ABSTRACT

Two new triterpenoids (1–2) were isolated and elucidated from the roots of *Gypsophila oldhamiana*, together with four known triterpenoids (3–6). Their structures were identified to be 3β-hydroxy-olean-13(18)-ene-23, 28-dioic acid (1), 3β, 12α-dihydroxy-23-carboxyolean-28, 13β-olide (2), 3β, 16α-dihydroxy-23-oxoolean-13(18)-en-28-oic acid (3), gypsogenin (4), quillaic acid (5) and gypsogenic acid (6) by spectral methods. All compounds were tested for their cytotoxicities against human tumour cell lines (lung cancer H460 and gastric cancer SGC-7901) and for their antiangiogenic effects using a zebrafish model. All compounds showed interesting antiangiogenic activities and the significant cytotoxicities against H460.

1. Introduction

*Gypsophila oldhamiana* Miq. known as ‘xia cao’ in China belongs to the Caryophyllaceae family. It is distributed in the north regions of China. Its roots have been used as a traditional Chinese medicine to treat fever, consumptive disease, infantile malnutrition and so on (The Chinese Medicine Dictionary 1977). The roots of *Gypsophila* species are an especially rich source of triterpenoid saponins (Hostettmann & Marston 1995; Yotova et al. 2012) and have some alkaloids (Zhang et al. 2015). The triterpenoid saponins from *Gypsophila* species

CONTACT Jing-Yong Sun yaowusu018@163.com

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/14786419.2015.1107060

© 2015 Taylor & Francis
were reported to have cytotoxic activities (Bai et al. 2007; Arslan et al. 2012; Arslan et al. 2013), α-glucosidase inhibition activities (Yao et al. 2010) and so on. The aglycone of the above-mentioned compounds are gypsogenin, gypsogenic acid and quillaic acid (Böttger & Melzig 2011), as well as vaccaric acid (Luo & Kong 2006) and segetalic acid (Yao et al. 2010). In previous studies, we found that G. oldhamiana is also a rich source of triterpenoids (Bai et al. 2007; Wu et al. 2011). As part of a continuing effort to discover secondary metabolites from local medicinal plants, the major constituents of the cytotoxic fraction from the extract of G. oldhamiana were investigated. Herein, in this study, we report the isolation and structure elucidation of two new triterpenoids (1–2), together with four known compounds (3–6) (Figure 1). Additionally, the compounds 1–6 were also investigated for their cytotoxic activities against human tumour cell lines (lung cancer H460 and gastric cancer SGC-7901) and for their antiangiogenic effects using a zebra fish model.

2. Results and discussion

The methanol extract of the air-dried roots of G. oldhamiana was fractionated into petroleum ether (60–90 °C) fraction, EtOAc fraction, n-BuOH fraction and water fraction. Then, the above four fractions were tested for their cytotoxic activities against human tumour cell lines (lung cancer H460), and the IC50 values of the petroleum ether (60–90 °C) fraction, the EtOAc fraction, the n-BuOH fraction and the water fraction were 196.45, 12.87, 50.46 and 33.22 μg/mL, respectively. The cytotoxic activity of the EtOAc extract was the highest. So, the portion EtOAc was subjected to successive column chromatography to give compounds 1–6.

Compound 1 was isolated as white solid powder. Its molecular formula was determined to be C30H46O5 on the basis of negative HR-ESI-MS (m/z: 485.3272 [M − H]−, calcd for C30H45O5 485.3270), which was also confirmed by the 13C NMR and DEPT spectral data. The IR spectrum of 1 indicated absorption bands for hydroxyl (3156 cm⁻¹) and carbonyl (1706 cm⁻¹) functions. The 1H NMR spectrum of 1 displayed six methyl singlets at δ 1.64 (H-24), 0.98 (H-25), 1.16 (H-26), 1.18 (H-27), 0.96 (H-29), 0.85 (H-30) and a signal signable to one methine protons bearing a hydroxyl group at δ 4.74 (H-3, 1H, dd, J = 12 Hz, 4 Hz). The 13C NMR and DEPT spectra revealed the presence of six methyl carbons, eleven methylene carbons, three methine carbons and ten quaternary carbons. The 13C NMR spectra also demonstrated the presence of two carboxyl carbons at δ 179.7 (C-23), 178.1 (C-28), two sp² carbons of a tetrasubstituted olefin at δ 128.5 (C-13) and δ 136.9 (C-18), one oxymethine carbon δ 74.6 (C-3) and six methyl

Figure 1. Chemical structures of compounds 1–6.
carbons (δ 31.5, 23.6, 20.4, 17.2, 16.2, 11.3). Specifically, the spectroscopic data of 1 indicated a close relationship with 3β-16α-dihydroxy-23-oxoolean-13(18)-ene-28-oic acid (Chang et al. 1989) apart from the presence of a carboxyl group at C-23 position and the absence of a hydroxyl group at C-16 position. In the HMBC spectrum, correlation signals from δ 4.74 (H-3) to δ 27.2 (C-2), 53.7 (C-4), 179.7 (C-23) and 11.3 (C-24) were exhibited, which confirmed the location of the hydroxyl group at C-3. In the NOESY contour map, correlations between H-3 and H-5 were detected. This correlation enables to confirm that H-3 was α-oriented. Furthermore, the presence of a carboxyl group at C-23 position was also confirmed by the correlations between H-25 and H-24, H-26. The correlations between H-29 and H-30 and between H-27 and H-9, H-16 were also detected in the NOESY spectrum. From the above data, the structure of 1 was deduced to be 3β-hydroxyolean-13(18)-ene-23, 28-dioic acid.

Compound 2 was isolated as white solid powder. Its molecular formula was determined to be C_{30}H_{46}O_{6}. HR-ESI-MS spectrum of 2 measured in the negative ion mode gave a quasi-molecular ion peak at m/z: 501.3222 [M − H]^−, calcld for C_{30}H_{45}O_{6} 501.3218. The IR spectrum of 2 indicated absorption bands for hydroxyl (3491 cm \(^{-1}\)) and carbonyl (1754, 1696 cm \(^{-1}\)) functions. The absorption at 1754 cm \(^{-1}\) suggested a γ-lactone carbonyl group (Song et al. 2009). The \(^1\)H NMR spectrum of compound 2 displayed six methyl singlets at δ 1.66 (H-24), 0.98 (H-25), 1.30 (H-26), 1.57 (H-27), 0.87 (H-29), 0.93 (H-30) and signals corresponding to two methine protons bearing a hydroxyl group at δ 4.70 (H-3, dd, J = 12 Hz, 4 Hz) and 4.06 (H-12, d, J = 4 Hz). The \(^13\)C NMR and DEPT spectra revealed the presence of six methyl carbons, ten methylene carbons, five methine carbons and nine quaternary carbons. The \(^13\)C NMR spectra further demonstrated the presence of two carbonyl carbon at δ 179.7 (C-23) and δ 177.4 (C-28), three oxymethine carbons δ 74.6 (C-3), 68.5 (C-12) and 90.0 (C-13) and six methyl carbons (δ 11.1, 16.0, 17.1, 20.3, 21.9, 32.1). From the above data, we found the signals in NMR spectra arising from rings B, C, D and E of 2 were very similar to 3β, 12α-dihydroxy-23-oxoolean-28, 13β-olide (Ali et al. 2002; García-Granados et al. 2004; Csuk & Siewert 2011) except for the signals from the ring A, a carboxyl group at C-23 position, in 2 instead of those due to a methyl group at C-23 position. In the HMBC spectrum, correlation signals from δ 4.06 (H-12) to δ 22.7 (C-11), δ 43.5 (C-14), δ 52.4 (C-17) and δ 90.0 (C-13) and from H-12 and δ 3.15 (H-18, dd, J = 11 Hz, 4 Hz) to C-13 were exhibited, which confirmed the location of a hydroxyl and a hydroxyl lactone groups at C-12 and C-13. Furthermore, according to the signals of the HR-ESI-MS and the IR, we concluded that compound 2 was the γ-lactone (13 → 28). The HMBC cross-peaks of δ 4.70 (H-3) to δ 26.9 (C-2), 53.7 (C-4), 179.7 (C-23) and 11.1 (C-24) confirmed the location of the hydroxyl group at C-3. Additional correlations between H-3 and H-5 and between H-12 and H-26 were also detected in the NOESY map. These correlations enable to confirm that H-3 was α-oriented and H-12 was β-oriented. The presence of a carboxyl group at C-23 position was also confirmed by the correlations between H-25 and H-24, H-26 in the NOESY map. From the above data, the structure of 2 was deduced to be 3β, 12α-dihydroxy-23-carboxyolean-28, 13β-olide.

Compounds 3–6 known were isolated as white, amorphous powder. They were identified as 3β, 16α-dihydroxy-23-oxoolean-13(18)-en-28-oic acid (3) (Chang et al. 1989), gypsogenin (4) (Nie et al. 1989), quillaic acid (5) (Jacobsen et al. 1996) and gypsogenic acid (6) (Wang et al. 1983).

The cytotoxic activities of 1–6 against the growth of human tumour cell lines (lung cancer H460 and gastric cancer SGC-7901) were evaluated in vitro, using cisplatin as a positive control. All of compounds displayed cytotoxic activities against the human tumour cell lines.
Compounds 1–6 showed the significant cytotoxicities against H460 (IC\textsubscript{50} values of compounds 1–6 are 7.14, 9.90, 7.76, 14.02, 6.91 and 8.31 μM). Compounds 4 and 5 also presented the strong cytotoxicities against SGC-7901 (IC\textsubscript{50} values of 4 and 5 are 14.36 and 8.00 μM). Therefore, these triterpenoids of the EtOAc extract make a contribution to the cytotoxic activity of the roots of G. oldhamiana.

The antiangiogenic activities of compounds 1–6 were tested using a zebrafish model, in terms of the inhibition on the growth of intersegmental vessels, with PTK787 as a positive control. Accordingly, all compounds showed interesting antiangiogenic activities. At a 5 μg/mL concentration, compound 1 showed 100% inhibition on the growth of intersegmental vessels, as good as PTK787.

3. Experimental

3.1. General experimental procedures

The 1D and 2D NMR spectra were obtained on a Bruker Avance 400 spectrometer (\textsuperscript{1}H: 400 MHz, \textsuperscript{13}C: 100 MHz) with TMS as internal standard in C\textsubscript{6}D\textsubscript{5}N. Chemical shifts were given in values of ppm and coupling constants in Hertz. IR (KBr) spectra were recorded by Thermo Nicolet Nexus 670 FT-IR. HR-ESI-MS were obtained on a Bruker Daltonics Inc. APEXII spectrometer. Optical rotations were obtained in the solvents specified with a JASCO P-1020 polarimeter (JASCO, Tokyo, Japan). Preparative HPLC was conducted using a KNAUER K-501 HPLC pump and UV Detector K-2501 with a Agilent-C\textsubscript{18} column (21.2 × 150 mm, 7 μm). Analytical HPLC was recorded using Waters 2695 with a Waters-C\textsubscript{18} column (4.6 × 250 mm, 5 μm). Silica gel (200–300 mesh, Qingdao Marine Chemical Industry Factory, Qingdao, China), and Sephadex LH-20 (25–100 μm, Pharmacia Fine Chemical Co., Ltd. Uppsala, Sweden) were used for column chromatography. TLC was performed on precoated silica gel plates (Qingdao Haiyang Chemical Co.), using the solvent systems CHCl\textsubscript{3}–MeOH (9:1), and detection was obtained by 10% sulphuric acid/EtOH, followed by heating at 100 °C for triterpenoids.

3.2. Plant material

The roots of G. oldhamiana were collected from Laoshan Mountain in Qingdao, Shandong, China, in August 2000. They were identified by Prof Li-Li Sun in Shandong Academy of Chinese Medicine. A voucher specimen (No. S200008) was stored in the Institute of Materia Medica, Shandong Academy of Medical Sciences, Jinan, Shandong Province China.

3.3. Extraction and isolation

The air-dried roots (5 kg) of G. oldhamiana were extracted with methanol (MeOH) under refluxing three times. The methanol extract was evaporated in vacuo to obtain a yellow residue (1000 g). Then, the residue was dissolved by water (2 L) and extracted with petroleum ether (60–90 °C) (3 × 2.0 L), EtOAc (3 × 2.0 L) and n-BuOH (3 × 2.0 L) to obtain an petroleum ether (60–90 °C) extract (19 g), an EtOAc extract (45 g), a n-BuOH extract (165 g) and water extract (266 g). The portion EtOAc was subjected to silica gel column chromatography (CC SiO\textsubscript{2}, 200–300 mesh) with a gradient system of CHCl\textsubscript{3}–MeOH (100:0 → 80:20) to give ten fractions (F1–F10). F2 (4.1 g), eluted with CHCl\textsubscript{3}–MeOH (95:5), was chromatographed to
silica gel CC and eluted with a CHCl$_3$ and MeOH gradient from 100:0 to 100:10, giving six subfractions (F2.1–F2.6). Then, they were purified using Sephadex LH-20 CC and eluted with MeOH (each fraction 10 mL), and further purified by preparative HPLC to give 1 (15.7 mg), 3 (9.3 mg), 4 (7.2 mg), 5 (11.4 mg), 6 (8.2 mg). F3 (2.4 g), eluted with CHCl$_3$–MeOH (90:10), was applied to silica gel CC and eluted with a CHCl$_3$ and MeOH gradient from 100:0 to 100:15 to afford six subfractions (F3.1–3.6). F3.4 was further purified by preparative TLC with CHCl$_3$ and MeOH (85:15) to give 2 (7.5 mg).

### 3.4. Compound 1
White solid powder, m.p. 214–216 °C; [α]$_D^{20} = -95.6$ (c = 0.66, MeOH); IR (KBr) $\nu_{\max} = 3156, 2944, 1706, 1452, 1387, 1266, 1042$ cm$^{-1}$; positive HR-ESI-MS (calcd for C$_{30}$H$_{45}$O$_5$ [M – H]$^- 485.3270$, found 485.3272). $^1$H NMR (C$_5$D$_5$N, 400 MHz) $\delta$: 4.74 (H-3, 1H, dd, $J = 12, 4$ Hz), 2.06–2.00 (H-5, 1H, m), 1.73–1.66 (H-9, 1H, m), 1.64 (H$_{3-24}$ 3H, s), 0.98 (H$_{3-25}$ 3H, s), 1.16 (H$_{3-26}$ 3H, s), 1.18 (H$_{3-27}$ 3H, s), 0.96 (H$_{3-28}$ 3H, s), 0.85 (H$_{3-29}$ 3H, s); $^{13}$C NMR (C$_5$D$_5$N, 100 MHz) $\delta$: 38.6 (C-1), 27.2 (C-2), 74.6 (C-3), 53.7 (C-4), 51.1 (C-5), 20.9 (C-6), 34.4 (C-7), 43.9 (C-8), 50.6 (C-9), 36.2 (C-10), 21.3 (C-11), 24.7 (C-12), 128.5 (C-13), 41.3 (C-14), 32.7 (C-15), 35.6 (C-16), 48.0 (C-17), 136.9 (C-18), 40.7 (C-19), 32.1 (C-20), 36.5 (C-21), 26.9 (C-22), 179.7 (C-23), 11.3 (C-24), 16.2 (C-25), 17.2 (C-26), 20.4 (C-27), 178.1 (C-28), 31.5 (C-29), 23.6 (C-30).

### 3.5. Compound 2
White solid powder, m.p. 258–261 °C; [α]$_D^{20} = +28.2$ (c = 0.39, CH$_3$OH); IR (KBr) $\nu_{\max} = 3491, 2954, 1754, 1696, 1449, 1387, 1227, 1118$ cm$^{-1}$; positive HR-ESI-MS (calcd for C$_{30}$H$_{45}$O$_6$ [M – H]$^- 501.3218$, found 501.3222). $^1$H NMR (C$_5$D$_5$N, 400 MHz) $\delta$: 4.70 (H-3, 1H, dd, $J = 12, 4$ Hz), 2.02–1.95 (H-5, 1H, m), 1.57–1.50 (H-9, 1H, m), 4.06 (H-12, d, $J = 4$ Hz), 3.15 (H-18, dd, $J = 11$ Hz, 4 Hz), 1.66 (H$_{3-24}$ 3H, s), 0.98 (H$_{3-25}$ 3H, s), 1.30 (H$_{3-26}$ 3H, s), 1.57 (H$_{3-27}$ 3H, s), 0.87 (H$_{3-28}$ 3H, s), 0.93 (H$_{3-29}$ 3H, s); $^{13}$C NMR (C$_5$D$_5$N, 100 MHz) $\delta$: 38.6 (C-1), 26.9 (C-2), 74.6 (C-3), 53.7 (C-4), 50.9 (C-5), 17.8 (C-6), 33.9 (C-7), 41.3 (C-8), 48.9 (C-9), 35.8 (C-10), 22.7 (C-11), 68.5 (C-12), 90.0 (C-13), 43.5 (C-14), 34.1 (C-15), 20.3 (C-16), 52.4 (C-17), 38.0 (C-18), 35.4 (C-19), 29.1 (C-20), 34.9 (C-21), 27.1 (C-22), 179.7 (C-23), 11.1 (C-24), 16.0 (C-25), 17.1 (C-26), 20.3 (C-27), 177.4 (C-28), 32.1 (C-29), 21.9 (C-30).

### 3.6. Cytotoxicity assays
The procedure for the cytotoxic activity was performed according to the MTT method (Mosmann, 1983; Gerlier & Thomasset 1986). In this study, human tumour cell lines (lung cancer H460 and gastric cancer SGC-7901) were used. In brief, the human tumour cells were incubated in RPMI 1640 medium (Gibco Co., USA) containing 10% foetal bovine serum supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin in a 96-well plate at 37 °C for 24 h, while suspended cells were seeded with an initial density of 1 × 105 cells mL$^{-1}$. Then, the compounds were added and cells were cultured for 48 h at 37 °C, with cisplatinum as positive controls. Last, 10 μL of 5 mg/mL MTT (Sigma, St. Louis, MO, USA) in phosphate-buffered saline was added to each well, and the tumour cells were incubated for another 4 h at 37 °C in carbon dioxide incubator. The supernatant was collected from each well, and 200 μL of DMSO was added. The absorbance of the produced formazan was measured at 560 nm.
in a microplate reader. The inhibition (%) was calculated, and the IC\textsubscript{50} value defined as the concentration of sample necessary to inhibit the growth to 50% of the control was calculated by a modified Karber formula.

### 3.7. Antiangiogenesis assay

The compounds 1–6 dissolved by 100% DMSO were prepared. These solutions were diluted in sterile salt water (5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl\textsubscript{2}, 0.16 mM MgSO\textsubscript{4}) to obtain final solutions of various concentrations in 0.1% DMSO. Aliquots were placed into 96-well culture plate. Then, zebrafish embryos at 24 hpf (hours post-fertilisation) were transferred into 96-well culture plate. PTK787 was used as the positive control. All embryos were incubated at 28.5 °C. After 48 h of treatment, the intersegmental vessels of embryos were visualised with green fluorescent protein labelling and endogenous alkaline phosphatase staining. The antiangiogenic activities of compounds were calculated from the inhibition ratio of angiogenesis (Murphey & Zon 2006).

### 4. Conclusion

The structures of the two new triterpenoids and four known compounds were established by 1D and 2D NMR techniques, other spectroscopic evidence and comparison with the literature. Compounds 1–6 showed the significant cytotoxicities against H460 and interesting antiangiogenic activities. Compounds 4 and 5 also presented the strong cytotoxicities against SGC-7901. At a 10.29 μM concentration, compound 1 showed 100% inhibition on the growth of intersegmental vessels, as good as PTK787. Further investigations are ongoing in our laboratory.

### Supplementary material

Supplementary material relating to this article is available online, alongside Tables S1–S3 and Figures S1–S20.

### Disclosure statement

No potential conflict of interest was reported by the authors.

### Funding

This work was supported by Projects of Science and Technology Development Planning of Shandong Province [grant number 2013GSF11854] and Projects of Medical and Health Technology Development Program in Shandong Province [grant number 2013WS0351].

### References

Ali MS, Jahangir M, Hussan SS, Choudhary MI. 2002. Inhibition of \( \alpha \)-glucosidase by oleanolic acid and its synthetic derivatives. Phytochemistry 60:295–299.

Arslan I, Celik A, Choi JH. 2012. A cytotoxic triterpenoid saponin from underground parts of Gypsophila pilulifera Boiss. & Heldr. Fitoterapia. 83:699–703.
Arslan I, Celik A, Melzig MF. 2013. Nebulosides A-B, novel triterpene saponins from under-ground parts of Gypsophila arrostii Guss. var. nebulosa. Bioorg Med Chem. 21:1279–1283.

Bai H, Zhong Y, Xie YY, Wang YS, Liu L, Zhou L, Mu YL, Zuo CX. 2007. A major triterpenoid saponin from Gypsophila oldhamiana. Chem Biodivers. 4:955–960.

Böttger S, Melzig MF. 2011. Triterpenoid saponins of the Caryophyllaceae and Illecebraceae family. Phytochem Lett. 4:59–68.

Chang IS, Han YB, Woo WS, Kang SS, Lotter H, Wagner H. 1989. Sapogenins from Melandrium firmum. Planta Med. 55:544–547.

Csuk r, Siewert B. 2011. A convenient separation of ursolic and oleanolic acid. Tetrahedron Lett. 52:6616–6618.

García-Granados A, López PE, Melguizo E, Parra A, Simeó Y. 2004. Partial synthesis of C-ring derivatives from oleanolic and maslinic acids. Formation of several triene systems by chemical and photochemical isomerization processes. Tetrahedron 60:1491–1503.

Gerlier D, Thomasset N. 1986. Use of MTT colorimetric assay to measure cell activation. J Immunol Methods. 94:57–63.

Hostettmann K, Marston A. 1995. Saponins. In: Phillipon JD, Ayres DC, Baxter H, editors. Chemistry and pharmacology of natural products. Cambridge: Cambridge University Press; p. 326–327.

Jacobsen Ne, Kensil CR, Lim A, Wheeler DA, Powell MF, Fairbrother WJ. 1996. Structure of the saponin adjuvant QS-21 and its base-catalyzed isomerization product by 1H and natural abundance 13C NMR spectroscopy. Carbohydr Res. 280:1–14.

Jiangsu New Medical College. 1977. The Chinese Medicine Dictionary. Shanghai: Shanghai People’s Publishing House. 2170.

Luo JG, Kong LY. 2006. A pair of new nortriterpene saponin epimers from the roots of Gypsophila oldhamiana. Helv Chim Acta. 89:947–953.

Luo JG, Ma L, Kong LY. 2008. New triterpenoid saponins with strong α-glucosidase inhibitory activity from the roots of Gypsophila oldhamiana. Bioorg Med Chem. 16:2912–2920.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65:55–63.

Murphey rD, Zon Li. 2006. Small molecule screening in the zebrafish. Methods 39:255–261.

Nie RL, Tanaka T, Miyakoshi M, Kasai R, Morita T, Zhou J, Tanaka O. 1989. A triterpenoid saponin from Thladiantha hookeri var. pentadactyla. Phytochemistry 28:1711–1715.

Song SJ, Qiu F, Wu LJ. 2009. Infrared spectroscopy. In: Wu LJ, Qiu F, Kong LY, Liu YX, Song SJ, Lou HX, editors. Organic spectroscopic analysis. Beijing: China Medical Science Press; p. 74–77.

Wang DZ, Pu XY, Fu J, Yang TR. 1983. 13C NMR spectra of some natural triterpenes olean-12-enes. Acta Botanica Yunnanica. 5:437–442.

Wu HY, An K, Li HB, Wang J, Sun JY, Zong Y. 2011. Progress on chemical ingredients and pharmacological effects of Gypsophila oldhamiana. Food Drug. 5:213–216.

Yao S, Ma L, Luo JG, Wang JS, Kong LY. 2010. New triterpenoid saponins from the roots of Gypsophila paniculata L. Helv Chim Acta. 93:361–374.

Yotova M, Krasteva I, Nikolov S. 2012. Triterpenoid saponins from Genus Gypsophila L. (Caryophyllaceae). In: Koh r, Tay I, editors. Saponins: properties, applications and health benefits. New York: Nova Science Publishers; p. 99–122.

Zhang YM, Wang G, Lv HW, Luo JG, Kong LY. 2015. Two new β-carboline alkaloids from the roots of Gypsophila oldhamiana. Nat Prod Res. 29:1207–1211.