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Triterpenoids with α-glucosidase inhibitory activity and cytotoxic activity from the leaves of *Akebia trifoliata*†

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Ten pentacyclic triterpenoids including a new multiflorane triterpene acid, 2α,3β,23-trihydroxymultiflor-7-en-28-oic acid (**1**), and a new lupane triterpene monoglucoside named akebiaoside C (**2**), were obtained from the leaves of *Akebia trifoliata*. Their structures were elucidated by extensive spectroscopic analysis, and they were all isolated from the leaves of *A. trifoliata* for the first time. These compounds, except **4** and **5**, showed *in vitro* α-glucosidase inhibitory activity much stronger than acarbose. Especially, **2**, **3**, **6**, **8** and **10** displayed *in vitro* α-glucosidase inhibitory activity with IC_{50} values from 0.004 to 0.081 mM, which were close or even more potent than corosolic acid (IC_{50} 0.06 mM). Triterpenoids **1**, **8** and **10** were further revealed to show moderate *in vitro* cytotoxic activity against human tumor A549, HeLa and HepG2 cell lines, with IC_{50} values ranging from 26.5 to 51.9 μM. Compound **9** selectively showed *in vitro* cytotoxicity toward HeLa and HepG2 cell lines, with IC_{50} values of 81.49 and 73.47 μM, respectively. These findings provided new data to support that the leaves of *A. trifoliata* are a rich source in bioactive triterpenoids highly valuable to be developed for medicinal usage.

Introduction

Akebia trifoliata, traditionally a medicinal plant in China, is naturally widely distributed in eastern Asia countries like Japan, Korea, and China.[1] This perennial liana plant is capable of producing large edible fruits with tremendous potential as a new fruit crop.[2] As a traditional Chinese folk medicine, *A. trifoliata* has been used as a diuretic and an antiphlogistic with a long history.[3,4] In recent years, some phytochemical studies have been carried out on the stems and fruits of this plant, by which many chemical constituents including triterpenoids, phenolics and lignans were revealed, and some of them displayed significant bioactivities.[5–11]

A. trifoliata is typically also a deciduous plant with most of its leaves reproducible and collectable in large scale annually, implicating that the leaves of *A. trifoliata* might potentially be a promising source for some bioactive chemicals. Meanwhile, few phytochemical studies have been conducted on the leaves of this plant in the past decades. Very recently, with the aim of clarifying potential bioactive chemicals in the leaves of *A. trifoliata*, we initiated a phytochemical investigation on the leaves of this plant, by which two new triterpene saponins were firstly identified.[12] In continuation of this work, ten pentacyclic triterpenoids including a new multiflorane triterpene acid (**1**) and a new lupane triterpene monoglucoside (**2**) are here further obtained from the leaves of *A. trifoliata* (Fig. 1). Herein, we report the isolation and structural elucidation of these compounds, along with the tests of their *in vitro* α-glucosidase inhibitory activity and their cytotoxic activity against three human tumor cell lines.

Results and discussion

Compound **1** was obtained as a white amorphous powder with molecular formula $C_{30}H_{48}O_5$ as determined by HR-ESI-MS, *m*/*z* 511.3378 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3394), which requires seven degrees of unsaturation. The ^1H NMR spectrum of **1** (Table 1) showed signals recognizable for six tertiary methyls at δ_H 1.08 (3H, s), 1.07 (3H, s), 0.99 (3H, s), 0.95 (3H, s), 0.84 (3H, s) and 0.77 (3H, s), an oxymethylene at δ_H 3.45 (1H, d, J = 11.0 Hz, Ha-23) and 3.22 (1H, d, J = 11.0 Hz, Hb-23), two

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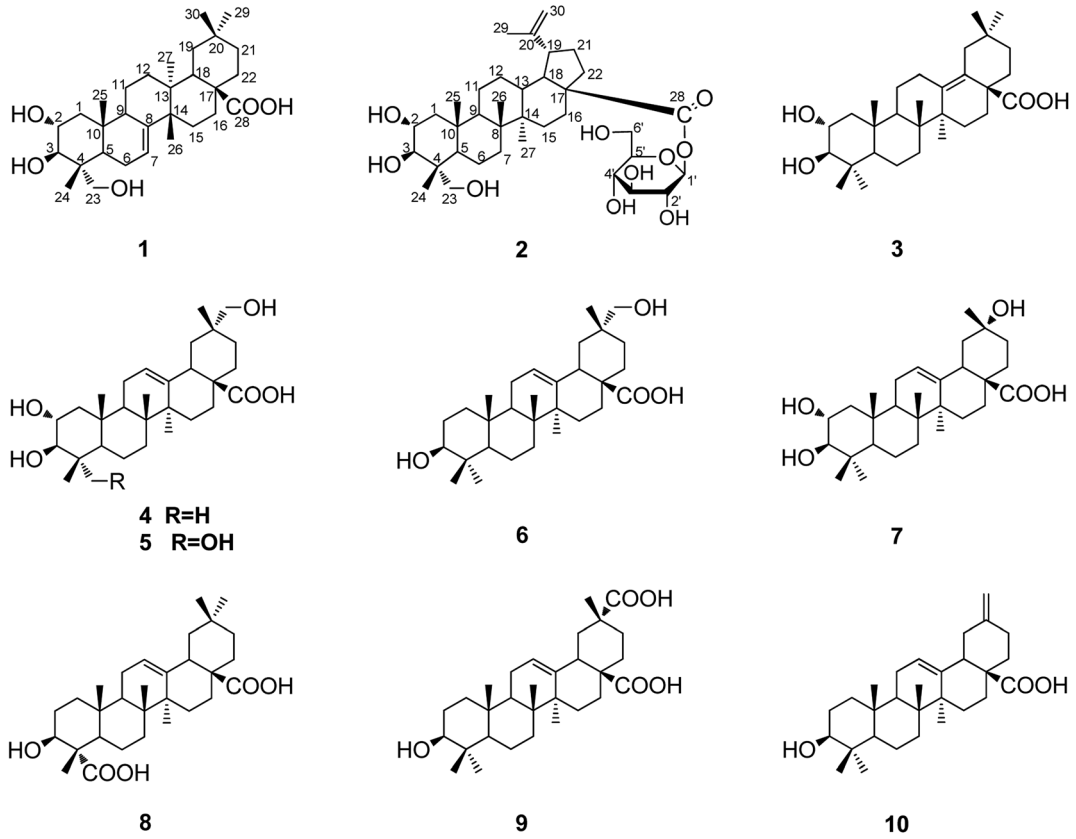


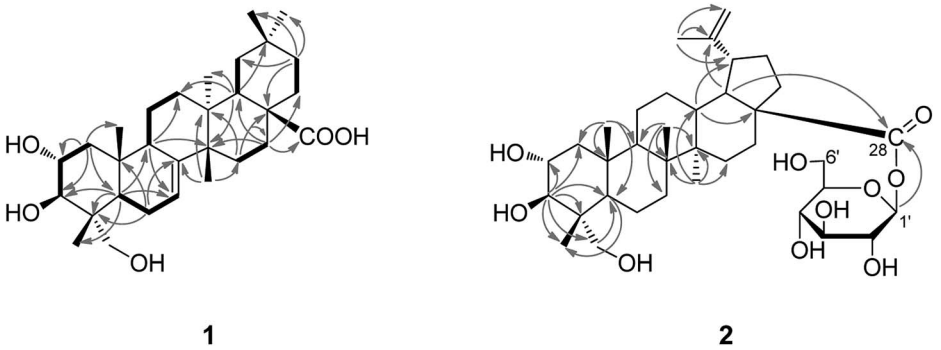
Fig. 1 Chemical structures of compounds **1–10**.

oxymethines at δ_H 3.68 (1H, m, H-2) and 3.36 (1H, d, $J = 9.3$ Hz, H-3), and an olefinic proton at δ_H 5.45 (1H, br.s, H-7). The ^{13}C-NMR (DEPT) spectra (Table 1) supported the above analysis, which indicated the presence of 30 carbons including six methyl groups, ten methylenes [with one oxygenated at δ_C 66.0 (C-23)], six methines [including an olefinic methine at δ_C 119.3 (C-7) and two oxymethines at δ_C 69.5 (C-2) and 78.1 (C-3)], and seven quaternary carbons [including an olefinic quaternary carbon at δ_C 149.3 (C-8) and a carboxyl carbon at δ_C 180.4 (C-28)]. These above findings, accounted for two of the seven degrees of unsaturation, supported that **1** is a pentacyclic triterpenoid with three –OH groups, a trisubstituted C=C bond, and a COOH group in the molecule. Coupled with HSQC spectral analysis, the three OH groups can each be located at δ_C 69.5 (C-2), 78.1 (C-3) and δ_C 66.0 (C-23), respectively, and all the other ^{1}H- and ^{13}C-NMR spectral data can be assigned as shown in Table 1. In the ^{1}H–^{1}H COSY spectrum, the observation of proton spin-coupling correlations of H-2 (δ_H 3.68) with H_2-1 (δ_H 1.95,

Table 1 The ^{1}H- and ^{13}C-NMR spectral data (in CD_3OD) of compound **1**[a]

No.	δ_C (**1**)	δ_H (**1**)	No.	δ_C (**1**)	δ_H (**1**)
1	46.5 CH_2	1.93 (m), 1.03 (m)	16	31.8 CH_2	2.15 (m), 1.62 (m)
2	69.5 CH	3.68 (m)	17	44.4 CH_2	—
3	78.1 CH	3.36 (d, 9.6)	18	42.6 CH	2.56 (m)
4	44.2 C	—	19	36.5 CH_2	1.41 (m), 1.22 (m)
5	43.2 CH	1.75 (m)	20	29.4 C	—
6	24.8 CH_2	2.08 (m), 1.95 (m)	21	35.1 CH_2	1.49 (m), 1.48 (m)
7	119.3 CH	5.45 (br.s)	22	34.7 CH_2	1.38 (m), 1.21 (m)
8	149.3 C	—	23	66.0 CH_2	3.45 (d, 10.8), 3.22 (d, 10.8)
9	50.7 CH	2.29 (m)	24	13.1 CH_3	0.77 (s)
10	37.0 C	—	25	15.2 CH_3	0.84 (s)
11	18.7 CH_2	1.62 (m), 1.53 (m)	26	29.5 CH_3	1.07 (s)
12	38.3 CH_2	1.73 (m), 1.61 (m)	27	26.1 CH_3	1.08 (s)
13	37.9 C	—	28	180.4 C	—
14	42.2 C	—	29	33.8 CH_3	0.95 (s)
15	34.5 C	1.81 (m), 1.72 (m)	30	31.7 CH_3	0.99 (s)

[a] Recorded at 600 MHz for ^{1}H- and at 100 MHz for ^{13}C-NMR data, δ in ppm and J in Hz.



1 2

Fig. 2 $^{1}H-^{1}H$ COSY (▬) of **1** and selected HMBC (⟶) of **1** and **2**.

3.45) and H-3 (δ_H 3.36), and of H-6 (δ_H 2.08, 1.95) with H-5 (δ_H 5.45) and H-7 (δ_H 1.75), permitted to establish the structural fragments of C-1–C-2–C-3 and C-5–C-6–C-7 (Fig. 2). Besides, $^{1}H-^{1}H$ COSY correlation signals corresponding to the structural fragments of C-9–C-11–C-12, C-15–C-16, C-18–C-19 and C-21–C-22 were also presented (Fig. 2). In the HMBC spectrum, the exhibition of $^{1}H-^{13}C$ long-range correlations of H-5 (δ_H 1.75) with C-3 (δ 78.1), C-9 (δ 50.7), C-23 (δ 66.0), C-24 (δ 13.1) and C-25 (δ 15.2), of H-1 (δ 1.95, 1.03) with C-5 (δ 43.4) and C-25 (δ 15.2), of H-7 with C-9, and of H-9 (δ 2.29) with C-5, revealed the connections of C-4 with C-5, C-23, Me-24; C-10 (δ 37.0) with C-1 (δ 46.5), C-5, C-9, Me-25 (δ 15.2), and the connection of C-9 with C-8 (δ 149.3). The correlations of Me-26 with C-8, C-13, C-15, of H-18 with C-12, C-14, C-27, C-28, of H_2-16 (δ_H 1.75) with C-18, C-22, C-28, and of H-15 with C-17, indicated the connections of C-13 with C-12, C-14, C-18, Me-27, the connections of C-14 with C-8, C-15, Me-26, and the connections of C-17 with C-16, C-18, C-22 and C-28. The observed HMBC correlations of H-18 with C-20, of H-21 with C-19, Me-29 and Me-30, indicated the linkages of C-20 with C-19, C-21, Me-29 and Me-30. Furthermore, the NOE correlations of H-2 with Me-24 (δ_H 0.77) and Me-25 (δ_H 0.84), and the presented proton spin-coupling constant of H-3 ($J_{H\text{-}2,H\text{-}3}$ = 9.6 Hz) supported the α- and β-orientation of the –OH groups at C-2 and C-3, respectively.[5] The NOE correlations of H-2 with Me-24, Me-25, and of H-5 with H-3, H_2-23 and H-9, confirmed the α-orientation of H-5, H-9 and 23-CH_2OH. The α-orientation of Me-27 and the β-orientation of H-18, Me-26, Me-30, were supported by significant NOE correlations of Me-27 with H-9, and of H-18 with Me-26, Me-30. Eventually, the whole structure of compound **1**, as shown in Fig. 1, was established as 2α,3β,23-trihydroxymultiflor-7-en-28-oic acid.

Compound **2**, $C_{36}H_{58}O_{10}$ (positive HR-ESI-MS showed $[M + Na]^+$ *m*/*z* 673.3916, calcd for $C_{36}H_{58}O_{10}Na$ 673.3922) was also obtained as a white powder. The ^{1}H and ^{13}C NMR spectra of **2** showed one sugar anomeric proton at δ_H 6.42 (Glc I-1) and an anomeric carbon at δ_C 95.3, suggesting the existence of a sugar moiety in the structure. Acid hydrolysis of **2** with 2 N HCl released the sugar unit from the molecule, which was identified to be a D-glucose as determined by GC-MS analysis of its chiral derivatives (see Experimental part). The detailed ^{1}H- and ^{13}C-NMR assignments of the D-glucose moiety in **2** (as listed in Table 2) were established by interpretation of combined HSQC and HMBC data. Apart from the signals due to the D-glucopyranose moiety, the remaining signals in the ^{1}H NMR spectrum for the aglycone of **2** were readily recognized for five tertiary methyls at δ_H 1.71 (3H, s), 1.15 (3H, s), 1.03 (3H, s), 0.97 (3H, s) and 0.92 (3H, s), two olefinic protons at δ_H 4.86, (1H, br.s) and 4.73 (1H, br.s), two oxymethine protons at δ_H 4.25 (1H, m) and 4.21 (1H, d, J = 9.3 Hz), and two protons for a hydroxymethylene group at δ_H 4.20 and 3.70 (each 1H, d, J = 10.4 Hz). The ^{13}C NMR spectrum indicated, besides the signals for the glucose moiety, 30 carbons for the aglycone unit, including five methyls, eleven methylenes [including an exomethylene at δ_C 109.9 (C-30), and a hydroxymethylene at δ_C 66.2 (C-23)], seven methines (including two oxygenated methines at δ_C 69.0 and 78.0), and seven quaternary carbons (including an olefinic quaternary carbon at δ_C 150.7 and a carboxyl carbon at δ_C 174.8). By comparison, it was found that the ^{1}H- and ^{13}C-NMR spectroscopic data (Table 2) of the aglycone of **2** were closely related to those of known compound hovenic acid (*i.e.* 2α,3β,23-trihydroxylup-20(29)-en-28-oic acid).[13] These findings supported **2**, as shown in Fig. 1, to be a monodesmoside saponin of 2α,3β,23-trihydroxylup-20(29)-en-28-oic acid with a D-glucose moiety linked at C-28 [14]. This deduction was consistent with the molecular formula of **2**, and well supported by the 2D NMR spectroscopic data. Coupled with HSQC and HMBC spectral analysis, the whole ^{1}H- and ^{13}C-NMR spectral data of **2** were assigned as shown in Table 2. In the HMBC spectrum, the $^{1}H-^{13}C$ long-range correlations of H-3 (δ_H 4.21) with C-1, C-2, C-4, C-5, C-24 and C-23 evidenced the direct linkage of C-4 with Me-24 and C-4 with C-23, and supported the location of a hydroxyl group at each of C-2, C-3 and C-23. The HMBC correlations of H_3-25 with C-1, C-5, C-9, C-10, of H_3-26 with C-7, C-8, C-9, C-14, and of H_3-27 with C-8, C-13, C-14, C-15, supported the locations of Me-25 at C-10, Me-26 at C-8, and Me-27 at C-14, respectively. The HMBC correlations of H_3-29 with C-19, C-20, C-30, of H-18 with C-20 and C-28, indicated the connections of C-20 with Me-29, C-19 and C-30, and the connection of C-17 with C-28. The $^{1}H-^{13}C$ long-range correlation of H-1′ (δ_H 6.42) with C-28 (δ_C 174.8) confirmed the glycoside linkage of the D-glucose moiety with the aglycone at C-28. Besides, the β-anomeric configuration of the D-glucose moiety was indicated by the coupling constant of $^{3}J_{H1',H2'}$ (8.2 Hz).[14,15] The presented proton spin-coupling constant of H-3 ($^{3}J_{H\text{-}2,H\text{-}3}$ = 9.3 Hz) supported the α- and β- configurations of the –OH groups at C-2 and C-3, respectively.[5] The stereochemistry of the 23-CH_2OH group

Table 2 The ^{1}H- and ^{13}C-NMR spectral data (in C_5D_5N) of compound **2**[a]

No.	δ_C (**2**)	δ_H (**2**)	No.	δ_C (**2**)	δ_H (**2**)
1	48.0 CH_2	2.34 (m), 1.32 (m)	19	47.3 CH_2	3.38 (m)
2	69.0 CH	4.25 (m)	20	150.7 C	—
3	78.0 CH	4.21 (d, 9.3)	21	30.7 CH_2	2.10 (m), 1.41 (m)
4	43.5 C	—	22	36.7 CH_2	2.17 (m), 1.47(m)
5	47.8 CH	1.75 (m)	23	66.2 CH_2	4.20 (d, 10.4), 3.70 (d, 10.4)
6	18.3 CH_2	1.68 (m), 1.41 (m)	24	14.0 CH_3	1.03 (s)
7	34.1 CH_2	1.52 (m), 1.31 (m)	25	18.0 CH_3	0.97 (s)
8	41.1 C	—	26	16.3 CH_3	1.15 (s)
9	50.8 CH	1.53 (m)	27	14.7 CH_3	0.92 (s)
10	38.4 C	—	28	174.8 C	—
11	21.1 CH_2	1.47 (m), 1.22 (m)	29	19.2 CH_3	1.71 (s)
12	25.8 CH_2	1.83 (m), 1.11 (m)	30	109.9 CH_2	4.86 (br.s), 4.73 (br.s)
13	38.2 C	2.64 (m)	1′	95.3 CH	6.42 d (8.2)
14	42.7 C	—	2′	74.2 CH	4.18 (m)
15	30.0 CH_2	2.02 (m), 1.16 (m)	3′	78.7 CH	4.30 (m)
16	32.1 CH_2	2.63 (m), 1.46 (m)	4′	70.9 CH	4.36 (m)
17	56.8 C	—	5′	79.3 CH	4.05 (m)
18	49.7 CH	1.70 (m)	6′	62.0 CH_2	4.46 (m), 4.41 (m)

[a] Recorded at 500 MHz for ^{1}H- and at 150 MHz for ^{13}C-NMR data, δ in ppm and J in Hz.

at C-4 was deduced as the α-configuration from the NOE correlation between H-2 and Me-24 in the NOESY spectrum. The α-iso-propenyl group at the C-19 position was evidenced by the observation of NOE correlations between H-13 (δ_H 2.64) and H-19 (δ_H 3.38). Therefore, the whole structure of compound **2** was identified as 2α,3β,23-trihydroxylup-20(29)-en-28-oic acid-*O*-β-D-glucopyranosyl ester, trivially named akebiaoside C.

The eight known compounds were identified as 2α,3β-dihydroxyolean-13(18)-en-28-oic acid (**3**),[16] 2α,3β,29-trihydroxyolean-12-en-28-oic acid (**4**),[11] stachlic acid A (**5**),[17] mesembryanthemoidigenic acid (**6**),[18] 2α,3β,20α-trihydroxy-29-norolean-12-en-28-oic acid (**7**),[19] gypsogenic acid (**8**),[20] serratagenic acid (**9**),[21] and akebonoic acid (**10**),[22] by comparison of their NMR and MS spectral data to those reported in literatures. These compounds were all obtained from the leaves of *A. trifoliata* for the first time.

These isolated triterpenoids were evaluated for their α-glucosidase inhibitory activity, with acarbose and corosolic acid used as two reference compounds. The resulting IC_{50} values, as listed in Table 3, indicated that all the compounds, except **4** and **5**, showed stronger the α-glucosidase inhibitory activity than acarbose (IC_{50} 0.409 mM). Especially, compounds **2**, **3**, **6**, **8** and **10** displayed the α-glucosidase inhibitory activity with IC_{50} values ranging from 0.004 to 0.081 mM, which were close or even more potent than corosolic acid (IC_{50} 0.06 mM). The results suggested that these compounds from the leaves of *A. trifoliata*, at least for **2**, **3**, **6**, **8** and **10**, were effective α-glucosidase inhibitors valuable to be developed as effective hypoglycemic agents for diabetes chemotherapy.[23] Comparison of the chemical structures and the α-glucosidase inhibitory activity of **6** *versus* **4** indicated that the addition of a hydroxyl group at C-2 had an obviously negative effect on the α-glucosidase inhibitory activity of the oleanane type triterpenes.

Compounds **1–10** were further tested for their *in vitro* cytotoxicity against human cancer cell lines A549, HeLa and HepG2, using a microdilution titre technique as described in the Experimental section. The resulting IC_{50} values are displayed in Table 4, compared to adriamycin as positive control. Compounds **1**, **8** and **10** were found to show moderate cytotoxicity against all the three cancer cell lines, with IC_{50} values ranging from 26.5 to 51.9 μM. Compound **9** showed weak cytotoxicity toward HeLa and HepG2 cell lines, with IC_{50} values 81.49 and 73.47 μM, respectively. While, no obvious cytotoxic activity was detected for the other compounds in this bioassay. Comparison of the chemical structures and the cytotoxic activity of **5** *versus* arjunolic acid[12] indicated a negative effect on the cytotoxicity of the oleanane type triterpenes when the Me-29 group was replaced by a $-CH_2OH$ group.

A. trifoliata is a liana plant widely distributed in Eastern Asia countries. As traditionally a medicinal plant, also a rapidly developing economic plant commercially for fruits in China, *A. trifoliata* has now been developed and cultivated in large scale in many places of China, including Hunan, Hubei, Jiangxi, Shaanxi, and Chongqing provinces.[1,3] Previously, phytochemical studies of this plant were mainly focused on the stems and fruits, by which structurally diverse triterpenes, triterpene saponins, and some other type of chemicals were identified. However, few studies were conducted on the leaves,[12] though the leaves of this plant were annually collectable in large scale. In a recent study, we have identified two new triterpene

Table 3 α-Glucosidase inhibitory activity of compounds **1–10**[a]

Compounds	IC_{50} (mM)	Compounds	IC_{50} (mM)
1	0.109 ± 0.003	**6**	0.042 ± 0.002
2	0.015 ± 0.001	**7**	0.367 ± 0.003
3	0.021 ± 0.002	**8**	0.081 ± 0.003
4	0.503 ± 0.004	**9**	0.342 ± 0.002
5	0.592 ± 0.007	**10**	0.009 ± 0.001
Acarbose	0.409 ± 0.006	Corosolic acid	0.060 ± 0.002

[a] Values represent mean ± SD (n = 3) based on three individual experiments.

Table 4 Cytotoxic activity of compounds **1–10** (IC_{50}, μM)[a]

Compounds	A549	HeLa	HepG2
1	27.58 ± 3.24	31.45 ± 2.38	38. 52 ± 5.63
2–7	>100	>100	>100
8	26.54 ± 7.52	43.63 ± 8.41	35.67 ± 7.50
9	>100	81.49 ± 16.50	73.47 ± 0.90
10	48.77 ± 8.56	27.82 ± 7.53	51.94 ± 5.37
Adriamycin	0.68 ± 0.06	0.48 ± 0.07	1.25 ± 0.04

[a] Values represent mean ± SD (n = 3) based on three individual experiments.

saponins from the leaves of *A. trifoliata*. The present findings further indicated that the leaves of this plant is rich in bioactive natural products valuable to be developed for medicinal usage. Among the chemicals here identified, **1** is a new multiflorane type triterpene. To the best of our knowledge, this is the first time for a multiflorane type triterpene isolated from *A. trifoliata*, suggesting that more so far unidentified triterpenoids would still exist in the leaves of *A. trifoliata* worthy of further investigation.

Materials and methods

General experimental procedures

Optical rotations were obtained on a Perkin-Elmer 341 polarimeter (Perkin-Elmer, Waltham, MA, USA) with MeOH as solvent. The 1D and 2D Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker Advance 600 instrument or Bruker Ascend-500 spectrometer (Bruker BioSpin GmbH). The positive and negative ESI-MS were collected on a MDS SCIEX API 2000 LC/MS/MS instrument (Applied Biosystems, Foster City, CA, USA) after the test solutions were directly injected into the ESI source by a syringe pump. HR-ESI-MS spectra were obtained on a Bruker maXis mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) in positive-ion mode. Preparative HPLC was conducted using a CXTH P3000 HPLC pump and a UV 3000 UV-Vis Detector with a Fuji-C18 column (10 μm to 100 A, ChuangXinTongHeng Science And Technology Co., Ltd, Beijing, China); the performance of MPLC (medium pressure liquid chromatography) achieved by using a CXTH P3000 HPLC pump, with a UV 3000 UV-Vis Detector and a ODS column (40 × 2.5 cm i.d, 50 μM, YMC Co. Ltd., Kyoto, Japan).

For column chromatography (CC), silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Qingdao, China), YMC ODS-A (50 μm, YMC Co. Ltd., Kyoto, Japan), and Sephadex LH-20 (Pharmacia Fine Chemical Co. Ltd., Uppsala, Sweden) were used. Those analytical grade petroleum ether (b.p. 60–90 °C), MeOH, AcOEt, $CHCl_3$, acetone, and *n*-butanol were purchased from Tianjin Fuyu Fine Chemical Industry Co. (Tianjin, China); HPLC grade MeOH was obtained from J&K Chemical Ltd. (Beijing, China); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), pyridine-d_5, DMSO-d_6, and α-glucosidase were purchased from Sigma Chemical Co. (Sigma-Aldrich, St. Louis, MO, USA). RPMI-1640 medium and fetal calf serum were from Gibco BRL (Gaithersburg, MD, USA). *p*-Nitrophenyl-α-D-glucopyranoside (PNPG) and acarbose were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and adriamycin was from Pfizer Italia SRL (Roma, Italy).

Plant materials

The leaves of *Akebia trifoliata*, collected in August 2014 at Sangzhi, Hunan Province in China, was authenticated by Prof. Fuwu Xing at the South China Botanical Garden, Chinese Academy of Sciences (CAS), and a voucher specimen (no. 20140815) was deposited at the Laboratory of Bioorganic Chemistry of South China Botanical Garden, CAS.

Extraction and isolation

Powders of air-dried leaves of *A. trifoliata* (3.5 kg) were extracted with 95% EtOH at room temperature for three times (each time 10 L for 2 days). The EtOH extracts were next combined and concentrated *in vacuo* to provide a dark brown residue, which was suspended in 3 L H_2O and then sequentially extracted by petroleum ether (3 L × 3), EtOAc (3 L × 3) and *n*-butanol (*n*-BuOH, 3 L × 3). The petroleum ether and EtOAc layers were evaporated *in vacuo* to yield a petroleum ether-soluble (31 g) and EtOAc-soluble (168 g) fractions. The petroleum ether-soluble fraction was subjected to silica gel CC (100 cm × 10.5 cm i.d.) using a gradient of petroleum ether–acetone (100 : 0–0 : 100, v/v) to provide nine fractions (E_1–E_9). Fraction E_4 (1.7 g) was further applied to a silica gel column using petroleum ether–acetone (10 : 1–6 : 1) elution and then using MPLC eluted with MeOH–H_2O (9.5 : 0.5, v/v) to yield **10** (2.2 mg). Fraction E_6 (290 mg) was purified by a Sephadex LH-20 column (160 × 3 cm i.d.) chromatography eluted with pure acetone to provide compound **3** (2.8 mg). Fraction E_8 (2.2 g) was passed through an MCI gel column (60 × 6 cm i.d.) for depigmentation. The resultant methanolic eluate (1.3 g) of E_8 was sequentially separated by MPLC using a gradient of MeOH–H_2O (6 : 4–10 : 0, v/v) to obtain eight subfractions ($E_{8\text{-}1}$–$E_{8\text{-}8}$). The subfraction $E_{8\text{-}5}$ was purified by silica gel CC eluted with $CHCl_3$–MeOH (10 : 0–9 : 1, v/v) to afford compounds **6** (3.2 mg) and **9** (2.1 mg).

The EtOAc-soluble fraction was subjected to silica gel CC (100 cm × 10.5 cm i.d.) eluted with $CHCl_3$–MeOH (97 : 3–0 : 100, v/v) to obtain ten fractions (F_1–F_{10}). Fraction F_5 (7.1 g), obtained on elution with $CHCl_3$/MeOH of 85 : 15 (v/v), was further subjected to silica gel CC (80 × 5 cm i.d.) eluted with $CHCl_3$–MeOH of increasing polarity (98 : 2–90 : 10, v/v) to obtain six subfractions ($F_{5\text{-}1}$–$F_{5\text{-}6}$). Subfraction $F_{5\text{-}3}$ (1.57 g) was separated by MPLC eluted with MeOH–H_2O (60 : 40–100 : 0, v/v) system at a flow rate of 10 mL min^{-1}, and further purified by a Sephadex LH-20 column (150 cm × 2.5 cm i.d) eluted with MeOH to afford compound **8** (2.5 mg). Fraction $F_{5\text{-}5}$ (2.3 g) was separated by MPLC eluted with MeOH–H_2O (30 : 70–80 : 20, v/v) at a flow rate of 10 mL min^{-1} to obtain subfractions $F_{5\text{-}5\text{-}1}$–$F_{5\text{-}5\text{-}6}$. Subfraction $F_{5\text{-}5\text{-}5}$ was purified by preparative HPLC with a Fuji-C18 column (10 μm to 100 A) eluted with MeOH–H_2O (73 : 27, v/v) at a flow rate of 8 mL min^{-1} to afford compounds **4** (t_R 53 min, 2 mg) and **1** (t_R 100 min, 2.4 mg). Fraction F_7 (22 g), obtained on elution with $CHCl_3$–MeOH (60 : 40, v/v), was further subjected to silica gel CC (100 cm × 10.5 cm i.d) eluted with a gradient of $CHCl_3$–MeOH (90 : 10–60 : 40, v/v) to obtain six subfractions ($F_{7\text{-}1}$–$F_{7\text{-}6}$).

Subfraction $F_{7\text{-}3}$ (2.8 g) was further separated by MPLC using a gradient of MeOH/H_2O (65 : 35–70 : 30, v/v) to afford compound **5** (16 mg) and **7** (2 mg). Fraction F_9 (3.1 g), obtained on elution with $CHCl_3$–MeOH (1 : 1, v/v), was subjected to a silica gel column (80 cm × 5 cm i.d), eluted with a gradient of $CHCl_3$–MeOH (9 : 1–5 : 5, v/v) to give subfractions $F_{9\text{-}1}$–$F_{9\text{-}8}$. The fraction $F_{9\text{-}4}$ (0.6 g) was separated by a silica gel column (80 × 7.5 cm i.d.) eluted with $CHCl_3$–MeOH (98 : 2–90 : 10, v/v) to yield four subfractions ($F_{9\text{-}4\text{-}1}$–$F_{9\text{-}4\text{-}4}$). Subfraction $F_{9\text{-}4\text{-}4}$ was first separated by MPLC with elution system of MeOH/H_2O (25 : 75–80 : 20, v/v) at a flow rate of 10 mL min^{-1}, and further purified by a Sephadex LH-20 column (150 cm × 2.5 cm i.d) eluted with 20% $CHCl_3$ in methanol (v/v) to afford compounds **2** (5 mg).

Compound 1. White amorphous powder. $[\alpha]_D^{20}$ + 9.0 (*c* 0.21, MeOH). ESI-MS (+) *m*/*z*: 511 $[M + Na]^+$; ESI-MS (−) *m*/*z*: 487 $[M - H]^-$. HR-ESI-MS (pos.) *m*/*z*: 511.3378 (calcd for $C_{30}H_{48}NaO_5$, 511.3394). For ^{1}H-NMR (600 MHz, CD_3OD) and ^{13}C-NMR (100 MHz, CD_3OD) data, see Tables 1 and 2

Compound 2. White amorphous powder. $[\alpha]_D^{20}$ −8.30 (*c* 0.53, MeOH). ESI-MS (+) *m*/*z*: 673 $[M + Na]^+$; ESI-MS (−) *m*/*z*: 649 $[M - H]^-$. HR-ESI-MS (pos.) *m*/*z*: 673.3916 (calcd for $C_{36}H_{58}NaO_{10}$, 673.3922). For ^{1}H-NMR (500 MHz, C_5D_5N) and ^{13}C-NMR (150 MHz, C_5D_5N) data, see Tables 1 and 2

Acid hydrolysis for the sugar unit of 2

Powders of 2.8 mg compound **2** was dissolved in 4 mL 2 M HCl and heated at 90 °C for 2 h. After cooling, the reaction mixture was extracted three times (each 4 mL) with EtOAc. The aqueous layer was then evaporated *in vacuo* to dryness to give a sugar-containing residue, which was reacted with L-cysteine methyl ester hydrochloride in C_5H_5N at 60 °C for 2 h. Subsequently, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added and stirred under reflux at 60 °C for 10 h. The supernatant was then analyzed by GC-MS technique using a GCMS-QP2010 PLUS instrument, equipped with a HP-5ms capillary column (30 m, 0.25 mm ID), conditioned at a constant helium flow rate of 46.5 cm s^{-1}, 1 μL injection volume, injector temperature at 230 °C, temperature program as 2 °C min^{-1} to 180 °C, then 20 °C min^{-1} to 280 °C. Electron ionization mode was set at 70 eV. The sugar unit derived from the hydrolysis of **2** was confirmed to be D-glucose by comparison of the retention time of the derivative with that of authentic D-glucose derivative (t_R 11.852 min) prepared *via* the same process.

α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity of compounds **1–10** were tested by using a method as we recently described in the literature,[12] with both acarbose and corosolic acid utilized as reference compounds. The resulting IC_{50} values of the tested compounds were listed in Table 3.

Cytotoxic assay

The cytotoxic activity of compounds **1–10** against human tumor A549, HeLa and HepG2 cell lines were assayed by using 96 well plates according to a literature MTT method with slight modification.[24] In brief, the cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2 at 37 °C. Each well of 96-well cell culture plates was seeded with 100 μL adherent cells (5 × 10^4 cell per mL) and placed in an atmosphere with 5% CO_2 at 37 °C for 24 h to form a monolayer on the flat bottoms. Subsequently, in each well, the supernatant was removed and 100 μL fresh medium and 100 μL medium containing one of the test compounds was added. Then the plate was incubated in 5% CO_2 atmosphere at 37 °C. After 3 days, 20 μL MTT at concentration 5 mg mL^{-1} in DMSO was added into each well and incubated for 4 h. Carefully, the supernatant in each well was removed and 150 μL DMSO was added. Then the plate was vortex shaken for 15 min to dissolve blue formazan crystals. The OD (optical density) value of each well was tested on a Genois microplate reader (Tecan GENios, Männedorf, Switzerland) at 570 nm. All the tests were conducted by three individual experiments and adriamycin was applied as a positive control. In a test, for each of the tumor cell lines, each of the test compounds was set at concentrations 50, 25, 12.5, 6.25, 3.125, 1.5625 μg mL^{-1}. The inhibitory rate of tumor cell growth was calculated by the formula: inhibition rate (%) = $(OD_{control} - OD_{treated})/OD_{control} \times 100\%$, and the IC_{50} values were calculated by SPSS 16.0 statistic software. The three tumor cell lines were purchased from the Kunming Institute of Zoology, CAS. The resulting IC_{50} values listed in Table 4 were based on three individual experiments and represented as means ± standard deviation (SD).

Conclusions

Ten pentacyclic triterpenoids, including a new multiflorane triterpene acid **1** and a new lupane triterpene monoglucoside **2**, were obtained from the leaves of *A. trifoliata*. Their structures were elucidated by extensive spectroscopic and chemical means. All the compounds were isolated from the leaves of *A. trifoliata* for the first time. These compounds, except **4** and **5**, were found to show the *in vitro* α-glucosidase inhibitory activity much stronger than acarbose. Especially, compounds **2**, **3**, **6**, **8** and **10** displayed *in vitro* α-glucosidase inhibitory activity with IC_{50} values close or even more potent than corosolic acid. Furthermore, compounds **1**, **8**, **9** and **10** selectively showed *in vitro* cytotoxicity against human tumor A549, HeLa and HepG2 cell lines. The present study support that the leaves of *A. trifoliata* is a highly valuable source rich in bioactive chemicals worthy to be developed in medicinal field.

Conflicts of interest

There are no conflicts to declare.

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