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Flavonol glycosides and phenylpropanoid glycosides with inhibitory effects on microglial nitric oxide production from *Neoshirakia japonica*

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ABSTRACT

Five new flavonol glycosides (**1–5**), one new phenylpropanoid glycoside (**6**), and nine known glycosides (**7–15**) were isolated from the stems and leaves of *Neoshirakia japonica*. The structures of the new compounds were determined by detailed analysis of spectroscopic data (HRESIMS, 1D and 2D NMR) and acid hydrolysis experiment. The antineuroinflammatory effects of all the isolates were evaluated by inhibiting NO production against LPS-induced BV-2 microglial cells. Compounds **1**, **8**, and **9** showed more potent inhibitory activities with IC_{50} values of 2.7, 5.5, and 4.1 μM, respectively, than that of the positive control minocycline (IC_{50} = 15.6 μM), while compounds **7** (IC_{50} = 17.0 μM) and **10** (IC_{50} = 24.3 μM) also displayed inhibitory activities to a certain degree.

1. Introduction

Sapium japonicum (revised as *Neoshirakia japonica*), as one species of the genus *Sapium* in the family Euphorbiaceae, is a shrub growing in moist places of forest or near streams and widely distributed in China, Japan, and North Korea [1]. Many species in the genus *Sapium* have been used as traditional medicines to treat eczema, scabies, detoxification, and detumescenece [1,2]. Meanwhile, previous phytochemical and pharmacological researches on this genus have revealed a wide array of diterpenoids, triterpenoids, flavonoids, lignanoids, and coumarins with significant biological activities [3].

Our previous study on chemical constituents and biological activity of the aerial parts of *S. discolor* (revised as *Triadica cochinchinensis*) and *S. rotundifolium* (revised as *Triadica rotundifolia*) acquired several antineuroinflammatory coumarinolignoids, diterpenoids, triterpenoids, and lignanoids [4–6], so in order to discover novel natural inhibitors of neuroinflammation, a chemical investigation on the stems and leaves of *N. japonica* was carried out. At last, six new (**1–6**) including five new flavonol glycosides (**1–5**) as well as one new phenylpropanoid glycoside (**6**) and nine known (**7–15**) compounds (Fig. 1) were isolated. Compounds **2–5** are rare 2″-*O*-galloyl-type kaempferol glycosides. Herein, the isolation and structural elucidation of these isolates, and their potential inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-induced BV-2 microglial cells were reported.

2. Material and methods

2.1. General experimental procedures

HRESIMS data were performed on Agilent 6545 Q-TOF LC-MS spectrometer. NMR spectra were carried out on Bruker AV-400 or AV-600 NMR spectrometer and chemical shifts were expressed in δ (ppm) and referenced to the solvent residual peak. Optical rotations were recorded by a JASCO P-2000 polarimeter. Silica gel (200–300 mesh, Qingdao Marine Chem. Co., Ltd., China), C_{18} reversedphase silica gel (50 μm, YMC, Japan), MCI gel (CHP20, 75–150 μm, MCC, Japan), and Sephadex LH-20 gel (Pharmacia Biotech, Sweden) were used for column chromatography (CC). The other testing instruments for the purified compounds and the materials used in the isolation were the same as previous [7,8].

2.2. Plant material

The stems and leaves of *N. japonica* were collected from Baise located in Guangxi Zhuang Autonomous Region of China (April 2019), and were authenticated by Prof. Shao-Qing Tang (College of Life Science, Guangxi

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Normal University). A voucher specimen (No. NJ-201904) has been deposited at the Collaborative Innovation Center for Guangxi Ethnic Medicine, Guangxi Normal University.

2.3. Extraction and isolation

The air-dried stems and leaves of *N. japonica* (20.5 kg) were extracted four times at the temperature of 70 °C (each for 3 h) by refluxing under the conditions of 90% EtOH-H_2O (4 × 75 L). The above filtrate was evaporated under reduced pressure to obtain the crude extract (2.5 kg), which was suspended in H_2O and sequentially partitioned with petroleum ether, EtOAc, and *n*-butyl alcohol. The EtOAc-soluble fraction (1100 g) was subjected to macroporous resin (D101) column chromatography (CC), eluting with EtOH/H_2O (30:70, 50:50, 80:20, and 95:5, *v*/v). Then, the EtOH/H_2O-80:20 fraction (121.0 g) was fractionated using MCI gel CC with MeOH/H_2O (30:70 to 100:0) to obtain seven fractions (A − G). Fractions C (14.6 g) and D (14.1 g) were separated by a silica gel (200–300 mesh) CC respectively, eluting with CH_2Cl_2/MeOH (80:1 to 1:1) of increasing polarity to obtain the following fractions: C1−C8 and D1−D8.

Fractions C3 (2100.0 mg) and C4 (800.0 mg) were subjected to Sephadex LH-20 columns (4 cm × 120 cm, MeOH, 0.8 mL/min) to yield the subfractions of C3a−C3d and C4a−C4d. Semi-preparative RP-HPLC purification of C3c (42.2 mg) with CH_3CN/H_2O/TFA (27:73:0.01, 6 mL/min) afforded **14** (11.6 mg, t_R 38.9 min) and **6** (3.1 mg, t_R 44.7 min). Subfraction C4d (31.7 mg) was purified using semi-preparative RP-HPLC with CH_3CN/H_2O/TFA (27:73:0.01, 6 mL/min) to yield **8** (5.7 mg, t_R 36.6 min) and **9** (4.1 mg, t_R 49.2 min).

Fractions D5 (1140.0 mg), D6 (725.0 mg), and D7 (960.0 mg) were fractioned over RP-C18 CC (MeOH/H_2O, 30:70 to 100:0) of decreasing polarity to obtain the subfractions of D5a−D5e, D6a−D6e, and D7a−D7f, respectively.

Fraction D5c (212.5 mg) was separated into five subfractions (D5c1−D5c5) via a Sephadex LH-20 column (2.6 cm × 120 cm, MeOH, 0.5 mL/min). Compound **15** (CH_3CN/H_2O, 30:70, 6 mL/min, 4.2 mg, t_R 48.3 min) was obtained via semi-preparative RP-HPLC from subfraction D5c2 (13.3 mg). Subfraction D6b (20.6 mg) were performed by semi-preparative RP-HPLC (CH_3CN/H_2O, 25:75, 6 mL/min) to obtain **10** (12.7 mg, t_R 50.2 min).

Fraction D6c (319.0 mg) was chromatographed on a Sephadex LH-20 column (2.6 cm × 120 cm, MeOH, 0.5 mL/min) to afford five subfractions (D6c1−D6c5). Furthermore, subfraction D6c4 (33.1 mg) was purified via semi-preparative RP-HPLC (CH_3CN/H_2O, 25:75, 6 mL/min), leading to the isolation of **1** (2.8 mg, t_R 64.1 min) and **12** (11.2 mg, t_R 68.7 min). Subfraction D6c5 (15.8 mg) was performed by semi-preparative HPLC (CH_3CN/H_2O, 25:75, 6 mL/min) to provide **7** (3.5 mg, t_R 72.5 min) and **11** (4.5 mg, t_R 80.9 min).

Subfractions D7c (42.0 mg) and D7d (39.2 mg) were purified using semi-preparative RP-HPLC to yield **3** (CH_3CN/H_2O/TFA, 27:73:0.01, 6 mL/min, 30.7 mg, t_R 62.5 min) and **2** (CH_3CN/H_2O, 27:73, 6 mL/min, 3.8 mg, t_R 69.5 min), respectively. Furthermore, semi-preparative RP-HPLC (CH_3CN/H_2O/TFA, 28.5:71.5:0.01, 6 mL/min) purification of subfraction D7e (87.0 mg) resulted in the isolation of **13** (4.6 mg, t_R 51.3 min), **4** (2.3 mg, t_R 54.7 min), and **5** (10.1 mg, t_R 58.4 min).

2.3.1. Kaempferol 3-O-(6″-O-feruloyl)-β-D-galactopyranoside (1)

Yellow amorphous powder; $[\alpha]_D^{20}$ −13.8 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 237 (4.47), 263 (4.46), 280.0 (4.44), 328 (4.47) nm; IR (KBr) ν_{max} 3445, 2938, 2869, 1655, 1609, 1507, 1449, 1385, 1181, 812 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; (+) HRESIMS *m/z* 647.1373 $[M + Na]^+$, calcd for $C_{31}H_{28}O_{14}Na$, 647.1371.

2.3.2. Kaempferol 3-O-(2″-O-galloyl-6″-O-feruloyl)-β-D-galactopyranoside (2)

Yellow amorphous powder; $[\alpha]_D^{20}$ −35.0 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 209 (4.51), 267 (4.20), 292 (4.16), 327 (4.15) nm; IR (KBr) ν_{max} 3435, 2929, 2860, 1661, 1607, 1516, 1451, 1385, 1203, 1178, 1039, 703 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; (+) HRESIMS *m/z*

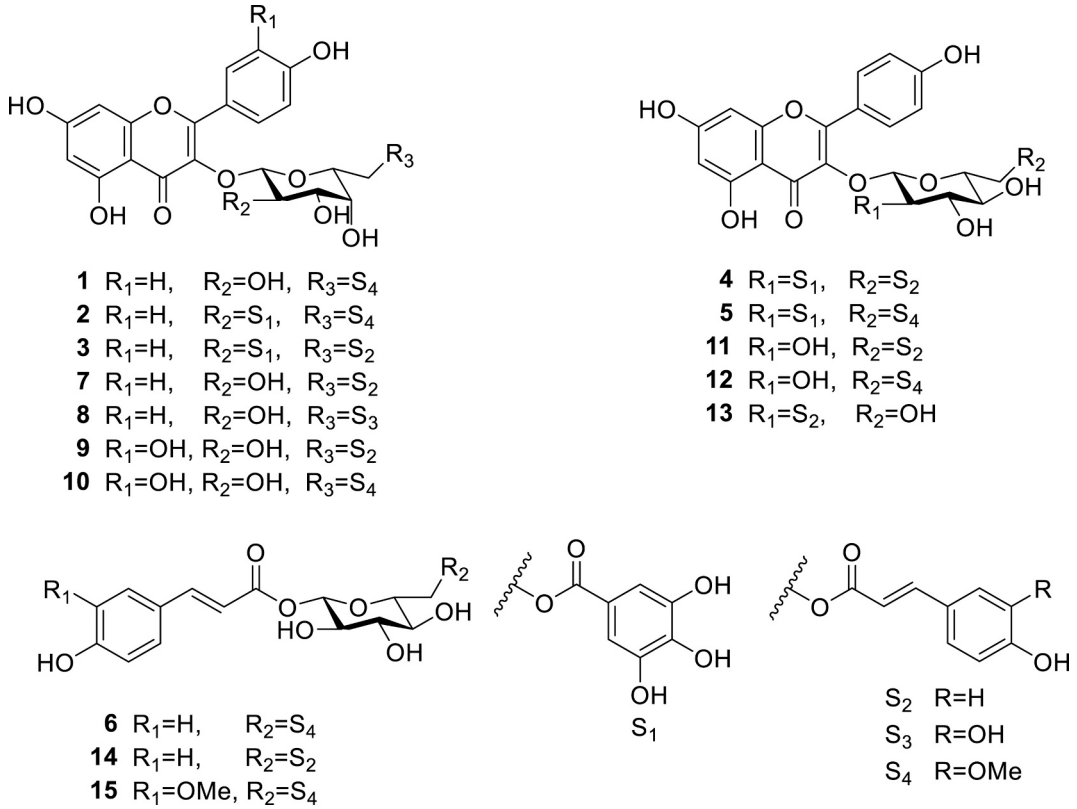

Fig. 1. Chemical structures of compounds **1–15**.

Table 1
^{1}H and ^{13}C NMR data of compounds **1–5**.

NO.	**1**[a]		**2**[a]		**3**[b]		**4**[a]		**5**[b]	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
2	159.2		158.2		158.3		158.8		158.8	
3	135.5		134.6		134.6		134.3		134.3	
4	179.6		179.4		179.4		179.1		179.1	
5	162.9		163.0		163.0		163.0		163.0	
6	99.9	6.10, br s	99.8	6.02, d (1.8)	99.9	6.07, br s	99.8	6.07, d (1.8)	99.8	6.03, d (1.8)
7	165.9		165.6		165.6		165.6		165.5	
8	94.7	6.29, br s	94.5	6.21, d (1.8)	94.6	6.25, br s	94.6	6.27, d (1.8)	94.6	6.23, d (1.8)
9	158.4		158.2		158.3		158.3		158.3	
10	105.5		105.7		105.7		105.7		105.7	
1′	122.6		122.7		122.7		122.8		122.8	
2′	132.4	8.05, d (9.0)	132.1	7.92, d (9.0)	132.1	7.92, d (8.8)	132.1	7.93, d (8.4)	132.1	7.92, d (8.8)
3′	116.1	6.85, d (9.0)	116.3	6.81, d (9.0)	116.3	6.81, d (8.8)	116.1	6.84, d (8.4)	116.1	6.83, d (8.8)
4′	161.6		161.4		161.4		161.4		161.3	
5′	116.1	6.85, d (9.0)	116.3	6.81, d (9.0)	116.3	6.81, d (8.8)	116.1	6.84, d (8.4)	116.1	6.83, d (8.8)
6′	132.4	8.05, d (9.0)	132.1	7.92, d (9.0)	132.1	7.92, d (8.8)	132.1	7.92, d (8.4)	132.1	7.92, d (8.8)
1″	105.0	5.12, d (7.8)	100.8	5.61, d (8.1)	100.8	5.61, d (8.0)	100.2	5.71, d (8.1)	100.2	5.72, d (8.0)
2″	72.9	3.80, m	74.2	5.45, dd (9.6, 8.1)	74.2	5.45, dd (10.0, 8.0)	75.8	5.14, dd (9.6, 8.1)	75.8	5.14, dd (9.6, 8.0)
3″	74.9	3.57, dd (9.6, 3.0)	73.2	3.85, dd (9.6, 3.0)	73.2	3.84, m	76.2	3.70, dd (9.6, 9.0)	76.3	3.70, dd (9.6, 8.8)
4″	70.3	3.82, m	70.7	3.90, m	70.7	3.89, m	72.1	3.43, dd (9.6, 9.0)	72.1	3.44, dd (9.6, 8.8)
5″	74.8	3.76, dd (8.4, 3.6)	75.1	3.86, m	75.0	3.85, m	76.0	3.55, m	76.0	3.57, m
6″	64.4	4.39, dd (11.4, 8.4)	64.2	4.47, dd (11.4, 8.4)	64.2	4.41, dd (11.2, 8.4)	64.0	4.36, dd (11.4, 1.8)	64.0	4.35, dd (11.6, 2.0)
		4.12, dd (11.4, 3.6)		4.16, dd (11.4, 4.2)		4.19, dd (11.2, 4.0)		4.23, dd (11.4, 7.2)		4.28, dd (11.6, 6.4)
1‴	127.6		127.6		127.0		127.0		127.6	
2‴	111.5	7.02, d (1.2)	111.5	7.04, d (1.2)	131.2	7.28, d (8.4)	131.2	7.31, d (8.4)	111.5	7.07, d (1.6)
3‴	149.2		149.3		116.8	6.79, d (8.4)	116.8	6.81, d (8.4)	149.3	
4‴	150.5		150.6		161.2		161.2		150.5	
5‴	116.4	6.78, d (8.4)	116.4	6.80, d (8.4)	116.8	6.79, d (8.4)	116.8	6.81, d (8.4)	116.4	6.81, d (8.0)
6‴	124.1	6.89, d (8.4, 1.2)	124.2	6.89, dd (8.4, 1.2)	131.2	7.28, d (8.4)	131.2	7.31, d (8.4)	124.3	6.91, dd (8.0, 1.6)
7‴	146.8	7.37, d (16.2)	146.9	7.39, d (16.2)	146.6	7.40, d (15.6)	146.6	7.40, d (15.6)	146.8	7.39, d (15.6)
8‴	114.9	6.08, d (16.2)	114.8	6.10, d (16.2)	114.6	6.05, d (15.6)	114.6	6.06, d (15.6)	114.9	6.10, d (15.6)
9‴	168.7		168.6		168.7		168.7		168.7	
1⁗			121.6		121.6		121.5		121.6	
2⁗			110.6	7.16, s	110.6	7.16, s	110.6	7.18, s	110.6	7.18, s
3⁗			146.4		146.4		146.4		146.4	
4⁗			139.9		139.9		139.9		139.9	
5⁗			146.4		146.4		146.4		146.4	
6⁗			110.6	7.16, s	110.6	7.16, s	110.6	7.18, s	110.6	7.18, s
7⁗			168.0		168.0		167.7		167.8	
3‴-OMe	56.4	3.87, s	56.4	3.89, s					56.4	3.91, s

[a] In MeOH-d_4, ^{1}H NMR at 600 MHz, ^{13}C NMR at 150 MHz.
[b] In MeOH-d_4, ^{1}H NMR at 400 MHz, ^{13}C NMR at 100 MHz.

777.1657 $[M + H]^+$, calcd for $C_{38}H_{33}O_{18}$, 777.1661.

2.3.3. *Kaempferol 3-O-(2″-O-galloyl-6″-O-p-coumaroyl)-β-D-galactopyranoside* ***(3)***

Yellow amorphous powder; $[\alpha]_D^{20}$ −46.8 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 211 (4.44), 267 (4.18), 279 (4.15), 314 (4.18) nm; IR (KBr) ν_{max} 3378, 2957, 1694, 1656, 1606, 1513, 1449, 1360, 1179, 832 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; (+) HRESIMS *m/z* 747.1556 $[M + H]^+$, calcd for $C_{37}H_{31}O_{17}$, 747.1556.

2.3.4. *Kaempferol 3-O-(2″-O-galloyl-6″-O-p-coumaroyl)-β-D-glucopyranoside* ***(4)***

Yellow amorphous powder; $[\alpha]_D^{20}$ −99.5 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 209 (4.46), 270 (4.16), 293 (4.16) nm; IR (KBr) ν_{max} 3454, 2935, 2869, 1644, 1513, 1448, 1384, 1186, 1061, 847 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; (+) HRESIMS *m/z* 747.1556 $[M + H]^+$, calcd for $C_{37}H_{31}O_{17}$, 747.1556.

2.3.5. *Kaempferol 3-O-(2″-O-galloyl-6″-O-feruloyl)-β-D-glucopyranoside* ***(5)***

Yellow amorphous powder; $[\alpha]_D^{20}$ −100.8 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.61), 267 (4.26), 292 (4.22), 328 (4.23) nm; IR (KBr) ν_{max} 3174, 2941, 2864, 1697, 1655, 1606, 1513, 1449, 1359, 1179, 1084, 842 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; (−) HRESIMS *m/z* 777.1666 $[M + H]^+$, calcd for $C_{38}H_{33}O_{18}$, 777.1661.

2.3.6. *1-O-p-coumaroyl-6-O-feruloyl-β-D-glucopyranoside* ***(6)***

White amorphous power; $[\alpha]_D^{20}$ +13.8 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.68), 231 (4.69), 316 (4.82) nm; IR (KBr) ν_{max} 3447, 1696, 1633, 1606, 1515, 1444, 1260, 1173, 1070, 830 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; (+) HRESIMS *m/z* 525.1369 $[M + Na]^+$, calcd for $C_{25}H_{26}O_{11}Na$, 525.1367.

Table 2
^{1}H and ^{13}C NMR data of compound **6**.

NO.	**6**		NO.	**6**	
	δ_C	δ_H (*J* in Hz)		δ_C	δ_H (*J* in Hz)
1	95.7	5.60, d (7.8)	8′	114.3	6.37, d (15.6)
2	74.0	3.46, dd (9.0, 7.8)	9′	167.6	
3	77.9	3.49, t (9.0)	1″	127.7	
4	71.3	3.44, t (9.0)	2″	111.6	7.20, d (1.8)
5	76.3	3.68, m	3″	149.4	
6	64.4	4.51, dd (12.0, 1.8)	4″	150.7	
		4.33, dd (12.0, 5.4)			
1′	127.0		5″	116.4	6.80, d (8.4)
2′	131.4	7.47, d (8.4)	6″	124.3	7.07, dd (8.4, 1.8)
3′	116.9	6.80, d (8.4)	7″	147.2	7.63, d (15.6)
4′	161.7		8″	115.1	6.40, d (15.6)
5′	116.9	6.80, d (8.4)	9″	169.1	
6′	131.4	7.47, d (8.4)	3″-OMe	56.4	3.88, s
7′	148.1	7.72, d (15.6)			

In MeOH-d_4, ^{1}H NMR at 600 MHz, ^{13}C NMR at 150 MHz.

2.4. Acid hydrolysis of compounds 1–6

Each of **1–6** (each 1.0 mg) was added to 1.0 mL of 8% hydrochloric acid. The reaction mixture was refluxed at 80 °C for 6 h and extracted with EtOAc (3 × 2 mL) to remove the aglycone. The aqueous layer was subjected to silica gel CC (EtOAc/EtOH/H_2O, 7:4:1) to obtain the sugar fraction. The optical rotation of sugar fraction was afforded by JASCO P-2000 polarimeter. The positive optical rotation of the sugar were compared with those of literature data, confirming the sugar to be D-configuration [7].

2.5. NO production measurements and cell viability assays

The antineuroinflammatory activities of all isolates were evaluated by Griess reaction and MTT assays including the tests of NO production and cell viability in lipopolysaccharide (LPS) induced BV-2 microglial cells, as described in our previous study [6,9].

3. Results and discussion

3.1. Structural identification

Compound **1** was isolated as a yellow, amorphous powder with a sodium adduct ion at *m/z* 647.1373 $[M + Na]^+$ (calcd for 647.1371) from the positive HRESIMS data, which together with the ^{13}C NMR data indicated that it has a molecular formula of $C_{31}H_{28}O_{14}$. The presence of hydroxy (3445 cm^{-1}), ester carbonyl (1655 cm^{-1}), and aromatic ring (1609, 1507 cm^{-1}) functionalities was evident from its IR absorption peaks. The ^1H NMR spectrum of **1** (Table 1) exhibited the presence of an AX spin system [δ_H 6.29 (1H, br s, H-8) and 6.10 (1H, br s, H-6)], an AA′BB′ spin system [δ_H 8.05 (2H, d, J = 9.0 Hz, H-2′, 6′) and 6.85 (2H, d, J = 9.0 Hz, H-3′, 5′)], an ABX spin system [δ_H 7.02 (1H, d, J = 1.2 Hz, H-2‴), 6.89 (1H, dd, J = 8.4, 1.2 Hz, H-6‴), and 6.78 (1H, dd, J = 8.4 Hz, H-5‴)], a *trans*-double bond [δ_H 7.37 (1H, d, J = 16.2 Hz, H-7‴) and 6.08 (1H, d, J = 16.2 Hz, H-8‴)], a sugar unit (δ_H 3.57–5.12), and a methoxy group [δ_H 3.87 (3H, s)]. Its ^{13}C NMR spectrum displayed 25 resonances aside from those of a sugar unit (δ_C 105.0, 74.9, 74.8, 72.9, 70.3, and 64.4), characteristic for a *trans*-olefinic bond (δ_C 114.9 and 146.8), a methoxy (δ_C 56.4), and 22 sp^2 carbons (δ_C 94.7–179.6) (Table 1). These spectral properties showed many similarities with those of **8** [10], implying **1** also being a flavonol glycoside.

Subsequently, its 2D NMR spectra strongly supported the above inference. The structure of the sugar moiety could be fully confirmed by the ^1H—^1H COSY correlations of H-1″ (δ_H 5.12)/H-2″ (δ_H 3.80)/H-3″ (δ_H 3.57)/H-4″ (δ_H 3.82)/H-5″ (δ_H 3.76)/H_2-6″ (δ_H 4.39 and 4.12) in Fig. 2 together with its HSQC spectrum. Additionally, the large $^3J_{1'',\,2''}$ value (7.8 Hz) and the small $^3J_{3'',\,4''}$ value (3.0 Hz) (Table 1) proven that it was a β-configured galactose. The prominent HMBC cross peaks (Fig. 2) from the nonequivalent methylene protons H_2-6″ and two olefinic protons H-7‴ (δ_H 7.37)/H-8‴ (δ_H 6.08) to the carbonyl C-9‴ (δ_C 168.7), from H-7‴ to C-2‴ (δ_C 111.5)/C-6‴ (δ_C 124.1), and from 3‴-OMe (δ_H 3.87)/H-2‴ (δ_H 7.02)/H-5‴ (δ_H 6.78) to C-3‴ (δ_C 149.2) indicated that a feruloyl moiety was esterified with 6-OH of the sugar. The other 14 aromatic carbon signals and one carbonyl were assigned to a kaempferol skeleton including a 5,7-dihydroxy A-ring and a 1,4-disustituted B-ring, based on the significant HMBC correlations from H-6 (δ_H 6.10) to C-5/C-7/C-8/C-10, from H-8 (δ_H 6.29) to C-6/C-7/C-9/C-10, as well as from H-2′/H-6′ (δ_H 8.05) to C-2, the ^1H-^1H COSY correlations (Fig. 2) of H-2′/6′ and H-3′/5′ (δ_H 6.85) together with its molecular. Meanwhile, the obvious HMBC correlation of the anomeric proton [δ_H 5.12 (1H, d, J = 7.8 Hz)] and C-3 implied that the sugar was connected at C-3 of the kaempferol unit. To established the absolute configuration of the galactopyranosyl unit, acid hydrolysis was carried out and the sugar moiety was deduced to be D-galactose based on the positive optical rotation [11]. Therefore, **1** was established as kaempferol 3-*O*-(6″-*O*-feruloyl)-β-D-galactopyranoside.

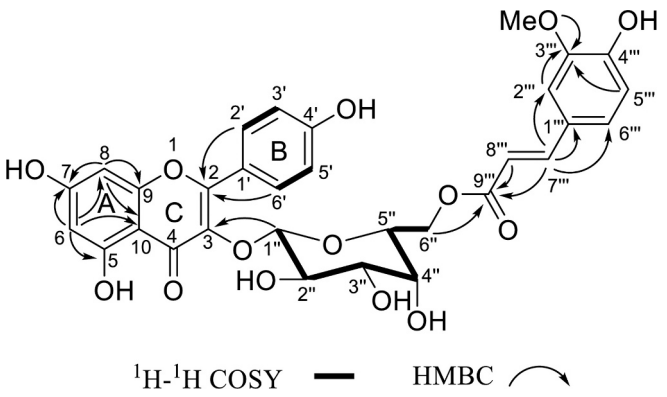

Fig. 2. Key $^1H-^1H$ COSY and HMBC correlations of **1**.

The HRESIMS data of **2** showed a hydrogen adduct ion at *m/z* 777.1657 $[M + H]^+$ (calcd for 777.1661), consistent with the molecular formula of $C_{38}H_{32}O_{18}$. Its ^1H and ^{13}C NMR data (Table 1) showed a high similarity to those of **1**, except for the presence of a galloyl moiety [δ_C 168.0, 146.4 (2C), 139.9, 121.6, and 110.6 (2C); δ_H 7.16 (2H, s)]. In the HMBC spectrum (Fig. 3), the obvious cross peaks from H-2″ (δ_H 5.45) and H-2⁗/6⁗ (δ_H 7.16) to C-7⁗ (δ_C 168.0) indicated that the galloyl unit was esterified with 2-OH of the sugar. The chemical shifts of the sugar group, the coupling constants of H-1″/H-2″ (8.1 Hz) and H-3″/H-4″ (3.0 Hz), together with acid hydrolysis and optical rotation experiments supported the presence of β-D-galactopyranosyl unit. Finally, the structure of **2** was determined to be kaempferol 3-*O*-(2″-*O*-galloyl-6″-*O*-feruloyl)-β-D-galactopyranoside.

Compound **3**, a yellow amorphous powder, has a molecular formula of $C_{37}H_{30}O_{17}$, which was deduced by the positive-ion HERSIMS (*m/z* 747.1556 $[M + H]^+$, calcd for 747.1556) and ^{13}C NMR data. The data of its ^1H and ^{13}C NMR spectra together with the HSQC spectrum indicated the existent of characteristic moieties including a galactose group, a flavonol core with 5,7-disubstituted A-ring and 1,4-disustituted B-ring, a galloyl group, an olefinic bond, and an aromatic ring. The obvious difference was the disappearance of the methoxy and 1,3,4-tirsubstituted aromatic ring as well as the presence of another 1,4-disubstituted aromatic ring, compared with **2**, speculating that a coumaroyl unit in **3** replaced the feruloyl unit in **2**. The above deduction and the location of these moieties were further verified by the significant HMBC correlations, especially the correlations from H-1″ (δ_H 5.61) to C-3 (δ_C 134.6), from H_2-6″ (δ_H 4.41 and 4.19)/H-7‴ (δ_H 7.40)/H-8‴ (δ_H 6.05) to C-9‴ (δ_C 168.7), from H-2‴ (δ_H 7.28) to C-7‴ (δ_C 146.6), and from H-2″ (δ_H 5.45)/H-2⁗ (δ_H 7.16) to C-7⁗ (δ_C 168.0). The result of acid hydrolysis defined the existence of D-galactose. The structure of **3** was assigned as kaempferol 3-*O*-(2″-*O*-galloyl-6″-*O*-*p*-coumaroyl)-β-D-galactopyranoside.

Compound **4** was obtained as a yellow amorphous power. Its molecular formula was the same as **3**, as confirmed through its HRESIMS ion at *m/z* 747.1556 $[M + H]^+$ (calcd for 747.1556) and ^{13}C NMR spectroscopic data. From the 1D and 2D NMR data, it was observed that the only difference of **3** and **4** in structure was the geometry of the sugar moiety, particularly a series of large coupling constants of H-1″/H-2″ (8.1 Hz), H-2″/H-3″ (9.6 Hz), H-3″/H-4″ (9.0 Hz), and H-4″/H-5″ (9.6 Hz) in **4** whereas the relatively small coupling constant of H-3″/4″ in **3**, supporting that **4** possessed a β-glucopyranosyl unit. The D-configuration was determined by acid hydrolysis experiment including TLC and the positive optical rotation results. Thus, the structure of **4** was concluded to be kaempferol 3-*O*-(2″-*O*-galloyl-6″-*O*-*p*-coumaroyl)-β-D-glucopyranoside.

Compound **5** had the identical molecular formula of $C_{38}H_{32}O_{18}$ with **2**, as was evident from its ^{13}C NMR and positive-ion HRESIMS data (*m/z* 777.1666 $[M + H]^+$, calcd for 777.1661). Inspection of its ^1H and ^{13}C NMR data (Table 1) and 2D NMR spectra suggested that its structure

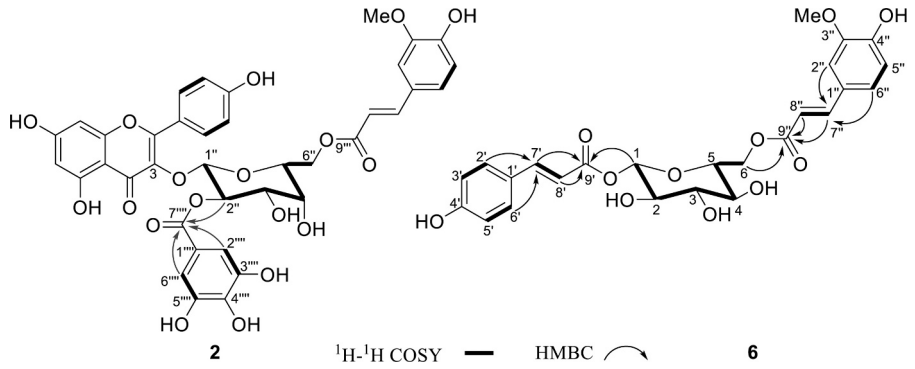

Fig. 3. Key ^{1}H–^{1}H COSY and HMBC correlations of **2** and **6**.

shared close similarities with **2** except for the resonances of sugar region. However, the oxymethines from the sugar group had relatively large coupling constants of H-1″/H-2″ (8.0 Hz), H-2″/H-3″ (9.6 Hz), H-3″/H-4″ (8.8 Hz), and H-4″/H-5″ (9.6 Hz) and almost identical chemical shifts with those of **4**, which implied the existence of a *β*-glucopyranosyl unit with D-configuration proven by the experiment of acid hydrolysis. Consequently, **5** was deduced as kaempferol 3-*O*-(2″-*O*-galloyl-6″-*O*-feruloyl)-*β*-D-glucopyranoside.

The molecular formula of **6** was ascertained as $C_{25}H_{26}O_{11}$ based on its ^{13}C NMR data and positive HRESIMS ion at *m/z* 525.1369 $[M + Na]^+$ (calcd for 525.1367). The absorption bands of hydroxy (3447 cm^{-1}), carbonyl (1696 cm^{-1}), and aromatic ring (1606, 1515 cm^{-1}) were observed in its IR spectrum. Meanwhile, the UV spectrum showed maximum absorption at 316 nm. In its ^{1}H NMR spectrum, the signals of seven aromatic protons assignable to a 1,4-disubstituted aromatic ring [δ_H 7.47 (2H, d, J = 8.4 Hz) and 6.80 (2H, d, J = 8.4 Hz)] and a 1,3,4-trisubstituted aromatic ring [δ_H 7.20 (1H, d, J = 1.8 Hz), 7.07 (1H, dd, J = 8.4, 1.8 Hz), and 6.80 (1H, d, J = 8.4 Hz)], two pairs of *trans*-olefinic protons [δ_H 7.72 (1H, d, J = 15.6 Hz), 7.63 (1H, d, J = 15.6 Hz), 6.40 (1H, d, J = 15.6 Hz), and 6.37 (1H, d, J = 15.6 Hz)], one set of sugar protons (δ_H 3.44–5.60 Hz), and a methoxy [δ_H 3.88 (3H, s)] exhibited obviously. Its ^{13}C NMR data and HSQC spectrum suggested that **6** possessed two carbonyls (δ_C 169.1 and 167.6), a six-carbon sugar unit (δ_C 95.7, 77.9, 76.3, 74.0, 71.3, and 64.4), a methoxy (δ_C 56.4), and the other 16 sp^2 carbons arising from two aromatic rings and two double bonds.

The ^{1}H—^{1}H COSY correlations of oxymethylene and oxymethine protons in Fig. 3 and their coupling constants as shown in Table 2 revealed the presence of a *β*-D-glucopyranosyl moiety, as also verified through acid hydrolysis method. Based on the HMBC spectrum of **6**, the significant cross peaks from H-1 (δ_H 5.60)/H-7′ (δ_H 7.72)/H-8′ (δ_H 6.37) to C-9′ (δ_C 167.6), from H_2-6 (δ_H 4.51, 4.33)/H-7″ (δ_H 7.63)/H-8″ (δ_H 6.40) to C-9″ (δ_C 169.1), from H-2′ (δ_H 7.47)/H-6′ (δ_H 7.47) to C-7′ (δ_C 148.1), and from H-2″ (δ_H 7.20)/H-6″ (δ_H 7.07) to C-7″ (δ_C 147.2) as shown in Fig. 3 indicated that a *p*-coumaroyl group and a feruloyl group were connected with 1-OH and 6-OH of the sugar, respectively. Thus, the structure of **6** was illustrated as 1-*O*-*p*-coumaroyl-6-*O*-feruloyl-*β*-D-glucopyranoside.

The isolated known compounds were identified as kaempferol 3-*O*-(6″-*O*-*p*-coumaroyl)-*β*-D-galactopyranoside (**7**) [12], kaempferol 3-*O*-(6″-*O*-caffeoyl)-*β*-D-galactopyranoside (**8**) [10], quercetin 3-*O*-(6″-*O*-*p*-coumaroyl)-*β*-D-galactopyranoside (**9**) [13], quercetin 3-*O*-(6″-*O*-feruloyl)-*β*-D-galactopyranoside (**10**) [14], kaempferol 3-*O*-(6″-*O*-*p*-coumaroyl)-*β*-D-glucopyranoside (**11**) [15], kaempferol 3-*O*-(6″-*O*-feruloyl)-*β*-D-glucopyranoside (**12**) [16], kaempferol 3-*O*-(2″-*O*-*p*-coumaroyl)-*β*-D-glucopyranoside (**13**) [11], 1,6-di-*O*-*p*-coumaroyl-*β*-D-glucopyranoside (**14**) [17], and 1,6-di-*O*-feruloyl-*β*-D-glucopyranoside (**15**) [18] by comparison of their MS and NMR data with literature results.

3.2. Anti-inflammatory effects for intervention of NO production in LPS-induced BV-2 cells

The cytotoxicities of all the isolated compounds against BV-2 microglial cells were tested using MTT method, and none showed obvious cytotoxicity at a dosage of 50 μM. Then, their inhibitory effects on nitric oxide (NO) release in LPS-induced BV-2 cells were measured by the Griess reaction [6,9]. As shown in Table 3, compounds **1**, **8**, and **9** exhibited exceptionally potent antineuroinflammatory activities according to their inhibitory effects on NO production with IC_{50} values of 2.7 ± 0.6, 5.5 ± 0.2, and 4.1 ± 0.2 μM, respectively, comparable to that of a known antineuroinflammatory inhibitor, minocylcine (IC_{50} = 15.6 ± 1.8 μM). Compounds **7** and **10** also showed weak inhibitory activities with IC_{50} values of 17.0 ± 0.7 and 24.3 ± 1.7 μM, while the other compounds were inactive (IC_{50} > 50 μM). A preliminary structure-activity relationship analysis implied that a galactopyranose group contributed more to the influence on NO inhibitory activity than a glucopyranose moiety (e.g., **1** vs **12** and **7** vs **11**). Meanwhile, the activity results of compounds **8–10** existing a galactopyranose unit supported the above deduction.

4. Conclusion

In summary, the research on the chemical components of *N. japonica* obtained 15 compounds including four rare 2″-*O*-galloyl-type kaempferol glycosides, a new 6″-*O*-feruloyl-type kaempferol glycoside, a new phenylpropanoid glycoside, and nine known compounds. The structures of the new compounds were elucidated by a series of NMR spectroscopic data. The absolute configurations of sugar in **1–6** were established via acid hydrolysis experiment and the comparison to literature data. The preliminary anti-inflammatory tests (Fig. 4) indicated that compounds **1** and **7–10** shared obviously inhibitory activities on NO production in LPS-induced BV-2 cells with IC_{50} values ranging from 2.7 to 24.3 μM, comparing with the positive control minocycline (IC_{50} = 15.6 ± 1.8 μM). These isolates enriched the structure types of flavonoid glycoside from *N. japonica*, in the meantime, above activity results demonstrated that

Table 3
Effects of compounds **1–15** on NO production in LPS-induced BV-2 microglial cells.

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
1	2.7 ± 0.6	**9**	4.1 ± 0.2
2	>50	**10**	24.3 ± 1.7
3	>50	**11**	>50
4	>50	**12**	>50
5	>50	**13**	>50
6	>50	**14**	>50
7	17.0 ± 0.7	**15**	>50
8	5.5 ± 0.2	minocycline[a]	15.6 ± 1.8

[a] Positive control.

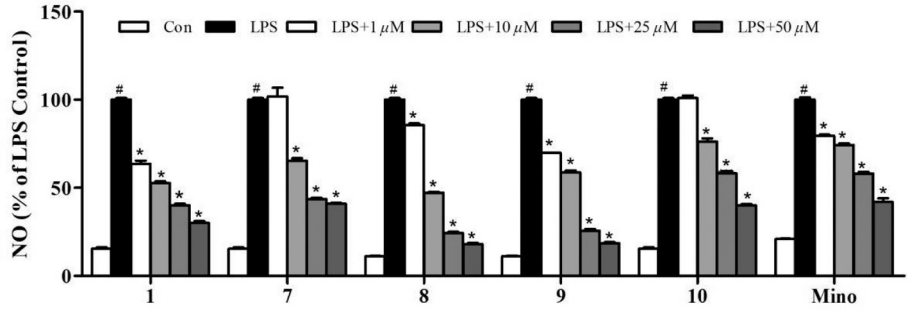

Fig. 4. The effects of compounds **1** and **7–10** on NO release in LPS-induced BV-2 cells. (Data were expressed as means ± SEM ($n = 3$); Significance: $^{*}P < 0.001$ compared to LPS group, $^{\#}P < 0.001$ compared to control group; MINO = minocycline.)

kaempferol or quercetin galactopyranosides deserved to be further investigated as anti-inflammatory agents.

Declaration of Competing Interest

The authors declare that no competing financial interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2021.104877.

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