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A new oleanane type pentacyclic triterpenoid saponin from the husks of *xanthoceras sorbifolium* bunge and its neuroprotection on PC12 cells injury induced by $A\beta_{25\text{-}35}$

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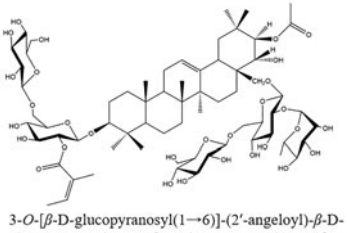
ABSTRACT

A new triterpenoid saponin (**1**), 3-*O*-[*β*-D-glucopyranosyl(1→6)]-(2′-angeloyl)-*β*-D-glucopyranosyl-28-*O*-*β*-D-glucopyranosyl(1→6)[*α*-L-rhamnopyranosyl(1→2)-*β*-D-glucopyranosyl]-21-*O*-acetyl-16-deoxybarringtogenol C, together with four known saponins (**2**∼**5**) were isolated from the husks of ***Xanthoceras sorbifolium* Bunge**. Their structures were characterized by HR-ESI-MS, 1D-NMR, 2D-NMR spectra and chemical methods. Compound **1** exhibited excellent neuroprotection on PC12 cells injury induced by $A\beta_{25\text{-}35}$ at the doses of 150 μmol/L and 200 μmol/L. The cell viabilities were (76.18 ± 2.09) % and (76.02 ± 3.20) %, respectively.

The husks of *Xanthoceras sorbifolium* Bunge



3-*O*-[*β*-D-glucopyranosyl(1→6)]-(2′-angeloyl)-*β*-D-glucopyranosyl-28-*O*-*β*-D-glucopyranosyl(1→6)[*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranosyl]-21-*O*-acetyl-16-deoxybarringtogenol C

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1. Introduction

Xanthoceras sorbifolium Bunge, a shrub belonging to the family of Sapindaceae (Chen et al. 1985), is widely distributed in Inner Mongolia, Liaoning, Gansu and other regions of China. The dry stems and branches of *X. sorbifolium* called "Wen Guan Mu" are

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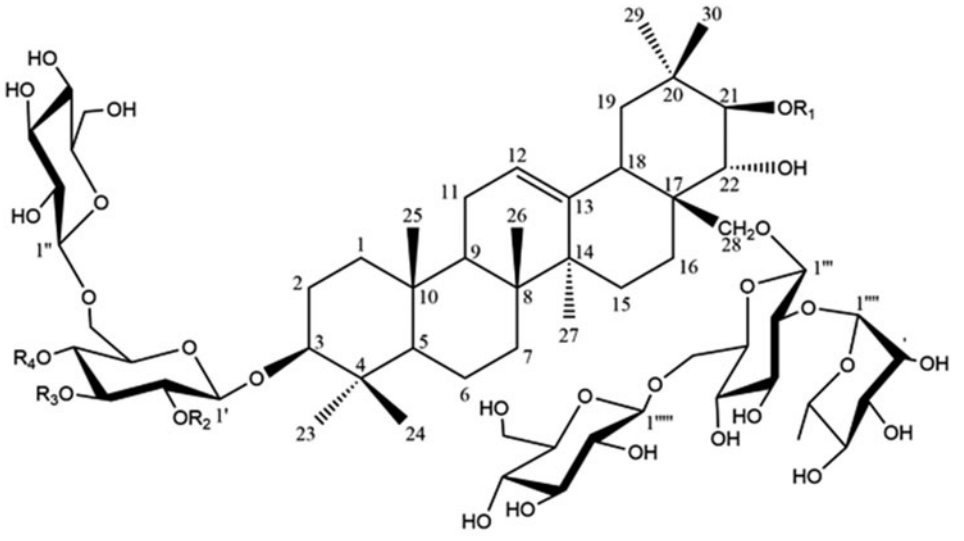
mainly used as a folk medicine for the treatment of rheumatism and gout. In recent reports, the extract and some ingredients of *X. sorbifolium* exhibited diverse biological activities including anti-inflammatory, anti-HIV (Du et al. 2016), antitumor (Ren et al. 2015) and improvement of learning and memory abilities (Li et al. 2016). However, the husks of this plant, always regarded as byproduct in the past, contain plenty of pentacyclic triterpenoid saponins. Saponins can be divided into three different types, known as R1 barrigenol, barringtogenol C and 16-deoxybarringtogenol C according to their nuclear structures. Xanthoceraside which belongs to the type of R1 barrigenol, is the richest saponin in the husks. It has different kinds of bioactivities especially in the aspect of anti-Alzheimer's disease (AD) (Ling et al. 2011; Jin et al. 2014). As to a series of 16-deoxybarringtogenol C derivatives isolated from *X. sorbifolium* husks (Li et al. 2013; Wang et al. 2018), they exhibited nearly no cytotoxicity against tumor cells due to the glycosidation at C-28 (Li et al. 2013). Moreover, researches about 16-deoxybarringtogenol C are insufficient due to the low content in the husks. Nevertheless, in our recent study (Rong et al. 2018), two isolated 16-deoxybarringtogenol C compounds 2 and 4 had been proved to have significant neuroprotection on PC12 cells. We speculated that other 16-deoxybarringtogenol C type saponins may have the similar bioactivity. Therefore, this paper mainly focused on a new isolated 16-deoxybarringtogenol C saponin from the husks of *X. sorbifolium*. Neuroprotection on $A\beta_{25\text{-}35}$ induced PC12 cells injury of compound 1 was also tested.

2. Results and discussion

Compound 1 was obtained as a white powder and its molecular formula was determined as $C_{67}H_{108}O_{30}$ by positive HR-ESI-MS ion at m/z 719.3373 1/2 $[M+2Na]^+$ (calcd for $C_{67}H_{108}O_{30}NaNa$, 1438.6721). The absorption of IR (KBr) spectrum in 3425 and 1647 cm^{-1} proved the presence of hydroxyl and carbonyl group, respectively. The nature of sugars were identified as D-glucose and L-rhamnose by acid hydrolysis, compared their retention times with authentic samples. ^{1}H-NMR (600 MHz; C_5D_5N) spectrum displayed seven characteristic angular methyl signals in high field at δ 0.89, 0.97, 1.00, 1.03, 1.06, 1.15, 1.19 (each 3 H, s) which gave direct correlations in the HSQC spectrum with carbon resonances at δ 17.1, 16.2, 29.9, 28.3, 16.9, 20.5, 26.5, respectively. In addition, one olefinic proton at δ 5.48 (1 H, brs), together with typical olefinic carbons at δ 124.7 and 142.3 in ^{13}C-NMR (600 MHz; C_5D_5N) spectrum revealed that it might be an olean-12-ene triterpenoid saponin. A group of angeloyl signals at δ 6.01 (1 H, d, $J=7.3$ Hz), 2.10 (3 H, d, $J=7.3$ Hz), 2.01 (3 H, s); an acetyl group signal at δ 2.18 (3 H, s); five anomeric proton signals of sugars at δ 4.93 (1 H, d, $J=8.7$ Hz, H-1′), 5.09 (1 H, d, $J=6.7$ Hz, H-1″), 4.58 (1 H, d, $J=7.3$ Hz, H-1‴), 6.54 (1 H, brs, H-1⁗), 5.01 (1 H, d, $J=8.7$ Hz, H-1⁗′) indicated that there are four glucopyranosyls and one rhamnopyranosyl in compound 1. ^{13}C-NMR (600 MHz; C_5D_5N) spectrum showed a pair of carbon signals of acetyl at δ 171.5 and 21.4, as well as five anomeric carbon signals of sugars at δ 104.2 (C-1′), 105.6 (C-1″), 103.7 (C-1‴), 100.6 (C-1⁗), 105.9 (C-1⁗′). Besides, a carbonyl signal at δ 167.4 and a pair of olefinic carbons at δ 128.8 and 138.2 were obtained to determine the presence of one angeloyl group. The above data established that compound 1 was a 16-deoxybarringtogenol C saponin with five sugar



	R1	R2	R3	R4
1	Ac	Ang	H	H
2	H	H	Ang	Ac
3	H	Ang	H	H
4	H	H	Ang	H
5	H	H	H	Ang

Figure 1. **Structures of compounds 1-5.**

units, one angeloyl and an acetyl group. From the HMBC spectrum, the long-range correlations between δ 1.03 (H-23), δ 0.89 (H-24) and δ 89.4 (C-3); δ 1.00 (H-29), δ 1.15 (H-30) and δ 79.7 (C-21) supported the location of four important angular methyl groups. The sequencing of the glycoside chains were authenticated by the HMBC long-range correlations of δ 4.93 (H-1′) to δ 89.4 (C-3), δ 4.58 (H-1‴) to δ 74.2 (C-28) which indicated two glucosyl moieties were assigned to C-3, C-28, respectively. Similarly, the correlations of δ 5.09 (H-1″) with δ 70.3 (C-6′), δ 5.01 (H-1⁗) with δ 70.5 (C-6‴) confirmed the connectivity of other glucosyl units. The attachment of the rhamnose was evidenced by the NOE interactions between the anomeric proton of the rhamnose δ 6.54 (H-1⁗) and δ 4.19 (H-2‴) of glucose in the NOESY spectrum and the deshielding of δ 80.3 (C-2‴) of glucose proved the rhamnose was substituted at C-2‴. The allocation of the angeloly group to glucose was deduced by the correlation in the HMBC spectrum between δ 5.65 (H-2′) and δ 167.4 (C-1⁗⁗), the NOESY correlations between δ 2.01 (H-5⁗⁗) with δ 1.03 (H-23), δ 0.89 (H-24) further confirmed the angeloyl was linked at C-2′. The HMBC correlation of δ 5.37 (H-21) and δ 171.5 (C-1⁗⁗⁗) was observed, establishing the acetyl group was substituted at C-21, the same conclusion with regard to the location of acetyl group was also drawn by the NOESY correlations between δ 2.18 (H-2⁗⁗⁗) with δ 5.37 (H-21), δ 1.00 (H-29), δ 1.15 (H-30). We can also find that the new compound exhibited similar spectral features of the

known compound 3 except the occurence of the acetyl group. One apparent difference was that the proton signal ascribed to H-21 was shifted downfield from δ 4.15 (1 H, m) to δ 5.37 (1 H, d, $J = 9.8$ Hz), suggesting that the new compound shared the same skeleton and sugar moieties with compound 3, however, the 21-hydroxy group has been acetylated. Furthermore, C-21 was shifted downfield to 79.7, whereas C-22 was shifted upfield to 69.5 clearly. The configurations of H-21 and H-22 were ascertained as α and β orientations by analyzing NOESY spectrum with the correlations between δ 5.37 (H-21) and δ 1.00 (H-29); δ 4.42 (H-22) and δ 1.15 (H-30), respectively. Combined with the data of HSQC and ^{1}H-^{1}H COSY spectra, the structure of compound 1 was univocally characterized as 3-*O*-[β-D-glucopyranosyl(1→6)]-(2′-angeloyl)-β-D-glucopyranosyl-28-*O*-β-D-glucopyranosyl(1→6)[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl]-21-*O*-acetyl-16-deoxybarringtogenol C.

Four known compounds were identified as sorbifoside B (2) (Ling et al. 2011), 3-*O*-[β-D-glucopyranosyl(1→6)]-(2′-angeloyl)-β-D-glucopyranosyl-28-*O*-β-D-glucopyranosyl (1→6)[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl]-16-deoxybarringtogenol C (3) (Xiao et al. 2013), 3-*O*-[β-D-glucopyranosyl(1→6)]-(3′-angeloyl)-β-D-glucopyranosyl-28-*O*-β-D-glucopyranosyl(1→6)[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl]-16-deoxybarringtogenol C (4) (Chan et al. 2015), sorbifoside C (5) (Ling et al. 2011) by comparing their NMR data with those reported in the literature.

In addition, the new compound was assayed for neuroprotection on PC12 cells injury induced by A$\beta_{25\text{-}35}$. It showed significant effect against neurotoxicity at the concentration of 150 μmol/L and 200 μmol/L. Furthermore, the highest cell viability was (76.18 ± 2.09) % at the concentration of 150 μmol/L. The cell viability of positive control (Hup-A) was (78.74 ± 2.02) % at the concentration of 1 μmol/L (Figure 1).

3. Experimental

3.1. General experimental procedure

1D, 2D-NMR spectra were obtained by Bruker AV-600 spectrometers (Bruker, Switzerland) using TMS as an internal standard. IR spectrum was obtained by IFS-55 Fourier Transform Infrared (FT-IR) spectrometer (Bruker, Switzerland). Melting point was obtained by B-540 melting point (BUCHI, Switzerland). Specific rotation was measured by Anton-Paar MCP 200 polarimeter (Anton-Paar, China). Rotation spectra were measured by LC-NetII/ADC HPLC equipped with ORD-4090 as the detector and Shodex Asahipak NH2P-50 4E (NO. N1490155) as the column. HR-ESI-MS data was obtained by TripleTOF^{TM} 5600 quadrupole-time-of-flight hybrid mass spectrometer (Sciex, Redwood City, CA, USA) equipped with an Agilent 1260 HPLC (Billerica, 156 USA). PHPLC was performed on a Shimazu LC-8A pump equppied with a SPD-10A VP detector using the YMC column (250 mm × 20 mm, 5 μm, YMC, Japan). C_{18}-PREP (50 μm, nakalai tesque, Japan). Macroporous resin D101 (Cangzhou Baoen chemical factory, Hebei, China), silica gel (100 ∼ 200, 200 ∼ 300, Qingdao Ocean chemical factory, Shandong, China). The PC12 cells were obtained from BOSTER Biological Technology (Wuhan, China), H-DMEM was purchased from HYclone (SH30022.01, Lot.B10201637), FBS was obtained from Gibco (10099-141 Lot. 1715752) and A$\beta_{25\text{–}35}$ was obtained from Sigma Aldrich (St Louis, MO, USA).

3.2. Plant materials

The husks of *Xanthoceras sorbifolum* Bunge were obtained from Chifeng City (Inner Mongolia, China), and identified by Prof. Dali Meng (Department of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang, China). A voucher specimen (WGG-1110) was deposited in the School of Traditional Chinese Materia Medica, Shenyang Parmaceutical University, Shenyang, China.

3.3. Extraction and isolation

Dried husks of *X. sorbifolium* (9.5 kg) were well crushed and extracted with 70% ethanol (ETOH) (8, 6, $4 \times 3 \times 2$ h). The filtrate was concentrated to 10 L and centrifugated at 4000 r/min for 10 min, the supernatant was eluted with H_2O, 50% EtOH, 70% EtOH, 95% EtOH on D101 macroporous resin. The 50% EtOH fraction (164.4 g) was subjected to silica gel and eluted with CH_2Cl_2-MeOH (100:0-0:100) to get 6 fractions (A1-A6). Fraction A4 (25.7 g) was then subjected to ODS eluted with MeOH-H_2O (10:90-100:0) to obtain 7 fractions (B1-B7). Fraction B6 (4.8 g) was purified by PHPLC (MeCN: H_2O: HCOOH, 31: 69: 0.1) to get compound 1 (12 mg), compound 2 (5 mg), compound 3 (12 mg). Fraction B5 (3.5 g) was purified by PHPLC (MeCN: H_2O: HCOOH, 28: 72: 0.1) to yield compound 4 (15 mg), compound 5 (25 mg).

3.4. Compound 1

White powder (methanol); mp 220 ~ 230 °C;$[\alpha]^{20}_D$= -12.3 (*c* 0.065 MeOH); IR (KBr) V_{max} (cm^{-1}): 3425, 2922, 1647, 1631, 1401, 1269, 1008; HR-ESI-MS *m/z* 719.3373 1/2 $[M + 2Na]^+$ (calcd for $C_{67}H_{108}O_{30}NaNa$, 1438.6721); The data of ^{1}H-NMR and ^{13}C-NMR were listed in the Table S1.

3.5. Acid hydrolysis of compound 1

The compound 1 (3 mg) was hydrolysed in HCl (2 M, 5 mL) and refluxed in oil bath for 8 hours at 90 °C. After cooling, the hydrolysate was then extracted with $CHCl_3$ (8 mL × 3). The aqueous layer was evaporated repeatedly under vacuum to remove HCl. The residue was dissolved in water (1 mL) and analyzed by LC-NetII/ADC HPLC equipped with ORD-4090 as the detector and Shodex Asahipak NH2P-50 4E (NO. N1490155) as the column. The chromatographic conditions: flow rate, 1.0 mL/min; mobile phase, MeCN: H_2O (3:1); column temperature, 30 °C. The sugar fractions of compound 1 were determined as D-glucose and L-rhamnose by comparing their retention times with the standard samples ($t_{D\text{-glucose}} = 8.48$ min, $t_1 = 8.37$ min; $t_{L\text{-rhamnose}} = 5.53$ min, $t_2 = 5.56$ min).

3.6. Neuroprotective effect on A$\beta_{25\text{-}35}$ reduced PC 12 cells injury

PC12 cells were incubated with 10% FBS and H-DMEM medium containing 100 IU/mL penicillin as well as 100 g/mL streptomycin in a CO_2 incubator (37 °C, saturated 5% CO_2, 95% air) and observed under an inverted microscope. Culture medium was

changed every day. Cells in ogarithmic phase were used for the experiment. The cultured PC12 cells were seeded on a 96 - well multiplates (1×10^5 cells/mL), after 24 h, the PC12 cells were randomly divided into 7 groups. The normal group and the model group were only added culture medium, meanwhile, the experiment groups were added 50 μmol/L, 100 μmol/L, 150 μmol/L, and 200 μmol/L of compound 1 and 1 μmol/L Hup-A, respectively. After 24 h, except for the normal group, 20 μmol of $A\beta_{25\text{-}35}$ was added and incubated for another 24 h.

5 mg/mL MTT 20 μL was then added into per well and incubated for 4 h. Supernatant was discarded and added 120 μL DMSO to per well to dissolve the crystals. The OD values were measured at 490 nm.

Cell viability was expressed as mean ± standard deviation and the normal group was regarded as 100%. The data were analyzed by one-way analysis of variance (ANOVA) in SPSS 19.0 Statistic software. Homogeneity of variance test was tested and significance level was 0.05.

$$\text{Cell viability } (\%) = \text{OD test}/\text{OD control} \times 100\%.$$

4. Conclusion

In this study, from 50% EtOH extract of the husks of *X. sorbifolium*, a new 16-deoxybarringtogenol C type saponin as well as four known ones were isolated and identified. The new compound shares the basic skeleton of 16-deoxybarringtogenol C with five sugar moieties, meanwhile, one angeloyl and an acetyl group were substituted at C-2′ and C-21, respectively. It showed significant protection against $A\beta_{25\text{-}35}$ induced neurotoxicity. We speculated that 16-deoxybarringtogenol C type saponins can be used as bioactive substances of the husks against AD. Moreover, this work accumulated material basis of the husks of *X. sorbifolium* for further research in treating Alzheimer's disease.

Disclosure statement

No potential conflict of interest was reported by the authors.

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