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One-step Separation of Three Flavonoids from *Poacynum hendersonii* by High-speed Counter-current Chromatography

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ABSTRACT:

Introduction – Owing to them having the same traditional name, the leaves of *Apoacynum venetum* and *Poacynum hendersonii* are used indiscriminately in some areas of China. Although a series of studies have been conducted on *Apoacynum venetum*, there are only a few studies on *Poacynum hendersonii*.

Objective – To develop an efficient method for the preparative isolation and purification of flavonoids from the leaves of *Poacynum hendersonii* by high-speed counter-current chromatography (HSCCC).

Methodology – Powdered *Poacynum hendersonii* lead was extracted three times with 75% ethanol at 60 °C for 3 h. The distribution constant (K_D) was measured to select an optimal two-phase solvent system for HSCCC separation. The purities of the target compounds were tested using HPLC and their structures were identified by ^{1}H-NMR and ^{13}C-NMR.

Results – Using a two-phase solvent system composed of *n*-butanol–petroleum ether–0.5% acetic acid (5:3:5, v/v), three main flavonoids, i.e. isoquercitrin, quercetin-3-*O*-sophoroside and quercetin-3-*O*-(6″-*O*-malonyl)-*β*-D-glucoside, were separated from 240 mg crude sample in a one-step separation by using HSCCC method. After further purification with a Sepdex-LH20 column, 5.7 mg isoquercitrin (LC purity 98.72%), 4.9 mg quercetin-3-*O*-sophoroside (LC purity 99.06%) and 7.4 mg quercetin-3-*O*-(6″-*O*-malonyl)-*β*-D-glucoside (LC purity 99.31%) were obtained, respectively.

Conclusion – The optimised high-speed counter-current chromatography method is fast, simple and efficient for the preparative separation of flavonoids from the leave of *Poacynum hendersonii*.

Keywords: high-speed counter-current chromatography; *Poacynum hendersonii*; isoquercitrin; quercetin-3-*O*-sophoroside; quercetin-3-*O*-(6″-*O*-malonyl)-*β*-D-glucoside

Introduction

Because of the same traditional name (Luobuma) and their high contents of quercetin glycosides, the leaves of *Apoacynum venetum* and *Poacynum hendersonii* have been indiscriminately used for making tea and medicine. According to the *Pharmacopeia of People's Republic of China* (Pan *et al.*, 2005), soup made from the leaves of *Apoacynum venetum* can be used in treating cardiac disease, hypertension, nephritis and neurasthenia. Modern research on *Apoacynum venetum* has also revealed that the ethanolic extract of *Apoacynum venetum* leaves exhibits a series of pharmacological activities, such as antioxidative (Sandei *et al.*, 1994; Shi *et al.*, 2009; Yokozawa and Nakagawa, 2004; Shirai *et al.*, 2005), antihypertensive (Kim *et al.*, 2000; Tagawa *et al.*, 2004), antidepressant effects (Butterweck *et al.*, 2003; Yokozawa and Nakagawa, 2004), cholesterol-lowering (Grundmann *et al.*, 2007) and anti-aging activity (Zhang, 2004). The main active components of this extract are flavones and flavan-3-ols (Kim *et al.*, 1998; Yokozawa and Nakagawa, 2004; Zhang *et al.*, 2006). However, there are only a few studies focusing on the chemical components (Wei *et al.*, 2008), pharmacological effects (Gu *et al.*, 1989; Qian *et al.*, 1990) and clinical effects of *Poacynum hendersonii*. Therefore, the isolation and purification of bioactive constituents from the leaves of *Poacynum hendersonii* are of great importance for further studying its biological, pharmacological and clinical effects.

High-speed counter-current chromatography (HSCCC), first introduced by Ito in the early 1970s (Ito and Bowman, 1970), is a support-free liquid–liquid partition chromatographic technique (Ito, 1991). Compared with the traditional liquid–solid separation methods, HSCCC could eliminate the complications resulting from the solid support matrix such as irreversible adsorptive sample loss and deactivation, tailing of solute peaks and contamination (Ito, 2005). Therefore, HSCCC has been widely used for the preparative separation of natural products such as traditional Chinese medicinal herbs (Deng *et al.* 2009; Yang *et al.*, 2009a; Guo *et al.*, 2010).

In this paper, the preparative isolation of isoquercitrin, quercetin-3-*O*-sophoroside and quercetin-3-*O*-(6″-*O*-malonyl)-*β*-D-glucoside (Fig. 1) using HSCCC is described. The upper phase of *n*-butanol–petroleum ether–water (0.5% acetic acid; 5:3:5, v/v) was used as the stationary phase while the lower phase of *n*-butanol–petroleum ether–water (0.5% acetic acid) (5:3:5, v/v) served as flowing phase.

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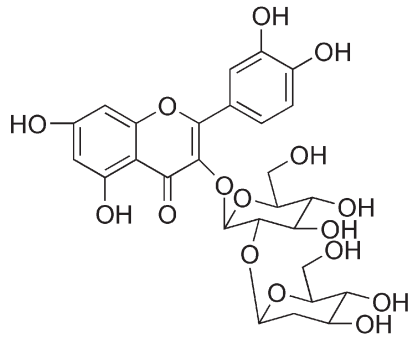
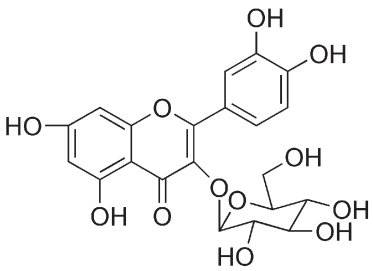
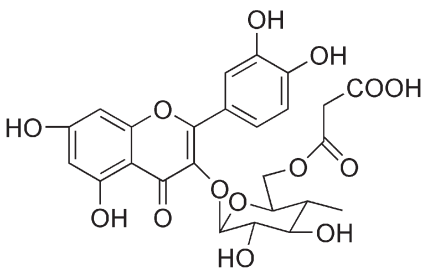


Quercetin-3-*O*-sophoroside



Isoquercitrin



Quercetin- 3-*O*-(6"-*O*-malonyl)
-β-D-glucoside

Figure 1. Chemical structures of the three analysed flavonoids of *Poacynum hendersonii*.

Experimental

Apparatus

This work was performed on a TBE-300A high-speed counter-current chromatography (Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation columns connected in series (inner diameter of the tubing is 1.6 mm and the total volume is 260 mL) and a 20 mL sample loop. The revolution radius was 5 cm and the β value of the multilayer coil varied from 0.5 at internal to 0.8 at the external terminal. An HX 1050 constant temperature circulating implement (Beijing Boyikang Laboratory Instrument Co. Ltd, Beijing, China) was used to control the separation temperature. A model NS-1007 constant flow pump (Beijing Institute of New Technology Application, China) was used to elute the mobile phase while the continuous monitoring of the effluent was achieved with a model 8823A-UV Monitor at 254 nm (Beijing Institute of New Technology Application). A manual six-port valve with a 20 mL loop was used as the injection valve and the data were collected with a Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Co. Ltd, Hangzhou, China.

Analyses were performed on an Agilent 1100 LC system equipped with a G1311A solvent delivery unit, 1315B UV–vis photodiode array detector, G1332A degasser and Agilent HPLC workstation. The analytical column was a Spherigel ODS C_{18} column (250 × 4.6 mm i.d., 5 µm). A FZ102 plant disintegrator (Taisite Instrument Company, Tianjin, China) was used for the disintegration of sample. Identification of the HSCCC peak fractions was carried out by ^{1}H-NMR and ^{13}C-NMR spectra (Varian Mercury 400).

Reagents and materials

All solvents used for preparation of crude sample and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). Methanol used for HPLC was chromatographic grade (Yucheng Chemical plant, Yucheng, China).

Leaves of *Poacynum hendersonii* were collected from the county of Golmud, Qinghai, China in August 2008.

Preparation of crude sample from *Poacynum hendersonii*

Air-dried leaves of *Poacynum hendersonii* were pulverised to 350 µm-size powder using an FZ102 plant disintegrator. About 1 kg of pulverised sample was extracted with 5 L of 75% ethanol at 60 °C for 3 h under reflux. The extraction procedure was repeated three times. All the extracts were combined and evaporated to dryness under reduced pressure at 60 °C. In total about 185 g of crude extract was obtained, which was stored in a refrigerator for the subsequent HSCCC separation.

Selection of the two-phase solvent system

The two-phase solvent systems were selected according to the distribution constant (K_D) of target components in a variety of solvent systems. The K_D values were determined as the following: first, different ratios of *n*-butanol–methanol–water (0.5% acetic acid), *n*-butanol–ethyl acetate–water (0.5% acetic acid) and *n*-butanol–petroleum ether–water (0.5% acetic acid) were prepared and equilibrated in a separation funnel at room temperature. Then 3 mg sample was added to 4.0 mL solution that was composed of equal volumes of upper and lower phases and the solution was then mixed thoroughly. After equilibrium was

Table 1. The distribution constant (K_D) of the three analysed flavonoids in various two-phase solvent systems

No	Solvent system	Volume ratio	K_1	K_2	K_3
	n-Butanol–ethyl acetate–water (0.5%acetic acid)	5:5:5	4.35	6.73	24.10
1	*n*-Butanol–light petroleum–water (0.5% acetic acid)	5:0.5:5	7.50	1.88	9.40
2	*n*-Butanol–light petroleum–water (0.5% acetic acid)	5:1:5	4.22	1.01	8.64
3	*n*-Butanol–light petroleum–water (0.5% acetic acid)	5:1.5:5	2.49	0.61	7.28
4	*n*-Butanol–light petroleum–water (0.5% acetic acid)	5:2:5	1.74	0.42	5.07
5	*n*-Butanol–light petroleum–water (0.5% acetic acid)	5:2.5:5	1.00	0.25	4.08
6	*n*-Butanol–light petroleum–water (0.5% acetic acid)	5:3:5	1.14	0.64	2.58
7	*n*-Butanol–light petroleum–water (0.5% acetic acid)	5:3.5:5	1.26	1.37	1.93

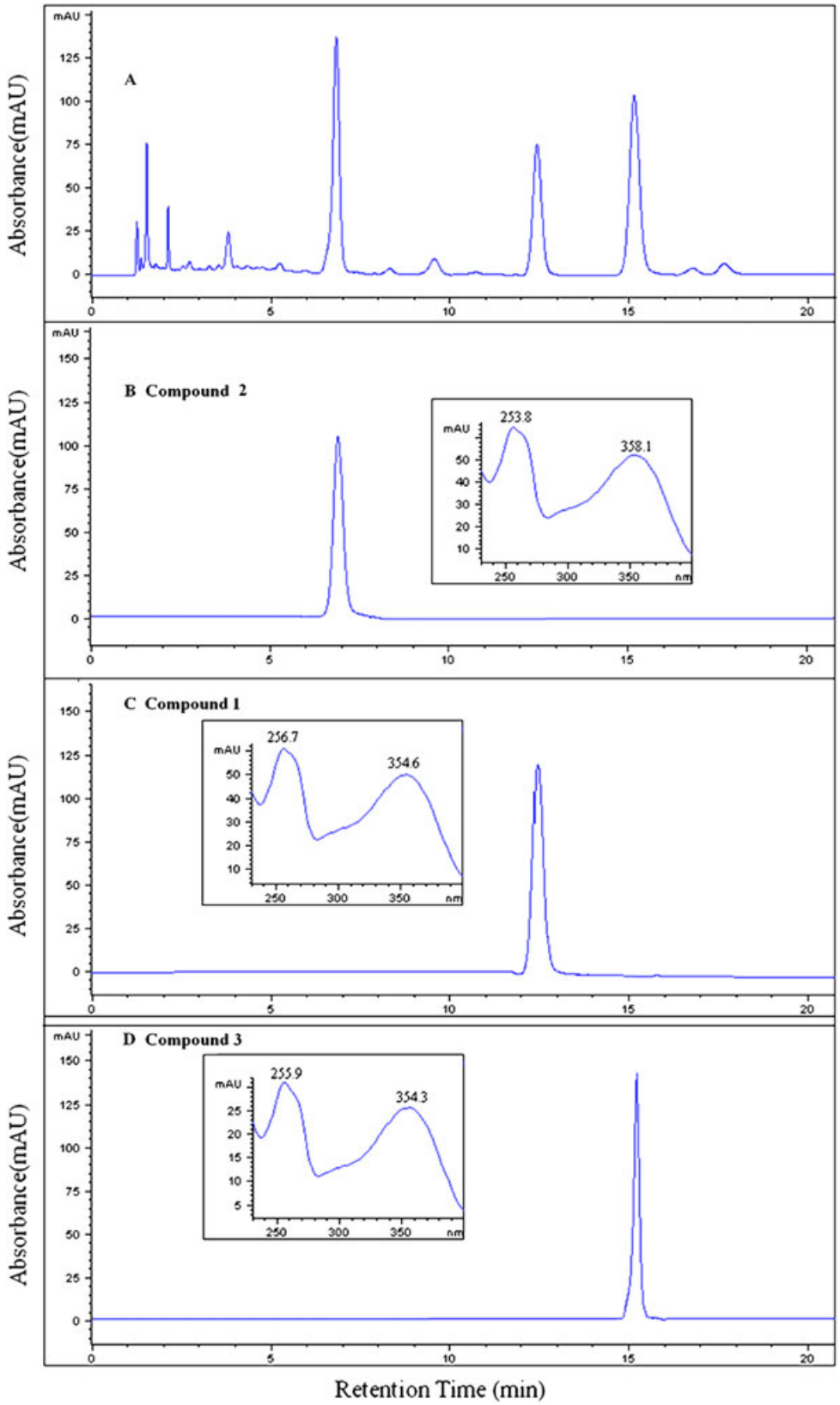


Figure 2. HPLC chromatograms of crude extract and HSCCC peak fractions. (A) Crude extract from *Poacynum hendersonii*; (B–D) purified HSCCC peak fractions I–III in Fig. 3. Conditions: column, Spherigel ODS C_{18} column (250 × 4.6 mm i.d., 5 μm); column temperature, 25 °C; mobile phase, methanol–water in gradient mode (methanol, 0–20 min, 20–70%); flow-rate, 1.0 mL/min; detection wavelength, 254 nm.

established, the two phases were analysed by HPLC. The peak area of the upper phase was recorded as A_U and that of the lower phase was A_L. The K_D values (A_U/A_L) were calculated as shown in Table 1. Finally, a mixture of *n*-butanol–petroleum ether–water (0.5% acetic acid) (5:3:5, v/v) was chosen. This solvent mixture was thoroughly equilibrated in a separating funnel at room temperature and the two phases were separated and degassed by sonication for 10 min shortly before use.

HSCCC separation procedure

Preparative HSCCC was performed using a two-phase solvent system composed of *n*-butanol–petroleum ether–water (0.5% acetic acid) (5:3:5, v/v) in the head to tail elution mode: first, the multi-layer coil column was entirely filled with the upper phase as stationary phase and the apparatus was rotated at 850 rpm. Then the lower phase was pumped into the column at a flow-rate of 1.5 mL/min as mobile phase. After hydrodynamic equilibrium was reached (about half an hour later), 240 mg of crude sample was dissolved in a 5 mL mixture of equal upper phase and lower phase solution and injected into the separation column. The temperature was controlled at 30 °C in the separation procedure. Because the maximum absorptions of the three quercetin glycosides were around 254 nm (Fig. 2), the effluent from the outlet of the column was continuously monitored at 254 nm. Each peak fraction was manually collected according to the chromatogram and evaporated under reduced pressure.

HPLC analysis and identification of HSCCC peak fractions

The crude sample of *Poacynum hendersonii* and each peak fraction from the HSCCC were analysed by HPLC with Spherigel ODS C_{18} column (250 × 4.6 mm i.d., 5 μm). The mobile phase was a linear gradient of methanol (A) and pure water (B) as follows: A:B (20:70, v/v) to A:B (70:30, v/v) in 20 min. The flow-rate was 1.0 mL/min and the effluent was monitored at 254 nm. Crude samples contained several flavonoids, including quercetin-3-*O*-sophoroside (retention time 6.93 min), isoquercitrin (retention time 12.38 min), quercetin-3-*O*-(6″-O-malonyl)-β-D-glucoside (retention time 15.17 min), and other constituents are shown in Fig. 2(A).

Results and Discussions

Selection of two-phase solvent system and other conditions of HSCCC

In an HSCCC separation, the first and most important step is to select a appropriate two-phase solvent system and the following factors should be considered: (1) the settling time of the solvent system should be short (i.e. <30 s) (Oka *et al.*, 2002); (2) the retention of the stationary phase should be satisfactory; and (3) the distribution constant of the target compound should be between 0.4 and 2.5 (Friesen and Pauli, 2005). If the K_D value is too small, the analytes will be eluted close to the solvent front with poor resolution, while the higher K_D value tends to lead to expectation of a good resolution but will cause broad peaks and a prolonged elution time.

According to the large polarity of these three quercetin glycosides, three kinds of solvent systems including *n*-butanol at different volume ratios were selected to separate these three similar compounds. When *n*-butanol–methanol–water (0.5% acetic acid) was tested, the solvents could not delaminate even when methanol was at a low ratio. However, if *n*-butanol–ethyl acetate–water (0.5% acetic acid) was used, the K_D value was much larger than 1, therefore *n*-butanol–petroleum ether–water (0.5% acetic acid), which had lower polarity, was tested. The K_D values of three target compounds are given in Table 1. For systems 1–5, the K_D values for compound 3 were too large, while for system 7, compounds **1** and **2** had similar K_D values. For system 6, the K_D values were between 0.5 and 2.5, and the separation factors between these three flavonoids were large enough for these three flavonoids to have large resolution from each other [$\alpha_{12} = 1.78$ (K_{D1}/K_{D2}), $\alpha_{31} = 2.26$ (K_{D3}/K_{D1})]; therefore, system 6 was satisfactory for the separation of crude sample.

The influences of flow-rate of mobile phase, the separation temperature and revolution speed were also investigated. Different flow-rates (1.0, 1.5, 2.0 and 2.5 mL/min) of mobile phase were examined in the present study. The results indicated that decreasing flow speed could improve separation to some extent, but the chromatogram peaks would be extended. Considering these two aspects, the flow-rate of mobile phase was set to 1.5 mL/min. On the other hand, the revolution speed of the coil tube had great influence on the retention of stationary phase, and the use of a high rotary speed could increase the volume of stationary phase retained in the column. The retentions of stationary phase at the rotary speeds of 700, 750, 800, 850 rpm were 57.2, 59.8, 62.4 and 64.1%, respectively, when the flow-rate and temperature were set to 1.5 mL/min and 30 °C. Therefore, the final revolution speed was set to 850 rpm. Also, the temperature had significant influence on the distribution constant (K_D), retention percentage of stationary phase and the mutual solvency of the two-phase. After testing

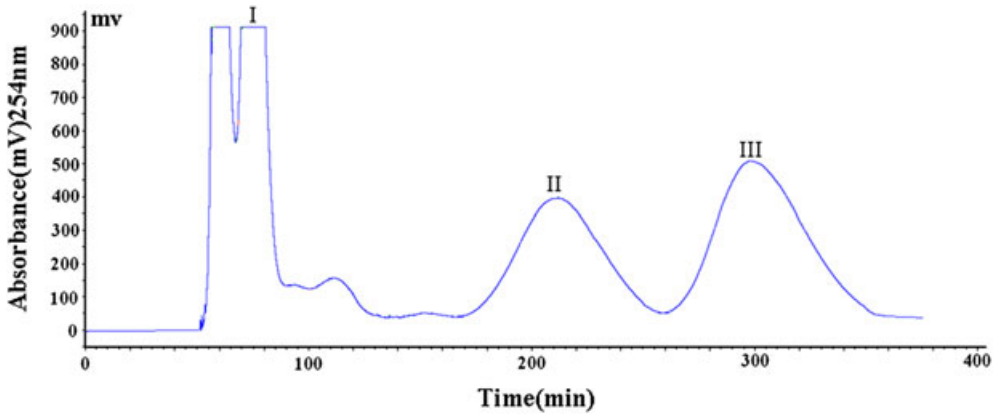


Figure 3. HSCCC chromatograms of the sample from *Poacynum hendersonii*. Solvent systems: *n*-butanol–petroleum ether–water (0.5% acetic acid; 5:3:5); flow-rate, 1.5 mL/min; revolution speed, 850 rpm; detection wavelength, 254 nm; 240 mg crude sample; I, isoquercitrin; II, quercetin-3-*O*-sophoroside; III, quercetin −3-*O*-(6″-*O*-malonyl)-β-D-glucoside. The retention of stationary phase was 64.1%.

at 20, 25, 30, 35 and 40 °C, the best results could be obtained when the separation temperature was controlled at 30 °C.

HSCCC separation

The crude sample (240 mg) was first dissolved in a 5 mL mixture of equal upper phase and lower phase and then separated according to the procedure described above under the optimised conditions. Figure 3 shows the chromatogram obtained from 240 mg of the crude sample from *Poacynum hendersonii* by HSCCC using the two-phase solvent system *n*-butanol–petroleum ether–water (0.5% acetic acid; 5:3:5, v/v). The retention of the stationary phase was 64.1%, and the total separation time was about 6 h. Fractions I–III were collected, and these three compounds were further purified in a Sephadex LH-20 column. After drying, the fraction from peak I yielded 4.9 mg compound **2** (LC purity 99.06%), while the fractions from peaks II and III yielded 5.7 mg compound **1** (LC purity 98.72%) and 7.4 mg compound **3** (LC purity 99.31%), respectively, which were determined at 210, 254, 280 and 360 nm (Fig. 2B–D).

Structural identification

The chemical structures of the compounds were identified by ^{1}H-NMR, ^{13}C-NMR. The compounds corresponding to peaks I–III were quercetin-3-*O*-sophoroside, isoquercitrin and quercetin-3-*O*-(6″-*O*-malonyl)-*β*-D-glucoside, respectively. The detailed data were as follows.

Isoquercitrin (compound 2). ^{1}H-NMR (400 MHz, DMSO-d6, TMS), *δ*, ppm, 3.07–5.29 (sugar protons), 5.47 (1 H, d, *J* = 7.2, H-1″), 6.20 (1 H, d, *J* = 2.1, H-6), 6.40 (1 H, d, *J* = 2.1, H-8), 6.84 (1 H, d, *J* = 9.0, H-5′), 7.58 (1 H, dd, H-6′), 7.56 (1 H, d, H-2′), 9.24 (1 H, s, 4′-OH), 9.74 (1 H, s, 3′-OH), 10.86 (1 H, s, 7-OH), 12.65 (1 H, s, 4′-OH); ^{13}C-NMR (100 MHz, DMSO-d6, TMS) *δ*, ppm: 61.97 (C-6″), 70.13 (C-4″), 74.29 (C-2″), 76.69 (C-3″), 77.81(C-5″), 91.02 (C-8), 98.85 (C-6), 101.01 (C-1″), 104.18 (C-10), 115.40 (C-2′), 116.39 (C-5′), 121.37 (C-1′), 121.82 (C-6′), 133.50 (C-3), 145.02 (C-3′), 148.66 (C-4′), 156.36 (C-9), 156.52 (C-2), 161.45 (C-5), 164.32 (C-7),177.65 (C-4) (Yang *et al.*, 2009b).

Quercetin-3-*O*-sophoroside (compound 1). ^{1}H-NMR (400 MHz, DMSO-d6) δ_H, ppm, 12.67 (1 H, s, 5-OH), 10.8(1 H, s, 7-OH), 9.74 (1 H, s , 3′-OH), 9.23 (1 H, s, 4′-OH), 7.60 (1 H, dd, *J* = 8.4 Hz, H-6′), 7.53 (1 H, d, H-2′), 6.85 (1 H,d, *J* = 8.4 Hz, H-5′), 6.39 (1 H, d, H-8), 6.18 (1 H,d, H-6), 5.69 (1 H, d, *J* = 6.3 Hz, H-1″), 4.59 (1 H, d, *J* = 7.2 Hz, H-1‴); ^{13}C-NMR (100 MHz, DMSO-d6, TMS) *δ*, ppm: 156.69 (C-2), 133.4 (C-3), 177.91 (C-4), 161.73 (C-5), 98.46 (C-6), 164.46 (C-7), 93.89 (C-8), 155.99 (C-9), 104.66 (C-10), 121.59 (C-1′), 115.85 (C-2′), 145.28 (C-3′), 148.96 (C-4′), 116.53 (C-5′), 122.31 (C-6′), 99.07 (C-1″), 83.21 (C-2″), 77.01 (C-3″), 70.03 (C-4″), 77.22 (C-5″), 61.14 (C-6‴), 104.40 (C-1‴), 74.86 (C-2‴), 77.01 (C-3‴), 70.03 (C-4‴), 77.98 (C-5‴), 61.14 (C-6‴) (Wei *et al.*, 2008).

Quercetin-3-*O*-(6″-*O*-malonyl)-*β*-D-glucoside (compound 3). ^{1}H-NMR (400 MHz, DMSO-d6) δ_H, ppm, 3.09 (2 H, s, malonyl), 3.15–3.40 (overlap, H-2″-H-5″), 4.00 (1 H, dd, *J* = 11.5, 5.7 Hz, H-6″A), 4.20 (1 H, d, *J* = 11.5 Hz, H-6″B), 5.31 (1 H, d, *J* = 6.3 Hz, H-1″), 6.21 (brs, H-6), 6.41 (brs, H-8), 6.84 (1 H, d, *J* = 8.0 Hz, H-5′), 7.52 (1 H, br s, H-2′), 7.52 (1 H, d, *J* = 8.0 Hz, H-6′), 12.59 (1 H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d6) d: 41.3 (CH2 malonyl), 63.7 (C-6″), 69.7 (C-4″), 74.1 (C-2″, 5″), 76.4 (C-3″), 93.7 (C-8), 98.8 (C-6), 101.2 (C-1″), 104.0 (C-10), 115.3 (C-2′), 116.3 (C-5′), 121.2 (C-1′), 121.7 (C-6′), 133.3 (C-3), 144.9 (C-3′), 148.6 (C-4′), 156.5 (C-2 or C-9), 156.7 (C-2 or C-9), 161.3 (C-5), 164.3 (C-7), 166.7 (CO malonyl), 167.9 (CO malonyl), 177.5 (C-4) (Kamata *et al.*, 2008).

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