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New 23, 27-dihydroxy-oleanane-type triterpenoid saponins from *Anemone Raddeana* Regel

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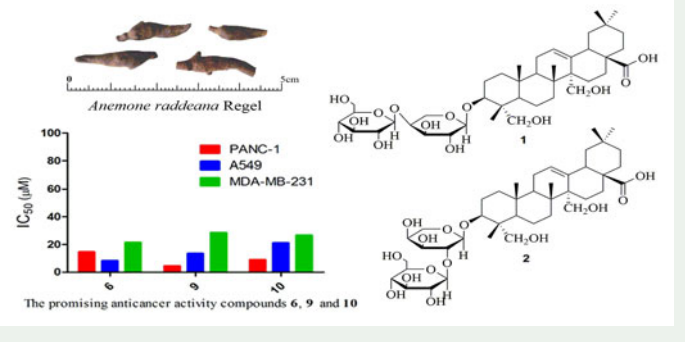
ABSTRACT

Two new 23, 27-dihydroxy-oleanane-type triterpenoid saponins named Raddeanoside Rf and Raddeanoside Rg (**1** and **2**), along with thirteen known triterpenoid saponins (**3**–**15**) were isolated from the rhizome of *Anemone raddeana* Regel. Their structures were determined by chemical and spectral analysis, including 1 D, 2 D NMR data and HRESIMS. The type of aglycone 23, 27-dihydroxy oleanolic acid is extremely rare in natural products. In addition, the anti-cancer activity for all the compounds were evaluated. Compounds **9** and **10** exhibited significant cytotoxicity with IC_{50} values of 4.47 and 8.97 μM against human pancreatic cancer lines (PANC-1), while compound **6** with IC_{50} value of 8.19 μM against human lung lines (A549). The possible structure-activity relationships of these triterpenoid saponins were also tentatively discussed.



The promising anticancer activity compounds **6**, **9** and **10**

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Anemone raddeana Regel; triterpenoid saponins; cytotoxicity

1. Introduction

The genus *Anemone* consists about 150 species distributed throughout the world. About 17 species of *Anemone* in China are traditional herbal medicines (Editorial Committee of the Flora of China 1980; Zhang et al. 2019; Zhou et al. 2007).

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Liangtoujian, which is recorded in pharmacopeia of the People's Republic of China, is a well-known traditional Chinese medicine for the treatment of rheumatism and pain. It is derived from the dried rhizome of *Anemone raddeana* Regel (National Pharmacopoeia Committee 2015). Modern research has proved that *A. raddeana* has various pharmacological action such as anti-inflammatory, antipyretic and antitumor (Guan et al. 2015; Lu et al. 2009). Phytochemical research showed triterpenoid saponin was the major bioactive components of *A. raddeana* (Zhang et al. 2017). Some of them have marked antitumor activity, such as Pulsatilla Saponin D (Jang et al. 2014) and Pulsatilla saponin A (Tong et al. 2017).

Therefore, it is necessary to conduct a more in-depth study of the anticancer activities of triterpenoid saponin in *A. raddeana*. As part of a continuous investigation, two new 23,27-dihydroxy oleanolic acid-type compounds named Raddeanoside Rf and Raddeanoside Rg (**1** and **2**), together with thirteen known ones (**3**–**15**) were isolated and elucidated. It is worth noting that the type of aglycone 23, 27-dihydroxy oleanolic acid is extremely rare in natural products and the methyl group at C-27 oxidation to hydroxymethyl group is considered as an impossible reaction due to steric hindrance and reactive selectivity. The anti-cancer activity for all the compounds were evaluated. Compounds **6**, **9** and **10** exhibited significant cytotoxicity (Table S1).

2. Results and discussion

The rhizome of *A. raddeana* afforded two new compounds (**1**–**2**) and thirteen known compounds (**3**–**15**) (Figure 1) by repeated column chromatography and preparative reversed-phase high-performance liquid chromatography, as described in experimental section. The known compounds were identified by comparison of experimental and reported spectroscopic data as Mateglycoside D (**3**) (Tiwari and Singh 1978), Eleutheroside K (**4**) (Liao et al. 2001), Hederacholichiside E (**5**) (Liao et al. 1999), Hederacolchiside A1 (**6**) (Li et al. 2018), oleanolic acid 3-*O*-β-D-glucopyranosyl(1→4)-α-L-arabinopyranoside (**7**) (Li et al. 1990), Raddeanoside 22 (**8**) (Fan et al. 2010), Raddeanoside 23 (**9**) (Fan et al. 2010), oleanolic acid 3-*O*-β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→2)[β-D-glucopyranosyl(1→4)]-α-L-arabinopyranoside (**10**) (Lv et al. 2016), Pulsatiloside D (**11**) (Liao et al. 1999), Leontoside B (**12**) (Li et al. 1990), Leontoside A (**13**) (Li et al. 1990), Raddeanoside 20 (**14**) (Lu et al. 2009), Raddeanoside 12 (**15**) (Lu et al. 2002). The new triterpenoids were identified by comprehensive spectroscopic analyses (1 D and 2 D NMR, HRESIMS). The sugar residues were identified by HPLC analysis after hydrolysis.

Compound **1** was isolated as a white, amorphous powder. The positive results of Molish and Liebermann-Burchard test suggested that **1** might be a triterpenoid glycoside or a steroidal glycoside. The HRESIMS (negative ion mode) showed the protonated pseudo-molecular ion peak at *m/z* 827.4470 $[M + HCOOH\text{-}H]^-$ (calcd. for $C_{42}H_{67}O_{16}$, 827.4429), corresponding to the molecular formula $C_{41}H_{66}O_{14}$. The ^1H NMR spectrum (Table S2) exhibited five angular methyl groups at δ_H 1.03, 1.02, 0.91, 0.89 and 0.88. The ^{13}C NMR spectrum (Table S2) showed 41 carbon signals, of which 30 were the aglycone. Compared with hederagenin (23-hydroxy oleanolic acid), the angular methyl single C-27 (δ_C 26.0) disappeared and a single at δ_C 65.2 appeared (Wang et al. 2014). The data

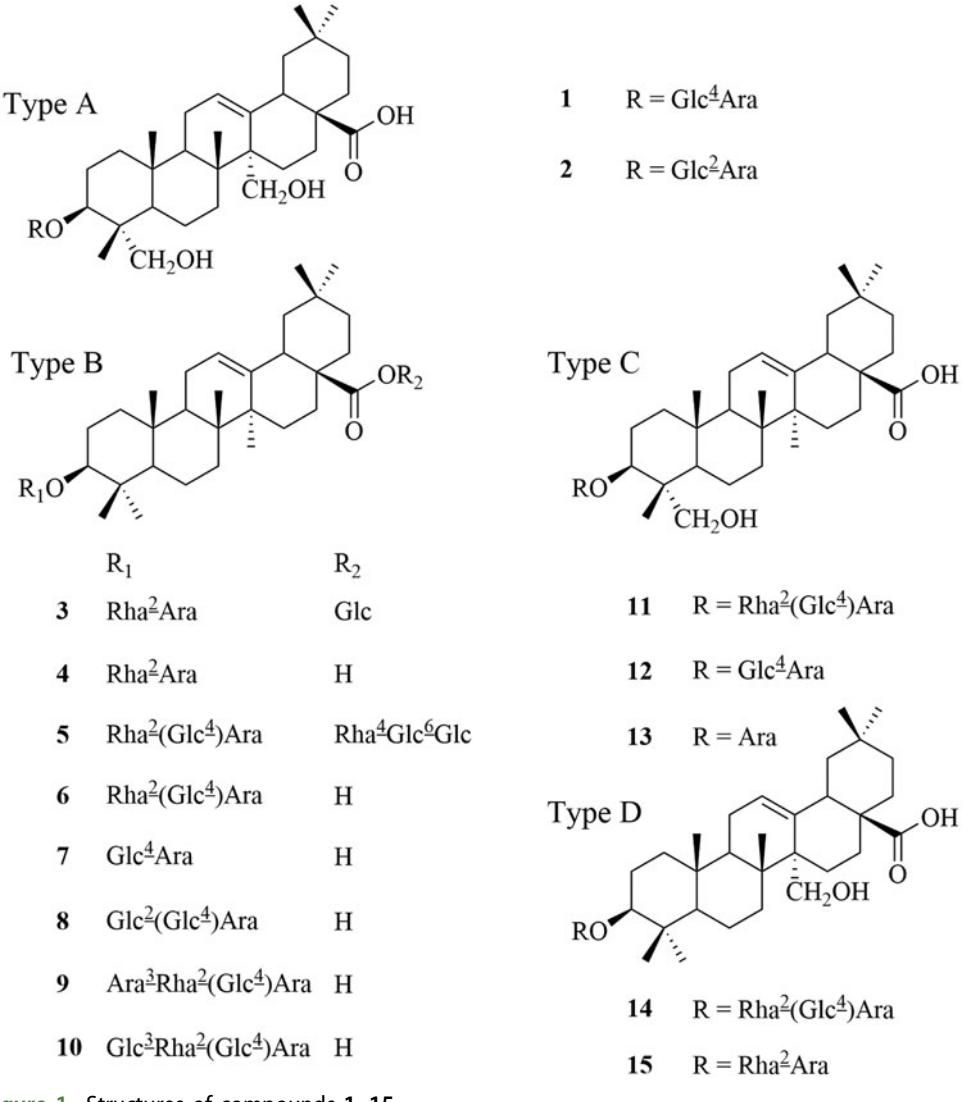

Figure 1. Structures of compounds **1**–**15**.

hinted that C-27 may be a hydroxymethyl group. The above data showed that the aglycone of compound **1** maybe 23, 27-dihydroxy oleanolic acid. The structure was further confirmed in HMBC spectrum by the correlation (Figure S1). The long-range correlations were observed between H_2-23 (δ_H 4.24, 3.66, *d*, $J = 10.9$ Hz) of aglycone and C-3 (δ_C 83.2), C-4 (δ_C 44.2), C-5 (δ_C 48.2) of the aglycone. Meanwhile, the correlations were observed between H_2-27 (δ_H 3.82, 4.09, *d*, $J = 12.1$ Hz) of aglycone and C-8 (δ_C 41.2), C-13 (δ_C 141.0), C-14 (δ_C 48.7), and C-15 (δ_C 24.7) of the aglycone. The above data confirmed the aglycone of compound **1** was 23,27-dihydroxy oleanolic acid.

On acid hydrolysis of compound **1**, L-arabinose (L-Ara) and D-glucose (D-Glc) were identified by comparison with retention time of their standard sugar derivatives on HPLC. Meanwhile, according to the 2 D NMR (^{1}H-^{1}H COSY, HSQC, HMBC and TOCSY) spectra, an arabinose unit (δ_C 107.1, 74.0,75.0, 80.4 and 67.1) and a glucose unit (δ_C

107.1, 76.2, 78.7, 72.0 79.1 and 63.2) were confirmed. On the base of terminal proton signals and anomeric carbons, the sugar moieties were determined as β for D-glucopyranose and α-configuration for L-arabinopyranose (Pei et al. 2011).

The correlations between the anomeric signal at δ_H 4.94 (Ara H-1) and δ_C 83.2 (C-3) of the aglycone, revealed a substitution at C-3 of the aglycone by an arabinose unit. Meanwhile, a long-range correlation between δ_H 5.17 (Glc H-1) and δ_C 80.4 (Ara C-4) indicated that the (1→4) linkage between these two sugars. Detailed analysis of the ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, TOCSY, HSQC, HMBC and DEPT data indicated that Glc and Ara are unambiguously supported the glyosidic linkages. Hence, compound **1** was elucidated as 23,27-dihydroxy oleanolic acid 3-O-β-D-glucopyranosyl(1→4)-α-L-arabinopyranoside, and it was named Raddeanoside Rf.

Compound **2** was obtained as a white, amorphous powder. The negative ion mode HRESIMS of **2** showed the pseudo-molecular ion at *m/z* 827.4479 $[M + HCOOH\text{-}H]^-$ (calcd. for $C_{42}H_{67}O_{16}$, 827.4429), in accordance with the molecular formula $C_{41}H_{66}O_{14}$. By comparison of the ^{13}C NMR signals of **2** with those of **1** (Table S2), the aglycone was consistent with 23,27-dihydroxy oleanolic acid. It should be noted that there is a missing of chemical shift in ^{13}C NMR of C-28 for compound **2**. It's a common phenomenon that sometimes there's no response with quaternary carbons in the ^{13}C NMR. However, the chemical shift of C-28(181.7) can be easily confirmed clearly with the help of HMBC correlation. A long-range correlation between δ_H 1.75 (C-16)) and δ_C 181.7 (C-28) was observed. With the same method as compound **1**, the sugar units were confirmed as one α-L-arabinopyranose and one β-D-glucopyranose unit. In the HMBC experiment, the long-range correlation between the anomeric proton at δ_H 5.26 (Glc H-1) and the carbon at δ_C 80.5 (Ara C-2) indicated that the glucose (Glc) was attached to position 2 of the arabinose. Detailed analysis of the data indicated that Glucose and Arabinose are unambiguously supported glyosidic linkages (Figure S1). Hence, compound **2** named Raddeanoside Rg was elucidated as 23,27-dihydroxy oleanolic acid 3-O-β-D-glucopyranosyl(1→2)-α-L-arabinopyranoside.

All the isolated compounds in present word were tested for their inhibitory activity against three human cancer cell lines by MTT method (Table S1). Among them, compounds **9** and **10** exhibited significant cytotoxicity with IC_{50} values of 4.47 and 8.97 μM against human pancreatic cancer lines (PANC-1), while compound 6 with IC_{50} value of 8.19 μM against human lung lines (A549). The inhibitory effects of all the monomers on MDA-MB-231 cells were weaker than those of the other two tumor cells. Only compounds **6**, **9** and **10** showed moderate activity to MDA-MB-231 cells with the values of 21.35, 28.52, and 26.65 μM, respectively. The results hinted that the sensitivity of different tumor cell lines to the above monomer was quite different.

The aglycone of all the isolated compounds could be divided into four types according to hydroxymethyl substitution position (Figure 1), 23,27-dihydroxy oleanolic acid (type A), oleanolic acid (type B), 23-hydroxy oleanolic acid (type C) and 27-hydroxy oleanolic acid (type D). The saponins with the aglycone of type B displayed stronger cytotoxic activity than those possessing the same sugar chain but the other type of aglycone (Table S1 and Figure 1). The presence of hydroxyl group at C-23 (type C) or C-27 (type D) would reduce the activity, especially hydroxyl group at C-27.

When both existed at the same time (type A), activities were further weakened or even disappeared.

The cytotoxicities of above saponin were also closely related to their sugar moiety. Comparing the anti-proliferative activity of compounds **3** and **5** with **4** and **6**, respectively, we hypothesized that the free carboxyl groups at C-28 played an important role for cytotoxic activity. The results are consistent with literature (Wang, et al. 2014). Compounds **6** and **9** possessing the Rha(1→2)[Glc(1→4)]Ara had a better activity than diglycosidic compounds **4** and **7**. Meanwhile they showed stronger activity than compound **8** which linked a Glc(1→2)[Glc(1→4)]Ara. These implied that the presence of rhamnopyranose linked at C-2 or glucopyranose linked at C-4 of the arabinpyranosyl group was propitious to enhance the cytotoxic activity. According to the data of **4**, **6**, **9** and **10**, we confirmed that the extension of Rha(1→2)Ara group was important for antitumor activity. It's in accordance with the report of Raddeanin A (Ma et al. 2018) and Pulsatilla Saponin D (Chen et al. 2018).

3. Experimental section

3.1. General

Optical rotations were measured on a MCP200 polarimeter (Anton Paar, Graz, Austria). IR spectra were obtained on a Shimadzu ftir-8400s spectrophotometer (Shimadzu Corporation, Tokyo, Japan). HRESIMS experiments were performed on a Micro TOF spectrometer (Bruker Co., Karlsruhe, Germany). NMR spectra were recorded at 600 MHz for ^{1}H and 150 MHz for ^{13}C on Bruker AV-600 instruments (Bruker Co., Billerica, MA, USA). Preparative HPLC was conducted using a Shimadzu LC-10A instrument with an SPD-10A detector (Shimadzu, Kyoto, Japan) and YMC-Pack ODS-A column (250 mm × 20 mm, 5 mm). HPLC method was conducted using an Agilent 1260 (Agilent Technologies, Inc., Santa Clara, USA) with DAD detector. A Thermo ODS-2 Hypersil analytical column (250 mm × 4.6 mm, 5 μm, Thermo Scientific Co., USA) was used. 2-Methylphenyl Isothiocyanate and L-cysteine methyl ester hydrochloride were purchased from Sigma Co. (Shanghai, China). All of the cell lines, including human pancreatic cancer lines (PANC-1), human lung lines (A549) and human breast adenocarcinoma (MDA-MB-231) cell lines were obtained from the American Type Culture Collection (ATCC). All the medium was purchased from Gibco company (Shanghai, China).

3.2. Plant material

The rhizome of *Anemone raddeana* Regel was collected in Liaoning Province (China) and authenticated by Prof. Qishi Sun (Department of Medicinal Plant, Shenyang Pharmaceutical University). A voucher specimen (No. 001875) was deposited in the Herbarium of Shenyang Pharmaceutical University.

3.3. Extraction and isolation

Air-dried and powered rhizome of *A. raddeana* (4.0 kg) was refluxed with 75% EtOH for three times. The combined alcohol extracts were concentrated under reduced pressure to afford a residue (520 g), which was suspended in H_2O (10 L) and partitioned successively by petroleum ether (10 L × 3), chloroform (10 L × 3), EtOAc (10 L × 3), and n-BuOH (15 L × 3). The n-butanol fraction (210 g) was eluted with EtOAc-MeOH-H_2O (10:1:0-30:10:5 gradient system) to afford eight fractions (B1–B8). Fr.B4 (38.2 g) was separated by silica gel column chromatography (7.5 × 60 cm) with Dichlone-MeOH-H_2O as the mobile phase (7:1:0.1-6:4:0.5) to afford eight subfractions Fr.B4-1-Fr.B4-8. Fr.B4-1 (20 mg) was further purified by a preparative HPLC using MeOH: H_2O (82:18) to yield compound **13** (4 mg, t_R 102.6 min). Fr.B4-2 was purified by a preparative HPLC using MeOH: H_2O (85:15) to yield compounds **15** (10 mg, t_R 47.0 min) and **4** (30 mg, t_R 152.4 min). Compound **7** (22 mg, t_R 122.6 min) was obtained from Fr.B4-3 (52 mg) by a preparative HPLC eluted with MeOH: H_2O (85:15). Fr.B4-4-1 (60 mg) was further purified by a preparative HPLC using MeOH: H_2O (74:26) to yield compounds **1** (8 mg, t_R 67.1 min), **2** (7 mg, t_R 77.6 min) and **3** (5 mg, t_R 214.4 min). Compound **12** (106 mg, t_R 104.1 min) was obtained from Fr.B4-4-2 (300 mg) by a preparative HPLC eluted with MeOH: H_2O (78:22). Compound **6** (400 mg, t_R 127.6 min) was obtained from Fr.B4-5 (650 mg) by a preparative HPLC eluted with MeOH: H_2O (82:18). Fr.B4-6 (300 mg) was purified by a preparative HPLC using MeOH: H_2O (82:18) to yield compound **14** (171 mg, t_R 43.7 min). Fr.B4-7 (180 mg) was eluted by a preparative HPLC using MeOH: H_2O (77:23) to yield compounds **8** (29 mg, t_R 214.9 min), **9** (45 mg, t_R 236.2 min), **10** (7 mg, t_R 253.6 min), and **11** (25 mg, t_R 98.1 min). Fr.B4-8 (104 mg) was purified by a preparative HPLC using MeOH: H_2O (72:28) to yield compound **5** (20 mg, t_R 77.1 min).

3.3.1. Raddeanoside Rf (1)

White amorphous solid; $[\alpha]_D^{20}$ +52.7 (*c* 0.125, MeOH); IR (KBr): ν_{max} (cm^{-1}): 3435, 2945, 1638, 1075; ^1H and ^{13}C NMR (C_5D_5N, 600 MHz and 150 MHz) data, see Table S2; HRESIMS *m/z* 827.4470 $[M + HCOOH\text{-}H]^{-1}$ (calcd. for $C_{42}H_{67}O_{16}$, 827.4429).

3.3.2. Raddeanoside Rg (2)

White amorphous solid; $[\alpha]_D^{20}$ +34.8 (*c* 0.125, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3427, 2943, 1641, 1072. ^1H and ^{13}C NMR (C_5D_5N, 600 MHz and 150 MHz) data, see Table S2; HRESIMS *m/z* 827.4479 $[M + HCOOH\text{-}H]^{-1}$ (calcd. for $C_{42}H_{67}O_{16}$, 827.4429).

3.4. Determination of the absolute configuration of the sugars

Compounds **1** and **2** (2.0 mg) were hydrolyzed with 2 M HCl (5.0 mL), heated for 6 h at 90 °C and extracted with $CHCl_3$ (3 × 5.0 mL). Then, the aqueous layer was concentrated in vacuo and concentrated to dryness to give a residue, which was dissolved in pyridine (1.0 mL), and then L-cysteine methyl ester hydrochloride (3.0 mg) was added to the solution. The mixture was heated at 60 °C for 1 h, then 2-Methylphenyl Isothiocyanate was added to the reaction mixture and further reacted at 60 °C for 60 min. The reaction product was analyzed by HPLC and detected by UV detector (at 250 nm). The reaction mixture was analyzed with a Thermo ODS-2 Hypersil analytical

column. The elution was performed by a gradient system of water-phosphoric acid as solvent A (1000:0.5) and acetonitrile as solvent B. The gradient program was carried out as follows: 20–40% B in 0–50 min. The flow rate was kept 1 mL/min and the column temperature was at 30 °C. The sugar parts from compounds **1** and **2** were compared to an authentic sugar sample: 15.70 min (L-glucose); 16.62 min (D-glucose); 17.75 min (L-arabinose); 18.63 min (D-arabinose).

3.5. Cytotoxicity assay

Cytotoxic effects of all compounds against PANC-1, A549, and MDA-MB-231 cell lines were measured by MTT assay (Han et al. 2017). The cells were seeded into 96-well culture plates (Costar, USA) at a density of 6×10^3 cells per well. After overnight incubation, the cells were treated with various concentrations of compounds for 72 h. Then 20 μL MTT solution was added to each well and incubated for an additional 4 h at 37 °C. The supernatant was removed by centrifugation, followed by the addition of 150 μL DMSO. The absorbances at 570 nm were measured for the cells using Thermo microplate reader. The IC_{50} values of the test compounds were calculated with SPSS 19.0 software.

4. Conclusion

In present work, two new 23, 27-dihydroxy-oleanane-type triterpenoid saponins named Raddeanoside Rf and Raddeanoside Rg (**1** and **2)**, along with 13 known oleanane-type triterpenoid saponins (**3**–**15**) were isolated and identified from the rhizome of *Anemone raddeana* Regel. In addition, the anti-cancer activity for all the compounds were evaluated. Compounds **6**, **9** and **10** exhibited significant cytotoxicity. Meanwhile, the possible structure-activity relationships of these triterpenoid saponin compounds were also tentatively discussed.

Disclosure statement

The authors declare no conflicts of interest.

Funding

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