Fitoterapia xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Nitric oxide inhibitory flavonoids from traditional Chinese medicine formula Baoyuan Decoction

Xiaoli Ma, Qian Yu, Xiaoyu Guo, Kewu Zeng, Mingbo Zhao, Pengfei Tu, Yong Jiang *

State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, People's Republic of China

ARTICLE INFO

Article history:
Received 4 March 2015
Accepted in revised form 9 April 2015
Accepted 12 April 2015
Available online xxxx

Keywords:
Flavonoids
Baoyuan Decoction
Traditional Chinese medicine formula
NO inhibition
UPLC-Q-trap-MS

ABSTRACT

Three new flavonoid glycosides, (3*R*)-(+)-isomucronulatol-2′-*O*-*β*-D-glucopyranoside (**1**), (3*R*)-(−)-isomucronulatol-7-*O*-*β*-D-apiofuranosyl(1 → 2)-*β*-D-glucopyranoside (**2**), and (2*S*)-(−)-7,8-dihydroxylflavanone-4′-*O*-*β*-D-apiofuranosyl(1 → 2)-*β*-D-glucopyranoside (**3**), along with eight flavanones (**4**, **8**, **10**, **12**, **15**, **16**, **21**, and **24**), four isoflavones (**5**, **11**, **13**, and **23**), four chalcones (**6**, **14**, **17**, and **18**), two isoflavans (**19–20**), one flavone (**7**), one flavonol (**9**), and one dihydrochalcone (**22**) were isolated from Baoyuan Decoction (BYD), a traditional Chinese medicine formula. The structures of the new compounds were established by detailed analysis of NMR and HRESIMS spectroscopic data, and their absolute configurations were determined by electronic circular dichroism (ECD) data. The inhibitory effects of the isolates were evaluated on nitric oxide production in lipopolysaccharide activated RAW 264.7 macrophage cells. Compounds **6**, **9**, and **10** showed the significant inhibitory activities, with IC_{50} values of 1.4, 13.8, and 9.3 μM, respectively, comparable to or even better than the positive control, quercetin (IC_{50}, 16.5 μM). The assignment of these isolated flavonoids was achieved using UPLC-Q-trap-MS, and the results suggested that they were originated from *Astragalus membranaceus* and *Glycyrrhiza uralensis*.

1. Introduction

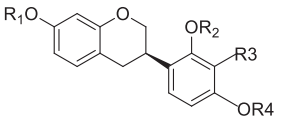
In recent years, traditional Chinese medicines (TCMs) have attracted more and more attention in many countries due to their high efficacy and low toxicity [1]. TCM prescription is a formula consisting of several single herbs at an intrinsic mass ratio, containing hundreds or thousands of chemical components [2]. The pharmacological effects of TCM formula are principally based on the synergic effects of multi-ingredients of each herb contained and their combined actions on different targets [3,4].

Baoyuan Decoction (BYD), a well-known traditional TCM formula, being composed of *Astragalus membranaceus* (Fisch.) Bunge. (Leguminosae), *Panax ginseng* C. A. Mey (Araliaceae), *Glycyrrhiza uralensis* Fisch. (Leguminosae), and *Cinnamomum cassia* Presl. (Lauraceae), has been used for the treatment of aplastic anemia, chronic renal failure, coronary heart disease, etc. [5–7]. In our preliminary analytical work, the flavonoids and saponins were found to be the main constituents contained in BYD (data not shown). Flavonoids, as one of the important natural products, exhibited diverse biological activities for the treatment of cardiovascular diseases, such as improving blood vessels elasticity, affecting coronary artery expansion, reducing blood pressure, antioxidation, and antiplatelet aggregation [8,9]. In order to make clear the flavonoids constitution in BYT and search for the active flavonoids, the water extract of BYD was studied to afford three new flavonoid glycosides (**1–3**), along with eight flavanones (**4**, **8**, **10**, **12**, **15**, **16**, **21**, and **24**), four isoflavones (**5**, **11**, **13**, and **23**), four chalcones (**6**, **14**, **17**, and **18**), two isoflavans (**19–20**), one flavone (**7**), one flavonol (**9**), and one dihydrochalcone (**22**). Their structures (Fig. 1) were established by detailed analysis of NMR and HRESIMS spectroscopic data, and the absolute configurations were determined by electronic circular dichroism (ECD) data. The inhibitory effects of the isolates were evaluated on nitric oxide

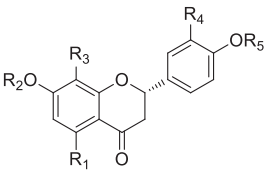
* Corresponding author. Tel./fax: +86 10 82802719.
E-mail address: yongjiang@bjmu.edu.cn (Y. Jiang).

http://dx.doi.org/10.1016/j.fitote.2015.04.011
0367-326X/

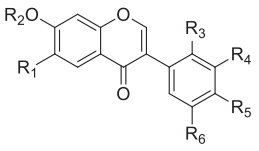
Please cite this article as: Ma X, et al, Nitric oxide inhibitory flavonoids from traditional Chinese medicine formula Baoyuan Decoction, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.04.011



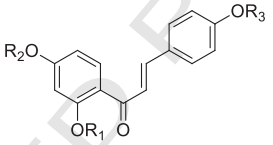
1 R_1=H, R_2=Glc, R_3=OCH3, R_4=CH_3
2 R_1=R_4=H, R_3=OCH_3, R_2=Glc (2→1) Api
19 R_1=R_2=R_3=R_4=H
20 R_1=Glc, R_2=H, R_3=OCH_3, R_4=CH_3



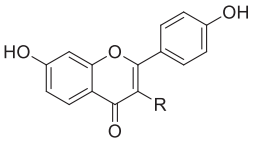
3 R_1=R_2=R_4=H, R_3=OH, R_5= Glc (2→1) Api
4 R_1=R_2=R_3=R_4=R_5=H
8 R_1=R_2=R_3=R_5=H, R_4=OH
10 R_2=R_3=R_5=H, R_1=R_4=OH
12 R_1=R_2=R_3=R_4=H, R_5=Glc
15 R_2=R_3=R_4=H, R_1=OH, R_5=Glc
16 R_1=R_2=R_4=R_5=H, R_3=OH
21 R_1=R_3=R_4=H, R_2=R_5=Glc
24 R_1=R_2=R_3=R_4=H, R_5=Glc (2→1) Api



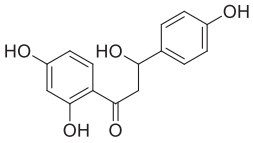
5 R_1=R_2=R_3=R_6=H, R_4=OH, R_5=OCH_3
11 R_1=R_3=R_4=R_6=H, R_2=Glc, R_5=OCH_3
13 R_1=R_3=R_6=H, R_2=Glc, R_4=OH, R_5=OCH_3
23 R_1=R_2=R_4=H, R_3=OGlc, R_5=OCH_3, R_6=OH



6 R_1=R_2=R_3=H
14 R_1=R_2=H, R_3=Glc
17 R_1=R_2=H, R_3=Glc (2→1) Api
18 R_1=R_3=H, R_2=Glc (2→1) Api



7 R =H
9 R=OH



22

Fig. 1. Chemical structures of isolated compounds **1**–**24** from BYD.

(NO) production in lipopolysaccharide (LPS) activated RAW 264.7 macrophage cells. Herein, the isolation and structure elucidation of the new compounds, and their sources and NO production inhibitory activities are reported.

2. Experimental

2.1. General experimental procedures

Melting points were determined on an X-4 micro melting point apparatus (Beijing Fukai Tech Instruments Co., Beijing, China) and were incorrect. The FT-IR spectra were measured using a Nicolet NEXUS-470 infrared spectrometer. NMR spectra were obtained on a Varian 500 spectrometer or a Bruker 400 spectrometer, with deuterated solvent as reference. HRESIMS spectra were recorded on a Waters Xevo G2 Q-TOF mass spectrometer equipped with electrospray ionization source in negative mode. ECD spectra were measured on a JASCO J-810 spectropolarimeter. Analytical HPLC was performed on an Agilent 1100 HPLC system, equipped with a photodiode array detector and an Agilent ZORBAX SB-Aq column (250 × 4.6 mm, 5 μm). Semi-preparative HPLC was carried out on an Agilent 1200 instrument, using an Agilent ZORBAX SB-Aq column (250 × 10 mm, 5 μm), detected with a variable-wavelength (190 to 600 nm) detector (VWD). Column chromatography (CC) was performed with macroporous resin AB-8 (Cangzhou Bon Adsorber Technology Co.), silica gel (100–200 mesh or 200–300 mesh, Qingdao Haiyang Chemical Works), ODS (Merck), and Sephadex LH-20 (Pharmacia Co.). Analytical grade solvents were purchased from Beijing Chemical Factory. D-Apiose solution and D-glucose were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant materials

The dried roots of *Panax ginseng*, the decoction pieces of *Glycyrrhiza uralensis* and *Astragalus membranceus*, and the barks of *Cinnamomum cassia* were purchased from Anguo

Please cite this article as: Ma X, et al, Nitric oxide inhibitory flavonoids from traditional Chinese medicine formula Baoyuan Decoction, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.04.011

traditional Chinese medicine market (Hebei Province, China). The plant materials were authenticated by Prof. P.F. Tu. The voucher specimens (PG-AG-20130312; GU-AG-20130312; AM-AG-20130312; CC-AG-20130312) were deposited in the herbarium of Modern Research Center for Traditional Chinese Medicine, Peking University, Beijing, China.

2.3. Sample preparation

For extraction and isolation: the dried roots of *A. membranceus* (30 kg), *P. ginseng* (10 kg), *G. uralensis* (10 kg), and the bark of *C. cassia* (5 kg) were powdered and mixed together, and extracted with the deionized water (550 L × 3), each for two hours. The extract was filtered and concentrated in vacuo. The resulting residue was dissolved in H_2O and subjected to macroporous resin AB-8 column (30 L) eluting with water and aqueous ethanol solution (15%, 30%, 50%, and 95% EtOH) to give five fractions, respectively.

The 50% aqueous ethanol eluate (623.3 g) was subjected to silica gel CC (6 kg) eluting with the gradient of CH_2Cl_2–MeOH–H_2O (95:5:0.5, 90:10:1, 85:15:1.5, 80:20:2, and 70:30:3) to give 10 subfractions, Frs. A–J. Compound **5** (22 mg) was furnished from Fr. B by recrystallization from CH_2Cl_2–MeOH mixture. Fr. C (38 g) was subjected to Sephadex LH-20 CC eluting with MeOH to give three fractions, Frs. C_1 (4 g), C_2 (19 g), and C_3 (8 g). Fr. C_2 was applied to silica gel CC (600 g) eluting with CH_2Cl_2–MeOH–H_2O (90:10:1, 85:15:1.5, 80:20:2, 70:30:3) to afford **4** (8 g) and **6** (56 mg). Fr. D (3 g) was subjected to Sephadex LH-20 CC eluting with MeOH to give 10 fractions, Frs. D_1–D_{10}. Fr. D_5 furnished **7** (8 mg) by recrystallization from CH_2Cl_2–MeOH mixture. Fr. D_7 (20 mg) was further chromatographed on ODS using 10–50% aqueous MeOH as eluents to afford **19** (5 mg). Fr. E (1.9 g) was subjected to Sephadex LH-20 CC eluting with MeOH to give six fractions, Frs. E_1–E_6. Compound **1** (15 mg) was precipitated from Fr. E_3. The remaining residue of Fr. E_3 was separated on ODS CC eluting with 10–80% aqueous MeOH to yield **8** (6 mg), **9** (4 mg), and **10** (5 mg). Fr. F (1.5 g) was applied to Sephadex LH-20 CC using MeOH as eluent to yield **11** (25 mg). Fr. G (2.5 g) was further chromatographed on silica gel CC (100 g) eluting with CH_2Cl_2–MeOH–H_2O (85:15:10) to give five fractions, Frs. G_1 (170 mg), G_2 (890 mg), G_3 (554 mg), G_4 (155 mg), and G_5 (20 mg). Fr.G_2 was further chromatographed on ODS CC eluting with 10–70% aqueous MeOH to afford **12** (500 mg). Frs. G_3, G_4, and G_5 were combined and applied to ODS MPLC CC, eluting with 5–70% aqueous MeOH to give **2** (4 mg), **13** (14 mg), **14** (8 mg), **15** (14 mg), and **16** (10 mg). Fr. I (3.4 g) was first applied to Sephadex LH-20 CC using MeOH as eluent to yield 17 fractions, Frs. I_1–I_{17}. Compounds **17** (750 mg) and **18** (6 mg) were yielded from Fr. I_7 by ODS MPLC employing MeOH–H_2O mixtures (3:7). Compound **20** (6 mg) was furnished from Fr. I_9 by semipreparative HPLC using ACN/H_2O (17:83) as mobile phase.

The 30% aqueous ethanol eluate (513.3 g) was subjected to silica gel CC (5 kg) eluting with the mixture of CH_2Cl_2–MeOH–H_2O (95:5:0.5, 90:10:1, 85:15:1.5, 80:20:2, and 70:30:3) to give 11 fractions, Frs. A–K. Fr. H was first applied to Sephadex LH-20 CC using MeOH as eluent to yield six fractions, Frs. H_1–H_6. Fr. H_3 (980 mg) was further chromatographed on ODS MPLC (10–50% aqueous MeOH) to afford **21** (30 mg) and **22** (19 mg). Fr. H_5 (1.49 g) was applied to silica gel CC (60 g) eluting with CH_2Cl_2–MeOH–H_2O (90:10:1, 85:15:1.5, 70:30:3) to afford **24** (15 mg). Fr. I was first applied to Sephadex LH-20 CC using MeOH as eluent to yield seven fractions, Frs. I_1–I_7. Fr. I_5 (47 mg) was further chromatographed on ODS MPLC (10–50% aqueous MeOH) to afford **23** (14 mg). Fr. K (5.0 g) was subjected to silica gel CC eluting with the mixture of CH_2Cl_2–MeOH–H_2O (95:5:0.5, 90:10:1, 85:15:1.5, 80:20:2, and 70:30:3) to yield 10 fractions, Frs. K_1–K_{10}. Fr. K_3 (56 mg) was further chromatographed on ODS MPLC (5–50% aqueous MeOH) to afford crude **3** (8 mg). Pure compound **3** (3 mg) was further purified by semipreparative HPLC using ACN/H_2O (11:89) as mobile phase.

2.3.1. (3R)-(+)-isomucronulatol-2′-O-β-D-glucopyranoside (*1*)

Colorless needles: mp 132–133 °C; $[\alpha]^{23}_D$ + 44 (*c* 0.25, MeOH); UV (MeOH) λ_{max}: 204, 280 nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$): 231 (−13.09), 280 (+4.39) nm; IR (KBr) ν_{max}: 3396, 2928, 1766, 1598, 1502, 1460, 1244, 1062, 845, 631 cm^{-1}; HRESI (−)-MS *m/z*: 509.1645 $[M + HCOO]^-$ (calcd for $C_{24}H_{29}O_{12}$ 509.1659); ^1H and ^{13}C NMR data, see Table 1.

2.3.2. (3R)-(−)-isomucronulatol-7-O-β-D-apiofuranosyl(1→2)-2)-β-D-glucopyranoside (*2*)

White amorphous powder; $[\alpha]^{23}_D$-60 (*c* 0.23, MeOH); UV (MeOH) λ_{max}: 204, 277 nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$): 232 (−14.01), 279 (+5.02) nm; IR (KBr) ν_{max}: 3396, 2925, 1767, 1504, 1463, 1375, 1244, 1049, 828, 631 cm^{-1}; HRESI (−)-MS *m/z*: 595.2031 $[M-H]^-$ (calcd for $C_{28}H_{35}O_{14}$ 595.2027); ^1H and ^{13}C NMR data, see Table 1.

2.3.3. (2S)-(−)-7,8-dihydroxylflavanone-4′-O-β-D-apiofuranosyl (1 → 2)-β-D-glucopyranoside (*3*)

Yellowish amorphous powder; $[\alpha]^{23}_D$-22 (*c* 0.25, MeOH); UV (MeOH) λ_{max}: 218, 289 nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$): 300 (−2.39) nm; IR (KBr) ν_{max}: 3402, 2942, 2245, 1647, 1600, 1513, 1361, 1057, 826, 629 cm^{-1}; HRESI (−)-MS *m/z*: 565.1558 $[M-H]^-$ (calcd for $C_{26}H_{29}O_{14}$ 565.1557); ^1H and ^{13}C NMR data, see Table 1.

For LC-MS analysis: the decoction pieces of *A. membranceus* (100 g) were crushed into fine powder and extracted twice with 1 L of distilled water. The extract was concentrated and the supernatant was freeze-dried. An aliquot (500 mg) of the freeze-dried powder was dissolved into 10.0 mL of water by vortexing mix for 1 min. After centrifugation at 9600 rpm for 10 min, the supernatant (5 mL) was subjected to a macroporous resin AB-8 column (60 mL) eluting subsequently with water, 15%, 50%, and 95% aqueous EtOH to give four eluents (AM0, AM15, AM50, and AM95), respectively. AM50 was concentrated to dryness under reduced pressure, and dissolved in MeOH (2 mL), and then filtered through a 0.22 μm membrane before LC/MS analysis. The sample preparation method was same for the pieces of *G. uralensis*, the crude drugs of *P. ginseng* and *C. cassia*, and the BYD water extract.

2.4. Acid hydrolysis

Compounds **1** (2.5 mg), **2** (1.4 mg), and **3** (2.4 mg) were respectively hydrolyzed with 2 M aqueous CF_3COOH (5 mL) by thermostat oil bath at 100 °C for 3 h. After the solvent was removed in vacuo with MeOH, and the residue was partitioned between CH_2Cl_2 and H_2O to give the aglycones of **1**–**3**, which

Please cite this article as: Ma X, et al, Nitric oxide inhibitory flavonoids from traditional Chinese medicine formula Baoyuan Decoction, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.04.011

Table 1
^{1}H and ^{13}C NMR data of compounds **1–3** (in DMSO-d_6, δ in ppm and J in Hz).

Position	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	4.29 dd (3.2, 10.0) 3.81 t (10.0)	69.6	4.26 d (10.3) 4.00 t (10.3)	71.0	5.51 dd (12.4, 2.6)	43.8
3	3.60 m	30.3	3.45 m	33.5	3.14 dd (12.4, 16.8) 2.70 dd (16.8, 2.6)	79.3
4	2.80 dd (15.6, 11.6) 2.66 dd (15.6, 4.0)	30.9	2.99 dd (15.9, 10.5) 2.85 dd (15.9, 4.6)	31.3		191.0
5	6.84 d (8.2)	130.0	6.98 d (8.4)	131.2	7.18 d (8.6)	117.9
6	6.27 dd (8.2, 2.2)	107.8	6.59 dd (8.4, 2.5)	110.0	6.52 d (8.6)	110.3
7		156.4		158.3		153.0
8	6.18 d (2.2)	102.6	6.51 d (2.5)	105.4		133.0
9		154.9		156.4		151.6
10		112.9		117.7		114.8
1′		128.7		122.2		133.3
2′		147.6		149.6	7.49 d (8.6)	128.6
3′		141.2		137.6	7.05 d (8.6)	116.4
4′		152.0		153.2		157.7
5′	6.79 d (8.7)	108.6	6.47 d (8.7)	104.4	7.05 d (8.6)	116.4
6′	6.90 d (8.7)	121.9	6.78 d (8.7)	122.8	7.49 d (8.6)	128.6
3′-OCH_3	3.73 s	60.5	3.79 s	61.1		
4′-OCH_3	3.77 s	55.8	3.81 s	56.3		
1″	4.86 d (6.1)	103.4	4.92 d (7.4)	101.0	4.96 d (7.4)	99.1
2″		77.5		78.7		76.5
3″		74.0		78.0		77.4
4″		70.2		71.4		70.5
5″		76.4		78.6		77.5
6″	3.69 dd (10.9, 3.9) 3.38 dd (6.0, 10.9)	61.3	3.88 dd (2.0, 12.2) 3.69 dd, (5.5, 12.2)	62.5	3.69 dd (2.0, 11.5) 3.45 dd (5.0, 11.5)	61.1
1‴			5.46 d (1.4)	110.8	5.36 s	108.7
2‴				78.1	3.74 s	76.2
3‴				80.8		79.8
4‴			4.05 d (9.6) 3.78 d (9.6)	75.5	3.95 d (9.2) 3.65 d (9.2)	74.5
5‴			3.55 d (3.7)	66.1	3.32 s	64.8

were used for the ECD determination, along with the sugar fractions. The sugar fractions of **1–3** were respectively passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA; with 40% MeOH), and then analyzed by HPLC (MeCN–H_2O 85:15; flow rate, 1.0 mL/min; CHIRALYSER-MP optical rotation detector). D-glucose (t_R 19.7 min, positive optical rotation) was detected from **1**, while D-apiose (t_R 7.70 min, positive optical rotation) and D-glucose (t_R 19.7 min, positive optical rotation) were detected from **2** and **3**.

2.5. Cell culture and NO measurement

The macrophage RAW 264.7 cells were obtained from Peking Union Medical College Cell Bank (Beijing, China), cultured in DMEM containing 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and grown at 37 °C with 5% CO_2 in fully humidified air. Cells were plated at a density of 2×10^5 cells/well in 96-well culture plates and stimulated with LPS (1 μg/mL) in the presence or absence of the test compound for 24 h. All compounds were dissolved in DMSO and further diluted with sterile PBS. Nitrite (NO_2^-) accumulation in the medium was used as an indicator of NO production, which was measured by adding the Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring optical density at 550 nm. All experiments were performed in triplicate. NO production by LPS stimulation was designated as 100% for each experiment. Quercetin (Sigma, 98.0% HPLC) was employed as a positive control. Cell viability was evaluated by MTT assay.

2.6. Liquid chromatography and mass spectrometry

UPLC analysis was performed with a Waters (Milford, MA) ACQUITY UPLC system equipped with a quaternary solvent manager, sample manager, column-heating compartment, and photodiode array detector. This system was controlled by Waters Empower software. An ACQUITY UPLC™ Cortecs column, 100 × 2.1 mm, 1.6 μm (Waters) was employed for chromatographic separation. The mobile phase was 0.05% formic acid in water as eluent A and 0.05% formic acid in acetonitrile as eluent B. The elution program was as follows: 0.0–8.0 min, 2%–15% B; 8.0–17.0 min, 15%–25% B; 17.0–22.0 min, 25%–40% B; 22.0–24.0 min, 40%–60% B; 24.0–25.0 min, 60%–100% B; 25.0–26.0 min, 100%–100% B; 26.0–26.01 min, 100%–2% B; 26.01–31.0 min, 2% B. The flow rate was 0.4 mL/min.

Mass spectrometry analysis was performed on an ABSciex API 4500 triple quadrupole-linear ion trap mass spectrometer (Q-trap), equipped with an ESI-Turbo Ion-Spray interface, operating in negative ion mode and controlled by Analyst 1.6.1 software (AB Sciex). The ESI source operation parameters were as follows: source temperature 550 °C; ion spray voltage

Please cite this article as: Ma X, et al, Nitric oxide inhibitory flavonoids from traditional Chinese medicine formula Baoyuan Decoction, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.04.011

(IS) 4500 V; ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set at 55, 55, and 35.0 psi, respectively; the collision gas (CAD) was high.

3. Results and discussion

Compound **1** was obtained as colorless needles. Its molecular formula was determined as $C_{23}H_{28}O_{10}$ from the ^{13}C NMR data and HRESIMS ion at *m/z*: 509.1645 $[M + HCOO]^-$ (calcd for $C_{24}H_{29}O_{12}$ 509.1659). The IR spectrum of **1** displayed the absorption bands at 3396 cm^{-1} (hydroxy group), 2928 cm^{-1} (methyl group), and 1598, 1502, and 1460 cm^{-1} (aromatic group). The ^1H NMR data of **1** showed the presence of a group of ABX coupled phenyl signals at δ_H 6.84 (1H, d, J = 8.2 Hz, H-5), 6.27 (1H, dd, J = 8.2, 2.2 Hz, H-6), and 6.18 (1H, d, J = 2.2 Hz, H-8), two *ortho*-coupled aromatic protons at δ_H 6.90 (1H, d, J = 8.7 Hz, H-6′) and 6.79 (1H, d, J = 8.7 Hz, H-5′), two methoxy groups at δ_H 3.77 (3H, s) and 3.73 (3H, s), and an anomeric proton at δ_H 4.86 (1H, d, J = 6.1 Hz, H-1″). The five other characteristic aliphatic signals at δ_H 4.29 (1H, dd, J = 3.2, 10.0 Hz, H-2a), 3.81 (1H, t, J = 10.0 Hz, H-2b), 3.60 (1H, m, H-3), 2.80 (1H, dd, J = 11.6, 15.6 Hz, H-4a), and 2.66 (1H, dd, J = 4.0, 15.6 Hz, H-4b) suggested that **1** is an isoflavan glycoside. The ^{13}C NMR data of **1** showed 23 carbon signals (Table 1), including 12 aromatic carbons, 3 aliphatic carbons, 2 methoxy carbons, and a group of glucosyl carbons. On acid

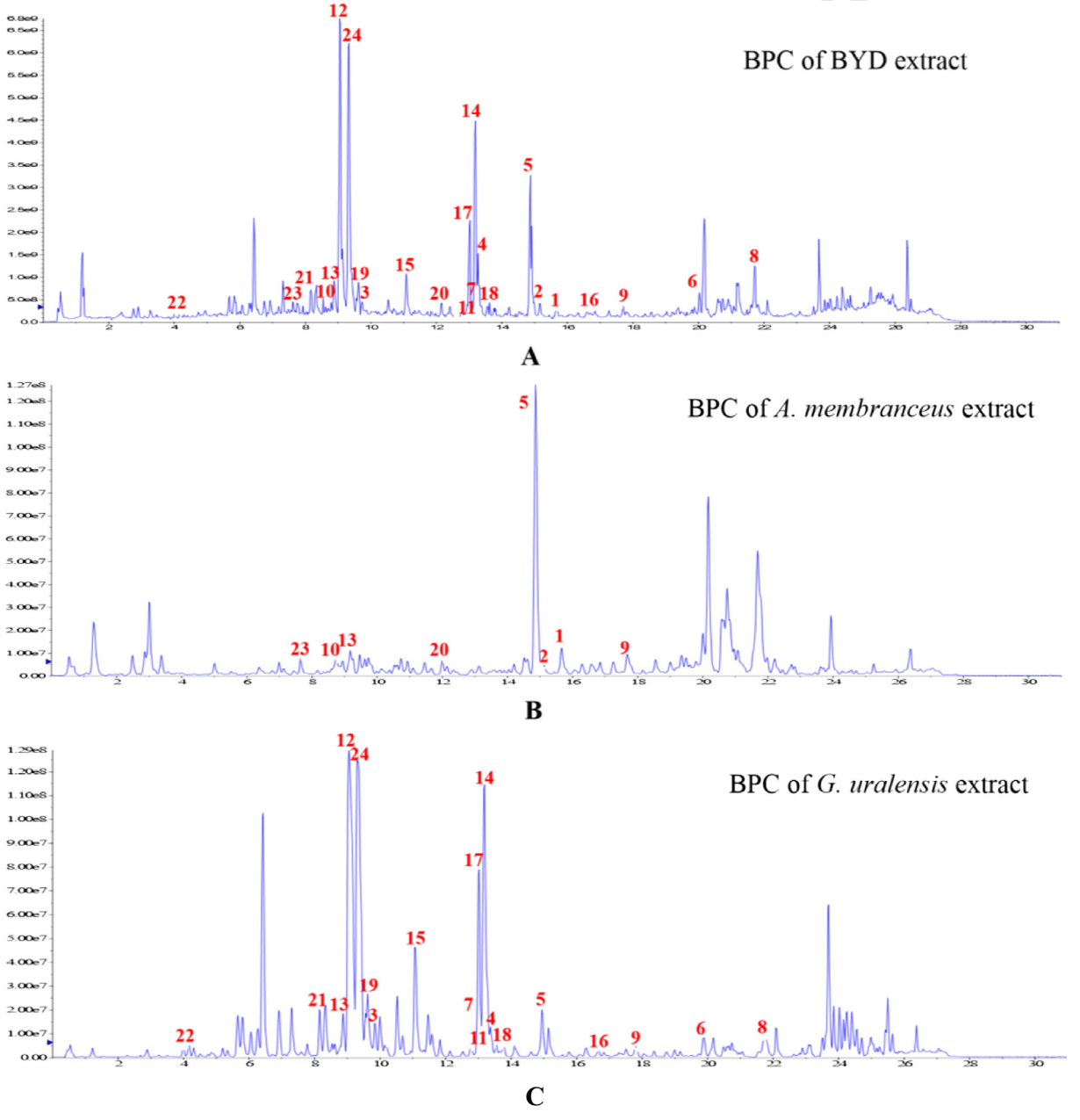


Fig. 2. UPLC-Q-trap-MS base peak ion chromatograms (BPC) of BYD extract (**A**), *A. membranceus* extract (**B**), and *G. uralensis* extract (**C**), in negative ion mode.

Please cite this article as: Ma X, et al, Nitric oxide inhibitory flavonoids from traditional Chinese medicine formula Baoyuan Decoction, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.04.011

hydrolysis, **1** yielded D-glucose after HPLC separation and optical rotation measurement. The coupling constant of the anomeric proton ($J = 6.1$ Hz) confirmed the β-configuration of the glucosyl. The HMBC correlations of the anomeric proton of glucosyl H-1″/C-2′, H-3/C-1′, C-2′, C-6′, OCH_3-2′/C-2′, and OCH_3-3′/C-3′ suggested that the linkage positions of the glucosyl and the two methoxy groups are at C-2′, 3′, and 4′, respectively. The ECD data of the aglycone (Supplementary data) produced by acid hydrolysis exhibited a negative CE in 231 nm (^1L_a) and a positive CE in 280 nm (^1L_b), indicating the 3*R* configuration for **1** [10]. Thus, compound **1** was determined as (3*R*)-(+)-isomucronulatol-2′-*O*-β-D-glucopyranoside.

Compound **2** was obtained as a white amorphous powder. Its molecular formula was determined as $C_{28}H_{36}O_{14}$ from the ^{13}C NMR and HRESIMS data (*m/z*: 595.2031 [M-H]$^-$, calcd for $C_{28}H_{35}O_{14}$ 595.2027). The IR spectrum of **2** displayed absorption bands at 3396 cm^{-1} (hydroxy group), 2925 cm^{-1} (methyl group), and 1616, 1505, and 1463 cm^{-1} (aromatic group). The NMR data of **2** (Table 1) were similar to those of **1** except for a group of additional apiosyl signals [δ_H 5.46 (1H, d, $J = 1.5$ Hz, H-1′), δ_C 110.8]. Acid hydrolysis afforded glucose and apiose, whose configurations were determined to be D by the optical rotation measurement after HPLC separation. The *J* value (7.4 Hz) of the anomeric proton of glucosyl suggested a β-D configuration, and the comparison of ^{13}C NMR spectroscopic data with literature values supported the β-D apiosyl [12]. The glucosyl moiety was deduced to be linked at C-7 of the aglycone from the HMBC correlation of H-1″ to C-7, and the apiosyl moiety was determined to be connected at C-2′ of glucosyl according to the HMBC correlation of H-1‴ to C-2″. The absolute configuration of C-3 was determined as 3*R* by the ECD data [10,13]. Thus, compound **2** was defined as (3*R*)-(−)-isomucronulatol-7-*O*-β-D-apiofuranosyl(1 → 2)-β-D-glucopyranoside.

Compound **3** was obtained as a yellowish amorphous powder. Its molecular formula was determined as $C_{26}H_{30}O_{14}$ from the ^{13}C NMR and HRESIMS data (*m/z*: 565.1558 [M-H]$^-$, calcd for $C_{26}H_{29}O_{14}$ 565.1557). The IR spectrum of **3** displayed the absorption bands at 3402 cm^{-1} (hydroxyl group), 1647 cm^{-1} (conjugated carbonyl group), and 1600, 1513, and 1462 cm^{-1} (aromatic group). The ^{1}H NMR data of **3** showed the presence of three characteristic aliphatic signals of flavanone at δ_H 3.14 (1H, dd, $J = 16.8$, 12.4 Hz, H-3a), 2.70 (1H, dd, $J = 16.8$, 2.6 Hz, H-3b), and 5.51 (1H, dd, $J = 12.4$, 2.6 Hz, H-3), a group of AA′BB′ coupled aromatic signals at δ_H 7.49 (2H, d, $J = 8.6$ Hz, H-2′, 6′), 7.05 (2H, d, $J = 8.6$ Hz, H-3′, 5′), two AB *ortho*-coupled aromatic protons at δ_H 7.18 (1H, d, $J = 8.6$ Hz, H-6′), 6.52 (1H, d, $J = 8.6$ Hz, H-5′), and two anomeric proton signals at δ_H 5.36 (1H, s, H-1‴), 4.96 (1H, d, $J = 7.4$ Hz, H-1″). The ^{13}C NMR data (Table 1) showed 26 carbon signals, which also provided support that **3** is a flavanone diglycoside. The HMBC correlations of H-5/C-4, C-7, and H-6/C-5, C-7, C-8, C-10 suggested that A ring is 7,8-dihydroxylated. On acid hydrolysis, **3** yielded D-glucose and D-apiose, whose configurations were determined in the same way as in **2** (Supplementary data). The anomeric proton of glucosyl showed correlation with C-4′ and the anomeric proton of apiosyl showed correlation with C-2″ of glucosyl. The absolute configuration of C-2 was determined as 2*S* by the ECD data, which is accordance with the other natural flavanones [14]. Therefore, compound **3** was determined as (2*S*)-(−)-7,8-dihydroxylflavanone-4′-*O*-β-D-apiofuranosyl (1 → 2)-β-D-glucopyranoside.

By comparing the spectroscopic data with literature values, the 21 known compounds isolated in this investigation were identified as liquiritigenin (**4**) [15], calycosin (**5**) [16], isoliquiritigenin (**6**) [15], 7,4′-dihydroxyflavone (**7**) [17], 3′-hydroxyliquiritigenin (**8**) [18], resokaempferol (**9**) [19], eriodictyol (**10**) [20], ononin (**11**) [21], liquiritin (**12**) [22], calycosin-7-*O*-β-D-glucoside (**13**) [23], isoliquiritin (**14**) [22], 5-hydroxyliquiritin (**15**) [24], 8-hydroxyliquiritigenin (**16**) [25], isoliquiritin apioside (**17**) [22], licraside (**18**) [26], demethylvestitol (**19**) [27], (3*R*)-(−)-isomucronulatol-7-*O*-β-D-glucoside (**20**) [28], liquiritigenin-7,4′-*O*-β-D- diglucoside (**21**) [29], 1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl)-1- propanone (**22**) [30], 5′-hydroxy-4′-methoxyisoflavone-3′-*O*-β-D-glucoside (**23**) [31], and liquiritin apioside (**24**) [32].

In order to clarify the sources of these isolates, a UPLC-Q-trap-MS method was established to analyze the isolated flavonoids in BYD and in each composition plant. Finally, compounds **3**–**9**, **11**–**19**, **21**, **22**, and **24** were found to be originated from *G. uralensis*, while compounds **1**, **2**, **5**, **9**, **10**, **13**, **20**, and **23** were derived from *A. membranceus* (Fig. 2, Table 2).

BYD has been clinically used for the prevention and treatment of coronary heart disease [5,6]. There are lots of evidences to prove that the coronary heart disease is associated with the inflammation [33,34]. NO, a significant signaling molecule of inflammation, has been reported to be closely related to the progression of myocardial damage [35,36]. Thus, the NO inhibitory model has been used for screening the cardioprotective or anti-inflammation components and studying their mechanism of action [35–38]. Herein, in order to search for the potential cardioprotective and anti-inflammatory

Table 2
The sources of the isolated compounds from Baoyuan Decoction.

Compound	Formula	[M-H]$^-$	Source
1	$C_{23}H_{28}O_{10}$	463.2	A
2	$C_{28}H_{36}O_{14}$	595.2	A
3	$C_{26}H_{30}O_{14}$	565.1	G
4	$C_{15}H_{12}O_4$	255.1	G
5	$C_{16}H_{12}O_5$	283.1	A/G
6	$C_{15}H_{12}O_4$	255.1	G
7	$C_{15}H_{10}O_4$	253.0	G
8	$C_{15}H_{12}O_5$	271.1	G
9	$C_{15}H_{10}O_5$	269.0	A/G
10	$C_{15}H_{12}O_6$	287.1	A
11	$C_{22}H_{22}O_9$	429.1	G
12	$C_{21}H_{22}O_9$	417.1	G
13	$C_{22}H_{22}O_{10}$	445.1	A/G
14	$C_{21}H_{22}O_9$	417.1	G
15	$C_{21}H_{22}O_{10}$	433.1	G
16	$C_{15}H_{12}O_5$	271.1	G
17	$C_{26}H_{30}O_{13}$	549.2	G
18	$C_{26}H_{30}O_{13}$	549.2	G
19	$C_{15}H_{14}O_4$	257.1	G
20	$C_{23}H_{28}O_{10}$	463.2	A
21	$C_{27}H_{32}O_{14}$	579.2	G
22	$C_{15}H_{14}O_4$	273.1	G
23	$C_{22}H_{22}O_{11}$	461.1	A
24	$C_{26}H_{30}O_{13}$	549.2	G

"A" means that the compound was originated from *A. membranceus*; "G" means that the compound was originated from *G. uralensis*; "A/G" means that the compound was originated from both *A. membranceus* and *G. uralensis*.

Please cite this article as: Ma X, et al, Nitric oxide inhibitory flavonoids from traditional Chinese medicine formula Baoyuan Decoction, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.04.011

t3.1 **Table 3**
t3.2 NO inhibitory activity of the isolates.

t3.3 Compound	IC_{50} (μM)
t3.4 **6**	1.4
t3.5 **7**	37.1
t3.6 **9**	13.8
t3.7 **10**	9.3
t3.8 **14**	20.8
t3.9 Quercetin [a]	16.5

t3.10 [a] Positive control.

components from BYD, the flavanoids isolated were evaluated for their inhibitory effects against LPS-induced NO production in RAW264.7 macrophages. As shown in Table 3, compounds **6**, **9**, and **10** showed the significant inhibitory effects with IC_{50} values of 1.4, 13.8, and 9.3 μM, respectively, comparable to or even better than the positive control of quercetin (IC_{50} = 16.5 μM). Compounds **7** and **14** showed the moderate inhibitory effects with IC_{50} values of 37.1 and 20.8 μM, respectively. On the other hand, other isolated flavonoids did not show any inhibitory effects (IC_{50} > 100 μM). The preliminary structure–activity relationship (SAR) analysis showed that the phenolic hydroxyl is an important active functionality, the more the better, such as **10** ≫ **8**, **16**; **9** > **7**. The compound with chalcone skeleton showed the best inhibitory activity when the phenolic hydroxyl number is equal, such as **6** > **9** ≫ **8**, **19**. Moreover, the glycosidation will lead to the activity decreasing obviously, such as only **14** showed a moderate inhibitory activity, and the other glycosides all lost the activities. The better inhibition effect of NO production presented by compounds **6**, **9**, and **10** in macrophages cells indicates that these several compounds may have potential value in the treatment or prevention of coronary heart disease associated with inflammation [35,36].

Q4 4. Uncited reference

[11]

Acknowledgments

This work was financially supported by the National Natural Sciences Foundation of China (No. 81222051) and the National Key Technology R&D Program "New Drug Innovation" of China (Nos. 2012ZX09301002-002-002 and 2012ZX09304-005).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2015.04.011.

References

[1] Normile D. The new face of traditional Chinese medicine. Science 2003; 299:188–90.
[2] Li P, Qi LW, Wen XD, Sheng LH. Methods for the elucidation of bioactive components and quality control of traditional Chinese medicines. Chin J Nat Med 2007;5:1–9.
Q6 [3] Posters. Clin Microbiol Infect 2010;16(Supplement 2):133–634.
[4] Xie B, Gong T, Tang M, Mi D, Zhang X, Liu J, et al. An approach based on HPLC-fingerprint and chemometrics to quality consistency evaluation of Liuwei Dihuang Pills produced by different manufacturers. J Pharm Biomed 2008;48:1261–6.
[5] Wang JL. The experimental research of BaoYuan jia jian fang decoction treating coronary heart disease and angina pectoris. J MuDanJiang Med Coll 2005;26:8–12.
[6] Sun C, Han Y, Wu Q. Clinical efficacy of Baoyuan Qingxue particles containing different cinnamic aldehyde content on treatment of coronary heart disease in elderly. Chin J Gerontol 2013;33:4692–3.
[7] Wang X, Gao G, Hu J. Effect of Baoyuan decoction on cellular immune function after mending surgery for ventricular septal defect of infants and preventive effect on pulmonary infection. J HaiNan Med Coll 2012;18: 235–7.
[8] Chang C, Wang G, Zhang L, Tsai W, Chen R, Wu Y, et al. Cardiovascular protective flavonolignans and flavonoids from *Calamus quiquesetinervius*. Phytochemistry 2010;71:271–9.
[9] Harnafi H, Amrani S. Flavonoids as potent phytochemicals in cardiovascular diseases prevention. Pharmacogn Rev 2007;1:193–202.
[10] Slade D, Ferreira D, Marais JPJ. Circular dichroism, a powerful tool for the assessment of absolute configuration of flavonoids. Phytochemistry 2005; 66:2177–215.
[11] Giorgio E, Parrinello N, Caccamese S, Rosini C. Non-empirical assignment of the absolute configuration of (−)-naringenin, by coupling the exciton analysis of the circular dichroism spectrum and the *ab initio* calculation of the optical rotatory power. Org Biomol Chem 2004;2:3602–7.
[12] Çaliş İ, Saracoğlu İ, Başaran AA, Sticher O. Two phenethyl alcohol glycosides from *Scutellaria orientalis* subsp. *pinnatifida*. Phytochemistry 1993;32:1621–3.
[13] Villinski JR, Bergeron C, Cannistra JC, Gloer JB, Coleman CM, Ferreira D, et al. Pyrano-isoflavans from *Glycyrrhiza uralensis* with antibacterial activity against *Streptococcus mutans* and *Porphyromonas gingivalis*. J Nat Prod 2014;77:521–6.
[14] Tchuendem MHK, Douanla PD, Tabopda TK, Tchinda AT, Tamze V, Nkengfack AE, et al. Two new glycosides from *Duboscia macrocarpa* Bocq. Phytochem Lett 2014;10:1–4.
[15] Piccinelli AL, Campo Fernandez M, Cuesta-Rubio O, Márquez Hernández I, De Simone F, Rastrelli L. Isoflavonoids isolated from cuban propolis. J Agric Food Chem 2005;53:9010–6.
[16] Fedoreyev SA, Bulgakov VP, Grishchenko OV, Veselova MV, Krivoschekova OE, Kulesh NI, et al. Isoflavonoid composition of a callus culture of the Relict Tree *Maackia amurensis* Rupr. et Maxim. J Agric Food Chem 2008;56: 7023–31.
[17] Park Y, Moon B, Lee E, Lee Y, Yoon Y, Ahn J, et al. ^{1}H and ^{13}C-NMR data of hydroxyflavone derivatives. Magn Reson Chem 2007;45:674–9.
[18] Gerardo M. Phenolic derivatives from fruits of *Dipteryx lacunifera* Ducke and evaluation of their antiradical activities. Helv Chim Acta 2008;91: 2159–67.
[19] Kim H, Moon B, Ahn J, Lim Y. Complete NMR signal assignments of flavonol derivatives. Magn Reson Chem 2006;44:188–90.
[20] Huang D, Chung C, Kuo Y, Lin Y, Chiang W. Identification of compounds in adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) seed hull extracts that inhibit lipopolysaccharide-induced inflammation in RAW 264.7 macrophages. J Agric Food Chem 2009;57:10651–7.
[21] Ryu YB, Kim JH, Park S, Chang JS, Rho M, Bae K, et al. Inhibition of neuraminidase activity by polyphenol compounds isolated from the roots of *Glycyrrhiza uralensis*. Bioorg Med Chem Lett 2010;20:971–4.
[22] Fu B, Li H, Wang X, Lee FSC, Cui S. Isolation and identification of flavonoids in licorice and a study of their inhibitory effects on tyrosinase. J Agric Food Chem 2005;53:7408–14.
[23] Du X, Bai Y, Liang H, Wang Z, Zhao Y, Zhang Q, et al. Solvent effect in ^{1}H NMR spectra of 3′-hydroxy-4′-methoxy isoflavonoids from *Astragalus membranaceus* var. *mongholicus*. Magn Reson Chem 2006;44:708–12.
[24] Sachiko EKN. Preparation and taste of certain glycosides of flavanones and of dihydrochalcones. Biosci Biotechnol Biochem 1994;58:1479–85.
[25] Edenharder R, Keller G, Platt KL, Unger KK. Isolation and characterization of structurally novel antimutagenic flavonoids from Spinach (*Spinacia oleracea*). J Agric Food Chem 2001;49:2767–73.
[26] Isao K, Saponin, Sapogenol. On the constituents of the roots of *Glycyrrhiza* Q7 *uralensis* Fischer from Xinjiang, China. chemical structures of licorice-saponin L3 and isoliquiritin apioside. Chem Pharm Bull 1993;41:1567–72.
[27] Naoki A, Hiroji S, Sadao S. Antifungal stress compounds from Adzuki Bean, *Vigna angularis*, treated with cephalosporium gregatum. Agric Biol Chem 1987;2:349–53.
[28] Li W, Sun YN, Yan XT, Yang SY, Kim S, Lee YM, et al. Flavonoids from *Astragalus membranaceus* and their inhibitory effects on LPS-stimulated pro-inflammatory cytokine production in bone marrow-derived dendritic cells. Arch Pharm Res 2014;37:186–92.
[29] Derese S, Barasa L, Akala HM, Yusuf AO, Kamau E, Heydenreich M, et al. 4′-Prenyloxyderrone from the stem bark of *Millettia oblata* ssp. *teitensis* and the antiplasmodial activities of isoflavones from some *Millettia* species. Phytochem Lett 2014;8:69–72.
[30] Rafi MM, Vastano BC, Zhu N, Ho C, Ghai G, Rosen RT, et al. Novel polyphenol molecule isolated from Licorice root (*Glycrrhiza glabra*)

induces apoptosis, G2/M cell cycle arrest, and Bcl-2 phosphorylation in tumor cell lines. J Agric Food Chem 2002;50:677–84.
[31] Shen Y, Feng Z, Jiang J, Yang Y, Zhang P. Dibenzoyl and isoflavonoid glycosides from *Sophora flavescens*: inhibition of the cytotoxic effect of D-galactosamine on human hepatocyte HL-7702. J Nat Prod 2013;76: 2337–45.
[32] Asano T, Ishihara K, Morota T, Takeda S, Aburada M. Permeability of the flavonoids liquiritigenin and its glycosides in licorice roots and davidigenin, a hydrogenated metabolite of liquiritigenin, using human intestinal cell line Caco-2. J Ethnopharmacol 2003;89:285–9.
[33] Dregan A, Charlton J, Chowienczyk P, Gulliford MC. Chronic inflammatory disorders and risk of type 2 diabetes mellitus, coronary heart disease, and stroke: a population-based cohort study. Circulation 2014;130:837–44.
[34] Gao S, Wang L, Liu W, Wu Y, Yuan Z. The synergistic effect of homocysteine and lipopolysaccharide on the differentiation and conversion of RAW 264.7 macrophages. J Inflamm 2014;11:13–9.
[35] Li YC, Luo Q, Ge LS, Chen YH, Zhou ND, Zhang T, et al. Ivabradine inhibits the production of proinflammatory cytokines and inducible nitric oxide synthase in acute coxsackievirus B3-induced myocarditis. Biochem Biophys Res Commun 2013;431:450–5.
[36] Shigeru I, Michiaki H, Toshio N, Shinji A, Takashi S, Hiroshi I, et al. Nitric oxide contributes to the progression of myocardial damage in experimental autoimmune myocarditis in rats. Circulation 1997;95:489–96.
[37] Lv HN, Wang S, Zeng KW, Li J, Guo XY, Ferreira D, et al. Anti-inflammatory coumarin and benzocoumarin derivatives from *Murraya alata*. J Nat Prod 2015;78:279–85.
[38] Jiang Y, Zeng KW, David B, Massiot G. Constituents of *Vigna angularis* and their in vitro anti-inflammatory activity. Phytochemistry 2014;107:111–8.

Please cite this article as: Ma X, et al, Nitric oxide inhibitory flavonoids from traditional Chinese medicine formula Baoyuan Decoction, Fitoterapia (2015), http://dx.doi.org/10.1016/j.fitote.2015.04.011