to contain two β-D-glucopyranosyl units and one α-L-rhamnopyranosyl unit on the basis of the remaining monosaccharide carbon signals. The HSQC and HSQC-TOCSY experiments led to unambiguous assignments of the carbons in each monosaccharide unit. The linkage of the sugars with the aglycone and the sequence in the sugar chains were established by HMBC correlations. Thus, the

structure of **2** was determined as oleanolic acid 3-*O*-*β*-D-glucopyranosyl-(1 → 4)-*β*-D-ribopyranosyl-(1 → 3)-*α*-L-rhamnopyranosyl-(1 → 2)[*α*-L-rhamnopyranosyl-(1 → 6)-*β*-D-glucopyranosyl-(1 → 4)]-*α*-L-arabinopyranosyl-28-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*β*-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester, and named clematomandshurica saponin G.

Compound **3** was obtained as white amorphous powder and its molecular formula $C_{76}H_{124}O_{39}$ was determined by HR-ESI-MS (*m/z* = 1683.7637 $[M + Na]^+$ calcd. for $C_{76}H_{124}O_{39}Na$: 1683.7617), which was supported by the ^{13}C-NMR data (**Tables 2** and **3**). Acid hydrolysis with 1 M HCl gave oleanolic acid, together with L-arabinose, L-rhamnose, D-ribose, and D-glucose. The ^1H- and ^{13}C-NMR spectra implied **3** was a bisdesmosidic saponin with oleanolic acid as the aglycone. Comparison of the ^1H- and ^{13}C-NMR data of **3** with those of **2** revealed that the terminal L-rhamnose (RhaI) in the saccharide chain at C-3 of **3** disappeared. Accordingly, the structure of **3** was identified as oleanolic acid 3-*O*-*β*-D-glucopyranosyl-(1 → 4)-*β*-D-ribopyranosyl-(1 → 3)-*α*-L-rhamnopyranosyl-(1 → 2)[*β*-D-glucopyranosyl-(1 → 4)]-*α*-L-arabinopyranosyl-28-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*β*-D-glucopyranosyl-(1 → 6)-*β*-D-glucopyranosyl ester, and named clematomandshurica saponin H.

Compound **4** was obtained as white amorphous powder and its molecular formula $C_{70}H_{114}O_{34}$ was determined by HR-ESI-MS (*m/z* = 1499.7257 $[M + H]^+$ calcd. for $C_{70}H_{115}O_{34}$: 1499.7264), which was supported by the ^{13}C-NMR data (**Tables 2** and **3**). The ^1H- and ^{13}C-NMR data indicated **4** to be a saponin composed of an oleanolic acid and seven monosaccharide units. Acid hydrolysis with 1 M HCl gave oleanolic acid, together with L-arabinose, L-rhamnose, D-ribose, and D-glucose. The coupling constants (5.5–8.0 Hz) of the anomeric protons and ^{13}C-NMR signals of the monosaccharide units suggested the presence of *α*-arabinose, *β*-glucose, *α*-rhamnose, and *β*-ribose. Comparison of the molecular formula and NMR data of **4** with those of **3** revealed that the absence of a terminal glucopyranosyl unit and the linkage of the sugars was established by HMBC correlations and by comparison with literature values [14]. Accordingly, the structure of **4** was identified as oleanolic acid 3-*O*-*β*-D-ribopyranosyl-(1 → 3)-*α*-L-rhamnopyranosyl-(1 → 2)[*β*-D-glucopyranosyl-(1 → 4)]-*α*-L-arabinopyranosyl-28-*O*-*α*-L-rhamnopyranosyl-(1 → 4)-*β*-D-glucopyranosyl-(1 → 6)-*β*-D-glucopyranosyl ester, and named clematomandshurica saponin I.

Compound **5** was obtained as white amorphous powder and its molecular formula $C_{74}H_{112}O_{32}$ was determined by HR-ESI-MS (*m/z* = 1535.7026 $[M + Na]^+$ calcd. for $C_{74}H_{112}O_{32}Na$: 1535.7029). The ^1H- and ^{13}C-NMR spectra indicated the aglycone of **5** was oleanolic acid (**Tables 2** and **4**). Acid hydrolysis with 1 M HCl gave oleanolic acid, together with L-arabinose, L-rhamnose, D-ribose, and D-glucose. The coupling constants (5.5–8.0 Hz) of the anomeric protons suggested the arabinopyranosyl moiety was *α*-configured and the glucopyranosyl and ribopyranosyl moieties were *β*-configured. In the ^{13}C-NMR spectrum of **5**, the C-3 and C-28 carbon signals were observed at *δ* 88.7 and 180.0, respectively, implying that **5** was a monodesmosidic triterpene saponin. Detailed analyses of HSQC, HMBC, and HSQC-TOCSY allowed the unambiguous assignments of the carbon signals in each monosaccharide unit. The linkage of the sugars was established by HMBC correlations. Further analysis of the NMR data (**Table 6**) of **5** revealed the presence of a 3-hydroxy-4-methoxycinnamoyl group (isoferuloyl, IF) [4], which was confirmed by HMBC correlations of IF-H-6 (*δ* 7.10) and IF-C-4 (*δ* 150.9), and of MeO (*δ* 3.73) and IF-C-4 (*δ* 150.9). The correlation observed between GlcI-H-2 (*δ* 5.72) and the carbonyl carbon (*δ* 166.7) indicated the isoferuloyl moiety was linked to GlcI-C-2 (*δ* 74.5). From the above evidence, the structure of **5** was established as oleanolic acid 3-*O*-*α*-L-rhamnopyranosyl-(1 → 6)-(2-*O*-isoferuloyl-*β*-D-glucopyranosyl)-(1 → 4)-*β*-D-ribopyranosyl-(1 → 3)-*α*-L-rhamnopyranosyl-(1 → 2)[*β*-D-glucopyranosyl(1 → 4)]-*α*-L-arabinopyranoside, and named clematomandshurica saponin J.

Compound **6** was obtained as white amorphous powder and its molecular formula $C_{68}H_{102}O_{27}$ was determined by HR-ESI-MS (*m/z* = 1389.6263 $[M + K]^+$ calcd. for $C_{68}H_{102}O_{27}K$: 1389.6240). Comparison of the molecular formula and NMR data of **6** with those of **5** revealed that the terminal D-glucose (GlcII) in the sugar chain at C-3 of **6** was absent. Accordingly, the structure of **6** was identified as oleanolic acid 3-*O*-*α*-L-rhamnopyranosyl-(1 → 6)-(2-*O*-isoferuloyl-*β*-D-glucopyranosyl)-(1 → 4)-*β*-D-ribopyranosyl-(1 → 3)-*α*-L-rhamnopyranosyl-(1 → 2)-*α*-L-arabinopyranoside, and named clematomandshurica saponin K.

The cell growth inhibitory effects of compounds **1**-**7** were tested against the human prostate cancer PC-3 cell line *in vitro*. The concentrations to inhibit 50 % of cell growth (GI_{50}) are shown in **Table 7**. Compounds **5, 6**, and **7** exhibited antiproliferative effects with GI_{50} values of 1.29, 1.50, and 0.71 µM, respectively. No significant inhibitory activity was observed for compounds **1**-**4**. Compounds **5**–**7** are all monodesmosidic triperpene saponins with a free carboxyl at the C-17 position. It may be assumed that the free 28-carboxyl group can increase cell growth inhibition.

Table 7 GI_{50} values of compounds **1**–**7** that inhibit human prostate cancer PC-3 cell growth.

Compounds	GI_{50} ± SE (µM)
1	> 100
2	> 100
3	> 100
4	> 100
5	1.29 ± 0.29
6	1.50 ± 0.48
7	0.71 ± 0.38
Podophyllotoxin	0.62 ± 0.29

The data shown are means ± SE of three independent experiments. PC-3 cells were treated with the compounds for 4 days

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Conflict of Interest

There are no conflicts of interest among all authors.

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