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ISOLATION AND IDENTIFICATION OF ANTIOXIDANTS FROM *SOPHORA JAPONICA*

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A new flavonol triglycoside, kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside (**1**), as well as two known kaempferol 3-*O*-[α-L-rhamnopyranosyl-(1 → 6)]-[β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside (**2**) and kaempferol 3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside (**3**), were isolated from the *n*-BuOH extract of the pericarps of *Sophora japonica* by bioassay-guided fractionation. The structure of compound **1** was established by UV, IR, MS, and one- and two-dimensional NMR spectroscopy, including DEPT, NOESY, DQF–COSY, TOCSY, HMQC, and HMBC experiments. Compounds **1**–**3** showed antioxidative activity in DPPH and cytochrome-c assay using HL-60 cell system.

Keywords: *Sophora japonica*; Flavonol glycosides; Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside; DPPH

INTRODUCTION

The fruits of *Sophora japonica* L. (Leguminosae) are commonly used as hemostatics in traditional Chinese medicine, and flavonoids were discovered as hemostatic constituents from the buds of *S. japonica* [1]. The *n*-BuOH extract of the pericarps of *S. japonica* showed antioxidant activity in preliminary DPPH assays. This prompted us to search for antioxidant compounds from this plant. Bioassay-guided fractionation of the *n*-BuOH extract led to the isolation of flavonol glycosides **1**–**3**: kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside (**1**), as well as two known kaempferol 3-*O*-[α-L-rhamnopyranosyl-(1 → 6)]-[β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside (**2**) and kaempferol 3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside (**3**) (Fig. 1). Compound **1** is a new flavonol triglycoside. Compounds **2** and **3** were isolated from this plant for the first time. Here, we report the isolation and structural elucidation, and biological activities of the three flavonol triglycosides isolated from the pericarps of *S. japonica*.

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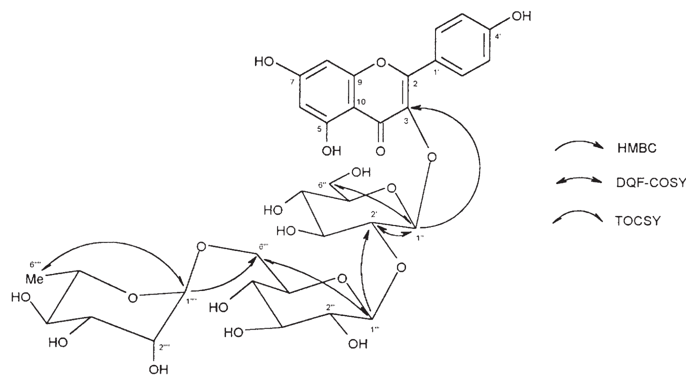

FIGURE 1 Structure of **1** and key correlations observed in HMBC (H → C), DQF–COSY, and TOCSY NMR experiments.

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RESULTS AND DISCUSSION

Compound **1** was obtained from the *n*-BuOH-soluble part of the 95% ethanol extract of *S. japonica*. The IR spectrum of compound **1** showed strong absorption bands at 3404 (OH), 1658 (α,β-unsaturated C=O), 1608, 1503 (C=C, aromatic), and broad bands at 1160–1000 cm^{-1} indicating its glycosidic nature. Its UV spectral data suggested the presence of flavonoid. Its ^1H and ^{13}C NMR spectra showed the presence of a kaempferol moiety and three sugar residues. NMR of **1** further confirmed the presence of one rhamnose (signals at δ 0.95 in ^1H NMR and at δ 17.63 in ^{13}C NMR for the methyl group) and two glucose residues. TLC after acid hydrolysis with appropriate reference compounds also indicated the presence of kaempferol as aglycone, and glucose and rhamnose. The ^1H NMR showed three anomeric signals at δ 5.54 (d, $J = 7.0$ Hz), 4.59 (d, $J = 7.7$ Hz) and 4.32 (brs). Its ^1H NMR spectrum suggested the presence of 3-substituted kaempferol glycoside with three free aromatic hydroxyl groups at 5-position (δ 12.63), 7-position (δ 10.97), 4′-position (δ 10.16). The chemical shifts of C-2 and C-3 (δ 156.49 and δ 132.73, respectively) also indicated C-3 substitution of kaempferol moiety [2].

A methyl doublet, observed at δ 0.95 in the ^1H NMR spectrum of **1**, was assigned to 6-position protons of rhamnose residue. All ^1H and ^{13}C NMR signals of the rhamnosyl moiety could be assigned based on the two-dimensional NMR spectral data (Table I). A TOCSY experiment showed a correlation between 6-position proton signal of rhamnose residue and the anomeric proton at δ 4.32, demonstrating that they belonged to the same spin system. The anomeric proton of rhamnosyl residue showed a long-range correlation with a ^{13}C NMR signal at δ 66.08, corresponding to two protons at δ 3.22 and δ 3.63 in the HMQC spectrum, and the ^{13}C NMR signal at δ 66.08 showed a triplet in the DEPT spectrum, which indicated the ^{13}C NMR signal at δ 66.08 was 6-position carbon signal of a glucosyl, and the rhamnosyl was linked to position 6 of the glucose unit. All ^1H and ^{13}C NMR signals of the glucosyl moiety could be assigned based on the two-dimensional NMR spectral data (Table I). A TOCSY experiment showed a correlation between 6-H signal of the glucose and the anomeric proton at δ 4.59, demonstrating that they belonged to the same spin system. The β-configuration of the anomeric carbon was evident from the coupling constant of H-1‴ ($J = 7.7$ Hz) observed in the ^1H NMR spectrum [3]. In this way, the β-rutinosyl residue could be characterized unambiguously. The ^{13}C NMR signals of the β-rutinosyl residue are also as same as that of flavonol β-rutinoside in literature [4].

The anomeric proton of the glucose residue, observed at δ 4.59, showed a long-range correlation with a ^{13}C NMR signal at δ 82.19, corresponding to a proton at δ 3.46 in the

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TABLE I ^{1}H and ^{13}C NMR assignments for **1** in DMSO-d_6

	^{13}C NMR		^{1}H NMR	
No.	δ (ppm)	*mult.*	δ (ppm), *mult. J* (Hz)	*HMBC*
2	156.49	s		7.99 (H-2′,6′)
3	132.73	s		5.54 (H-1″)
4	177.33	s		6.38 (H-8)
5	161.22	s		6.18 (H-6)
6	98.85	d	6.18 (d, 1.7)	6.38 (H-8)
7	164.62	s		6.18 (H-6), 6.38 (H-8)
8	93.80	d	6.38 (d, 1.7)	6.18 (H-6)
9	156.23	s		6.38 (H-8)
10	103.69	s		6.18 (H-6), 6.38 (H-8)
1′	120.97	s		6.90 (H-3′,5′)
2′,6′	130.99	d	7.99 (d, 8.8)	6.90 (H-3′,5′), 7.99 (H-2′,6′)
3′,5′	115.22	d	6.90 (d, 8.8)	6.90 (H-3′,5′), 7.99 (H-2′,6′)
4′	159.83	s		6.90 (H-3′,5′), 7.99 (H-2′,6′)
5-OH			12.63 (s)	
7-OH			10.97 (s)	
4′-OH			10.16 (s)	
1″	98.26	d	5.54 (d, 7.0)	3.46 (H-2″)
2″	82.19	d	3.46 (m)	4.59 (H-1‴), 3.47 (H-3″)
3″	76.40	d	3.47 (m)	3.46 (H-2″), 5.54 (H-1″)
4″	69.73	d	3.16 (m)	3.15 (H-5″)
5″	77.03	d	3.15 (m)	3.16 (H-4″)
6″	60.86	t	3.51 (m), 3.61 (m)	3.15 (H-5″)
1‴	103.95	d	4.59 (d, 7.7)	3.08 (H-2‴), 3.46 (H-2″)
2‴	74.29	d	3.08 (m)	3.19 (H-3‴)
3‴	76.52	d	3.19 (m)	3.08 (H-2‴)
4‴	69.54	d	3.15 (m)	3.23 (H-5‴)
5‴	75.60	d	3.23 (m)	3.22, 3.63 (H-6‴), 4.59 (H-1‴)
6‴	66.08	t	3.22 (m), 3.63 (m)	4.32 (H-1⁗), 3.23 (H-5‴)
1⁗	100.39	d	4.32 (brs)	3.21 (H-5⁗), 3.22, 3.63 (H-6‴), 3.23 (H-2⁗)
2⁗	70.55	d	3.23 (m)	3.36 (H-3⁗), 4.32 (H-1⁗)
3⁗	70.29	d	3.36 (m)	3.07 (H-4⁗)
4⁗	71.81	d	3.07 (m)	0.95 (H-6⁗), 3.21 (H-5⁗), 3.36 (H-3⁗)
5⁗	68.18	d	3.21 (m)	0.95 (H-6⁗), 3.07 (H-4⁗), 4.32 (H-1⁗)
6⁗	17.63	q	0.95 (d, 6.2)	3.07 (H-4⁗), 3.21 (H-5⁗)

HMQC spectrum. The latter signal showed a ^{1}H–^{1}H correlation, observed in the DQF–COSY experiment, with the third anomeric proton at δ 5.54, assigned to H-1″ of the second glucosyl moiety. Therefore, glycosylation of the rutinose at the C-2″ position became evident. Since compound **1** is a 3-monosubstituted kaempferol derivative, the second glucosyl residue was attached to C-3 of kaempferol, which was confirmed by a long-range ^{13}C–^{1}H correlation observed in a HMBC experiment between the ^{13}C NMR signal at δ 132.73 (C-3) and the ^{1}H NMR signal at δ 5.54 (H-1″). Two-dimensional NMR allowed the assignment of all ^{1}H and ^{13}C NMR signals of the second glucosyl moiety (Table I). The β-configuration of the anomeric carbon was evident from the coupling constant of H-1″ ($J = 7.0$ Hz) observed in the ^{1}H NMR spectrum [3]. This showed the presence of 3-sophorosyl residue. The ^{13}C NMR signals of the 3-glucosyl residue are also as same as that of flavonol 3-sophoroside in literature [4]. Therefore, compound **1** was identified as kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside, which is a new compound. The structure was also confirmed by a $[M-H]^-$ peak at *m/z* 755 in the negative FABMS, consistent with a molecular formula $C_{33}H_{40}O_{20}$.

Compounds **2** and **3** were identified as kaempferol 3-*O*-[α-L-rhamnopyranosyl-(1 → 6)]-[β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside (**2**) [5] and kaempferol 3-*O*-β-D-

TABLE II Antioxidant activity of flavonol glycosides from *S. japonica* pericarps

Compounds	*DPPH assay* (IC_{50})*		*Cytochrome-c reduction assay* (IC_{50})*	
	μg/ml	μM	μg/ml	μM
1	19.3	25.5	19.5	25.8
2	19.1	25.3	19.4	25.7
3	20.1	26.6	20.5	27.1
Gallic acid	3.6	21.2	3.0	17.6

* Results are expressed as IC_{50} values (μg/ml and μM). Data for active compounds were mean of triplicates. Gallic acid used for positive control.

glucopyranosyl-(1 → 2)-β-D-glucopyranoside-7-*O*-α-L-rhamnopyranoside (**3**) [6,7] by comparing their physical and spectral data with the literature values.

For the screening and evaluation of antioxidant activity of pure compounds and/or plant extracts, DPPH and cytochrome-c reduction assays were adopted. Compounds **1**–**3** exhibited inhibitory activity against TPA-induced free radical formation in a HL-60 cell culture system and showed free radical scavenging activity in the DPPH assay (Table II).

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EXPERIMENTAL

General Experimental Procedures

Melting points were determined by an Ellectrothermal 9200 micro melting point apparatus and are not corrected. Optical rotations were measured on a Perkin–Elmer model 241 polarimeter. UV and IR spectra were measured on a Shimadzu UV-1601 and on a Perkin–Elmer 983, respectively. All NMR spectra were run on a Bruker DRX-400 instrument operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, using standard pulse sequences. Chemical shifts are reported on the δ scale in parts per million downfield from TMS. Carbon multiplicities were determined in DEPT-135 and DEPT-90 experiments. All two-dimensional NMR spectra were recorded using pulsed field gradients. $^{1}H-^{1}H$ correlations were observed in double quantum filtered (DQF) COSY and TOCSY experiments. One-bond $^{13}C-^{1}H$ correlations were observed in a HMQC experiment. Long-range $^{13}C-^{1}H$ correlations were observed in HMBC experiments. FABMS spectra were obtained on a JEOL JMS DX-303HF mass spectrometer. TLC was carried out on precoated Si gel 60 F_{254} plates (Merck), developed with EtOAc–HOAc–HCOOH–H_2O (30:0.9:1.1:8, v/v, upper phase, and 10:1:1:2), *n*-BuOH–HOAc–H_2O (4:1:5, upper phase), and for sugars EtOAc–HOAc–MeOH–H_2O (13:4:3:3). 1% methanolic $AlCl_3$ was used to visualize isoflavonoids; thymol in H_2SO_4 (0.5 g thymol in 95 ml EtOH and 5 ml H_2SO_4), followed by heating the plates to 120°C for 15–20 min, to visualize sugars. Column chromatography was performed on Si gel (Marine Chemical Factory in Qingdao), Sephadex LH-20 (Pharmacia), and RP-18 (Shimadzu).

Plant Material

Fruits of *S. japonica* L. were collected from mature trees, growing in Nanjing, China, in November 1998, and identified by Prof. Luoshan Xu, China Pharmaceutical University. A voucher specimen (No. CPUT-981120) has been deposited in the herbarium of China Pharmaceutical University.

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Extraction and Isolation

Dried and powdered pericarps of *S. japonica* (9.0 kg) were extracted three times with 80% MeOH using ultrasonic apparatus for 3 h, the solvent was removed under reduced pressure, and the residue dissolved in hot water. This residue was left in the refrigerator overnight and filtered. The filtrate was partitioned against $CHCl_3$, EtOAc, and *n*-BuOH, successively. The *n*-BuOH-soluble fraction was concentrated and subjected to Si gel column chromatography eluting with $CHCl_3$–MeOH (25:1) followed by stepwise addition of MeOH to yield 15 fractions. Fraction 9 (32.5 g) was subjected to Si gel ($CHCl_3$–MeOH, 10:3), and Sephadex LH-20 (MeOH) chromatography and purified by HPLC (RP_{18}, 4 μm, 260 nm, MeOH–1% acetic acid, 18:82; **1**: $t_R = 9.96$ min; **2**: $t_R = 8.72$ min and **3**: $t_R = 12.82$ min) to give compounds **1** (20 mg), **2** (43 mg) and **3** (52 mg).

For acid hydrolysis, a solution of compound **1** in 5 ml 6% HCl was heated for 3 h. The reaction mixture was extracted with EtOAc. The EtOAc fraction (aglycone) and the aqueous fraction (sugars) were concentrated until dryness for identification.

Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside (1): Yellow crystals. m.p. 184°C; $[\alpha]_D^{25} = -132°$ (MeOH, *c* 0.001). Negative FAB MS (*m/z*): 755 $[M-H]^-$, 609 $[M-rha-H]^-$, 447 $[M-rha-glc-H]^-$, and 285 $[aglycone-H]^-$. UV spectra λ_{max}^{MeOH} nm: 348, 298, 267, 258. IR ν_{max}^{KBr} cm^{-1}: 3404, 2973, 2921, 1658, 1608, 1503, 1450, 1416, 1360, 1301, 1279, 1260, 1200–1000. ^1H- and ^{13}C NMR spectral data are shown in Table I.

Antioxidant Assay

DPPH assay was performed essentially according to the modified method of Kirby and Schmidt [8]: 95 μl of 3.2×10^{-4} M of DPPH solution in absolute EtOH and 5 μl of sample solution in DMSO were mixed in a 96-well plate. The optical density was measured at 515 nm after incubation of the plate for 1 h at 37°C. The DPPH control contained no sample but was otherwise identical. The cytochrome-c reduction assay was performed according to Sharma *et al.* [9]. HL-60 cells were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 1% penicillin–streptomycin at 37°C in humidified atmosphere at 5% CO_2 in air. Differentiation was induced by seven-day treatment with 1.3% DMSO, and the cells were cultured in a 96-well plate (1×10^6 cells per well) in HBSS. After the addition of TPA (8 μM) to induce free radical formation, cytochrome-c (160 μM) and samples were added. The cells were incubated for 1 h at 37°C, and antioxidant activity was determined by monitoring absorbance at 550 nm. The same reaction mixture, without the HL-60 cells, was used as a blank control.

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