Three new isoflavone glycosides from Tongmai granules

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Three new isoflavone glycosides, 3′-methoxydaidzein-7,4′-di-*O*-β-D-glucopyranoside (**1**), biochanin A-8-*C*-β-D-apiofuranosyl-(1 → 6)-*O*-β-D-glucopyranoside (**2**), daidzein-7-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranoside (**3**), and a new natural isoflavone glycoside, daidzein-7-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranoside (**4**) were isolated along with 18 known isoflavones from the EtOAc and *n*-BuOH fractions of the aqueous extraction of Tongmai granules. All the isoflavones were obtained and determined for the first time from Tongmai granules. The structures of these compounds were elucidated by spectral methods. It was confirmed that the compounds **1**–**4** were originally from Puerariae Lobatae Radix based on HPLC-DAD analysis of the crude drug extract. The isoflavones isolated were tested for their antioxidative activities by measuring the capacities of scavenging the 2,2′-diphenyl-1-picrylhydrazyl radical.

Keywords: Tongmai granules; 3′-methoxydaidzein-7,4′-di-*O*-β-D-glucopyranoside; biochanin A-8-*C*-β-D-apiofuranosyl-(1 → 6)-*O*-β-D-glucopyranoside; daidzein-7-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranoside; DPPH radical-scavenging activity

1. Introduction

Tongmai granules, consisting of the water extracts of three herbs Puerariae Lobatae Radix, Salviae Miltiorrhizae Radix et Rhizoma, and Chuanxiong Rhizoma, are a formula created under the theory of traditional Chinese medicine to invigorate the 'blood' and dispel 'blood stasis', which arises from poor blood circulation. Water extraction of *Pueraria* radix has an effect on the activity of hepatic antioxidant enzymes and lipid profile in ethanol-treated rats. It can contribute to alleviating the adverse effect of ethanol ingestion by enhancing the lipid metabolism as well as the hepatic antioxidant defense system [1].

To find the substantial foundation of therapy, we first studied the chemical constituents of Tongmai granules. In our recent research on chemical constituents, three new isoflavone glycosides, 3′-methoxydaidzein-7,4′-di-*O*-β-D-glucopyranoside (**1**), biochanin A-8-*C*-β-D-apiofuranosyl-(1 → 6)-*O*-β-D-glucopyranoside (**2**), daidzein-7-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranoside (**3**), and a new natural isoflavone glycoside, daidzein-7-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranoside (**4**) were isolated along with 18 known isoflavones (Figures 1 and 2). Their structures were determined by the HR-MS and NMR
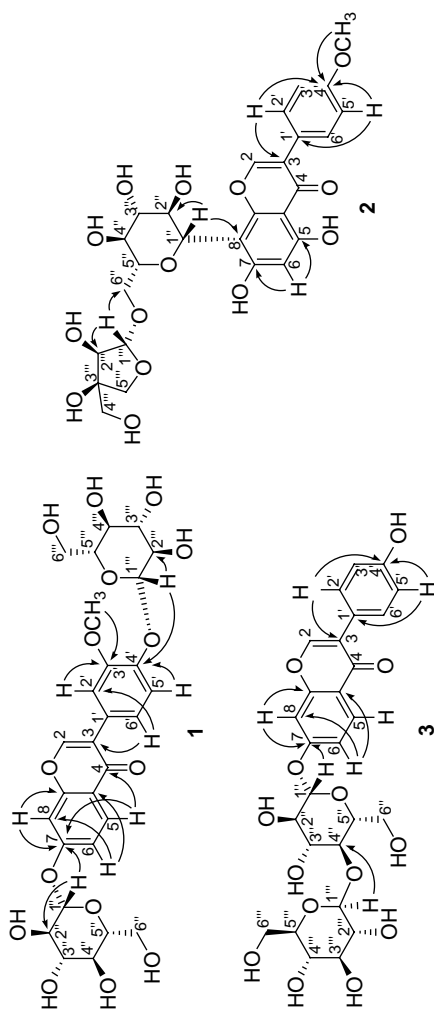
*Corresponding author. Email: xwyang@bjmu.edu.cn

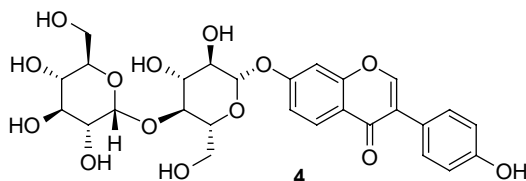Figure 1.   Structures and key HMBC correlations of compounds **1**–**3**.

Figure 2. Structure of compound **4**.

spectroscopic analysis. It was confirmed that compounds **1**–**4** were originally from Puerariae Lobatae Radix based on HPLC-DAD analysis of the crude drug extract. The isoflavones isolated were tested for their antioxidative activities by measuring the capacities of scavenging the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical, using butylated hydroxytoluene (BHT) and ascorbic acid as standards [2,3].

2. Results and discussion

2.1 Characterization of isoflavones

Compound **1** was obtained as a white amorphous powder. Its molecular formula was determined as $C_{28}H_{32}O_{15}$ on the basis of the HR-ESI-MS (positive ion mode) data at m/z 609.1821 $[M + H]^+$. The IR spectrum showed characteristic absorption bands for hydroxyl (3413 cm^{-1}) and carbonyl (1733 cm^{-1}) groups, and aromatic ring (1648, 1553, and 1511 cm^{-1}). The characteristic absorption maxima in the UV spectrum (257, 287, and 304 nm) and the proton singlet at δ_H 8.47 (H-2) in the ^{1}H NMR spectrum indicated **1** to be an isoflavone [4]. ESI-TOF-MS (positive ion mode) showed quasimolecular ions $[M + H]^+$ at m/z 609 and $[M + Na]^+$ at m/z 631, and fragment ions at m/z 447 $[M + H-162]^+$ and 285 $[M + H-162-162]^+$ were also observed. The loss of two 162 units suggested the presence of two *O*-hexosyl groups. The mass difference of 284 between **1** and the two hexoses suggested that the aglycone moiety of **1** was perhaps substituted with two hydroxyls and one methoxyl group. In

the ^{1}H NMR spectrum, the signal at δ_H 8.05 (1H, d, $J = 8.8$ Hz) was assigned to H-5; the signals at δ_H 7.24 (1H, d, $J = 2.0$ Hz) and 7.23 (1H, d, $J = 2.4$ Hz) were assigned to H-2' and H-8, respectively; the signal at δ_H 7.15 (1H, dd, $J = 2.4$, 8.8 Hz) was assigned to H-6; the signals at δ_H 7.14 (1H, d, $J = 8.8$ Hz) and 7.10 (1H, dd, $J = 2.0$, 8.8 Hz) were assigned to H-5' and H-6', respectively; and the signal at δ_H 3.79 (3H, s) was ascribed to a methoxyl group. Therefore, it can be concluded that **1** was a 7,3',4'-trisubstituted isoflavone glycoside. The ^{13}C NMR spectrum of **1** exhibited 12 aliphatic carbon signals due to two hexosyl moieties. These were in good agreement with the published data for the sugar moieties of daidzein-7,4'-*O*-glucopyranoside [4]. The ^{13}C NMR spectrum of the aglycone moiety of **1** was quite similar to the published data of 3'-methoxydaidzin except for the B-ring [5]. A comparison of the ^{1}H and ^{13}C NMR spectral data between **1** and published data of 3'-methoxydaidzin [5] indicated that H-2', H-5', and H-6' were upfield shifted by $\Delta\delta$ 0.05, 0.31, and 0.08, respectively; C-1' and C-3' were upfield shifted by $\Delta\delta$ 2.8 and 1.4, respectively; and C-4' was downfield shifted by $\Delta\delta$ 0.2, suggesting that **1** was 4'-*O*-glucopyranoside of 3'-methoxydaidzin. In the HMBC spectrum, correlations of H-1'' (Glu H-1) at δ_H 5.11 with C-7 (δ_C 161.4) and C-2'' (δ_C 73.2) were observed (Figure 1), showing that one *O*-glucosyl unit was attached to C-7, and correlation of H-1''' (Glu H-1) at δ_H 4.95 with C-4' (δ_C 146.3) was observed (Figure 1), suggesting that another *O*-glucosyl group was attached to

C-4′. Meanwhile, the HMBC experiment showed the correlations of the protons at δ_H 3.79 (OCH$_3$) and 7.24 (H-2′) with C-3′ at δ_C 148.5, which indicated that the OCH$_3$ group was attached to C-3′. The β-configurations of the two glucosyl groups were confirmed by the large *J* values (7.2 and 7.2 Hz, respectively) of their anomeric protons due to axial interaction. Based on the above evidence, **1** was characterized as 3′-methoxydaidzein-7,4′-di-*O*-β-D-gluco-pyranoside (Figure 1). The ^{1}H and ^{13}C NMR spectral data are listed in Tables 1 and 2. The signals were confirmed by means of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra.

Compound **2** was obtained as a white amorphous powder. The molecular formula, C$_{27}$H$_{30}$O$_{14}$, was determined by HR-ESI-MS at *m/z* 579.1709 [M + H]$^+$. The IR spectrum showed characteristic absorption bands for hydroxyl (3366 cm^{-1}) and carbonyl (1651 cm^{-1}) groups, and aromatic ring (1615, 1579, and 1510 cm^{-1}). The absorption bands at 263 and 326 nm in the UV spectrum and the proton singlet at δ_H 8.42 (H-2) in the ^{1}H NMR spectrum suggested **2** to be an isoflavone. ESI-TOF-MS (positive ion mode) showed [M + H]$^+$ at *m/z* 579 and [M + Na]$^+$ at *m/z* 601, and fragment ions at *m/z* 447 [M + H-132]$^+$ and 327 [M + H-132-120]$^+$ were also observed in the spectrum. The loss of 132 units indicated the presence of one *O*-pentosyl group, and the strong loss of 120 units indicated the presence of one *C*-hexosyl group [6]. The mass difference of 284 between **2** and the disaccharide unit (a hexose and a pentose) indicated that the aglycone moiety of **2** was substituted with two hydroxyl groups and one methoxyl group. In the ^{1}H NMR spectrum, the signals at δ_H 6.99 (2H, d, *J* = 8.8 Hz) and 7.51 (2H, d, *J* = 8.8 Hz) were assigned to H-3′, -5′ and H-2′, -6′, respectively; the signals at δ_H 6.31 (1H, s) and 13.16 (1H, s) were assigned to H-6 and 5-OH, respectively; and the signal at δ_H 3.78 (3H, s) was ascribed to a methoxyl group. Therefore,

it can be concluded that **2** was a 5,7,8,4′-tetrasubstituted isoflavone. The ^{13}C NMR spectrum of **2** exhibited 11 aliphatic glycosidic carbon signals due to one pentosyl and one hexosyl moiety. These were in good agreement with the published data for the sugar moiety of daidzein-8-*C*-β-D-apiofuranosyl-(1 → 6)-*O*-β-D-gluco-pyranoside and genistein-8-*C*-β-D-apio-furanosyl-(1 → 6)-*O*-β-D-glucopyrano-side [4]. Thus, the sugar moiety of **2** was deduced to be β-D-apiofuranosyl-(1 → 6)-*O*-β-D-glucopyranoside. In the HMBC spectrum, correlations of H-1″ (Glu H-1) at δ_H 4.65 with C-8 (δ_C 104.5) and C-2″ (δ_C 70.5) were observed (Figure 1), showing that the *C*-glucosyl group was attached to C-8. Meanwhile, the HMBC spectrum showed the correlations of the protons at δ_H 3.78 (OCH$_3$) and 7.51 (H-2′,6′) with C-4′ (δ_C 159.1), and the proton at δ_H 4.77 (Api H-1) with the carbons at δ_C 68.4 (Glu C-6), 75.7 (Api C-2), and 78.8 (Api C-3), which indicated that the OCH$_3$ group was attached to C-4′ and the apiosyl group was linked to C-6″ of the glucosyl group. The β-configuration of the glucosyl group was confirmed by the large *J* value (8.4 Hz) of its anomeric proton due to axial interaction. Based on the above evidence, **2** was elucidated as biochanin A-8-*C*-β-D-apiofuranosyl-(1 → 6)-*O*-β-D-glucopyra-noside (Figure 1). The ^{1}H and ^{13}C NMR spectral data are listed in Tables 1 and 2. The signals were confirmed by means of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra.

Compound **3** was obtained as a white amorphous powder. The molecular formula, C$_{27}$H$_{30}$O$_{14}$, was determined by HR-ESI-MS at *m/z* 579.1729 [M + H]$^+$. The IR spectrum showed characteristic absorption bands for hydroxyl (3392 cm^{-1}) and carbonyl (1742 cm^{-1}) groups, and aromatic ring (1628, 1577, and 1500 cm^{-1}). The absorption maxima at 261 and 303 nm in the UV spectrum and the proton singlet at δ_H 8.37 (H-2) in the ^{1}H NMR spectrum suggested **3** to be an isoflavone too.

Table 1. ¹H NMR (400 MHz) spectral data of **1**–**4** in DMSO-d_6.

No.	1 (*J* in Hz)	2 (*J* in Hz)	3 (*J* in Hz)	4 (*J* in Hz)
2	8.47 s	8.42 s	8.37 s	8.37 s
5	8.05 d (8.8)	–	8.04 d (8.8)	8.04 d (8.8)
6	7.15 dd (8.8, 2.4)	6.31 s	7.14 dd (8.8, 2.4)	7.14 dd (8.8, 2.4)
8	7.23 d (2.4)	–	7.24 d (2.4)	7.24 d (2.4)
2′	7.24 d (2.0)	7.51 d (8.8)	7.39 d (8.0)	7.39 d (8.0)
3′	–	6.99 d (8.8)	6.81 d (8.0)	6.81 d (8.0)
5′	7.14 d (8.8)	6.99 d (8.8)	6.81 d (8.0)	6.81 d (8.0)
6′	7.10 dd (8.8, 2.0)	7.51 d (8.8)	7.39 d (8.0)	7.39 d (8.0)
1″	5.11 d (7.2)	4.65 d (8.4)	5.19 d (7.6)	5.18 d (8.0)
2″	3.28 dd (7.2, 8.8)	3.92 dd (8.4, 8.8)	3.04 dd (7.6, 8.4)	3.06 dd (8.0, 9.2)
3″	3.29 t (8.8)	3.23 t (8.8)	3.46 t (8.4)	3.46 t (9.2)
4″	3.18 t (8.8)	3.13 t (8.8)	3.42 t (8.4)	3.43 t (9.2)
5″	3.45 ddd (2.0, 4.0, 8.8)	3.34 ddd (2.4, 4.4, 8.8)	3.69 ddd (3.2, 4.8, 8.4)	3.65 ddd (2.4, 4.4, 9.2)
6″	3.68 dd (2.0, 12.0) / 3.44 dd (4.0, 12.0)	3.91 dd (2.4, 10.8) / 3.34 dd (4.4, 10.8)	3.73 dd (3.2, 12.0) / 3.39 dd (4.8, 12.0)	3.74 dd (2.4, 11.2) / 3.42 dd (4.4, 11.2)
1‴	4.95 d (7.2)	4.77 d (2.4)	4.30 d (8.0)	5.07 d (4.0)
2‴	3.27 dd (7.2, 8.8)	3.73 d (2.4)	3.40 dd (8.0, 8.8)	3.39 dd (4.0, 9.2)
3‴	3.29 t (8.8)	–	3.15 t (8.8)	3.16 t (9.2)
4‴	3.18 t (8.8)	3.82 d (8.4) / 3.56 d (8.4)	3.06 t (8.8)	3.08 t (9.2)
5‴	3.45 ddd (2.0, 4.0, 8.8)	–	3.18 ddd (2.4, 4.8, 8.8)	3.18 ddd (2.4, 4.8, 9.2)
6‴	3.68 dd (2.0, 12.0) / 3.44 dd (4.0, 12.0)	3.29 d (12.0) / 3.31 d (12.0)	3.65 dd (2.4, 12.0) / 3.47 dd (4.8, 12.0)	3.65 dd (2.4, 12.0) / 3.48 dd (4.8, 12.0)
OCH₃	3.79 s	3.78 s	–	–
5-OH	–	13.16 s	–	–
4′-OH	–	–	9.69 s	9.54 s

Table 2. ^{13}C NMR (100 MHz) spectral data of **1**–**4** in DMSO-d_6.

No	1	2	3	4
2	153.9	154.1	153.2	153.3
3	123.4	121.7	123.6	123.7
4	174.6	180.4	174.7	174.8
5	127.0	161.1	126.9	127.0
6	115.6	99.3	115.5	115.6
7	161.4	162.9	161.2	161.3
8	103.4	104.5	103.3	103.4
4a	118.4	104.2	118.5	118.6
8a	157.0	156.4	157.2	157.3
1′	125.5	122.9	122.2	122.3
2′	113.5	130.2	130.0	130.1
3′	148.5	113.7	115.0	115.0
4′	146.3	159.1	157.0	157.0
5′	115.1	113.7	115.0	115.0
6′	121.2	130.2	130.0	130.1
1″	100.0	73.2	99.5	99.7
2″	73.2	70.5	73.3	73.6
3″	76.5	78.6	74.8	76.1
4″	69.6	70.5	79.6	79.0
5″	77.2	80.0	75.4	75.4
6″	60.6	68.4	61.0	60.8
1‴	100.0	109.0	103.1	100.7
2‴	73.1	75.7	72.8	72.5
3‴	76.8	78.8	76.5	73.3
4‴	69.6	73.2	70.0	69.9
5‴	77.0	63.0	76.8	72.7
6‴	60.6	–	60.2	60.2
OCH$_3$	55.8	55.2	–	–

ESI-TOF-MS (positive ion mode) showed [M + H]$^+$ at m/z 579, [M + Na]$^+$ at m/z 601, and [M + H-162-162]$^+$ at m/z 255, and ESI-TOF-MS (negative ion mode) showed [M − H]$^-$ at m/z 577 and [M − H-162-162]$^-$ at m/z 253. The loss of two 162 units indicated the presence of two O-hexosyl substituents. The mass difference of 254 between **3** and the two hexoses suggested that the aglycone moiety was substituted with two hydroxyl groups. The ^{1}H NMR spectrum showed the signals at δ_H 9.69 (1H, s, 4′-OH), 8.04 (1H, d, J = 8.8 Hz, H-5), 7.14 (1H, d, J = 8.8, 2.4 Hz, H-6), 7.24 (1H, d, J = 2.4 Hz, H-8), 7.39 (2H, d, J = 8.0 Hz, H-2′, -6′), and 6.81 (2H, d, J = 8.0 Hz, H-3′, -5′). These signals suggested **3** to be a 7,4′-disubstituted isoflavone. The presence of a signal

at δ_H 9.69 (1H, s, 4′-OH) indicated that the disaccharide unit (two hexosyl groups) was attached to C-7. The ^{13}C NMR spectrum exhibited 12 aliphatic carbon signals due to two hexosyl moieties. In the HMBC spectrum, correlations of H-1″ (Glu H-1) at δ_H 5.19 with C-7 (δ_C 161.2) and H-1‴ (Glu H-1) at δ_H 4.30 with C-4″ (δ_C 79.6) were observed (Figure 1), showing that the disaccharide unit was attached to C-7 and the sugar moiety was β-D-glucopyranosyl-(1 → 4)-O-β-D-glucopyranoside (β-cellobioside). The β-configurations of two glucosyl groups were confirmed by the large J values (7.6 and 8.0 Hz, respectively). Thus, it can be concluded that **3** was daidzein-7-O-β-D-glucopyranosyl-(1 → 4)-O-β-D-glucopyranoside (Figure 1). The ^{1}H and ^{13}C NMR spectral data were listed in Tables 1 and 2. The signals were confirmed by means of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra.

Compound **4** was obtained as a white amorphous powder. The molecular formula, C$_{27}$H$_{30}$O$_{14}$, was confirmed on the basis of the [M + H]$^+$ at m/z 579 and the [M + Na]$^+$ at m/z 601 in the ESI-TOF-MS spectra. The ^{1}H and ^{13}C NMR spectra of **4** were in good agreement with the published data of daidzein-7-O-α-D-glucopyranosyl-(1 → 4)-O-β-D-glucopyranoside [7], which was obtained by glycosylation of daidzein using the *Eucalyptus* cell cultures. Therefore, **4** was daidzein-7-O-α-D-glucopyranosyl-(1 → 4)-O-β-D-glucopyranoside (Figure 2). So far, it was first isolated from the crude drug extract. Thus, it was a new natural product. The ^{1}H and ^{13}C NMR spectral data of **4** are listed in Tables 1 and 2.

According to the biogenesis, compounds **1**–**4** were originally from Puerariae Lobatae Radix. This conclusion was further confirmed by HPLC-DAD analysis (Figure 3) of the extract of Puerariae Lobatae Radix.

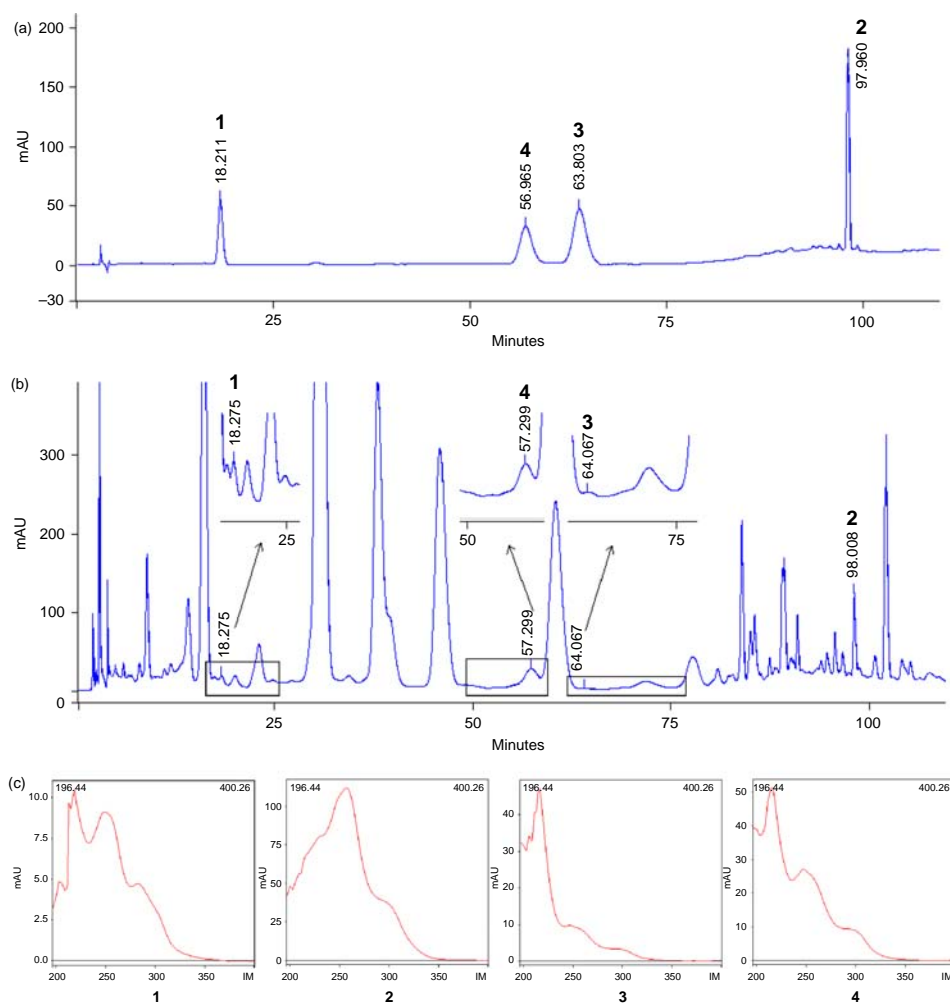In addition, the other isolated isoflavones from Tongmai granules were

Figure 3. HPLC profiles of **1**–**4** in Puerariae Lobatae Radix sample. (a) HPLC chromatogram of authentic substances **1**–**4**; (b) HPLC chromatogram of Puerariae Lobatae Radix; and (c) UV spectrum of **1**–**4** in Puerariae Lobatae Radix by HPLC-DAD analysis.

identified as daidzein (**5**) [4], sissotrin (5-hydroxylononin) (**6**) [8], ononin (**7**) [8], daidzin (**8**) [4], 3′-methoxypuerarin (**9**) [4], genistin (**10**) [4], puerarin (**11**) [4], formononetin-8-*C*-β-D-apiofuranosyl-(1 → 6)-*O*-β-D-glucopyranoside (**12**) [9], formononetin-7-*O*-β-D-apiofuranosyl-(1 → 6)-*O*-β-D-glucopyranoside (**13**) [10], lanceolarin (**14**) [11,12], kakkanin (**15**) [13], daidzein-7,4′-*O*-glucopyranoside (**16**) [4], mirificin (**17**) [4], 3′-hydroxy-puerarin (**18**) [4], 3′-methoxydaidzin (**19**) [5], formononetin-8-*C*-β-D-xylopyrano-syl-(1 → 6)-*O*-β-D-glucopyranoside (**20**) [9], genistein-8-*C*-apiofuranosyl-(1 → 6)-*O*-β-D-glucopyranoside (**21**) [4], and ambocin (**22**) [12].

2.2 DPPH radical-scavenging activities of isoflavones

Twenty-two isoflavones showed different DPPH radical-scavenging activities. Among these compounds, **9**, **18**, and **19** showed significant activities in a concentration-dependent manner with IC_{50}

values of 57.3 ± 1.2, 9.9 ± 0.6, and $66.4 \pm 2.3 \, \mu mol/l$, respectively. The rest 19 isoflavones showed DPPH radical-scavenging activities in high concentration ($IC_{50} > 1000 \, \mu mol/l$). The positive controls, BHT and ascorbic acid showed DPPH radical-scavenging activities in a concentration-dependent manner with IC_{50} values of 75.4 ± 6.9 and $16.5 \pm 1.6 \, \mu mol/l$, respectively. The above result clearly indicates that the DPPH radical-scavenging activities of the isoflavones are influenced by the molecular structure. Compounds with two *O*-phenolic hydroxyl groups substituted at C-3′ and 4′ of the molecule, such as **18**, can greatly enhance the DPPH radical-scavenging activities of isoflavones, and compounds with a methoxyl group and a hydroxyl group substituted at C-3′ and 4′, respectively, such as **9** and **19**, can also enhance the DPPH radical-scavenging activities, but slightly weaker.

3. Experimental

3.1 General experimental procedures

Optical rotation was determined on an Autopol III polarimeter (Rudolph Research Analytical, Flanders, NJ, USA). IR spectra were taken on a Thermo Nicolet Nexus 470 FT-IR spectrometer. UV spectra were obtained on a Varian Cary-300 ultraviolet-visible spectraphotometer in MeOH solution. Mass spectra were recorded on a Finnigan TRACE 2000 mass spectrometer (for EI-MS), a MDS SCIEX API ASTAR spectrometer (for ESI-MS), and a Bruker DALTONICS APEX IV Fourier transform ICR high-resolution mass spectrometer (for HR-ESI-MS). 1D and 2D NMR spectra were run on a Bruker AM-400 NMR spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) using DMSO-d_6 as solvents, with TMS as internal standard. Thin layer chromatography (TLC) was conducted on silica gel GF$_{254}$ plates (Qingdao Marine Chemical Co., Ltd, Qingdao, China), polyamide

membrane (Taizhou Si-Jia Biochemical Plastic Company, Zhejiang, China), and reversed-phase C$_{18}$ silica gel plates (Merck, Darmstadt, Germany). Open column chromatography (CC) was carried out using silica gel (200–300 mesh, Qingdao Marine Chemical Co., Ltd), polyamide (Taizhou Si-Jia Biochemical Plastic Company), macroporous resins AB-8 (Resin Factory of Nankai University, Tianjin, China), and Sephadex LH-20 (Pharmacia, Fine Chemicals, Inc., Piscataway, NJ, USA) as stationary phases. HPLC experiments used to monitor sample purification were carried out on reversed-phase chromatography with a P2000 pump, a SpectraSYSTEM UV3000 detector, and a Dikma Diamonsil™ C$_{18}$ column ($5 \, \mu m$, $4.6 \times 250 \, mm$ I.D.). Preparative HPLC was carried out on self-made industrial chromatography with two P3050 pumps, a UV3000 ultraviolet-visible detector, and a Daisogel C$_{18}$ column ($10 \, \mu m$, $30 \times 250 \, mm$, I.D.). HPLC detection used to confirm the presence of **1**–**4** in Puerariae Lobatae Radix was carried out on a Varian chromatographic system that included a Varian ProStar 230 HPLC pump, a Varian ProStar 330 PDA detector, and a Dikma Diamonsil™ C$_{18}$ column ($5 \, \mu m$, $4.6 \times 250 \, mm$ I.D.). The solvents used for HPLC experiments were either spectral grade or distilled prior to use. All the other solvents were analytical-grade commercial preparations. DPPH radical and ascorbic acid were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). BHT was purchased from DIMA Technology, Inc. (National City, CA, USA). Ninety-six-well plates were purchased from Corning Costar (Cambridge, MA, USA). UV$_{max}$ Kinetic Microplate reader (Molecular Dynamics, Inc., Sunnyvale, CA, USA) was used as a microplate reader.

3.2 Plant material

Puerariae Lobatae Radix, Salviae Miltiorrhizae Radix et Rhizoma, Chuanxiong Rhizoma, and the extract of Tongmai granules were obtained from Shenwei Medicine Ltd Company (Hebei, China). The voucher specimens have been deposited in Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University.

3.3 Extraction and isolation

Ten kilograms of Puerariae Lobatae Radix, 10 kg of Salviae Miltiorrhizae Radix et Rhizoma, and 10 kg of Chuanxiong Rhizoma were mixed together and then extracted with boiling water twice (first for 1.5 h and second for 1 h) to get the extract solution. The solution was concentrated *in vacuo* to make the relative density in the range of 1.18–1.22 and then precipitated with 65% ethanol for 24 h. The 65% ethanol-soluble part was subsequently filtered and concentrated *in vacuo*, and then partitioned successively with cyclohexane, EtOAc, and *n*-BuOH. The EtOAc-soluble portion (500 g) was then subjected to silica gel CC, eluting successively with CHCl$_3$, CHCl$_3$–MeOH (30:1 → 1:1), and MeOH. The combination of similar fractions on the basis of TLC analysis then afforded seven fractions (Fractions-E-1–E-7). Fraction-E-1 (16 g) was subjected to silica gel CC, eluting with cyclohexane–EtOAc (2:1) to provide compound **5** (612 mg). Fraction-E-3 (19 g) was subjected to silica gel CC, eluting with CHCl$_3$–MeOH (10:1) to provide compounds **6** (17 mg) and **7** (142 mg). Fraction-E-4 (36 g) and Fraction-E-6 (26 g) were isolated by the same method as Fraction-E-3 to yield compounds **8** (1358 mg), **9** (362 mg), and **10** (83 mg). Fraction-E-5 (95 g) was subjected to CC with silica gel, polyamide, and preparative HPLC to yield compounds **11** (1279 mg), **12** (126 mg), **13** (17 mg), **14** (15 mg), **15** (8 mg), and **2** (15 mg).

The *n*-BuOH-soluble fraction (520 g) was absorbed on macroporous resin AB-8, and then eluted successively with water, 20% ethanol, 30% ethanol, and 95% ethanol. The 20% ethanol fraction (20 g) was subjected to silica gel CC, eluting with CHCl$_3$–MeOH–H$_2$O (3:1:0.1). The combination of similar fractions on the basis of TLC analysis then afforded four fractions (Fractions-B-1–B-4). Fraction-B-2 (7 g) was subjected to polyamide CC, eluting with CHCl$_3$–MeOH (3:1) to yield compounds **16** (16 mg), **17** (773 mg), and **18** (809 mg). Fraction-B-3 (3 g) was isolated by the same method as above to yield compound **1** (18 mg). Thirty percent of ethanol fraction (38 g) was subjected to silica gel CC, eluting with CHCl$_3$–MeOH–H$_2$O (3:1:0.1). The combination of similar fractions on the basis of TLC analysis then afforded four fractions (Fractions-B-I–B-IV). Fraction-B-I (4 g) was purified over Sephadex LH-20 CC to give compound **19** (39 mg). Fraction-B-II (8 g) was subjected to Sephadex LH-20 CC and preparative HPLC to yield compounds **20** (63 mg) and **22** (64 mg). Fraction-B-III (5 g) was isolated by the same method as Fraction-B-II to yield compound **21** (32 mg). Fraction-B-IV (12 g) was isolated by the same method as Fraction-B-II to yield compounds **3** (13 mg) and **4** (130 mg).

3.3.1 3′-Methoxydaidzein-7,4′-di-O-β-D-glucopyranoside (1)

White amorphous powder (MeOH–H$_2$O); $[\alpha]_D^{25}$ −62.3 (*c* = 0.10, DMSO); UV λ_{MeOH} nm (log ε): 257 (3.27), 287 (2.98), 304 (2.77); IR (KBr) ν_{max} (cm^{-1}): 3413 (OH), 2930 (CH), 1733 (C=O), 1648 (aromatic), 1553, 1511, 1459, 1246, 1174, 1056; ^{1}H and ^{13}C NMR spectral data (Tables 1 and 2); ESI-MS (positive ion mode) *m/z* 609 [M + H]$^+$, 631 [M + Na]$^+$, 447 [M + H-Glc]$^+$; ESI-MS (negative ion mode) *m/z* 445 [M − H-Glc]$^-$; HR-ESI-MS *m/z* 609.1821 [M + H]$^+$ (calcd for C$_{28}$H$_{33}$O$_{15}$, 609.1814).

3.3.2 *Biochanin A-8-C-β-D-apiofuranosyl-(1 → 6)-O-β-D-glucopyranoside (2)*

White amorphous powder (MeOH–H₂O); $[\alpha]_D^{25}$ − 10.3 ($c = 0.45$, MeOH); UV λ_{MeOH} nm (log ε): 263 (3.59), 326 (2.58); IR (KBr) ν_{max} (cm⁻¹): 3366 (OH), 2943 (CH), 1651 (C═O), 1615 (aromatic), 1579, 1510, 1436, 1292, 1248, 1057; ¹H and ¹³C NMR spectral data (Tables 1 and 2); ESI-MS (positive ion mode) *m/z* 579 [M + H]⁺, 601 [M + Na]⁺, 447 [M + H-Api]⁺; HR-ESI-MS *m/z* 579.1709 [M + H]⁺ (calcd for C₂₇H₃₁O₁₄, 579.1708).

3.3.3 *Daidzein-7-O-β-D-glucopyranosyl-(1 → 4)-O-β-D-glucopyranoside (3)*

White amorphous powder (MeOH–H₂O); $[\alpha]_D^{25}$ − 39.6 ($c = 0.27$, MeOH); UV λ_{MeOH} nm (log ε): 261 (3.03), 303 (2.50); IR (KBr) ν_{max} (cm⁻¹): 3392 (OH), 2976 (CH), 1742 (C═O), 1628 (aromatic), 1577, 1500, 1450, 1259, 1131, 1056; ¹H and ¹³C NMR spectral data (Tables 1 and 2); ESI-MS (positive ion mode) *m/z* 579 [M + H]⁺, 601 [M + Na]⁺, 255 [M + H-Glc-Glc]⁺; ESI-MS (negative ion mode) *m/z* 577 [M − H]⁻, 253 [M − H-Glc-Glc]⁻; HR-ESI-MS *m/z* 579.1729 [M + H]⁺ (calcd for C₂₇H₃₁O₁₄, 579.1708).

3.3.4 *Daidzein-7-O-α-D-glucopyranosyl-(1 → 4)-O-β-D-glucopyranoside (4)*

White amorphous powder (MeOH–H₂O); $[\alpha]_D^{25}$ + 104.4 ($c = 0.16$, MeOH); ¹H and ¹³C NMR spectral data (Tables 1 and 2); ESI-MS (positive ion mode) *m/z* 579 [M + H]⁺, 601 [M + Na]⁺, 255 [M + H-2Glc]⁺.

3.4 **DPPH radical-scavenging activity**

The free radical scavenging activity of compounds **1**–**22** was determined using DPPH as a reagent with BHT and ascorbic acid used as standards according to the previously reported method [14]. In brief, 50 μl of 200 μmol/l DPPH radical in MeOH and 150 μl of each MeOH solution of compounds **1**–**22** at various concentrations (0.01–1000 μmol/l) were mixed in a 96-well microtiter plate. The reaction mixtures were incubated for 30 min in a dark room at room temperature. Scavenging capacity was read spectrophotometrically by monitoring the decrease in absorbance at 490 nm using a UVmax Kinetic Microplate Reader (Molecular Devices, Inc., Sunnyvale, CA, USA). Finally, the percentage of DPPH radical-scavenging effect was calculated with the following equation:

$$\text{DPPH radical-scavenging effect}\,(\%)$$
$$= \left[\frac{A_0 - A_1}{A_0}\right] \times 100\%,$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the samples and the standards.

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