

SUPPLEMENTARY MATERIAL

Characterization of flavonoid glycosides from rapeseed bee pollen using a combination of chromatography, spectrometry and nuclear magnetic resonance with step-wise separation strategy

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Abstract

To identify the structures of flavonoid glycosides in bee pollen collected from rapeseed plants (*Brassica napus* L.), we utilised an approach that combined liquid chromatography-diode array detector-electrospray ionization-mass spectrometry (LC-DAD-ESI-MS) and nuclear magnetic resonance (NMR) technology with a step-wise separation strategy. We identified four constituents of high purity in rape bee pollen samples: (1) quercetin-3-O- β -D-glucosyl-(2 \rightarrow 1)- β -glucoside, (2)

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kaempferol-3, 4'-di-O- β -D-glucoside, (3) 5, 7, 4'-trihydroxy-3'-methoxyflavone-3-O- β -D-sophoroside and (4) kaempferol-3-O- β -D-glucosyl-(2 \rightarrow 1)- β -D-glucoside. They were isolated and purified into a series of fractions using ODS chromatography and semi-preparative HPLC on a reverse-phase column. Subsequently, LC-DAD-ESI-MS enabled the identification of the four flavonoid glycosides via their ultra-violet absorbance wavelengths, molecular ions, and fragmentation information; the structures were then confirmed with ^1H and ^{13}C NMR techniques. This study will also provide useful reference standards for qualification and quantification of four flavonoid glycosides in natural products.

Keywords: Bee pollen; Step-wise separation strategy; Semi-preparative HPLC; LC-DAD-ESI-MS; NMR; Flavonoid glycosides

Experimental

Materials and methods

Preparation of crude sample

Rape pollen grains (900 g sample) were ground, and their constituents were extracted twice with 80% ethanol/pure water at a solid/liquid (S/L) ratio of 1 g/10 mL for 30 min using ultrasound-assisted extraction (at power 500 W and frequency 40 kHz) to obtain a combined ethanol extract. The resulting supernatant was passed through a filter paper to remove solid particles, and then concentrated with a rotary evaporator at 35–40 °C for 30 min. The residual solution was extracted successively with petroleum ether, ethyl acetate, n-butyl alcohol and pure water. The n-butyl alcohol extract was freeze-dried, and then this lyophilised powder was stored at -20 °C in darkness.

Octadecylsilyl (ODS) chromatography

Procedure 1: The lyophilised powder was dissolved in 20 mL methanol; the resulting solution and 20 g of octadecylsilyl (ODS) packing were placed together in a glass mortar and gently ground to obtain a homogeneous material. After natural evaporation of the solvent, the mixture was introduced into an 80-mL syringe barrel, pre-plugged with a filter disc and placed on a vacuum

manifold. The gradient elution conditions were as follows: solvent A (pure water), solvent B (methanol), 18–80% B (0–30min). The flow rate was controlled at 8 mL/min and the absorbance wavelength was set at 270 nm. Five fractions (A1, A2, A3, A4 and A5) were collected by an automatic sample collector, according to their absorbance fluctuation.

Procedure 2: This procedure was to further separate fraction A2, obtained in Procedure 1. The isocratic elution condition was changed as follows: solvent A (pure water), solvent B (acetonitrile), 14%B (0-15 min). The flow rate was controlled at 8 mL/min and the absorbance wavelength was set at 270 nm. Three fractions (B1, B2 and B3) were collected by an automatic sample collector, according to their absorbance fluctuation, and then stored at 4 °C in darkness.

Semi-preparative HPLC

To isolate the individual flavonoid glycosides, aliquots of extract B2 (prepared above) were subjected to a Shimadzu (Kyoto, Japan) preparative HPLC solvent delivery system equipped with an LC-6AD pump, a UV detector set at 254 nm and a FRC-10A fraction collector. The prep-HPLC separation was performed as follows: Shim-pack PREP-ODS (H)/KIT (9.4 × 250 mm, i.d. 5µm); the solvent system consisted of isocratic 30% MeOH (aq.); the eluent was pumped at 8 mL/min for about 120 min at 30 °C, and a 2-mL sample solution was injected through the sample injector; and flavonoid glycosides were detected at 254 nm. The peak fractions (C1, C2, C3 and C4) were collected separately from multiple chromatographic runs, and like fractions were combined according to their chromatograms. Then, the combined fraction extract was freeze-dried and stored at 0-4°C in darkness.

HPLC–DAD–ESI-MS analysis of the extract

An accurately weighed 10-mg sample of lyophilised powder (containing C1, C2, C3 and C4) was dissolved separately in 10 mL of a methanol/pure water (v: v=4:6) solution. The UV-vis absorption spectra and MS spectrum in full-scan mode of each target analyte were acquired simultaneously, using one automated injection under the instrument conditions detailed below.

HPLC-DAD Conditions: The Agilent LC 1200 system (Waldbronn, Germany) with a vacuum degasser (G1322A), a binary pump (G1312B), an autosampler (G1367D), a column compartment (G1316B) and a diode array detector (DAD, G1315C) was used to measure the UV spectra of flavonoid glycosides. The DAD detector was set to scan from 190 nm to 400 nm. The mobile phase consisted of acetonitrile/0.1% formic acid (70:30, v/v); the column temperature was maintained at 30 °C and the injection volume was 5 µL. An Agilent 6460 triple quadrupole tandem MS coupled to electrospray ionization (ESI) interface and Agilent Jet Stream Ion Focusing (Agilent Technologies, Palo Alto, USA) was used to acquire total ion chromatogram (TIC) and fragmentation information. The MS was operated in the positive ion mode. Tuning parameters were optimised for the target analytes: gas temperature 350 °C, drying gas flow: 6 L/min, nebulizer pressure 35 psi, Vcap voltage 3500 V, sheath gas temperature 350 °C, sheath gas flow 9 L/min, and nozzle voltage 1000 V. The system operation, data acquisition and analysis were controlled and processed by the MassHunter software.

¹H and ¹³C NMR analysis of the extract

The ¹H and ¹³C NMR spectra were recorded on a Varian Mercury-400 system (Palo Alto, CA, USA) operating at 400 and 300 MHz, respectively. An accurately weighed 10-mg sample of lyophilised powder (containing C1, C2, C3 and C4) was dissolved separately in 1 mL dimethyl sulfoxide-d₆ for ¹H and ¹³C NMR analysis and the results were processed using the Varian VNMR software.

Statistical analysis

The experimental data are expressed as mean ± SD of three assays in each case using OriginPro 9.0.

Table S1 ¹H-NMR and ¹³C-NMR result of component C1

No.	δ H(J/Hz)	δ C	No.	δ H(J/Hz)	δ C
2		155.5	6'	7.59 (1H,dd,8.8,2.0)	121.9
3		133.0	1''	5.68(1H,d,7.2)	98.0
4		177.4	2''		82.8
5		161.3	3''		76.6
6	6.16 (1H,d,2.0)	98.8	4''		69.6
7		164.6	5''		76.6
8	6.37(1H,d,2.0)	93.5	6''		60.7
9		156.3	1'''	4.59(1H,d,8.0)	103.8
10		104.2	2'''		74.4
1'		121.1	3'''		77.5
2'	7.54(1H,d,2.0)	115.4	4'''		69.6
3'		144.9	5'''		76.8
4'		148.6	6'''		60.6
5'	6.85(1H,d,8.8)	116.0			

Table S2 ¹H-NMR and ¹³C-NMR result of component C2

No.	δ H(J/Hz)	δ C	No.	δ H(J/Hz)	δ C
2		156.5	6'	8.11(2H,d,8.8)	130.6
3		133.7	1''	5.47(1H,d,7.2)	100.8
4		177.6	2''		74.2
5		161.3	3''		76.5
6	6.21(1H,s)	98.8	4''		69.9
7		164.3	5''		77.6
8	6.45(1H,s)	93.8	6''		60.9
9		155.6	1'''	5.01(1H,d,6.8)	100.0
10		104.1	2'''		73.2
1'		123.7	3'''		76.4
2'	8.11(2H,d,8.8)	130.6	4'''		69.6
3'	7.14(2H,d,8.8)	145.8	5'''		77.1
4'		159.2	6'''		60.6
5'	7.14(2H,d,8.8)	145.8			

Table S3 ¹H-NMR and ¹³C-NMR result of component C₃

No.	δH(J/Hz)	δC	No.	δH(J/Hz)	δC
2		156.1	5'	6.92 (2H,d,9.0)	115.3
3		132.6	6'	8.03 (2H,d, 9.0)	130.6
4		177.0	1''	5.70 (1H, d,7.2)	98.0
5		154.6	2''		82.4
6	6.14 (1H, s)	100.0	3''		74.3
7		156.1	4''		69.6
8		128.1	5''		77.4
9		148.5	6''		60.8
10		102.0	1'''	4.61(1H, d, 7.8)	104.0
11	3.78 (3H, s)	60.7	2'''		76.5
1'		121.2	3'''		77.5
2'	8.03 (2H,d, 9.0)	130.6	4'''		69.7
3'	6.92 (2H,d,9.0)	115.3	5'''		77.0
4'		159.9	6'''		60.5

Table S4 ¹H-NMR and ¹³C-NMR result of component C4

No.	δ H(J/Hz)	δ C	No.	δ H(J/Hz)	δ C
2		156.1	5'	6.90 (2H, d, J=9.0Hz)	115.1
3		133.3	6'	8.03 (2H, d, J=9.0Hz)	130.5
4		177.0	1''	4.60,5.68 (1H, d, J=7.5Hz)	104.7
5		161.5	2''		82.5
6		98.8	3''		77.8
7		163.1	4''		71.1
8	6.18,6.41(1H,d, J=2.5Hz)	94.8	5''		78.2
9		155.6	6''		62.2
10		104.1	1'''		101.0
11		60.7	2'''		75.5
1'		121.2	3'''		77.9
2'	8.03 (2H, d, J=9.0Hz)	130.5	4'''		71.3
3'	6.90 (2H, d, J=9.0Hz)	115.1	5'''		78.2
4'		159.1	6'''		62.6

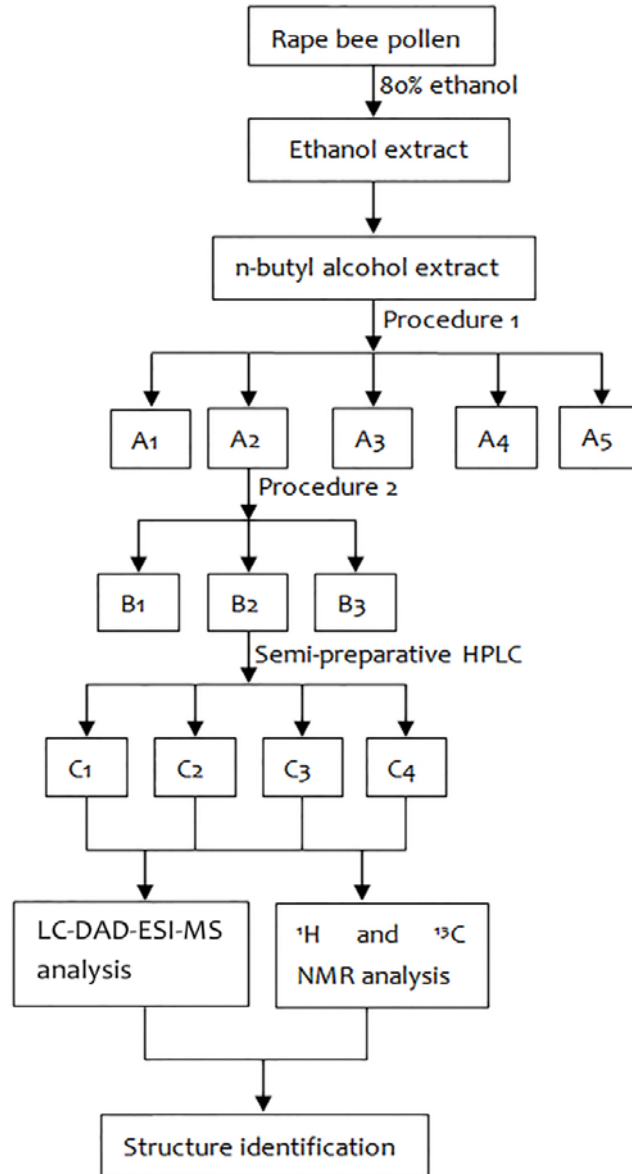


Figure S1. Extraction and purification protocols of flavonoid glycosides from rape bee pollen.

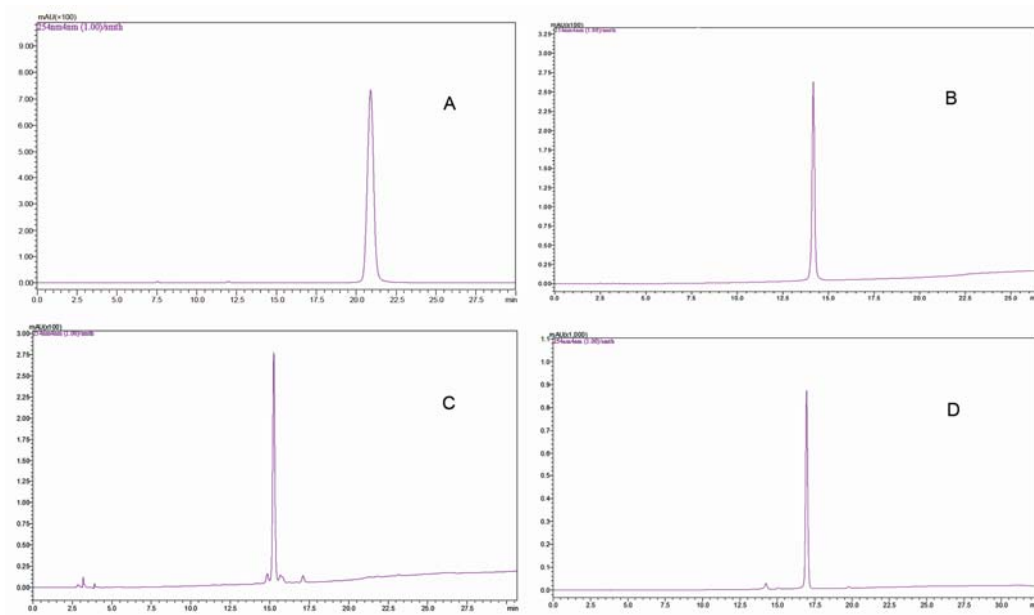


Figure S2. The chromatograms of four flavonoid glycosides. (A: quercetin-3-O- β -D-glucosyl-(2 \rightarrow 1)- β -glucoside, B: kaempferol-3, 4'-di-O- β -D-glucoside, C: 5, 7, 4'-trihydroxy-3'-methoxyflavone-3-O- β -D-sophoroside D: kaempferol-3-O- β -D-glucosyl-(2 \rightarrow 1)- β -D-glucoside).

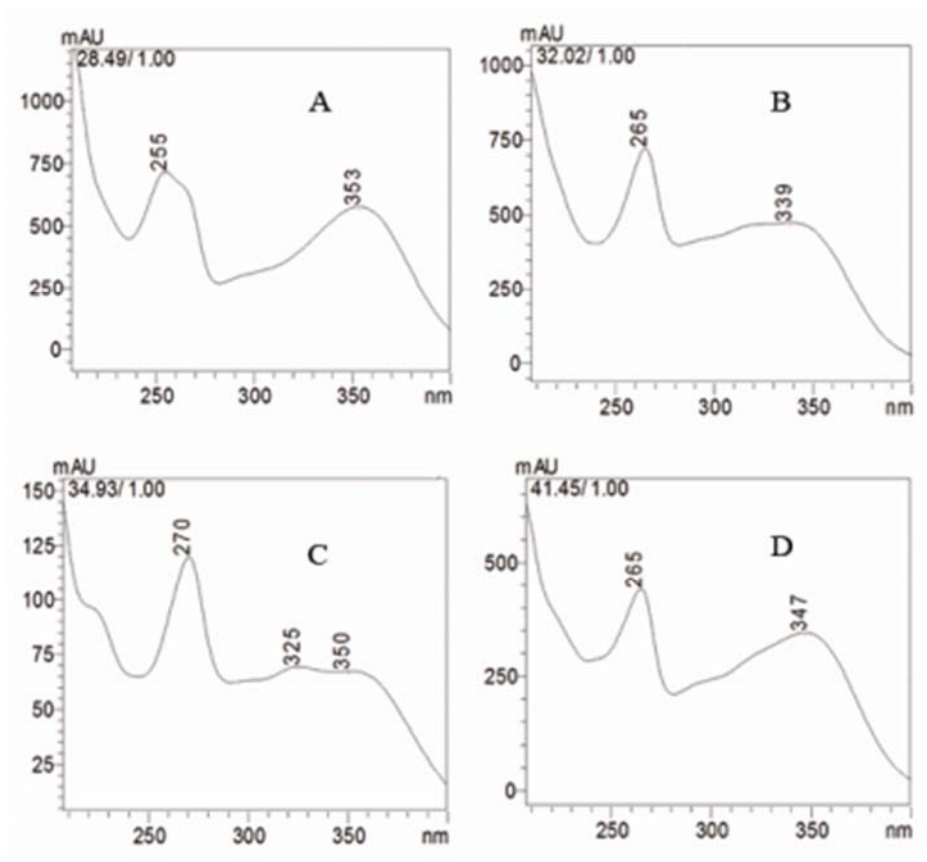


Figure S3. The UV absorption maxima of four flavonoid glycosides. (A: quercetin-3-O- β -D-glucosyl-(2 \rightarrow 1)- β -glucoside, B: kaempferol-3, 4'-di-O- β -D-glucoside, C: 5, 7, 4'-trihydroxy-3'-methoxyflavone -3-O- β -D-sophoroside D: kaempferol-3-O- β -D-glucosyl-(2 \rightarrow 1)- β -D-glucoside).

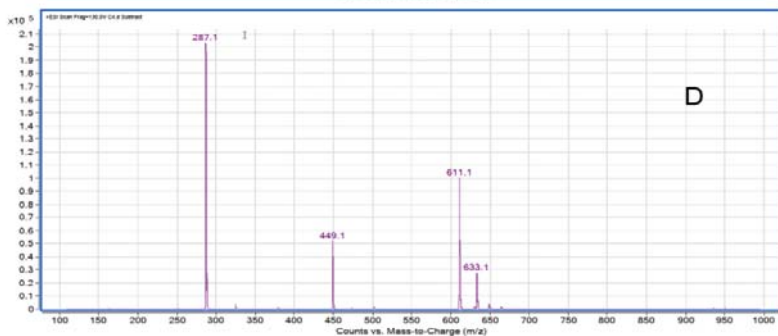
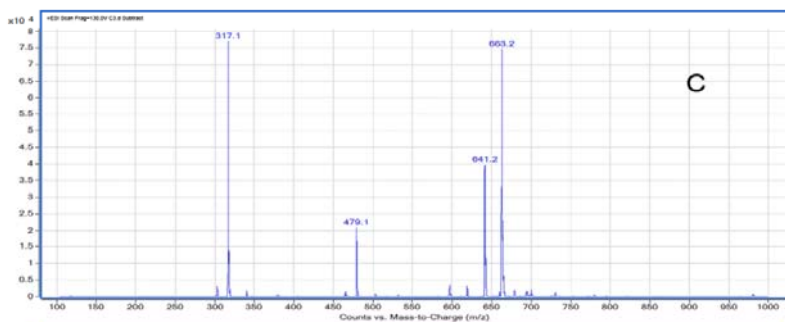
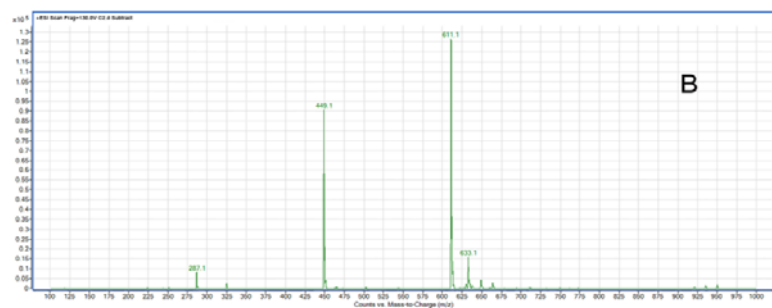
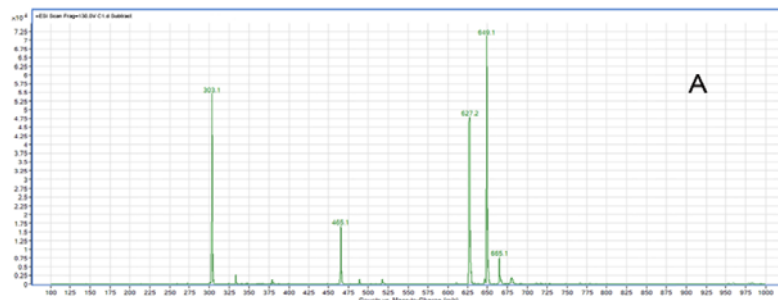


Figure S4. The mass spectra of four flavonoid glycosides. (A: quercetin-3-O- β -D-glucosyl-(2 \rightarrow 1)- β -glucoside, B: kaempferol-3, 4'-di-O- β -D-glucoside, C: 5, 7, 4'-trihydroxy-3'-methoxyflavone-3-O- β -D-sophoroside D: kaempferol-3-O- β -D-glucosyl-(2 \rightarrow 1)- β -D-glucoside).

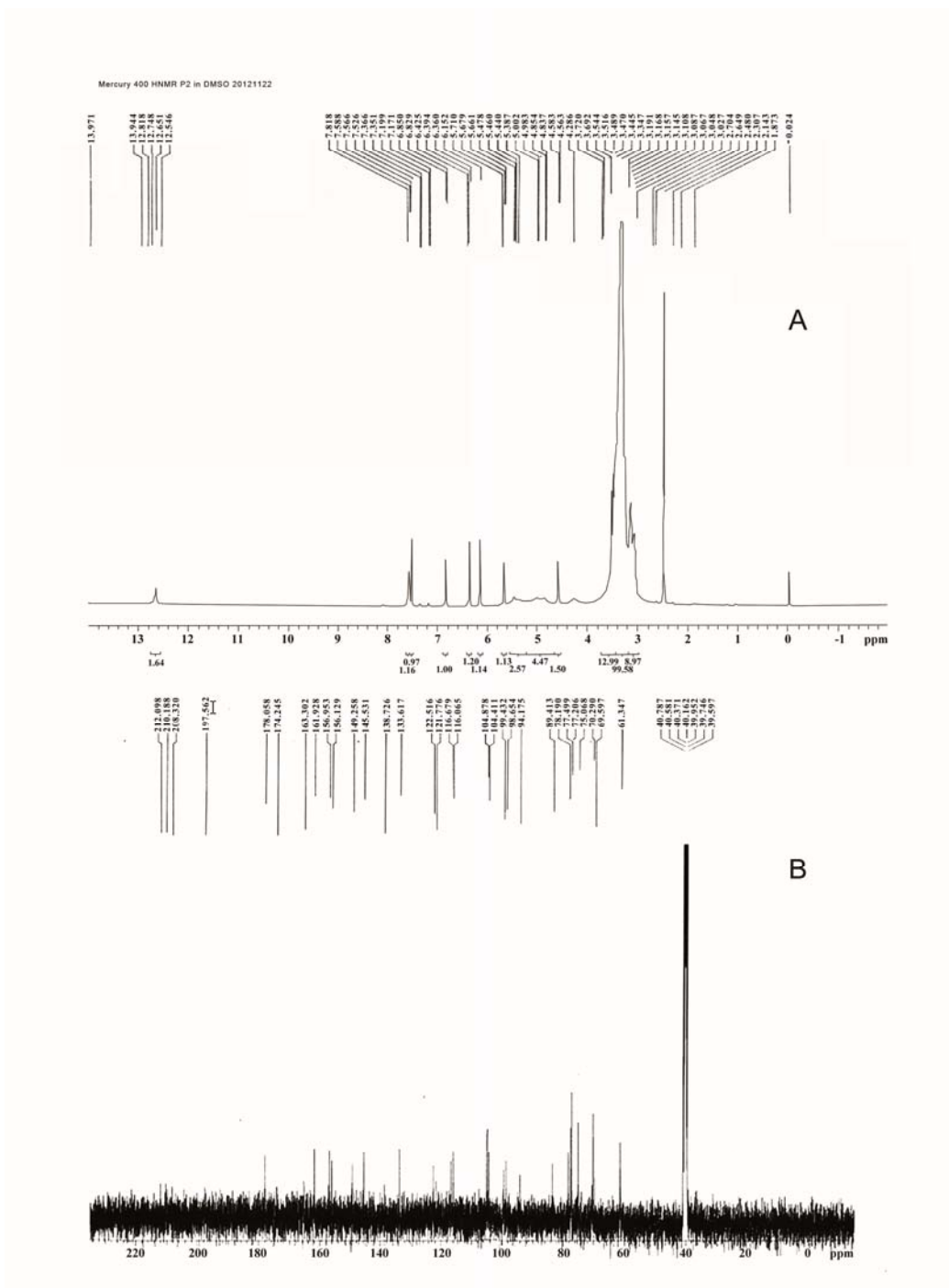


Figure S5. ¹H and ¹³C NMR spectra of quercetin-3-O-β-D-glucosyl-(2→1)-β-D-glucoside.

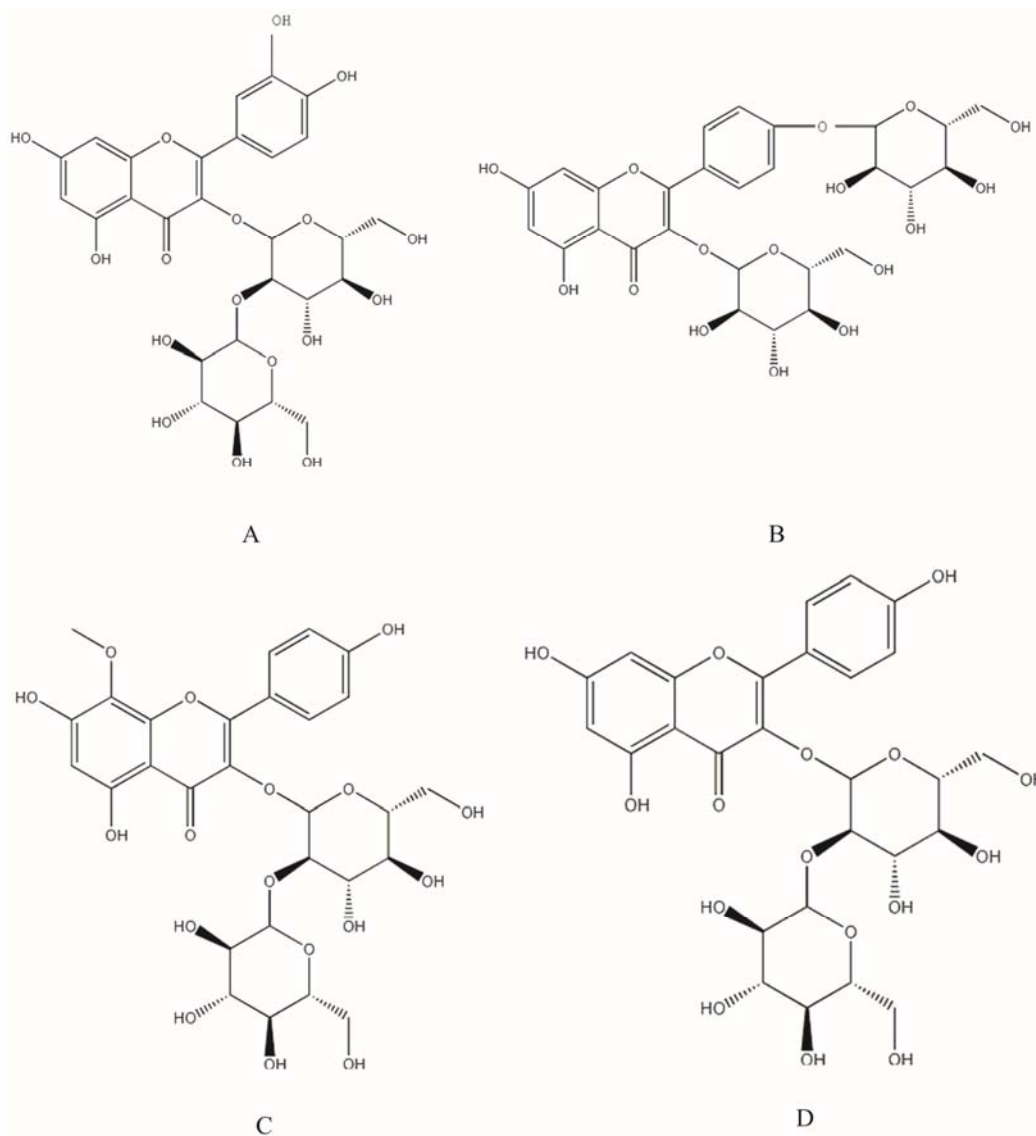


Figure S6. The structural formulas of four flavonoid glycosides. (A: quercetin-3-O- β -D-glucosyl-(2 \rightarrow 1)- β -D-glucoside, B: kaempferol-3, 4'-di-O- β -D-glucoside, C: 5, 7, 4'-trihydroxy-3'-methoxyflavone-3-O- β -D-sophoroside D: kaempferol-3-O- β -D-glucosyl-(2 \rightarrow 1)- β -D-glucoside).

