RESEARCH ARTICLE

Phenylethanol glycosides from the seeds of Aesculus chinensis var. chekiangensis

Nan Zhang1,2†, Di Liu1,2†, Shuxiang Wei1,2, Shijie Cao1,2, Xincheng Feng1, Kai Wang1, Liqin Ding1,2* and Feng Qiu1,2*

Abstract
Three new phenylethanol glycosides (1-3) and one known analogue (4) were isolated from the seeds of Aesculus chinensis Bge. var. chekiangensis. To the best of our knowledge, this represents the first isolation of phenylethanol glycosides from the genus of Aesculus, which enriched its chemical composition. Structure elucidations were performed via extensive NMR and HRESIMS data together with comparison with literature data. Thereafter, the isolated compounds were assayed for their neuroprotective activities against CoCl2-induced cytotoxicity in PC12 cells and compound 3 exhibited moderate activity.

Keywords: Aesculus chinensis Bge. var. chekiangensis (Hu et Fang) Fang, Phenylethanol glycosides, Neuroprotective activity

Introduction
The genus Aesculus, which belongs to the family Hippocastanaceae contains about 30 species found worldwide. The dried seeds of Aesculus chinensis Bge. var. chekiangensis (Hu et Fang) Fang, Aesculus chinensis Bge and Aesculus wilsonii Rehd are commonly used to treat chest and abdomen pain, dysentery and ague [1, 2] in traditional Chinese medicine. Previous studies on the genus of Aesculus revealed the presences of diverse secondary metabolites such as triterpenoids [3–7], flavonoids [8, 9], coumarins [10] and steroids [11]. And a number of pharmacological studies have suggested that A. chinensis exhibited beneficial effects on antitumor [12], neuroprotective [13], anti-inflammatory [14] and cardio-protective activities [15]. Nevertheless, compared to other species of Aesculus genus, the chemical investigation of Aesculus chinensis Bge. var. chekiangensis (Hu et Fang) Fang is limited. Our interests in cytotoxic and neuroprotective components from A. chinensis Bge. var. chekiangensis (Hu et Fang) Fang have led to the isolation of numerous new ones [16, 17]. As a continuous search for structurally novel compounds with diverse bioactivities, three new phenylethanol glycosides (1-3) and one known analog (4) were obtained (Fig. 1), which represent the first examples of phenylethanol glycosides obtained from the genus of Aesculus. Herein, the isolation, structure identification and biological evaluation of 1-4 are described.

Methods
General experimental procedures
The chemicals and material were similar to our previous researches [16, 17].

Plant material
The plant was the same batch of medicinal material as our previous reports [16, 17].

Extraction and isolation
The extracted method was the same to our previous studies [16, 17]. Chopped, dried seeds of A. chinensis Bge. (8.8 kg) were extracted with 70% ethanol, then...
partitioned via D101 resin column eluting with a step-wise gradient of H$_2$O-EtOH.

The 60% EtOH-H$_2$O part was loaded onto a silica gel column using CH$_2$Cl$_2$/CH$_3$OH (100:1 → 1:1) to yield 4 fractions (A–D). Fraction A was further separated by RP C$_{18}$ CC (MeOH-H$_2$O, 10:90 to 100:0) to give seven subfractions (A1–A7). The subfraction A3 was further sub-divided with an ODS RP-C$_{18}$ column (MeOH/H$_2$O, 10:90 to 100:0) to give seven subfractions (A3A–A3G). The subfraction A3G was applied to a Sephadex LH-20 column (MeOH), and then purified by recycling preparative HPLC with 40% MeOH/H$_2$O to yield compounds 2 (3.7 mg) and 3 (9.0 mg).

4-methoxy-phenylethanol-8-O-α-L-rhamnopyranosyl-(1→6)-β-d-glucopyranoside (1)
Brown amorphous powder; [a]$_D$ 7.3 (c 0.10, MeOH); Proton nuclear magnetic resonance ($^1$H-NMR) and carbon-13 nuclear magnetic resonance ($^{13}$C-NMR): Table 1; HR-ESI–MS: m/z 505.1918 [M+COOH]$^-$ (calculated for C$_{22}$H$_{33}$O$_{13}$, 505.1921).

4-methoxy-phenylethanol-8-O-β-d-glucopyranosyl-(1→2)-β-d-glucopyranoside (2)
Brown amorphous powder; [a]$_D$ 11.2 (c 0.11, MeOH); $^1$H-NMR and $^{13}$C-NMR: Table 1; HR-ESI–MS: m/z 521.1870 [M+COOH]$^-$ (calculated for C$_{22}$H$_{33}$O$_{14}$, 521.1870).

4-methoxy-phenylethanol-8-O-β-d-glucopyranosyl-(1→3)-β-d-glucopyranosyl (3)
Brown amorphous powder; [a]$_D$ 14.6 (c 0.10, MeOH); $^1$H-NMR and $^{13}$C-NMR: Table 1; HR-ESI–MS: m/z 683.2398 [M+COOH]$^-$ (calculated for C$_{28}$H$_{43}$O$_{19}$, 683.2399).

Hydrolysis and determination of absolute configuration of sugars
Compounds 1–3 (1.0 mg, respectively) was hydrolyzed with 2 M HCl (4.0 mL) at 90 °C for 2 h. Then the hydrolysed materials were disposed and tested by means of the procedure described in our previous work [16, 17].

Neuroprotective effect assay
Compounds 1–4 were assayed for their neuroprotective effects against CoCl$_2$-induced PC12 cell injury [18] by 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) method with trolox as the positive control according to our previously reported procedure [16, 17]. PC12 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum as well as 100 U/mL penicillin/streptomycin and were incubated at 37 °C with 5% CO$_2$. PC12 cells were placed into a 96-well plate at a density of 2×10$^4$ cells/well and kept there for 24 h. Cells were incubated with test compounds and trolox (10 μM) for 2 h. To induce an oxidative stress, 1 mM CoCl$_2$ was added to the cells and incubated for 24 h. Then, the supernatant was changed with 100 μL MTT solution (5 mg/mL) for 2.5 h, the plate was vibrated, and the absorbance at 490 nm was measured using a microplate reader.

Cytotoxicity assay
Cell viability was determined with the MTT method [19, 22]. The human hepatocellular carcinomas cells (HepG2), the human colorectal carcinoma cells (HCT-116) and the human gastric carcinoma cells (MGC-803) were purchased from ATCC. HepG2, MGC-803 and HCT-116 were respectively cultured in DMEM and RPMI-1640 mediums, which were supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO$_2$. HepG2, HCT-116, and MGC-803 cells (1×10$^4$) were seeded in 96-well tissue culture plates. Cells were treated in triplicate with five concentrations (50, 25, 12.5, 6.25 and 3.125 μM) of the tested compounds

Fig. 1 The structures of isolated compounds

![Fig. 1 The structures of isolated compounds](image-url)
Table 1 ¹H (600 MHz) and ¹³C (150 MHz) NMR data of 1–3 (δ in ppm, in DMSO-d₆)

| Position | 1          | 2          | 3          |
|----------|------------|------------|------------|
|          | δH         | δC         | δH         | δC         |
| 1        | 130.5      | 130.7      | 130.6      |
| 2        | 129.9      | 130.0      | 130.0      |
| 3        | 113.7      | 113.6      | 113.6      |
| 4        | 157.6      | 157.6      | 157.7      |
| 5        | 113.7      | 113.6      | 113.6      |
| 6        | 129.9      | 130.0      | 130.0      |
| 7        | 34.8       | 34.7       | 34.6       |
| 8        | 69.9       | 69.8       | 69.9       |

4-OCH₃  55.0, 3.71 (3H, s)  55.0, 3.71 (3H, s)  55.0, 3.71 (3H, s)

1' 103.0, 4.17 (1H, d, J=7.7 Hz)  101.3, 4.33 (1H, d, J=7.7 Hz)  101.6, 4.39 (1H, d, J=7.5 Hz)

2' 73.4, 2.94 (1H, t, J=8.5 Hz)  82.2, 3.23 (1H, dd, J=9.1, 7.7 Hz)  79.6, 3.44 (1H, m)

3' 76.7, 3.13 (1H, t, J=8.9 Hz)  76.7, 3.10 (1H, m)  86.2, 3.49 (1H, t, J=8.8 Hz)

4' 70.2, 2.99 (1H, t, J=9.1 Hz)  69.8, 3.10 (1H, m)  68.4, 3.19 (1H, m)

5' 75.4, 3.26 (1H, m)  76.1, 3.36 (1H, m)  76.1, 3.18 (1H, m)

6' 67.0, 3.81 (1H, dd, J=11.2, 2.0 Hz)  60.9, 3.65 (1H, m)  60.9, 3.65 (1H, m)

1'' 100.8, 4.59 (1H, d, J=1.2 Hz)  104.1, 4.41 (1H, d, J=7.8 Hz)  102.8, 4.55 (1H, d, J=8.0 Hz)

2'' 70.3, 3.60 (1H, m)  74.9, 3.00 (1H, dd, J=8.4, 7.8 Hz)  74.5, 2.94 (1H, dd, J=8.5, 5.9 Hz)

3'' 70.7, 3.42 (1H, m)  77.1, 3.08 (1H, m)  76.4, 3.19 (1H, m)

4'' 72.0, 3.17 (1H, t, J=9.1 Hz)  69.9, 3.10 (1H, m)  70.0, 3.62 (1H, m)

5'' 69.4, 3.45 (1H, m)  76.1, 3.16 (1H, m)  76.2, 3.16 (1H, m)

6'' 180.1, 1.12 (3H, d, J=6.2 Hz)  61.0, 3.65 (1H, m)  61.0, 3.67 (1H, m)  3.39 (1H, dd, J=11.8, 5.9 Hz)

1''' 103.4, 4.38 (1H, d, J=7.9 Hz)  73.7, 3.04 (1H, d, J=8.7 Hz)

2''' 76.9, 3.07 (1H, m)  76.0, 3.06 (1H, m)  76.8, 3.05 (1H, m)

3''' 67.8, 3.12 (1H, m)  60.7, 3.69 (1H, m)  3.45 (1H, m)

for 24 h, with 5-fluorouracil (5-FU) as positive control. Subsequently, 100 μL of MTT (5 mg/mL) was added and the cells were incubated for additional 2.5 h. Thereafter, the supernatant was discarded and 0.15 ml of DMSO was added to each well, then the plate was mixed on a microshaker for 10 min and read on a microplate reader at 490 nm.

Results and discussion

Compound 1 was obtained as brown amorphous powder with a molecular formula of C₂₂H₃₃O₁₁ deduced from its HR-ESI-MS spectrum (m/z 505.1918 [M+COOH]⁺, calcd. for C₂₂H₃₃O₁₁, 505.1921). The ¹H-NMR spectrum of compound 1 exhibited signals characteristic for a 1, 4-disubstituted benzene ring [δH 7.17 (2H, d, J=8.5 Hz, H-2, 6), 6.83 (2H, d, J=8.5 Hz, H-3, 5)], an ethoxy moiety [δH 2.78 (2H, dt, J=8.2, 6.3 Hz), 3.83 (1H, dt, J=8.2, 6.3 Hz)] as well as a O-methyl at δH 3.71 (3H, s) (Table 1). The heteronuclear multiple bond correlations (HMBC) (Fig. 2) of H-2 (δH 7.17) to C-4 (δC 157.6), C-6 (δC 129.9), C-7 (δC 34.8), H-3 (δH 6.83) to C-1 (δC 130.5), C-5 (δC 113.7), H-8 (δH 3.83) to C-1 (δC 130.5) and OCH₃ (δH 3.71) to C-4 (δC 157.6) indicated 1 contains a 4-methoxyphenylethanol moiety.

The two anomeric protons at δ 4.17 (1H, d, J=7.7 Hz), 4.59 (1H, d, J=1.2 Hz) correlated with carbons at δ 103.0 and 100.8 in heteronuclear single quantum coherence (HSQC) spectrum, respectively, indicated a disaccharide residue. Acid hydrolysis of 1 liberated d-glucose and l-rhamnose, which were identified by HPLC analysis after derivatization [21, 22]. The β-orientation of the glucopyranosyl unit was deduced from the coupling
constant \((J=7.7 \text{ Hz}, \text{H-1}')\). The \(\alpha\)-anomeric configuration of rhamnose was determined from the absence of nuclear overhauser effect spectroscopy (NOESY) correlations between protons H-1 and H-3/H-5. The \(\beta\)-d-glucose was attached to the 4-methoxy-phenylethanol nucleus at C-8, evidenced by the HMBC correlation between H-1' (\(\delta_H 4.17\)) to C-8 (\(\delta_C 69.9\)). In addition, the downfield chemical shift of C-6' (\(\delta_C 67.0\)) of the glucose coupled with the cross peak of H-1'' of 2. The NMR data is 162 mass units more than that of 1. Based on these data, compound 1 was concluded to be 4-methoxy-phenylethanol-8-O-\(\alpha\)-L-rhamnopyranosyl-(1 \(\rightarrow\) 6)-\(\beta\)-d-glucopyranoside.

The elemental formula of compound 2 was confirmed to be C\(_{21}\)H\(_{32}\)O\(_{12}\) with one oxygen more than that of 1 according to the [M + COOH]\(^+\) ion peak at \(m/z\) 521.1870 in its HRESIMS spectrum. The \(^1\)H and \(^13\)C NMR data of 2 revealed a close resemblance to 1 except for the corresponding signals to the two sugar units. The other known one, phenylethanol-8-O-\(\alpha\)-L-rhamnopyranosyl-\((1 \rightarrow 6)-\beta\)-d-glucopyranoside (4) were also obtained and identified by NMR analysis and comparison with literature data [23].

All compounds (1–4) were tested in three human cancer cell lines, HepG2, HCT-116 and MGC-803, using 5-FU as the positive control. However, they did not show obvious cytotoxicity (IC\(_{50}\) > 50 \(\mu\)M).

The neuroprotective effects of 1–4 were also evaluated in CoCl\(_2\)-induced PC12 cell damage [24] by MTT assay. According to the references [25, 26] and our study, the positive control, trolox, exhibited statistically significant neuroprotective effect at 10 \(\mu\)M (Fig. 3). Therefore, the concentration of 10 \(\mu\)M was selected for the cytotoxic and neuroprotective evaluation of these compounds. First, the cytotoxic activity of compounds 1–4 against PC12 cell line was tested and none of them showed cytotoxicity at 10 \(\mu\)M (Additional file 1: Fig. S16). Subsequently, 10 \(\mu\)M compounds were bioassayed for their neuroprotective properties. And according to Fig. 3, compound 3 exhibited moderate activities against CoCl\(_2\)-induced PC12 cell injury.

**Conclusion**

In this paper, three new phenylethanol glycosides (1-3) and one known compound (4) were obtained from the seeds of *A. chinensis* Bge. var. *chekiangensis*, which represents the first isolation of phenylethanol glycosides from the genus of *Aesculus*. The findings also
provided more insights into the chemotaxonomy of the Aesculus genus. Besides, the neuroprotective activities of the phenylethanol glycosides were also evaluated and compound 3 exhibited statistically significant neuroprotective activity.

**Supplementary information**

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s13065-020-00685-3.

![Figure S16](image)

**Fig. 3** Neuroprotective activities of compounds 1-4 (10 μM) against CoCl₂-induced cell death in PC12 cells. The data (cell viability, measured by MTT assay) are expressed as mean±SD. Three independent experiments were performed. Trolox was used as the positive control at 10 μM. Compared with CoCl₂ treated group, *P<0.05, **P<0.01

**Abbreviations**

DMDSO-d₆: Deuterated dimethyl sulfoxide; ¹H-NMR: Proton nuclear magnetic resonance; ¹³C-NMR: Carbon-13 nuclear magnetic resonance; HMBC: Heteronuclear multiple bond correlation; HSQC: Heteronuclear single quantum coherence; NOESY: Nuclear overhauser effect spectroscopy; HepG2: The human hepatocellular carcinoma cells; HCT-116: The human colorectal carcinoma cells; MGC-803: The human gastric carcinoma cells; MTT: 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide; 5-FU: 5-Fluorouracil.

**Authors’ contributions**

NZ conceived and designed the experiments. NZ tested cytotoxicity and their neuroprotective activities. Molecules 24:4063–4071. for the isolation and elucidation the structures. NZ interpreted the data and wrote the manuscript. LD and FQ were the project leaders organizing and guiding the experiment. All authors read and approved the final manuscript.

**Funding**

This work was financially supported by the State Key Program of National Natural Science of China (Grant No. 81430095).

**Availability of data and materials**

All other datasets generated for this study are included in the article and Additional file 1.

**Competing interests**

No potential conflict of interest was reported by authors.

**Author details**

¹ School of Chinese Materia Medica, Tianjin University of Traditional Chinese Medicine, No. 10 Poyanghu Road, West Area, Tuanbo New Town, Jinghai Dist, Tianjin 301617, People's Republic of China. ² Tianjin State Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China.

Received: 8 February 2020   Accepted: 16 April 2020

Published online: 22 April 2020

**References**

1. Zhang X, Zhao J, Cui Y, Liu X, Ma C, Hattori M, Zhang L (1999) Anti-HIV-1 protease triterpenoid saponins from the seeds of Aesculus chinensis. J Nat Prod. 62(11):1510–1513
2. Zhang Z, Li S, Zhang S, Geurtsen D (2006) Triterpenoid saponins from the fruits of Aesculus pavia. Phytochemistry 67(8):794–794
3. Zhao J, Yang XW, Cui YX, Liu XH, Ouyangz SH (1999) A new triterpenoid oligoglycoside escin IVe from the seeds of Aesculus Chinensis. Chin Chem Lett 10(6):473–476
4. Jie G, Xiu WY (2004) Studies on Triterpenoid Saponins of seeds of Aesculus chinensis Bunge var. chekiangensis (Hu et Fang). J Chin Pharm Sci. 13(2):87–91
5. Zhang Z, Kauzo K, Jia Z, Nikaido T, Gao D, Zheng J (1999) New saponins from the seeds of Aesculus chinensis. Chem Pharm Bull 47(11):1515–1520
6. Yang X, Zhao J, Cui Y, Zhang L (1999) A pair of new geometrically isomeric Triterpenoid Saponins from the seeds of Aesculus chinensis. Chin Chem Lett 11:925–928
7. Cheng JT, Chen ST, Guo C, Jiao MJ, Cui WJ, Wang SH (2018) Triterpenoid Saponins from the seeds of Aesculus chinensis and their cytotoxicities. Nat Prod Bioprospect 8(1):47–56
8. Ireneusz K, Bogdan J, Barbara S, Anna S, Sonia P, Cosimo P, Federico F, Chlodwig F, Wieslaw O, 1997 Antitumoral activity of some compounds from Aesculus hippocastanum L. J Ethnopharmacol 57:266–273
9. Wei F, Ma S, Ly M, But PP, Khan IA (2004) Antiviral flavonoids from the fruits of Aesculus hippocastanum. J Nat Prod 67(4):650–653
10. Niu X, Wang Y, Li W, Zhang H, Wang X, Mu Q, He Z, Yao H (2015) Esculin exhibited ant-inflammatory activities in vivo and regulated TNF-α and IL-6 production in LPS-stimulated mouse peritoneal macrophages in vitro through MAPK pathway. Int Immunopharmacol 29(2):779–786
11. Zhang QL, Wu SQ, Lu XS (2009) Analysis of fatty acids composition in Aesculus chinensis seeds. Seed 28(8):53–55
12. Patolla JM, Raju J, Swamy MV, Rao CV (2006) Beta-escin inhibits colonic aberrant crypt foci formation in rats and regulates the cell cycle growth by inducing p21(waf1/cip1) in colon cancer cells. Mol Cancer Ther 5:1459–1466
13. Peng C, Fang K, Gong J (2016) Aescin reduces oxidative stress and provides neuroprotection in experimental traumatic spinal cord injury. Free Radical Bio Med 99:405–417
14. Matsuda H, Li Y, Murakami T, Ninomiya K, Araki N, Yoshikawa M (1997) Production of furanocoumarin oligoglycoside escin IVe from the seeds of Aesculus chinensis (Hu et Fang) Fang. J Chin Pharm Sci. Bunge var. chekiangensis (Hu et Fang). J Chin Pharm Sci. Bunge var. chekiangensis (Hu et Fang). J Chin Pharm Sci. Bunge var. chekiangensis (Hu et Fang). J Chin Pharm Sci. Bunge var. chekiangensis (Hu et Fang). J Chin Pharm Sci. Bunge var. chekiangensis (Hu et Fang). J Chin Pharm Sci. Bunge var. chekiangensis (Hu et Fang). J Chin Pharm Sci.
18. Tan YZ, Yong Y, Dong YH, Wang RJ, Li HX, Zhang H, Guo DL, Zhang SJ, Dong XP, Xie XF (2016) A new secoiridoid glycoside and a new sesquiterpenoid glycoside from Valeriana jatamansi with neuroprotective activity. Phytochem Lett 17:177–180
19. Elreadi MZ, Eid S, Ashour ML, Tahraii A, Wink M (2013) Modulation of multidrug resistance in cancer cells by chelidonine and Chelidonium majus alkaloids. Phytomedicine 20:282–294
20. Xia YZ, Yang L, Wang ZD, Guo C, Zhang C, Geng YD, Kong LY (2015) Schisandrin a enhances the cytotoxicity of doxorubicin by the inhibition of nuclear factor-kappa B signaling in a doxorubicin-resistant human osteosarcoma cell line. RSC Adv 5:13972–13984
21. Tanaka T, Nakashima T, Ueda T, Kenji T, Iako K (2007) Facile discrimination of aldose enantiomers by reversed-phase HPLC. Chem Pharm Bull 55:899–901
22. Zhang N, Huang WX, Xia GY, Oppong MB, Ding LQ, Li P (2018) Methods for determination of absolute configuration of monosaccharides. Chin Herb Med 10:14–22
23. Nakamura S, Zhang Y, Nakashima S, Oda Y, Matsuda H (2016) Structures of aromatic glycosides from the seeds of cassia auriculata. Chem Pharm Bull 64(7):970–974
24. Zou W, Zeng J, Zhuo M, Xu W, Sun L, Wang J (2002) Involvement of caspase-3 and p38 mitogen-activated protein kinase in cobalt chloride-induced apoptosis in PC12 cells. J Neurosci Res 67:837–843
25. Li GL, Hong G, Li XY, Zhang Y, Xu ZP, Mao LN, Feng XZ, Liu TJ (2018) Synthesis and activity towards Alzheimer’s disease in vitro: tacrine, phenolic acid and ligustrazine hybrids. Eur J Med Chem 148:238–254
26. Lubica H, Licht A, Sandig G, Manuela J, Zdena D, Tilman G (2003) Standardized extracts of flavonoids increase the viability of PC12 cells treated with hydrogen peroxide: effects on oxidative injury. Arch Toxicol 77:22–29

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.