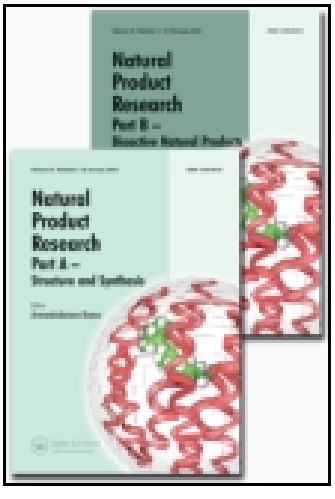

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A new oleanane-type triterpenoidal saponin from *Pulsatilla chinensis*

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A new oleanane-type triterpenoidal saponin, 3-*O*-β-D-glucopyranosyl-hederagenin 23-*O*-α-D-ribofuranoside (**1**), was isolated from the roots of *Pulsatilla chinensis*, and its structure was established on the basis of the spectral data. Compounds **2**, **7** and **8** showed moderate cytotoxic activity by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide assay.

Keywords: *Pulsatilla chinensis*; Ranunculaceae; triterpenoidal saponins; 3-*O*-β-D-glucopyranosyl-hederagenin 23-*O*-α-D-ribofuranoside; cytotoxic activity

1. Introduction

The roots of *Pulsatilla chinensis* (Ranunculaceae) have been used in traditional Chinese medicine for treatment of intestinal amoebiasis, malaria, vaginal trichomoniasis and bacterial infections (Editorial Committee of Chinese Pharmacopeia 2010). During the course of our investigation for cytotoxic agents from the roots of *P. chinensis*, a new oleanane-type triterpenoidal saponin, 3-*O*-β-D-glucopyranosyl-hederagenin 23-*O*-α-D-ribofuranoside (**1**), together with seven known saponins (Figure 1), oleanolic acid 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside (**2**) (Yang et al. 2012), pastuchoside D (**3**) (Mshvildadze et al. 2004), hederacolchiside E (**4**) (Zhang et al. 2000), 23-*O*-α-D-ribofuranosyl-hederagenin 28-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester (**5**) (Liao et al. 2001), 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl-oleanolic acid 28-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl ester (**6**) (Chen et al. 2009), oleanolic acid 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 4)]-α-L-arabinopyranoside (**7**) (Schenkel et al. 1991) and oleanolic acid 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside (**8**) (Mimaki et al. 1999), was obtained. In this paper, we described the isolation and structural elucidation of the new compound, as well as the evaluation of cytotoxic activities of all isolated saponins against A549, SGC-7901 and HL-7702 human tumour cell lines.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder, and its positive-ion HR-ESI-MS spectrum showed a quasi-molecular ion peak at *m/z* 789.4362 $[M + Na]^+$ (calcd 789.4401), indicating the molecular formula of $C_{41}H_{66}O_{13}$. In the ^1H NMR spectrum (Table 1), six methyl

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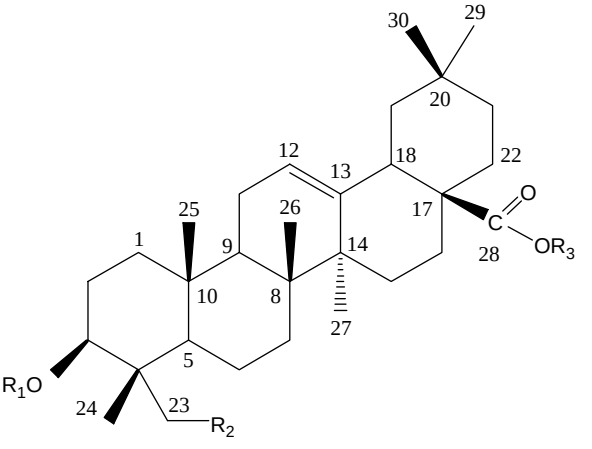

	R_1	R_2	R_3
1	glc	O-rib	H
2	rha (1→6) glc (1→4) glc (1→3) rha (1→2) ara	H	H
3	rha (1→2)[glc (1→4)] ara	OH	H
4	rha (1→2) [glc (1→4)]ara	OH	rha (1→4)glc (1→6) glc
5	H	O-rib	rha (1→4) glc (1→6) glc
6	glc (1→4) glc (1→3) rha (1→2) ara	H	glc (1→6)glc
7	glc (1→3) rha(1→2)[glc(1→4)] ara	H	H
8	glc(1→4) glc (1→3) rha (1→2) ara	H	H

glc: β-D-glucopyranosyl,rha: α-L-rhamnopyranosyl,ara: α-L-arabinopyranosyl,rib: α-D-ribofuranosyl

Figure 1. Structures of compounds **1**–**8**.

singlets were observed at δ_H 1.33 (3H, s, Me-27), 1.17 (3H, s, Me-24), 1.10 (3H, s, Me-30), 1.04 (3H, s, Me-29), 1.03 (3H, s, Me-26) and 0.87 (3H, s, Me-25). The ^{1}H and ^{13}C NMR spectra showed the characteristic signals for a triterpenoidal saponin, especially one olefinic proton at δ_H 5.55 (1H, brs, H-12), two olefinic carbons at δ_C 122.4 (C-12), 145.0 (C-13) and a carboxylic group at δ_C 180.1 (C-28). The aglycone was identified as hederagenin by comparing the NMR spectral data with those of pastuchoside D (Mshvildadze et al. 2004), and the downfield shifts at

Table 1. *In vitro* cytotoxicity of compounds **1**–**8**.

No.	IC_{50} (μM)[a]		
	A549	SGC-7901	HL-7702
1	30.25 ± 2.53	35.35 ± 0.84	30.27 ± 8.79
2	9.89 ± 0.17	7.15 ± 1.45	8.83 ± 2.49
3	>100	>100	>100
4	>100	>100	>100
5	>100	>100	>100
6	>100	>100	>100
7	8.11 ± 0.30	5.41 ± 0.80	9.42 ± 2.23
8	7.33 ± 0.95	7.13 ± 0.77	6.32 ± 1.77
Fluorouracil	1.16 ± 0.13	1.55 ± 0.18	0.86 ± 0.11

Note: [a]Results were calculated from three independent experiments and are shown as mean ± SD.

δ 85.7 (+12.2 ppm, C-3) and δ 78.4 (+9.5 ppm, C-23) indicated that compound **1** was a bisdesmosidic saponin (Zhang et al. 2002). The ^{1}H NMR spectrum also showed two anomeric protons at δ_H 5.29 (1H, d, $J = 8.0$ Hz) and 5.37 (1H, d, $J = 6.0$ Hz), giving HSQC correlations with anomeric carbons at δ_C 105.5 (C-1 of glucose) and 103.8 (C-1 of ribose) indicating the presence of two sugar units in compound **1**. The types of sugar units were identified as D-ribose and D-glucose upon acid hydrolysis and gas chromatography (GC) analysis (Gao et al. 2011). The glycosidic side chain located at C-3 of the aglycone was identified as β-D-glucopyranosyl, which was confirmed by the HMBC correlation between δ_H 3.35 (H-3 of aglycone) and δ_C 105.5 (C-1 of glucose), as well as correlation between δ_H 5.29 (H-1 of glucose) and δ_C 85.7 (C-3 of aglycone), and the β-configuration of glucopyranosyl group was identified on the observation of the large $^3J_{H\text{-}1,H\text{-}2}$ coupling constant. Another glycosidic side chain at C-23 was elucidated as α-D-ribofuranosyl, which was evidenced by the HMBC correlation between δ_H 3.40, 4.01 (H-23 of aglycone) and δ_C 103.8 (C-1 of ribose), as well as correlation between δ_H 5.37 (H-1 of ribose) and δ_C 78.4 (C-23 of aglycone) and the α-configuration of ribofuranosyl group was indicated by correlations between δ 5.37 (H-1 of ribose) and δ 4.76 (H-3 of ribose) in the NOESY spectrum (Liao et al. 2001). Consequently, compound **1** was elucidated as 3-*O*-β-D-glucopyranosyl-hederagenin 23-*O*-α-D-ribofuranoside.

Cytotoxic activity of compounds **1**–**8** were evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay and expressed as IC_{50} values. The results indicated that only compounds **2**, **7** and **8** showed moderate cytotoxic activity, with IC_{50} values of 9.89 ± 0.17, 8.11 ± 0.30 and 7.33 ± 0.95 μM for A549 cell line; 7.15 ± 1.45, 5.41 ± 0.80 and 7.13 ± 0.77 μM for SGC-7901 cell line and 8.83 ± 2.49, 9.42 ± 2.23 and 6.32 ± 1.77 μM for HL-7702 cell line. Our results suggested that saponins might be, at least in part, responsible for the proposed therapeutic effect of roots of *P. chinensis*.

3. Experimental

3.1. *General experimental procedures*

Melting points were determined by the XT5 micro-melting point apparatus (XT5, Beijing families instrument light instrument plant, China) and were uncorrected. Optical rotations were obtained on a Perkin-Elmer model 241 polarimeter. IR spectra were taken on a Perkin-Elmer 983 G spectrometer. ^{1}H, ^{13}C and 2D NMR spectra were recorded on a Varian Inova 500 spectrometer in C_5D_5N using tetramethylsilane as internal standard. HR-ESI-MS spectra were determined on a Micromass Q-TOF2 spectrometer. Semi-preparative HPLC was carried out on a column of octadecyl bonded silica (ODS) (250 mm × 9.4 mm i.d., Agilent Zorbax SB-C_{18}, Palo Alto, CA, USA) with a Waters 2996 detector, the flow rate was 2 mL min^{-1} and the wavelength for detection was 203 nm. Medium-pressure liquid chromatography (MPLC) was carried out on a column of ODS (460 mm × 26 mm i.d., Büchi Borosilikat 4.6, Flawil, Swiss). Precoated plates of silica gel used for TLC were obtained from Qingdao Marine Chemical Factory, Qingdao, China. Compounds on the TLC were coloured by 10% sulphuric acid alcohol solution. A549 lung carcinoma cell, SGC-7901 gastric carcinoma cell and HL-7702 hepatocellular carcinoma cell were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

3.2. *Plant material*

Dried roots of *P. chinensis* were collected from Suizhong City, Liaoning provinces in July 2010, and were authenticated by Professor Xiao-ran Li in Soochow University. A voucher specimen (No. 10-07-05-01) has been deposited in the herbarium of the College of Pharmaceutical Science, Soochow University.

3.3. *Extraction and isolation*

The dried plant material (1 kg) was extracted twice with 70% EtOH (10 L) under reflux. The solvent was subsequently removed under reduced pressure to give the residue (120 g), which was dissolved in distilled water and partitioned with petroleum ether, $CHCl_3$, EtOAc and *n*-BuOH. The *n*-BuOH partition (61.2 g) was dissolved in distilled water and passed through D101 macroporous resin column (3.0 × 60 cm, Xi'an Sunresin New Materials Co. Ltd, Xi'an, China), eluted with a gradient of aqueous EtOH (EtOH–H_2O 0%, 30%, 60%, 90%, each 2.5 L). The 60% EtOH eluate (15 g) was then separated into 10 fractions (fractions 1–10) by reversed-phase MPLC eluted with gradient mixtures of MeOH and H_2O [40:60 (2 L), 80:20 (5 L), 100:0 (2 L)] with a flow rate of 30 mL min^{-1}. Fraction 4 (38 mg) was separated by semi-preparative HPLC to afford compound **4** (23 mg), using MeOH–H_2O (70:30) as mobile phase. Compounds **3** (79 mg), **4** (114 mg), **5** (132 mg) and **6** (46 mg) were obtained from fraction 5 (741 mg) by semi-preparative HPLC eluted by MeOH–H_2O (71:29). Fraction 6 (267 mg) was separated by semi-preparative HPLC to afford compounds **3** (12 mg), **6** (37 mg) and **8** (31 mg) using MeOH–H_2O (72:28) as mobile phase. The separation of fraction 7 (276 mg) by repeated semi-preparative HPLC, using MeOH–H_2O (70:30) as mobile phase, led to compounds **1** (13 mg), **7** (43 mg) and **8** (37 mg). Fraction 9 (271 mg) was separated by semi-preparative HPLC to afford compounds **2** (53 mg) and **7** (51 mg), eluted by MeOH–H_2O–HCOOH (78:22:0.1).

3.3.1. *3-O-β-D-glucopyranosyl-hederagenin 23-O-α-D-ribofuranoside (**1**)*

White amorphous powder; mp > 300°C; $[\alpha]^{20} + 37.1$ (*c* 0.05, MeOH); IR (KBr) ν_{max} 3415, 2933, 1741, 1645, 1380 and 1041 cm^{-1}; ^{13}C NMR (125 MHz, C_5D_5N) δ: 39.0 (C-1), 23.9 (C-2), 85.7 (C-3), 37.2 (C-4), 51.6 (C-5), 18.0 (C-6), 33.4 (C-7), 39.9 (C-8), 48.0 (C-9), 37.5 (C-10), 23.7 (C-11), 122.4 (C-12), 145.0 (C-13), 42.1 (C-14), 28.4 (C-15), 23.7 (C-16), 46.8 (C-17), 42.3 (C-18), 46.7 (C-19), 31.1 (C-20), 34.4 (C-21), 32.7 (C-22), 78.4 (C-23), 13.9 (C-24), 16.6 (C-25), 17.4 (C-26), 26.2 (C-27), 180.1 (C-28), 33.4 (C-29), 23.9 (C-39), 105.5 (C-1 of glucose), 75.3 (C-2 of glucose), 78.6 (C-3 of glucose), 71.8 (C-4 of glucose), 78.5 (C-5 of glucose), 62.7 (C-6 of glucose), 103.8 (C-1 of ribose), 71.2 (C-2 of ribose), 70.6 (C-3 of ribose), 83.9 (C-4 of ribose), 64.5 (C-5 of ribose); ^1H NMR (500 MHz, C_5D_5N) δ: 0.99, 1.49 (2H, m, H-1), 1.40, 1.47 (2H, m, H-2), 3.35 (1H, dd, $J = 11.0$ and 5.5 Hz, H-3), 0.85 (1H, t, $J = 7.0$ Hz, H-5), 1.14, 1.34 (2H, m, H-6), 1.91, 2.13 (2H, m, H-7), 1.73 (1H, m, H-9), 1.65, 1.94 (2H, m, H-11), 5.55 (1H, brs, H-12), 1.22, 2.20 (2H, m, H-15), 2.04, 2.22 (2H, m, H-16), 3.39 (1H, m, H-18), 1.37, 1.89 (2H, m, H-19), 1.28, 1.52 (2H, m, H-21), 1.25, 1.45 (2H, m, H-22), 3.40, 4.01 (2H, d, $J = 10.5$ Hz, H-23), 1.17 (3H, s, H-24), 0.87 (3H, s, H-25), 1.03 (3H, s, H-26), 1.33 (3H, s, H-27), 1.04 (3H, s, H-29), 1.10 (3H, s, H-30), 5.29 (1H, d, $J = 8.0$ Hz, H-1 of glucose), 4.12 (1H, m, H-2 of glucose), 4.22 (1H, m, H-3 of glucose), 4.27 (1H, m, H-4 of glucose), 3.98 (1H, m, H-5 of glucose), 4.39, 4.58 (2H, m, H-6 of glucose), 5.37 (1H, d, $J = 6.0$ Hz, H-1 of ribose), 5.00 (1H, m, H-2 of ribose), 4.76 (1H, m, H-3 of ribose), 4.76 (1H, m, H-4 of ribose), 4.36, 4.69 (2H, dd, m, H-5 of ribose); HR-ESI-MS (positive-ion mode), $m/z = 789.4362$ $[M + Na]^+$ (calcd 789.4401).

3.4. *Acid hydrolysis of compound 1*

Compound **1** (about 4 mg) was added into a solution of water (1 mL) and 2 N aqueous CF_3COOH (2 mL), heated to 120°C under reflux conditions for 4 h. The mixture was diluted with water (2 mL) and then extracted with EtOAc (3 × 2 mL). The combined organic phase was washed with brine and evaporated to dryness to afford the aglycones. The aqueous phase was concentrated. Then dry pyridine (1 mL) and L-cysteine methyl ester hydrochloride (2 mg) were added into the residue. Each mixture was reacted at 60°C for 1 h, and 0.5 mL of (trimethylsilyl)

imidazole dissolved in H_2O was added, followed by heating to dryness at 60°C for 2 h. Each dried reactant was extracted with *n*-hexane (3 × 1 mL) and H_2O (1 mL, each). The *n*-hexane fraction was subjected to GC (column: Rtx-1, 0.25 mm i.d. 0.25 μm, length 30 m). The conditions of GC were flame ionization detector; column temperature 100–180°C (10°C min^{-1}) and 180–230°C (3°C min^{-1}); injector temperature 250°C; detector temperature 300°C and the carrier gas (N_2, 0.8 mL min^{-1}). Under these conditions, these sugars of each reactants were identified by comparison with authentic samples: t_R (min) 7.56 (D-ribose), 7.85 (L-ribose), 10.49 (D-glucose) and 11.10 (L-glucose).

3.5. *Cytotoxic activity*

To evaluate the cytotoxic activity of saponins from the roots of *P. chinensis* against A549, SGC-7901 and HL-7702 human tumour cell lines, the MTT colorimetric assay had been carried out with fluorouracil as positive control. The amount of formazan was determined by photometer at 570 nm. Cells were plated into 96-well flat-bottomed cultured plates at a concentration of 5×10^4 cells per well in complete Roswell Park Memorial Institute 1640 culture medium. Twenty-four hours after plating, the medium containing foetal calf serum was removed and test solutions were given to cells in various final concentrations such as 2.5, 5, 10, 20, 50 and 100 μg mL^{-1}. After incubation with drugs for 24 h, MTT solution was added to the wells and plates were incubated at 37°C for 4 h. Results were expressed as percentage of the absorbance in control cells compared to that in the drug-treated cells. IC_{50} values (50% inhibitory concentration) of compounds **1**–**8** are shown in Table 1.

4. Conclusions

A new oleanane-type triterpenoidal saponin, 3-*O*-β-D-glucopyranosyl-hederagenin 23-*O*-α-D-ribofuranoside (**1**), together with seven known saponins, ws isolated from the roots of *P. chinensis*, and compounds **2**, **7** and **8** showed moderate cytotoxic activities against human A549, SGC-7901 and HL-7702 human tumour cell lines.

Supplementary material

Supplementary material relating to this article is available online, alongside Figures S1–S8.

Acknowledgements

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