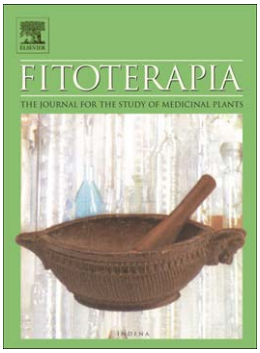

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6,8-Di-*C*-methyl-flavonoids with neuroprotective activities from *Rhododendron fortunei*

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ABSTRACT

Six 6,8-di-*C*-methyl-flavonoids, (2*R*,3*R*)-6,8-di-*C*-methyl-5,7,4′-trihydroxyflavanonol 7-*O*-*β*-D-glucopyranoside (**1**), (2*R*,3*R*)-6,8-di-*C*-methyl-5,7,4′-trihydroxyflavanonol 7-*O*-*β*-D-xylopyranosyl(1→6)-*β*-D-glucopyranoside (**2**), 6,8-di-*C*-methylkaempferol 7-*O*-*β*-D-glucopyranoside (**3**), (2*R*)-farrerol (**4a**), (2*R*/2*S*)-farrerol 7-O-*β*-D-glucopyranoside (**5**), and (2*R*/2*S*)-farrerol 7-*O*-*β*-D-xylopyranosyl(1→6)-*β*-D-glucopyranoside (**6**), and four known analogues, farrerol (**4b**), (2*R*,3*R*)-6,8-di-*C*-methyldihydrokaempferol (**7**), 6,8-di-*C*-methylkaempferol 7-*O*-*β*-D-glucopyranoside (**8**), and 6,8-di-*C*-methylkaempferol (**9**), were isolated from the twigs and leaves of *Rhododendron fortunei*. The structures of compounds **1**–**9** were determined by spectroscopic analyses (HRESIMS, 1D and 2D NMR, and CD) and chemical methods. Compounds **1**–**9** were evaluated for their neuroprotective effects on the human neuroblastoma SH-SY5Y cells apoptosis induced by hydrogen peroxide (H_2O_2) and amyloid *β* peptide (A*β*), respectively. Compounds **1**–**3** and **5**–**9** exhibited significant neuroprotective effects against H_2O_2-induced SH-SY5Y cell apoptosis, and compound **8** exhibited the strongest activity with a improvement of cell viability by about 30% at the concentration of 10 μM. Compounds **1**–**9** showed significant neuroprotective effects against A*β*-induced SH-SY5Y cell apoptosis.

1. Introduction

Alzheimer′s disease (AD) is a chronic neurodegenerative disease, and is the leading cause of dementia[1]. Oxidative stress-induced cell damage and the deposition of *β*-amyloid (A*β*) peptide into senile plaques in the extracellular space are the manifest pathological process of AD[2,3]. Natural products from plants, marine organisms, and bacteria have been reported to be an important source of Alzheimer's drug leads[4,5]. Flavonoids are widely distributed in plants, and are reported to possess neuroprotective effects by inhibiting the formation of reactive oxygen species (ROS), as well as by preventing A*β* aggregation and A*β*-induced toxicity[6,7]. Although flavonoids are abundant in nature, *C*-methyl-flavonoids having methylations on carbon are rare[8]. Literature survey revealed that *C*-methyl-flavonoids were mainly reported from plants of Caesalpiniaceae[9], Didieraceae[10,11], Dryopteridaceae[12], Ericaceae[13,14], Fabaceae[15], Myrtaceae [16–18], Onocleaceae[19], Pinaceae[8,20–23], and Ranunculaceae[24], and dem- onstrated anticancer[8], antifungal[15], antiviral[17], antiinflammatory[9], antibacterial[9], antioxidant[18], antimicrobial[24] , neuroprotective[7], peroxynitrite scavenging[20], and aldose reductase inhibitory[19] activities.

Rhododendron fortunei Lindil. (Ericaceae), an evergreen shrub or small tree, is native to China, and widely distributes in Anhui, Fujian, Guangdong, Guangxi, Guizhou, Henan, Hubei, Hunan, Jiangxi, Shaanxi, Sichuan, Yunnan, and Zhejiang provinces of China[25]. The leaves and flowers of *R. fortunei* are used as a folk medicine in China to treat skin ulcers, menstrual disorder, leucorrhea, hemorrhage of digestive tract, and cough[26]. Literature survey revealed limited phytochemical studies on *R. fortunei*. Preliminary phytochemical studies of our term on the twigs and leaves of *R. fortunei* led to the isolation of twelve known compounds, five flavonoids including a *C*-methyl-flavonoids, (2*R*, 3*R*)-6,8-di-*C*-methyldi- hydrokaempferol (**7**, in the reference the structure was determined as (2*R*, 3*S*)-6,8-di-*C*-methyldihydrokaempferol by mistake), a diterpenoid, and six phenylpropanoids[27]. Farrerol (**4b**), a well-known 6,8-di-*C*-methyl-flavonoid in the genus of *Rhododendron*, has been reported to possess the neuroprotective activity[7]. In order to discovery more 6,8-di-*C*-methyl-flavonoids with neuropective activity from *R. fortunei*, the chemical constituents of this plants were further investigated. In the follow-up study, six 6,8-di-*C*-methyl-flavonoids (**1**–**3, 4a**, **5**–**6**) and four known analogues (**4b**, **7**–**9**) (Figure 1) were obtained. Here, we described the isolation and structural determination of the ten 6,8-di-*C*-methyl-flavonoids, and their neuroprotective effects on the human neuroblastoma SH-SY5Y cells apoptosis induced by hydrogen peroxide (H_2O_2) and amyloid-*β*

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(Aβ) peptide, respectively.

2. Results and discussion

The dried twigs and leaves of *R. fortunei* were extracted by 95% EtOH. The extract was concentrated under reduced pressure to remove the EtOH, and successively extracted by petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH to yield four extraction fractions. Repeated chromatographic purification of the EtOAc and *n*-BuOH fractions by silica gel, RP C-18, Sephadex LH-20, and HPLC resulted in the isolation of ten *C*-methyl-flavonoids, of which six are new compounds. Comparing their NMR data with reported values, we assigned the sructures of the four known *C*-methyl-flavonoids to be farrerol (**4b**)[22], (2*R*,3*R*)-6,8-di-*C*-methyldi-hydrokaempferol (**7**)[21], 6,8-di-*C*-met-hylkaempferol 7-*O*-β-D-glucopyranoside (**8**)[23], and 6,8-di-*C*-methyl-kaempferol (**9**)[18].

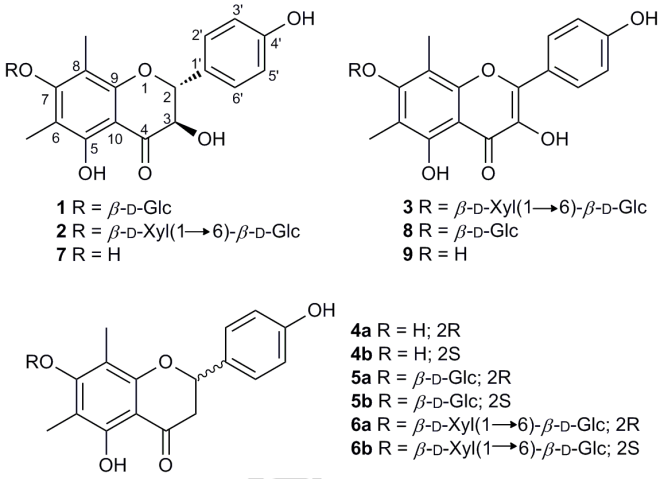

Figure 1. Chemical structures of 6,8-di-*C*-methyl-flavonoids **1**–**9**.

Compound **1** was obtained as a yellow amorphous solid. The molecular formula of **1** was determined to be $C_{23}H_{26}O_{11}$ by the quasi-molecular ion $[M + Na]^+$ at *m/z* 501.1354 (calcd for $C_{23}H_{26}O_{11}Na$, 501.1373) in the HRESIMS and the ^{13}C NMR data. The IR spectrum of **1** showed the presence of hydroxy (3381 cm^{-1}) and conjugated carbonyl (1630 cm^{-1}) groups. In addition, the strong absorption at 285 and 368 nm in the UV spectrum inferred **1** to be a flavonoid. The ^1H NMR spectrum of **1** (Table 1) exhibited resonances for a typical AA′BB′ coupling system, two oxymethines, a glucopyranose at 4.74 (d, *J* = 7.6 Hz, H-1″), 3.77 (dd, *J* = 5.2, 11.8 Hz, H-6″a), 3.66 (dd, *J* = 2.2, 11.8 Hz, H-6″b), 3.53 (t, *J* = 8.3 Hz, H-2″), 3.44 (t, *J* = 8.8 Hz, H-3″), 3.39 (t, *J* = 8.8 Hz, H-4″), and 3.18 (m, H-5″), and two methyl singlets. The ^{13}C NMR (Table 2) and DEPT spectra displayed resonances for a ketone carbonyl, eight substituted aromatic carbons, four aromatic methines, a glucopyranose unit (δ_C 105.5, C-1″; 78.3, C-5″; 78.0, C-3″; 75.9, C-2″; 71.6, C-4″; 62.8, C-6″), two oxymethines, and two methyls. The above NMR data suggested that compound **1** is a glycosylated derivatives of the known compound **7**[21]. The HSQC, ^1H-^1H COSY, and HMBC data were used to establish the planar structure of compound **1** (Figure 2). The HMBC correlations of 6-CH_3 (δ_H 2.15) with C-5 (δ_C 159.7), C-6 (δ_C 113.5), and C-7 (δ_C 163.2) and 8-CH_3 (δ_H 2.08) with C-7, C-8 (δ_C 112.3), C-9 (δ_C 159.1) indicated that these two methyl groups were located at C-6 and C-8. Moreover, the glucosidation position was deduced to be at C-7 based on the HMBC correlation of the anomeric proton H-1″ (δ_H 4.74) and C-7. To determine the absolute configuration of the glucopyranose, **1** was hydrolyzed by 2 M CF_3CO_2H, and the trimethylsilylthiazolidine derivatives of the hydrolysate were prepared. The absolute configuration of the glucose in **1** was determined to be D by comparing the GC retention time of the trimethylsilylthiazolidine derivatives of the hydrolysate with those of standard D- and L-glucose. The β-glucosyl linkage was deduced from the coupling constant *J* = 7.6 Hz of the anomeric proton H-1″ (δ_H 4.74, d). The large coupling constant *J* = 11.8 Hz between H-2 and H-3 revealed their *trans* relationship, and the strong positive Cotton effect at 353 nm in the CD spectrum suggested the absolute configuration of **1** was 2*R*,3*R*[21,28]. Thus, compound **1** was characterized as (2*R*,3*R*)-6,8-di-*C*-methyl-5,7,4′-trihydroxyflava-nonol 7-*O*-β-D-glucopyranoside.

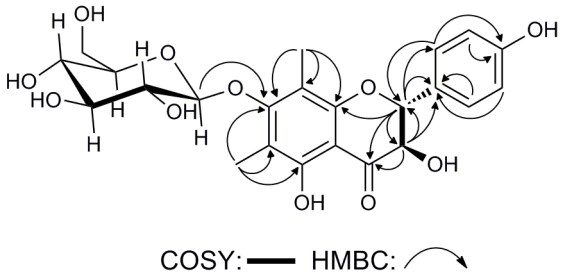

Figure 2. Key ^1H-^1H COSY and HMBC correlations of compound **1**.

Table 1 ^1H NMR Data (400 MHz) for Compounds **1**–**4**, *J* in Hz.

position	**1**[a]	**2**[b]	**3**[c]	**4**[a]
Aglycone				

2	4.98 d (11.8)	5.38 d (11.8)		5.29 dd (2.9, 12.8)
3*α*	4.56 d (11.8)	5.13 d (11.8)		2.71 dd (2.9, 17.1)
3*β*				3.05 dd (12.8, 17.1)
2′ 6′	7.37 d (8.5)	7.87 d (8.3)	8.10d (8.8)	7.32 d (8.5)
3′ 5′	6.84 d (8.5)	7.30 d (8.3)	6.96 d (8.8)	6.83 d (8.5)
Me-6	2.15 s	2.68 s	2.19 s	2.00 s
Me-8	2.08 s	2.55 s	2.40 s	1.99 s
Glc				
1″	4.74 d (7.6)	5.46 d (6.8)	4.65 d (7.7)	
2″	3.53 t (8.3)	4.34 overlap	3.35 overlap	
3″	3.44 t (8.8)	4.33 overlap	3.26 m	
4″	3.39 t (8.8)	4.29 m	3.22 m	
5″	3.18 m	4.12 m	2.99 t (8.6)	
6″	3.66 dd (2.2, 11.8)	4.30 m	3.53 dd (4.0, 11.0)	
	3.77 dd (5.2, 11.8)	4.75 d (10.8)	3.85 d (11.0)	
Xyl				
1‴		4.86 d (7.4)	4.07 d (7.4)	
2‴		3.93 t (8.1)	2.88 d (8.1)	
3‴		4.08 m	3.24 m	
4‴		4.17 m	3.20 m	
5‴		4.33 overlap	2.95 t (11.2)	
		3.65 t (10.6)	3.63 dd (5.2, 11.2)	

[a] Measured in methanol-d_4; [b] Measured in pyridine-d_6;
[c] Measured in DMSO-d_6.

Compoud **2** was obtained as a yellow amorphous powder. The $[M + Na]^+$ ion peak at *m/z* 633.1785 in the HRESIMS and ^{13}C-NMR data determined the molecular formula of **2** to be $C_{28}H_{34}O_{15}$ (calcd for $C_{28}H_{34}O_{15}Na$, 633.1795). The NMR data of **2** (Tables 1 and 2) were similar to those of **1** , and the major differences were that there is an additional xylose moiety (δ_H 4.86, H-1‴; 3.93, H-2‴; 4.08, H-3‴; 4.17, H-4‴; 4.33, 3.65, H-5‴; δ_C 106.1, C-1‴; 75.3, C-2‴; 78.7, C-3‴; 71.6, C-4‴; 67.5 C-5‴)[29] in **2**, and the H-6″(δ_H 4.30, 4.75) and C-6″(δ_C 70.3) of the glucose unit in **2** were deshielded more than those in **1**. Thus, this additional xylose moiety in **2** should be connected to the C-6″ of the glucose. The correlations from H-1‴ (δ_H 4.86) of the xylose to C-6″ (δ_C 70.3) of the glucose in the HMBC spectrum verified the linkage between these two sugar moieties to be 7-*O*-xylopyranosyl(1→6)-glucopyranose. The absolute configuration of the sugar moieties was identified as D-glucose and D-xylose by comparing the GC retention times of the trimethylsilyl-thiazolidine derivative of the hydrolysate with those of standards, D- and L-glucose, and D- and L-xylose. The *β*-linkage for two sugar units was deduced from the large coupling constants of the anomeric protons (J = 6.8 Hz for glucose and J = 7.4 Hz for xylose). The *trans*-relationship of H-2 and H-3 in **2** was determined by the large coupling constant J = 11.8 Hz, and the 2*R*,3*R* absolute configuration was suggested by the strong positive Cotton effect at 353 nm in the CD spectrum[21,28], which is almost the same as that of **1**. Thus, compound **2** was designated as (2*R*,3*R*)-6,8-di-*C*-methyl-5,7,4′-trihydroxyflavano-nol 7-*O*-*β*-D-xylopyranosyl(1→6)-*β*-D-glucopyranoside.

Table 1 1H NMR Data (400 MHz) for Compounds **1**–**4**, *J* in Hz.

position	1[a]	2[b]	3[c]	4[a]
Aglycone				
2	84.9	84.9	147.7	80.1
3	74.2	74.3	136.1	44.1
4	200.8	201.5	176.5	198.4
5	159.7	159.9	155.0	160.3
6	113.5	113.0	113.1	104.8
7	163.2	163.1	158.5	164.2
8	112.3	112.1	109.8	104.1
9	159.1	158.8	151.0	159.3
10	105.0	105.2	106.2	103.2
1′	129.5	129.6	122.0	131.5
2′ 6′	130.4	130.8	129.7	128.8
3′ 5′	116.3	116.8	115.6	116.3
4′	159.3	160.1	159.5	158.8
Me-6	9.3	10.1	9.0	7.4
Me-8	9.8	10.5	9.2	8.1
Glc				
1″	105.5	106.2	104.5	
2″	75.9	76.2	74.0	

3″	78.0	78.8	76.2
4″	71.6	71.9	69.7
5″	78.3	78.2	76.5
6″	62.8	70.3	68.5
Xyl			
1‴		106.1	103.7
2‴		75.3	73.2
3‴		78.7	75.7
4‴		71.6	69.5
5‴		67.5	65.5

[a] Measured in methanol-d_4; [b] Measured in pyridine-d_6;
[c] Measured in DMSO-d_6.

Compound **3** was isolated as a yellow amorphous powder. In conjunction with the ^{13}C NMR data, the HRESIMS data of **3** at *m*/*z* 631.1621 $[M + Na]^+$ suggested a molecular formula of $C_{28}H_{32}O_{15}$ (calcd for $C_{28}H_{32}O_{15}Na$, 631.1639), two mass units less than that of compound **2**. The resonances in the ^{1}H (Table 1) and ^{13}C (Table 2) NMR spectra of **3** were similar to those of **2**, except that two olifinic quaternary carbons (δ_C 147.7, 136.1) in **3** replaced two oxymethines (δ_H 5.38, 5.13; δ_C 84.9, 74.3) in **2**. So, **3** should be a 2,3-dehydro-derivative of **2**. The HMBC correlations from H-1‴ (δ_H 4.07, d) of the xylose to C-6″ (δ_C 68.5) of the glucose, and from H-1″ (δ_H 4.65, d) of the glucose to C-7 (δ_C 158.5) established the connections of these two sugar units to aglycone. The absolute configurations of these two sugar moieties were determined to be D-glucose and D-xylose by the GC analysis of the trimethylsilylthiazolidine derivatives of the hydrolysate and the standards, D- and L-glucose, and D- and L-xylose. The coupling constants of anomeric protons (δ_H 4.65, d, J = 7.7 Hz, H-1″; 4.07, d, J = 7.4 Hz, H-1‴) assgined the β-linkage of the D-glucose and D-xylose. Hence, compound **3** was identified as 6,8-di-*C*-methylkaempferol 7-*O*-β-D-xylopyranosyl (1→6)-β-D-glucopyranoside.

Compound **4** is a pair of racemate, while Compounds **5** and **6** are mixtures of two epimers, respectively. Before the isolation of compounds **5** and **6**, **4** was deemed to be an optically pure compound. The structural elucidation of compounds **5** and **6** hinted that **4** was likely to be a pair of racemate, which was confirmed by chiral separation and CD in the subsequent study.

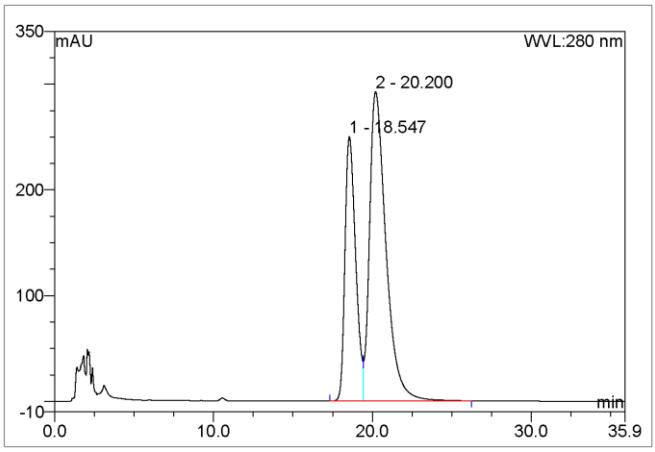

Figure 3. Chiral HPLC analysis of compounds **4a** (peak 1) and **4b** (peak 2).

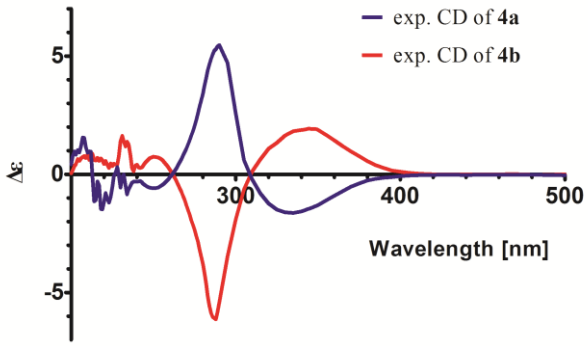

Figure 4. CD spectra of compounds 4a and 4b.

The molecular formula of compound **4**, $C_{17}H_{16}O_5$, was determined from the ^{13}C NMR data and HRESIMS data at *m*/*z* 301.1069 $[M + H]^+$ (calcd for $C_{17}H_{17}O_5$, 301.1076). Compound **4** was isolated as a pair of enantiomers. After many attempts, compound **4** was separaed to **4a** (0.4 mg, t_R = 18.5 min) and **4b** (0.6 mg, t_R = 20.2 min) by a chiral HPLC using a Chiralpack AD-RH column, eluting with

$CH_3CN–H_2O$ = 35:65; flow rate: 1.0 mL/min (Figure 3). Their CD spectra (Figure 4) are totally different, and are almost mirror images of each other. The absolute configuration of C-2 in compound **4a** was determined as *R* by a positive Cotton effect at 290 nm and a negative Cotton effect at 334 nm in the CD spectrum[22,28], while the 2*S* configuration in compound **4b** was determined by the negative Cotton effect at 290 nm and the positive Cotton effect at 335 nm in the CD spectrum. Thus, compound **4a** was established as (2*R*)-farrerol. In fact, natural farrerol, a mixture of *R* and *S* configuration with a ratio of 2:3, has been reported in the previous studies[30]. In this study, enantiomers were separated from each other and their physicochemical properties were supplemented.

Compound **5** was obtained as a yellow amorphous solid. The quasi-molecular ion $[M + Na]^+$ at *m*/*z* 485.1408 in the HRESIMS, together with the ^{13}C NMR data, suggested a molecular formula of $C_{23}H_{26}O_{10}$ (calcd for $C_{23}H_{26}O_{10}Na$, 485.1424). The NMR spectra indicated that compound **5** was isolated as a mixture of two epimers (**5a** and **5b**) at C-2, however, these two epimers could not be seperated by RP C_{18} or Chiralpack AD-RH HPLC. The NMR spectra exhibited resonances for two 6,8-di-*C*-methyl-5,7,4′-trihydroxy-flavanone moieties, and two glucopyranose units. The glucopyranose unit was located at C-7 on the basis of correlations of H-1″ (δ_H 4.73, d, *J* = 7.6 Hz; 4.74 , d, *J* = 7.6 Hz) and C-7 (δ_C 162.4, 162.6) in the HMBC spectrum. In order to determine the absolute configuration of the glucopyranose, compound **5** was hydrolyzed by 2 M CF_3CO_2H. The trimethyl-silylthiazolidine derivative of the hydrolysate showed a peak at 16.56 min in the GC, which was consistent with that of standard D-glucose (R_t = 16.63 min), rather than L-glucose (R_t = 17.29 min). Thus, the absolute configuration of the glucose was determined as D. The *β*-glucosyl linkage was deduced from the coupling constant *J* = 7.6 Hz of the anomeric protons H-1″. The aglycone, which was obtained from the cellulase hydrolysis of **5**, was identified to be a mixture of 2*R* and 2*S*-farrerol (**4a** and **4b**, respectively) by Chiralpack AD-RH HPLC and NMR analysis. Therefore, compound **5** was elucidated as (2*R*/2S)-farrerol 7-*O*-*β*-D-glucopyranoside.

SciFinder search showed that (2*R*)-farrerol 7-*O*-*β*-D-glucopyranoside was reported from *R. concinnum* by Zhao and their colleagues[31]. However, when we study the detailed structures in the paper we found that the authors incorrectly assgined the absolute configuration of their compound as (2*R*)-farrerol 7-*O*-*β*-D-glucopyranoside based on a negative Cotton effect at 270 nm and a positive Cotton effect at 306 nm in the CD spectrum. Actually, that CD stpectrum should suggest a 2*S* configuration of flavanonol according to the rules of Slade[22,28]. In particular, the authors claimed that the compound was known, and the data for the compound was the same as that of a compound in the reference[12]. In fact, there was a (2*S*)-farrerol 7-*O*-*β*-D-glucopyranoside, instead of (2*R*)-farrerol7-*O*-*β*-D-gluco-pyranoside, in their cited reference[12]. Therefore, the real (2*R*)-farrerol 7-*O*-*β*-D-glucopyranoside is never reported. Thus, in compund **5** the epimer (2*R*)-farrerol 7-*O*-*β*-D-glucopyranoside (**5a**) is new, while the other (**5b**) is known.

The HRESIMS data at *m*/*z* 617.1762 $[M + Na]^+$ and the ^{13}C NMR data determined the molecular formula of compound **6,** a yellow amorphous solid, to be $C_{28}H_{34}O_{14}$ (calcd for $C_{28}H_{34}O_{14}Na$, 617.1846). The NMR spectra of **6** revealed that there were two sets of resonances for a 6,8-di-*C*-methyl-5,7,4′-trihydroxy-flavanone, a glucopyranose, and a xylopyranose. Similar to those of compound **5**, the NMR data suggested that compound **6** was also isolated as a mixture of two epimers (**6a** and **6b**) at C-2, which could not be seperated by an RP C_{18} or a Chiralpack AD-RH HPLC. HMBC correlations from the anomeric protons H-1″ of the glucose to C-7, and from the anomeric protons H-1‴ of the xylose to C-6″ of the glucose established the connection of the glucose unit to C-7, and the xylose unit to C-6″ of the glucose unit, respectively. The aglycone, obtained from the cellulase hydrolysis of **6**, was identified to be a mixture of 2*R* and 2*S*-farrerol (**4a** and **4b**, respectively) by Chiralpack AD-RH HPLC analysis. To determine the absolute configuration of the glucopyranose and the xylopyranose, compound **6** was hydrolized by 2M CF_3CO_2H, and the trimethylsilylthiazolidine derivatives of the hydrolysate was prepared. Comparison the retention times of the trimethylsilylthiazolidine derivatives of hydrolysate with those of standards D- and L-glucose, and D- and L-xylose, assigned the absolute configuration of the glucose and xylose in compound **6** to be D. The *β*-linkage of the D-glucose and D-xylose in compound **6** were deduced from the coupling constants of anomeric protons of the glucose (δ_H 4.78, 4.77, d, *J* = 7.6 Hz, H-1″) and the xylose (δ_H 4.16, d, *J* = 7.0 Hz, H-1‴). Hence, the structure of compound **6** was eluciated as (2*S*/2*R*)-farrerol 7-*O*-*β*-D-xylopyranosyl(1→6)-*β*-D-glucopyranoside.

Since farrerol (**4b**) has been reported to possess the neuroprotective activity against A*β*-induced cell death[7], compounds **1**–**9** were evaluated for neuroprotective effects on human neuroblastoma SH-SY5Y cells using the MTT method. As shown in Table 3, compounds **1**–**3** and **5**–**9** exhibited significant neuroprotective effects against H_2O_2-induced SH-SY5Y cell apoptosis. Among them, 6,8-di-*C*-flavonols (**3**, **8**, and

Table 3 Neuroprotective effects of compounds 1–9 against hydrogen peroxide (H_2O_2 300 *μ*M) and amyloid-*β*-protein (A*β* 1.0 *μ*M)-induced human neuroblastoma SH-SY5Y cells apoptosis. Data (cell viability (%) assessed using MTT assay) are expressed as mean ± SEM based on three independent experiments.

	H_2O_2 (300 μM) Concentration of test compounds (μM)				A*β* (1.0 μM) Concentration of test compounds (μM)			
	0.1	1	10	Model[a]	0.1	1	10	Model[a]
Cell viability (%) Vitamin E[b]			82.1 ± 1.4*	73.9 ± 0.9			80.2 ± 1.0	73.3 ± 0.7
1	77.5 ± 2.0	79.4 ± 2.1	83.7 ± 2.0*	73.9 ± 0.9	92.8 ± 2.4$^{\#\#}$	91.9 ± 2.4$^{\#\#}$	94.2 ± 4.0$^{\#\#}$	76.1 ± 1.3
2	79.8 ± 2.3	82.2 ± 1.4*	82.2 ± 1.3*	73.9 ± 0.9	84.8 ± 3.3	86.1 ± 3.6	90.6 ± 2.5$^{\#}$	76.1 ± 1.3
3	98.7 ± 1.4**	101.9 ± 1.9**	103.7 ± 2.3**	76.4 ± 1.2	91.8 ± 2.1$^{\#\#}$	88.1 ± 1.2	90.4 ± 5.3$^{\#}$	77.0 ± 1.6
4	74.6 ± 0.7	77.3 ± 1.8	76.9 ± 2.1	71.0 ± 2.9	79.9 ± 4.8	83.2 ± 1.9$^{\#}$	85.4 ± 1.4$^{\#}$	73.3 ± 0.7
5	78.9 ± 1.8*	79.9 ± 0.9*	78.2 ± 1.5	71.0 ± 2.9	78.2 ± 1.8	83.1 ± 3.7	88.4 ± 3.5$^{\#}$	73.3 ± 0.7
6	75.6 ± 1.8	79.6 ± 0.5*	80.0 ± 2.4*	71.0 ± 2.9	80.9 ± 1.3$^{\#\#}$	84.4 ± 1.6$^{\#\#}$	87.1 ± 0.7$^{\#\#}$	73.3 ± 0.7
7	84.3 ± 0.9**	91.1 ± 3.4**	92.8 ± 1.6**	73.9 ± 0.9	96.9 ± 2.2$^{\#\#}$	85.6 ± 0.6	88.2 ± 4.3$^{\#}$	76.1 ± 1.3
8	98.7 ± 2.7**	104.8 ± 1.8**	107.8 ± 0.6**	76.4 ± 1.2	92.8 ± 1.6$^{\#\#}$	88.2 ± 1.8$^{\#}$	94.3 ± 3.6$^{\#\#}$	77.0 ± 1.6

9	85.0 ± 1.7*	87.2 ± 3.5*	89.0 ± 2.1*	76.4 ± 1.2	81.5 ± 2.0	90.2 ± 2.4##	93.8 ± 1.2##	77.0 ± 1.6

[a] Model group was only treated with H_2O_2 or Aβ

[b] Positive group

* $P < 0.05$ ** $P < 0.01$ compared with the H_2O_2-treated model group. # $P < 0.05$ ## $P < 0.01$ compared with the Aβ-treated model group

9) showed more potent neuroprotection than 6,8-di-*C*-flavanonols (**1**, **2**, and **7**) and 6,8-di-*C*-flavanones (**5** and **6**). Thus, a larger conjugated system in the molecules of flavonones may be beneficial for the neuroprotective activity against H_2O_2-induced SH-SY5Y cell apoptosis. Comparing the structures and activities it seems that the glycosidation at A ring of the 6,8-di-*C*-flavonoids does not affect the activity. In addition, compounds **1**–**9** displayed more significant neuroprotective effects against Aβ-induced SH-SY5Y cell apoptosis than the positive control, 10 μM vitamin E. Our bioassay data confirmed that 6,8-di-*C*-flavonoids possess potential neuroprotective effects[7].

3. Experimental section

3.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer PE-341 polarimeter (Perkin-Elmer, Waltham, MA, USA). UV spectra were recorded on a Varian Cary 50 UV/VIS spectrophotometer (Varian, Salt Lake City, UT, USA). CD spectra were measured with a Jasco J-810 spectrometer (Jasco, Easton, MD, USA). IR spectra were recorded on a Bruker Vertex 70 FT-IR spectrophotometer (Bruker, Karlsruhe, Germany). NMR spectra were recorded on Bruker AM-400 spectrometers (Bruker, Karlsruhe, Germany). Chemical shifts were given in ppm with reference to the residual methanol-d_4 (δ_H 3.31/δ_C 49.0), pyridine-d_5 (δ_H 8.74/δ_C 150.3), and DMSO-d_6 (δ_H 2.50/δ_C 39.5) signals. HRESIMS data were acquired using a Thermo Fisher LTQ XL LC/MS (Thermo Fisher, Palo Alto, CA, USA). Column chromatography was performed using silica gel (100−200 mesh and 200−300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), ODS (50 μm, YMC, Japan), and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden). Semipreparative HPLC was performed on an Agilent 1100 HPLC (Agilant, Santa Clara, CA, USA) with UV detector and an Ultimate XB-C_{18} (10 × 250 mm, 5 μm) column. The chiral HPLC isolation was accomplished by a Daicel Chiralpak AD-RH (4.6 × 150 mm, 5 μm; Daicel Chemical Ltd, Tokyo, Japan) on a Dionex Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA). Solvents were distilled prior to use, and spectroscopic grade solvents were used. TLC was carried out on precoated silica gel GF_{254} plates. GC analysis was carried out on an Agilent 7820A gas chromatography system (Agilant, Santa Clara, CA, USA).

3.2. Plant materials

Leaves of *R. fortunei* were collected at about 1500 m altitude, latitude 30°10′39.47″ North and longitude 109°44′42.93″ East in Changlinggang, Enshi, Hubei Province, People′s Republic of China, in June 2010, and authenticated by one of the authors (M He). A voucher specimen (No. 20100613) was deposited in the herbarium of Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, Tongji Medical College, Huazhong University of Technology and Science.

3.3. Extraction and isolation

The dried and powdered twigs and leaves of *R. fortunei* (6.0 kg) were extracted with 95% EtOH (30 L) at room temperature for four times. After the solvent was removed under reduced pressure, the EtOH extract (800 g) was suspended in H_2O and partitioned with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH, successively. The EtOAc partition fraction (31.3 g) was separated on a silica gel CC (200–300 mesh) eluted with $CHCl_3$–MeOH (from 1:0 to 3:1) to obtain six fractions (EA–EF). Fraction EB was subjected to ODS CC (50–100% MeOH) to obtain EB_1–EB_6. Subfraction EB_3 eluted by 65% MeOH was purified on a silica gel CC, eluting with petroleum ether–Me_2CO (3.5:1) to obtain Compound **7** (18 mg). Subfraction EB_4 eluted by 75% MeOH was fractionated on a silica gel CC, eluting with $CHCl_3$-MeOH (25:1) to give three fractions $EB_{4.1}$, $EB_{4.2}$, and $EB_{4.3}$. Fraction $EB_{4.1}$ was purified by Sephadex LH-20 and silica gel CC to afford compound **4** (9.6 mg), which was further isolated on a Chiralpack AD-RH (CH_3CN–H_2O = 35:65; flow rate 1.0 mL/min) to obtain compounds **4a** (0.4 mg, t_R = 18.5 min) and **4b** (0.6 mg, t_R = 20.2 min). Compound **9** (13.7 mg) was obtained by recrystallization from fraction $EB_{4.1}$ in MeOH. Fractions EE was subjected to ODS CC eluted with MeOH–H_2O (20:80→100:0) to afford seven subfractions EE_1–EE_7. The subfraction EE_3 was subjected to Sephadex LH-20 CC and then purified by semi-preparative HPLC (MeOH–H_2O = 41:59, flow rate: 2 mL/min) to obtain compound **1** (64 mg, t_R = 24.4 min). Compound **5** (8.2 mg, t_R = 23.6 min) was isolated from the subfraction EE_4 by repeated chromatography including silica gel CC, ODS, and Sephadex LH-20 and final semi-preparative HPLC (MeOH–H_2O = 54:46, flow rate 2 mL/min). The subfraction EE_5 was first fractionated by silica gel CC ($CHCl_3$–MeOH = 8:1) and further purified by semi-preparative HPLC (MeOH–H_2O = 62:38, flow rate: 2 mL/min) to afford compound **8** (15.4 mg, t_R = 21.0 min).

The *n*-BuOH extract (68.5 g) was subjected to silica gel CC eluted with $CHCl_3$–MeOH–H_2O (10:1:0→2:1:0.01) to give five fractions BA–BE. Fraction BD was separated by ODS CC eluted with MeOH–H_2O (20:80, 40:60, 60:40, 80:20, and 100:0) to give five fractions (BD_1–BD_5), and BD_2 was fractionated by silica gel CC again to three subfractions $BD_{2.1}$, $BD_{2.2}$, and $BD_{2.3}$. Compound **6** (110 mg, t_R = 28 min) was purified by semi-preparative (MeOH-H_2O = 43:57, flow rate: 2.5 mL/min) from subfraction $BD_{2.2}$. Subfraction $BD_{2.3}$ was further purified by repeated silica gel and Sephadex LH-20 CC to give compound **2** (1 g). Fraction BD_3 was subjected to repeated chromatography including silica gel CC, ODS, and Sephadex LH-20, and finally purified by semi-preparative HPLC (MeOH–H_2O = 60:40, flow rate: 2 mL/min) to obtain compound **3** (47 mg).

*(2R,3R)-6,8-di-C-methyldihydrokaempferol 7-O-β-D-glucopyr-anoside (**1**)*: Yellowish amorphous solid, [α]20 D +30.8 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 285 (4.23), 368 (3.60) nm; CD (*c* 0.44 mM, MeOH) 299 ($\Delta\varepsilon$ –5.68), 353 ($\Delta\varepsilon$ +2.60) nm; IR (film) ν_{max} 3381, 2922, 1630, 1518, 1450, 1362, 1271, 1174, 1107, 1070, 1039 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively; (+)-HRESIMS: *m/z* 501.1354 $[M+Na]^+$ (calcd for $C_{23}H_{26}O_{11}Na$, 501.1373).

*(2R,3R)-6,8-di-C-methyldihydrokaempferol 7-O-β-D-xylopyra-nosyl(1→6)-β-D-glucopyranoside (**2**)*: Yellowish amorphous solid, [α]20 D +0.4 (*c* 0.38, MeOH); UV (MeOH) λ_{max} (log ε) 283 (4.19)， 361 (3.55) nm; CD (*c* 0.26 mM, MeOH) 295 ($\Delta\varepsilon$ –5.98), 353 ($\Delta\varepsilon$ +2.88) nm; IR (film) ν_{max} 3371, 2924, 1628, 1518, 1448, 1364, 1271, 1173, 1107, 1067 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively; (+)-HRESIMS: *m/z* 633.1785 $[M+Na]^+$ (calcd for $C_{28}H_{34}O_{15}Na$, 633.1795).

*6,8-di-C-methylkaempferol 7-O-β-D-xylopyranosyl(1→6)-β-D-glucopyranoside (**3**)*: Yellow amorphous solid, [α]20 D –4.5 (*c* 0.20, MeOH : DMSO = 4:1); UV (MeOH) λ_{max} (log ε) 276 (4.31), 334 (4.24), 378 (4.23) nm; IR (KBr) ν_{max} 3402, 2923, 1641, 1612, 1555,

1511, 1478, 1419, 1381, 1339, 1299, 1281, 1174, 1120, 1069 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively; (+)-HRESIMS: *m/z* 631.1621 $[M+Na]^+$ (calcd for $C_{28}H_{32}O_{15}Na$, 631.1639).

*(2R)-farrerol (**4a**)*: Yellowish amorphous solid; [α]20 D –21.8 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 297 (4.11), 347(3.53) nm; CD (*c* 0.33 mM, MeOH) 290 ($\Delta\varepsilon$ +5.47), 334 ($\Delta\varepsilon$ –1.63) nm; IR (KBr) ν_{max} 3400, 2921, 1637, 1587, 1516, 1444, 1362, 1229, 1174, 1117, 1066, 1031 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2, respectively; (+)-HRESIMS: *m/z* 301.1069 $[M+H]^+$ (calcd for $C_{17}H_{17}O_5$, 301.1076).

*(2R/2S)-farrerol 7-O-β-D-glucopyranoside (**5**)*: Yellowish amorphous solid; UV (MeOH) λ_{max} (log ε) 283 (4.21), 363 (3.56) nm; IR (KBr) ν_{max} 3378, 2920, 1627, 1518, 1445, 1351, 1278, 1176, 1124, 1069, 1038 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 7.33(4H, d, *J* = 8.4 Hz, H-2′ and H-6′), 6.83 (4H, d, *J* = 8.4 Hz, H-3′ and 5′), 5.33-5.36 (2H, H-2), 4.74 and 4.73 (2H, d, *J* = 7.6 Hz, H-G1″), 3.79-3.75 and 3.68-3.64 (4H, H-G6″), 3.53 (2H, t, *J* = 8.3 Hz, H-G2″), 3.44 and 3.43 (2H, t, *J* = 8.8 Hz, H-G3″), 3.39 (2H, t, *J* = 8.8 Hz, H-G4″), 3.19-3.14 (2H, H-G5″), 3.15-3.08 and 2.82-2.76 (4H, H-3), 2.14 (6H, s, Me-6), 2.13 (6H, s, Me-8). ^{13}C NMR (CD_3OD, 100 MHz) δ 200.1 and 200.0 (C-4), 162.6 and 162.4 (C-7), 159.8 (C-5), 159.3 (C-9), 158.9 (C-4′), 131.3 (C-1′), 128.9 (C-2′ and C-6′), 116.4 (C-3′ and C-5′), 113.0 (C-6), 111.9 and 11.8 (C-8), 106.3 (C-10), 105.3 (C-G1″), 80.1 (C-2), 78.1 (C-G5″), 77.9 (C-G3″), 75.7 (C-G2″), 71.5 (C-G4″), 62.7 (C-G6″), 44.3 (C-3), 9.8 (Me-8), 9.1 (Me-6); (+)-HRESIMS: *m/z* 485.1408 $[M+Na]^+$ (calcd for $C_{23}H_{26}O_{10}Na$, 485.1424).

*(2R/2S)-farrerol 7-O-β-D-xylopyranosyl(1→6)-β-D-glucopyra-noside (**6**)*: Yellowish amorphous solid; UV (MeOH) λ_{max} (log ε) 282 (4.23), 363 (3.59) nm; IR (KBr) ν_{max} 3370, 2926, 1630, 1612, 1518, 1444, 1349, 1278, 1170, 1124, 1065, 1045 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 7.36 and 7.35 (4H, d, *J* = 8.5 Hz, H-2′ and H-6′), 6.83 (4H, d, *J* = 8.5 Hz, H-3′ and 5′), 5.44 and 5.37 (2H, dd, *J* = 2.7, 13.0 Hz, H-2), 4.78 and 4.77 (2H, d, *J* = 7.6 Hz, H-G1″), 4.16 (2H, d, *J* = 7.0 Hz, H-X1‴), 3.98-3.95 and 3.75-3.69 (4H, H- G6″), 3.82-3.77 and 3.07-3.02 (4H, H-X5‴), 3.55-3.50 (2H, H-G2″), 3.46-3.39 (4H, H-G5″ and H-X4‴), 3.37-3.35 (4H, H-G3″ and H-G4″), 3.28-3.13 and 2.80-2.73 (4H, H-3), 2.13 (6H, s, Me-6), 2.11 and 2.10 (6H, s, Me-8). ^{13}C NMR (CD_3OD, 100 MHz) δ 200.1 and 200.0 (C-4), 162.6 and 162.4 (C-7), 159.8 (C-5), 159.3 (C-9), 158.9 (C-4′), 131.3 (C-1′), 129.0 (C-2′ and C-6′), 116.3 (C-3′ and C-5′), 112.9 (C-6), 112.0 and 111.8 (C-8), 106.4 (C-10), 104.8 (C-G1″), 104.7 and 104.6 C-X1‴), 80.4 and 80.0 (C-2), 78.1 and 77.9 (C-G3″), 77.8 (C-G5″), 77.6 and 77.5 (C-X3‴), 75.8 (C-G2″), 74.8 and 74.7 (C-X2‴), 71.5 (C-G4″), 71.1 (C-X4‴), 69.4 and 69.2 (C-G6″), 66.7 and 66.6 (C-X5‴), 44.4 and 44.2 (C-3), 9.8 (Me-8), 9.2 and 9.1 (Me-6); (+)-HRESIMS: *m/z* 617.1762 $[M+Na]^+$ (calcd for $C_{28}H_{34}O_{14}Na$, 617.1846).

3.4. Acid hydrolysis of compounds 1–3, 5, and 6 and determina-tion of the absolute configuration of monosaccharides

Compounds **1** (2 mg), **2** (4 mg), **3** (4 mg), **5** (4 mg), and **6** (3.5 mg) were each refluxed in 2M CF_3CO_2H (MeOH–H_2O, 1:4, 2 mL) for 2 h. The reaction mixture was then evaporated under reduced pressure to dryness until neutral. The residue was diluted in H_2O (2 mL) and then extracted with EtOAc (2 mL × 3). The H_2O layer including the sugar component was evaporated, and the part of sugar residue was compared with the authentic sample by TCL (silica gel; $CHCl_3$–MeOH–H_2O, 6:4:1). The other residue and L-cysteine methyl ester hydrochloride (3 mg) were dissolved in 2 mL re-distilled dry pyridine and heated at 60 °C for 2 h. The reaction mixture was concentrated to dryness and then 400 μL trimethylsilyl imidazole was added to the residue, followed by incubation at 60 °C for 1 h[32]. Finally, the mixture was partitioned between *n*-hexane (600 μl) and H_2O (2 mL). The *n*-hexane fraction was detected by GC under the following conditions: capillary column, OV-17 (30 m × 0.32 mm × 0.5 μm, Lanzhou Zhongke Antai Analysis Technology Co., Ltd., China); the column temperature was raised from 220 °C to 250 °C at the rate of 2 °C/min and then maintained for 10 min; injection temperature, 250 °C; detector FID, detector temperature, 250 °C; carrier gas, N_2; split ratio, 10:1; flow rate, 1.0 mL/min. The reference D-xylose, and L-xylose, D-glucose, L-glucose derivatives were prepared by similar reaction, which showed retention times of 11.86, 12.51, 16.63, and 17.29 min, respectively. By comparing the retention times of the trimethylsilylthiazolidine derivatives of the hydrolysate and the standards, the monosaccharides in compounds **1** and **5** was determined to be D-glucose, and the monosaccharides in compounds **2**, **3**, and **6** were identified as D-xylose and D-glucose.

*3.5. Cellulase hydrolysis of compounds **5** and **6***

Compounds **5** (4 mg) and **6** (6 mg) were each hydrolyzed by cellulase (50 mg) in H_2O (10 mL) at 50 °C for 24 h. The reaction mixture was extracted by EtOAc (2 mL × 5). The aglycone in the EtOAc extraction was further analyzed by Chiralpack AD-RH HPLC.

*3.6. Neuroprotective activities of compounds **1**–**9** against H_2O_2- or Aβ-induced SH-SY5Y cells apoptosis*

The neuroprotective activities of compounds **1**–**9** against H_2O_2- or A*β*-induced apoptosis in human neuroblastoma SH-SY5Y cells were evaluated using the MTT method. The SH-SY5Y cells (100μl) were seeded into 96-well plates at a density of 4×10^4 cells/well in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 unit/mL penicillin and 100 μg/mL streptomycin in a humid atmosphere of 5% CO_2 at 37 °C and incubated for 24 h[3]. Then the cells were pre-treated with test compounds (10, 1, and 0.1 μM) and Vitamin E (10 μM, positive control) for 1 h before the addition of H_2O_2 or aggregated A*β* (3 days at 37 °C). After 24 h exposure to H_2O_2 or A*β*, 10 μL of MTT (5 mg/mL in PBS) were added to each well and incubated for 4 h at 37 °C in the dark. The formazan converted from MTT was dissolved in 100 μL DMSO in each well. The absorbance was measured at 570 nm in an ELISA plate reader. The results were presented as the percentage of survival relative to the controls. Data were expressed as means ± standard errors of the mean (SEM). Statistical analyses were performed using Dunnett-t test after analysis of variance.

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Competing financial interests

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data associated with this article including HRESIMS, 1D and 2D NMR spectra, IR, UV, and CD spectra, and GC analysis of sugar for compounds **1**–**6** are can be found online at http://

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Conflict of interest statement

The authors (Yongji Lai, Hong Zeng, Meijun He, Huiqin Qian, Zhaodi Wu, Zengwei Luo, Yongbo Xue, Guangmin Yao, Yonghui Zhang) declared that they have no conflicts of interest to this work (6,8-Di-*C*-methyl-flavonoids with neuroprotective activities from *Rhododendron fortunei*).

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Yonghui Zhang
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6,8-Di-*C*-methyl-flavonoids with neuroprotective activities from *Rhododendron fortunei*

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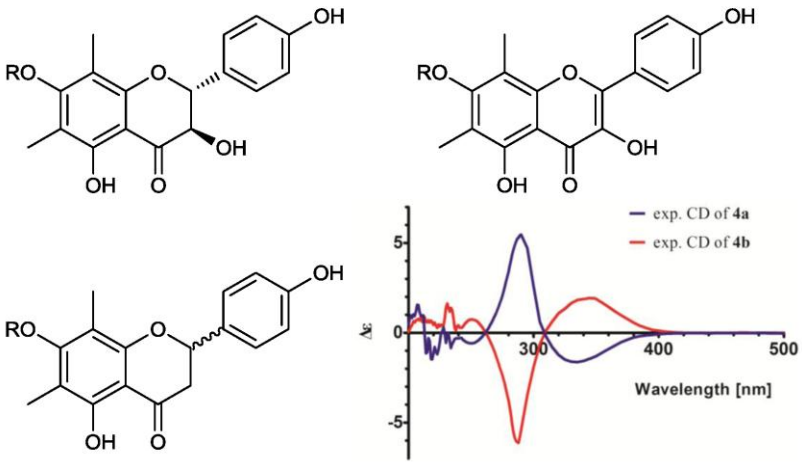

Six 6,8-di-*C*-methyl-flavonoids (**1**–**3, 4a**, **5**–**6**) and four known compounds (**4b**, **7**–**9**) were isolated. Their neuroprotective activities were evaluated.