Journal of Natural Medicines
https://doi.org/10.1007/s11418-019-01375-8

NOTE

Isolation, structure elucidation, tyrosinase inhibitory, and antioxidant evaluation of the constituents from *Angelica dahurica* roots

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Received: 3 October 2019 / Accepted: 16 November 2019

Abstract
Two undescribed phenolic compounds, angelicols A (**1**) and B (**2**) and one undescribed coumarin rhamnoside, angelicoside A (**3**), together with 17 known compounds (**4**–**20**) were isolated from the roots of *Angelica dahurica*. Their structures were characterized by physical data analyses such as NMR, HRESIMS, and X-ray diffraction. Compounds **2**, **3**, **5**, **6** and L-ascorbic acid (positive control) exhibited obvious DPPH radical scavenging activities with IC_{50} values of 0.36 mM, 0.43 mM, 0.39 mM, 0.44 mM, 0.25 mM, respectively. At a concentration of 25 μM, all compounds showed weaker tyrosinase inhibition activities (%inhibition < 5%) than kojic acid (26.00 ± 0.67%, IC_{50} = 44.29 ± 0.06 μM).

Keywords *Angelica dahurica* · Tyrosinase · X-ray diffraction · Antioxidant

Introduction

Angelica dahurica, a commonly used medicinal plant, belongs to the family Apiaceae. Its roots are widely utilized in Chinese medicine to treat various diseases such as toothache, headache, stuffy nose, supraorbital neuralgia, and nasosinusitis [1]. Previous investigations revealed that the major chemical constituents of the root part are coumarins [2–8]. Many of these coumarins showed significant cytotoxic [2], anti-inflammatory [5, 7], antioxidant [6], and tyrosinase inhibitory activities [9]. In addition, alkaloids [10], polysaccharides [11], and neoligans [12] have also been reported from *Angelica dahurica*. In a search for chemical constituents with strong anti-tyrosinase and antioxidant activities, the ethanol extracts of *Angelica dahurica* roots were explored, which provided three new compounds (**1**–**3**) and 17 known compounds (**4**–**20**) (Fig. 1). In the subsequent antioxidant and anti-tyrosinase assays, compounds **2**, **3**, **5**, and **6** exhibited obvious DPPH radical scavenging activities with IC_{50} values close to that of L-ascorbic acid.

Results and discussion

Compound **1** was obtained as cubic crystals. The molecular formula $C_{10}H_{14}O_3$, with four degrees of unsaturation, was established based on its quasi-molecular ion peak at *m/z* 183.1017 $[M+H]^+$ (calcd for $C_{10}H_{15}O_3$, 183.1016) in the HRESIMS spectrum. Its IR spectrum showed absorption bands for hydroxyl (3423, 3257 cm^{-1}) and aromatic ring (1610, 1596, 1513, 1448 cm^{-1}). The ^{1}H-NMR spectrum of **1** revealed the signals of an AA′BB′ spin system at δ 7.14 (dd, *J* = 7.6 Hz, H-5, H-7), 6.79 (d, *J* = 7.6 Hz, H-4 and H-8), which showed a 1,4-disubstituted phenyl ring. The proton signals at δ 3.46 (1H, dq, *J* = 14.8, 6.8 Hz, H-1′a), 3.35 (1H, dq, *J* = 14.8, 6.8 Hz, H-1′b), and 1.18 (3H, t, *J* = 6.8 Hz, H-2′), and the ^{13}C-NMR signals at δ 64.5 (C-1′) and 15.5 (C-2′) demonstrated the presence of an ethoxyl group. The remaining ^{1}H-NMR signals at δ 4.34 (1H, dd, *J* = 8.4, 3.6 Hz, H-1) and δ 3.64 (1H, dd, *J* = 11.2, 8.4 Hz,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11418-019-01375-8) contains supplementary material, which is available to authorized users.

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Published online: 26 November 2019

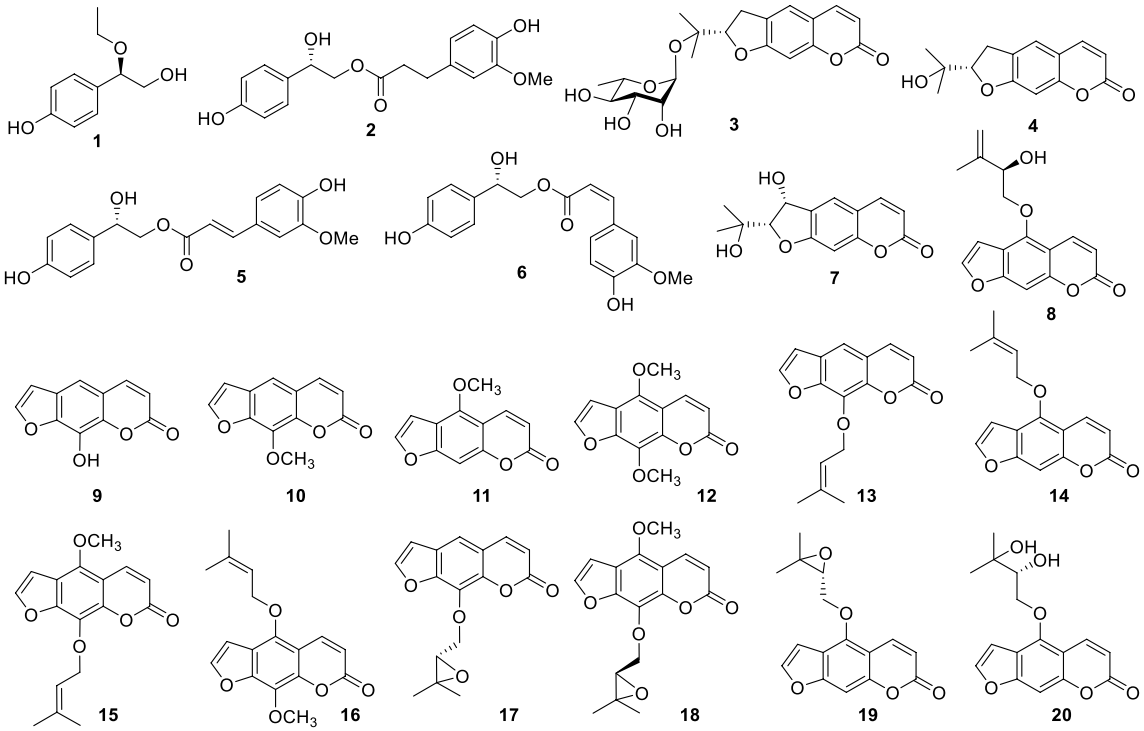

Fig. 1 Structures of compounds **1–20**

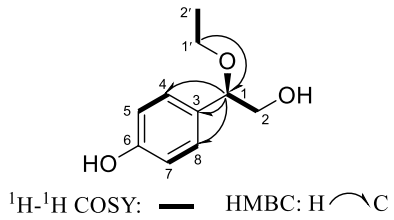

Fig. 2 Key ^{1}H-^{1}H COSY and HMBC correlations for compound **1**

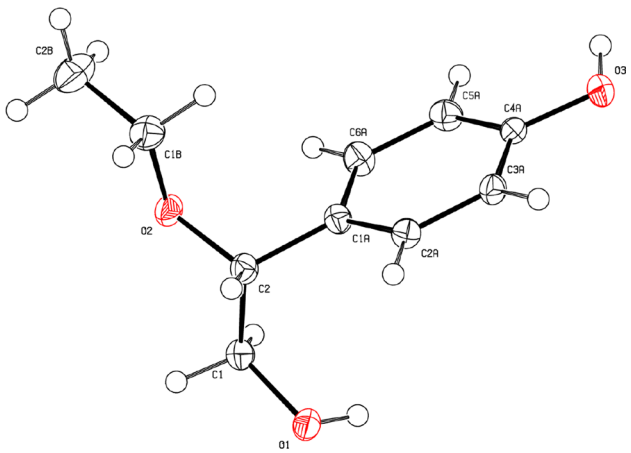

Fig. 3 ORTEP drawing of compound **1**

H-2a), δ 3.56 (1H, dd, J = 11.2, 3.6 Hz, H-2b) were ascribed to the moiety –O–$CHCH_2$–O–. The speculations above were confirmed by the key ^{1}H−^{1}H COSY, HSQC, and HMBC correlations shown in Fig. 2. The HMBC correlations from H-1′ to C-1 indicated that the ethoxyl group was connected with C-1. The HMBC correlations from H-1 to C-3, C-4, and C-8 suggested the –O–$CHCH_2$– was linked to C-3. To determine the absolute configuration of C-1, compound **1** was recrystallized carefully in acetone and the crystal was subjected to single-crystal X-ray diffraction analysis with Cu Kα radiation. The absolute configuration of compound **1** was assigned as 1*R* by the calculated Flack parameter of − 0.01(6) (Fig. 3). Therefore, the structure of compound **1** was identified as (1*R*)-ethoxyl-1-(4-hydroxyphenyl)ethane-1,2-diol, named as angelicol A.

Compound **2** was obtained as a colorless oil. Its molecular formula was established as $C_{18}H_{20}O_6$ based on the quasi-molecular ion peak at *m/z* 355.1158 $[M+Na]^+$ (calcd for $C_{18}H_{20}O_6Na$, 355.1158) in the HRESIMS spectrum, implying the presence of nine degrees of unsaturation. The IR absorption bands at 3409 and 1716 cm^{-1} were attributed to hydroxyl and carbonyl groups, respectively. The ^{1}H-NMR spectrum of **2** revealed signals of an aromatic ABX spin system at δ 6.65 (1H, d, J = 8.0 Hz, H-6′), 6.72 (1H, br d, J = 8.0 Hz, H-5′), 6.84 (1H, br s, H-2′), and an AA′BB′ system at δ 6.80 (2H, d, J = 8.0 Hz, H-5, H-7), 7.23 (2H, d, J = 8.0 Hz, H-4, H-8). The former indicated the presence of

a 1,3,4-trisubstituted aromatic ring, and the latter suggested a 1,4-disubstituted aromatic ring. In the ^{1}H-NMR and ^{1}H-^{1}H COSY spectra of **2**, the signals at δ 4.09 (2H, d, J = 5.6 Hz, H-2) and 4.81 (1H, t, J = 5.6 Hz, H-1) showed the existence of a –CH(OH)–CH_2O– subunit, while the signals at δ 2.58 (2H, t, J = 8.0 Hz, H-8′) and 2.81 (2H, t, J = 8.0 Hz, H-7′) indicated the existence of a –CH_2–CH_2– subunit. Based on the key HMBC signals such as correlations from H-1 to C-3, C-4, and C-8, from H-2 to C-9′ and correlations from H-7′ to C-1′, C-2′, and C-6′, the planar structure of **2** was established as shown in Fig. 4. Furthermore, compound **2** showed positive optical activity with $+[\alpha]_D^{20}$4.3° (c 0.09, CH_3OH) and the configuration of C-1 was proposed as *S* after comparison with the related analogs such as (*S*)-1-(4-hydroxyphenyl)ethane-1,2-diol [13], phomoaspardiol [14], phomopsuidiol [15], (*S*)-2-hydroxy-2-phenylethyl acetate [16], and (*R*)-2-hydroxy-2-phenylethyl acetate [17] (Fig. 5). Thus, the structure of compound **2** was elucidated as (1*S*)-2-*O*-dihydroferuloyl-1-(4-hydroxyphenyl)ethane-1,2-diol, named as angelicol B.

Compound **3** was also obtained as a colorless oil. Its molecular formula was established as $C_{20}H_{24}O_8$ based on the quasi-molecular ion peak at *m/z* 393.1552 $[M+H]^+$ (calcd for $C_{20}H_{25}O_8$, 393.1549) in the HRESIMS spectrum, implying the presence of nine degrees of unsaturation. The ^{1}H-NMR spectrum of **3** revealed the presence of a marmesin unit [δ 6.18 (1H, d, J = 9.6 Hz, H-3), 7.84 (1H, d, J = 9.6 Hz, H-4), 7.39 (1H, s, H-5), 6.70 (1H, s, H-8), 4.82 (1H, dd, J = 8.8, 8.4 Hz, H-2′), 3.26 (2H, m, H-3′), 1.37 (3H, s, H-5′), 1.31 (3H, s, H-6′)], and one L-rhamnose unit [δ 5.07 (1H, d, J = 1.2 Hz, H-1″), 3.63 (1H, dd, J = 1.2, 2.8 Hz, H-2″), 3.41 (1H, dd, J = 2.8, 9.6 Hz, H-3″), 3.30 (1H, dd, J = 9.6, 7.2 Hz, H-4″), 3.65 (1H, dq, J = 7.2, 6.4 Hz, H-5″), 1.15 (3H, d, J = 6.4 Hz, H-6″) (Table 1). The α-L-Rha was characterized by the small coupling constant $J_{1'',2''}$ (1.2 Hz) and supported by the key NOESY correlations from H-1′ to H-4″ and H-6″ (Fig. 6). The key HMBC correlation from H-1″ to C-4′ defined C-4′ of marmesin as the site of *O*-glycosylation. Moreover, the acid hydrolysis of **3** afforded marmesin (**4**) [18], which was also isolated as a natural product in this study. The $[\alpha]_D^{20}$ of **4** was measured to be + 11.7° (c 0.08, $CHCl_3$). Thus, the aglycone was established as marmesin, rather than its *R*-isomer nodakenetin ($[\alpha]_D^{20}$–25.4°) [19]. Based on the evidences described above, the structure of compound **3** was finally determined as shown in Fig. 6, named as angelicoside A.

By comparing their NMR data with those published literature values, the known compounds **4**–**20** were identified as follows: marmesin (**4**) [18], (1*S*)-2-*O*-*Z*-feruloyl-1-(4-hydroxyphenyl)ethane-1,2-diol (**5**) [20], (1*S*)-2-*O*-*E*-feruloyl-1-(4-hydroxyphenyl)ethane-1,2-diol (**6**) [20], xanthoarnol (**7**) [21], pangelin (**8**) [2], xanthotoxol (**9**) [22], xanthotoxin (**10**) [23], bergapten (**11**) [24], isopimpinellin (**12**) [23], imperatorin (**13**) [25], isoimperatorin (**14**) [26], phellopterin (**15**) [27], cnidilin (**16**) [28], heraclenin (**17**) [28], byakangelicol (**18**) [29], (+)-oxypeucedanin (**19**) [30],

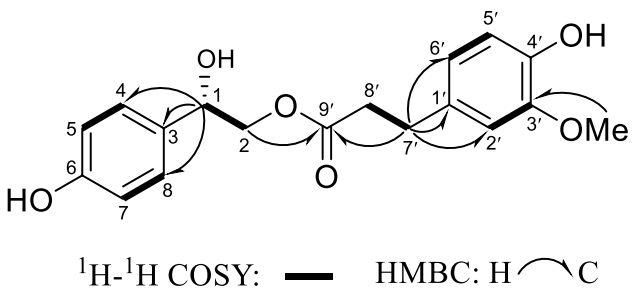

Fig. 4 Key ^{1}H-^{1}H COSY and HMBC correlations for compound **2**

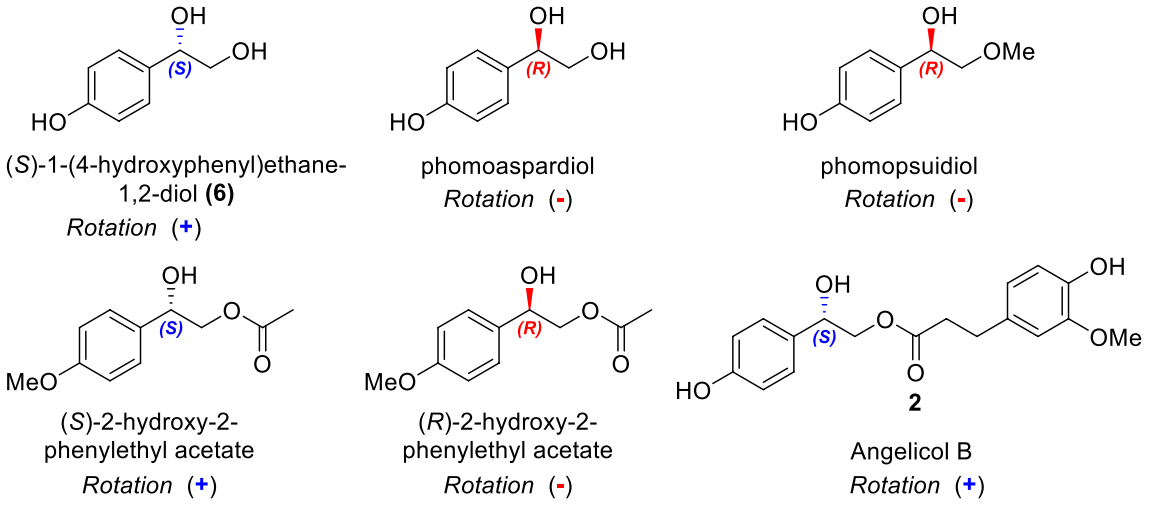

Fig. 5 Optical rotations of compound **2** and its analogs

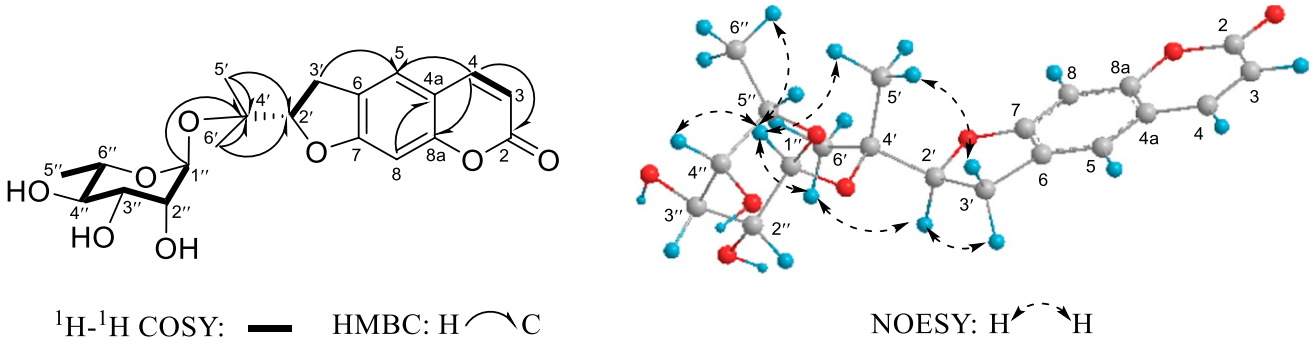

Fig. 6 Key ^{1}H-^{1}H COSY, HMBC, and NOESY correlations for compound **3**

Table 1 ^{1}H-NMR and ^{13}C-NMR (CD_3OD) of compound **3**

Position	δ_C	δ_H (mult, *J* in Hz)	Position	δ_C	δ_H (mult, *J* in Hz)
2	163.9		3′	30.6	3.26 (m)
3	112.3	6.18 (d, 9.6)	4′	79.1	
4	146.4	7.84 (d, 9.6)	5′	22.8	1.37 (s)
4a	114.2		6′	22.3	1.31 (s)
5	125.0	7.39 (s)	1″	96.3	5.07 (d, 1.2)
6	127.3		2″	73.6	3.63 (dd, 1.2, 2.8)
7	165.4		3″	72.4	3.41 (dd, 2.8, 9.6)
8	98.2	6.70 (s)	4″	74.1	3.30 (dd, 9.6, 7.2)
8a	157.1		5″	70.0	3.65 (dq, 7.2, 6.4)
2′	92.1	4.82 (dd, 8.8, 8.4)	6″	18.0	1.15 (d, 6.4)

Table 2 DPPH radical scavenging activity of compounds **1**–**20** and L-ascorbic acid

Compounds	DPPH radical scavenging activity (%)	Compounds	DPPH radical scavenging activity (%)
1	26.26 ± 0.46	**12**	21.74 ± 0.80
2	74.40 ± 0.35	**13**	23.62 ± 1.93
3	69.80 ± 0.36	**14**	20.34 ± 0.54
4	28.05 ± 0.42	**15**	25.12 ± 0.34
5	72.74 ± 0.30	**16**	22.79 ± 1.25
6	71.69 ± 0.28	**17**	24.70 ± 0.86
7	22.67 ± 0.48	**18**	25.15 ± 0.38
8	23.62 ± 1.93	**19**	34.30 ± 0.51
9	66.03 ± 0.30	**20**	44.26 ± 0.29
10	27.21 ± 0.44	L-ascorbic acid	83.32 ± 0.26
11	11.96 ± 0.42		

The radical scavenging effects of compounds **1**–**20** and L-ascorbic acid were measured at a concentration of 1 mM. Three independent experiments were performed and the results were expressed as mean ± SEMs

oxypeucedanin hydrate (**20**) [31], respectively. Compounds **1**–**3** and **5**–**6** were reported from *Angelica dahurica* for the first time.

The mushroom tyrosinase inhibitory activities of compounds **1**–**20** and kojic acid (positive control) were evaluated at a concentration of 25 μM. All isolated compounds showed weaker tyrosinase inhibition activities (%inhibition < 5%) than kojic acid (26.00 ± 0.67%, IC_{50} = 44.29 ± 0.06 μM).

DPPH radical scavenging assays were conducted to assess the radical scavenging effects of the 20 isolated compounds (**1**–**20**) at a concentration of 1 mM, with L-ascorbic acid as the positive control. The results were shown in Table 2. Compounds **2** and its two derivatives **5**–**6** exhibited considerable DPPH radical scavenging activities: 74.40 ± 0.35%, IC_{50} = 0.36 mM for **2**; 72.74 ± 0.30%, IC_{50} = 0.39 mM for **5**; 71.69 ± 0.28%, IC_{50} = 0.44 mM for **6**; 83.32 ± 0.26%, IC_{50} = 0.25 mM for L-ascorbic acid. Just as Moon's group has reported, compounds with a 4-hydroxy-3-methoxyl substituent on the phenyl ring usually exhibited obvious DPPH radical scavenging activity [32, 33]. Interestingly, the new glycoside **3** showed a good radical scavenging activity with 69.80 ± 0.36% inhibition, while its aglycone **4** showed a weak activity. Perhaps the sugar moiety played a key role in the process of DPPH radical scavenging.

Experimental section

General experimental procedures

Melting points (uncorrected) were measured on an X-5 microscopic melting point apparatus (Yuhua Instrument Inc., China). Optical rotations were determined on a Rudolph Autopol IV polarimeter (589 nm, 20 °C). FT-IR and UV spectra were determined using FTIR-650 and Puxi TU-1950 instruments, respectively. NMR spectra were recorded on a Bruker AM-400 spectrometer. High-resolution electrospray ionization mass spectra (HRESIMS) were carried out on a Waters Xevo G2-XS QTof spectrometer, a Thermo Fisher

Ultimate 3000 RSLC/Q spectrometer or a Bruker micrOTOF II spectrometer. The X-ray diffraction experiments were carried out on a Rigaku XtaLAB synergy four-circle diffractometer with Cu Kα radiation ($\lambda = 1.54184$ Å). Column chromatography (CC) was performed using silica gel (Qingdao Marine Chemical Inc., China), RP-C18 (50 μm, Fuji Silysia Chemical Ltd., Japan), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden). TLC was performed with silica gel 60 F254 (Yantai Chemical Industry Research Institute).

Plant material

The fresh roots of *Angelica dahurica* were collected in Yuzhou, People's Republic of China, in August 2018. The botanical identification was made by Prof. Lin Yang, School of Life Science and Engineering, Lanzhou University of Technology. A voucher specimen (SPH2018A) was deposited in the herbarium of School of Chemistry and Chemical Engineering, Xuchang University.

Extraction and isolation

The roots of *Angelica dahurica* (10 kg) were extracted with 95% EtOH at room temperature (3 × 50 L) to afford a crude extract of 523 g after evaporation of the solvent under vacuum. The extract was suspended in distilled H_2O and partitioned with EtOAc and *n*-BuOH. The EtOAc soluble portion (137 g) was subjected to silica gel CC using petroleum ether–EtOAc (100:0 to 1:2) as eluents to give nine fractions F1–F9. The fraction F1 (eluted by petroleum ether–EtOAc 100:1) was subsequently purified using RP-C_{18} CC (MeOH–H_2O, 85:15 to 100:0) to afford compounds **10** (3.3 mg), **11** (2.8 mg), **12** (4.1 mg), **13** (32.5 mg), **14** (24.7 mg), **15** (13.6 mg), **16** (7.8 mg), **18** (5.2 mg). The fraction F2 (eluted by petroleum ether–EtOAc 70:1) was further separated using Sephadex LH-20 column (CH_2Cl_2) to give compounds **8** (4.3 mg), **9** (3.5 mg), and **17** (4.4 mg). The fraction F5 (eluted by petroleum ether–EtOAc 50:1) was passed through a RP-C_{18} CC eluted with MeOH-H_2O (90:10) to give seven subfractions (F5-1 to F5-7). The subfraction F5-1 was chromatographed on the silica gel CC to give compounds **19** (5.0 mg) and **20** (11.6 mg). The subfraction F5-3 was purified by RP-C_{18} CC (MeOH–H_2O, 85:15) to give compounds **1** (10.7 mg) and **4** (7.9 mg). The subfraction F5-5 was purified by Sephadex LH-20 column (CH_2Cl_2–MeOH 1:1) to afford compounds **5** (6.7 mg) and **7** (14.8 mg). The fraction F8 (eluted by petroleum ether–EtOAc 3:1) was further separated using Sephadex LH-20 column (MeOH) to give four subfractions F8-1–F8-4. After further purification with RP-C_{18} CC, the subfraction F8-1 gave compound **2** (3.1 mg) and the subfraction F8-5 gave compounds **3** (8.4 mg) and **6** (5.2 mg).

Compound 1

Colorless cubic crystals. $+[\alpha]_D^{20}$3.9° (c 0.25, $CHCl_3$). mp 101 − 102 °C. IR (KBr) ν_{max} 3423, 3257, 1610, 1596, 1513, 1448 cm^{-1}. UV λ_{max} (MeOH) nm (log ε): 239 (2.8), 275 (2.8). HRESIMS *m/z* 183.1017 $[M+H]^+$ (calcd for $C_{10}H_{15}O_3$, 183.1016). ^{1}H-NMR ($CDCl_3$) δ: 1.18 (3H, t, *J* = 6.8 Hz, H-2′), 2.62 (1H, br s, OH), 3.35 (1H, dq, *J* = 14.8, 6.8 Hz, H-1′b), 3.46 (1H, dq, *J* = 14.8, 6.8 Hz, H-1′a), 3.56 (1H, dd, *J* = 11.2, 3.6 Hz, H-2b), 3.64 (1H, dd, *J* = 11.2, 8.4 Hz, H-2a), 4.34 (1H, dd, *J* = 8.4, 3.6 Hz, H-1), 6.09 (1H, br s, OH), 6.79 (d, *J* = 7.6 Hz, H-4, H-8), 7.14 (dd, *J* = 7.6 Hz, H-5, H-7). ^{13}C-NMR ($CDCl_3$) δ: 15.5 (C-2′), 64.5 (C-1′), 67.6 (C-2), 82.3 (C-1), 115.6 (C-4, C-8), 128.4 (C-5, C-7), 131.0 (C-3), 155.9 (C-6).

Compound 2

Colorless oil. $+[\alpha]_D^{20}$4.3° (c 0.09, CH_3OH). IR (KBr) ν_{max} 3409, 1716, 1616, 1513, 1448 cm^{-1}. UV λ_{max} (MeOH) nm (log ε): 225 (2.9), 278 (3.5). HRESIMS *m/z* 355.1158 $[M+Na]^+$ (calcd for $C_{18}H_{20}O_6Na$, 355.1158). ^{1}H-NMR ($(CD_3)_2CO$) δ: 2.58 (2H, t, *J* = 8.0 Hz, H-8′), 2.81 (2H, t, *J* = 8.0 Hz, H-7′), 3.82 (3H, s, OMe), 4.09 (2H, d, *J* = 5.6 Hz, H-2), 4.46 (1H, br s, OH), 4.81 (1H, t, *J* = 5.6 Hz, H-1), 6.65 (1H, br d, *J* = 8.0 Hz, H-6′), 6.72 (1H, br d, *J* = 8.0 Hz, H-5′), 6.80 (2H, d, *J* = 8.0 Hz, H-5, H-7), 6.84 (1H, br s, H-2′), 7.23 (2H, d, *J* = 8.0 Hz, H-4, H-8), 7.36 (1H, br s, OH), 8.33 (1H, br s, OH). ^{13}C-NMR ($(CD_3)_2CO$) δ: 31.2 (C-7′), 36.8 (C-8′), 56.2 (–OMe), 70.0 (C-2), 71.9 (C-1), 112.8 (C-2′), 115.7 (C-5′), 115.8 (C-5, C-7), 121.5 (C-6′), 128.4 (C-4, C-8), 133.1 (C-1′), 133.5 (C-3), 145.9 (C-4′), 148.3 (C-3′), 157.8 (C-6), 173.1 (C-9′).

Compound 3

Colorless oil. $[\alpha]_D^{20}$–66.1° (c 0.26, CH_3OH). IR (KBr) ν_{max} 3428, 1733, 1625, 1567, 1394 cm^{-1}. UV λ_{max} (MeOH) nm (log ε): 225 (4.2), 248 (3.6), 335 (3.9). HRESIMS *m/z* 393.1552 $[M+H]^+$ (calcd for $C_{20}H_{25}O_8$, 393.1549). ^{1}H-NMR and ^{13}C-NMR data (CD_3OD), see Table 1.

Acid hydrolysis of 3

The acid hydrolysis of **3** was conducted according to the literature with slight modifications [18]. Compound **3** (4.0 mg) was dissolved in 3.0% HCl (4 mL) and heated at 85 °C for 1 h. The reaction mixture was diluted with EtOAc, washed with water, dried (Na_2SO_4), concentrated and purified by the silica gel CC to give marmesin (**4**) [18] and L-rhamnose, $+[\alpha]_D^{20}$5.2° (c 0.05, H_2O) [reported: $+[\alpha]_D^{20}$2.4° (c 1, H_2O)] [34].

Single-crystal X-ray diffraction analysis of compound 1

The single-crystal diffraction data of compound **1** were collected on a Rigaku XtaLAB synergy four-circle diffractometer with Cu Kα radiation ($\lambda = 1.54178$ Å), with the CrysAlisPro software (version 1.171.39.34b) for data reduction and analysis. The single-crystal diffraction data were collected at 150 K and the structures were solved by direct methods and refined by full-matrix least-squares method on F2 using SHELX algorithms in Olex2. All non-hydrogen atoms were refined with anisotropic displacement parameters. All hydrogen atoms were generated geometrically. The X-ray crystallographic data have been deposited at the Cambridge Crystallographic Data Centre (CCDC, ref. No. 1955041).

Crystallographic Data of ***1****:* $C_{10}H_{14}O_3$, $M = 182.21$, monoclinic, Cc, $a = 12.3276(6)$ Å, $b = 5.0380(1)$ Å, $c = 17.4645(3)$ Å, $\alpha = \gamma = 90°$, $\beta = 96.324(1)$, $V = 1099.64(3)$ Å^3, $T = 150$ K, $Z = 4$, $D_{calcd} = 1.276$ g cm^{-3}, μ (Cu Kα) 0.77 mm^{-1}, crystal size $0.2 \times 0.2 \times 0.2$ mm^3, $F(000) = 392$. The final R_1 value is 0.030 ($wR_2 = 0.076$) for 1954 reflections [$I > 2\sigma(I)$]. Flack structure parameter: − 0.01 (6).

Mushroom tyrosinase inhibition assay

Mushroom tyrosinase inhibition assay was conducted according to the literatures [32, 35] with slight modifications. 10 µL of the tested compounds (**1**–**20**, 25 µM) and 20 µL of mushroom tyrosinase (1000 U/mL) in a 50 mM potassium phosphate buffer (pH 6.5) were added to 170 µL of an assay mixture containing a 10:10:9 ratio of 1 mM L-tyrosine solution, 50 mM potassium phosphate buffer (pH 6.5), and distilled water in a 96-well microplate. After incubation of the reaction mixture at 37 °C for 30 min, the absorbance of the mixture was measured at 450 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc.). The extent of inhibition from the samples was expressed as the concentration necessary for 50% inhibition (IC_{50}). Kojic acid (25 µM) was used as the control. Three independent experiments were performed. The %inhibition was determined by $[1 - (A_s/A_c)] \times 100$, where A_s is the absorbance of tested compound and A_c the non-treated control.

DPPH radical scavenging assay

A slightly modified version of a previously described DPPH radical scavenging assay was used [32]. Briefly, 20 µL of a sample solution (in DMSO, 1 mM) and 180 µL of 0.2 mM DPPH methanol solution were added to 96-well microplate. L-ascorbic acid was used as the positive control. Mixtures were then incubated for 30 min in the dark and antioxidant activities were determined by measuring absorbance at 517 nm using a Multiskan FC microplate reader. Experiments were performed in triplicate. The DPPH radical scavenging activity was calculated by radical scavenging activity (%) $= [1-(A_s/A_c)] \times 100$, where A_c is the absorbance of the non-treated control and A_s is the absorbance of tested compound.

Acknowledgements We are grateful to Lanxiang Dai for the collection of *Angelica dahurica* roots and Prof. Lin Yang at Lanzhou University of Technology for the authentication of the plant material. This work was financially supported by National Natural Science Foundation of China (no. 21702178), Key Scientific Research Program in Universities of Henan Province (no. 18A350010), Undergraduate Training Program for Innovation and Entrepreneurship of Henan Province (nos. 201910480011, 201910480017), Science and Technology Project of Henan Province (no. 182102311108), and Excellent Young Key Teacher Funding Project of Xuchang University (no. 2017).

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