Four new Flavonol Glycosides from the leaves of *Ginkgo biloba*

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Four new flavonol glycosides, 5, 7, 5′-trihydroxy-3′, 4′-dimethoxyflavonol-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (1), quercetin 3-O-(6-trans-feruloyl)-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside (2), kaempferol 3-O-(6-trans-caffeoyl)-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside (3), myricetin 3-O-(6-trans-p-coumaroyl)-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside (4), together with nine known flavonoids and two known lignans, were isolated from the leaves of *Ginkgo biloba*. Their structures were determined by extensive spectroscopic analyses. Their cardioprotective effects against H₂O₂-induced apoptosis in H9c2 cells were also evaluated. The flavonol glycosides had stronger activity than the acylated flavonol glycosides at the concentration of 50 µM.

Keywords: *Ginkgo biloba*; cardioprotective activity; acylated flavonol glycoside

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Table S1. The ¹H and ¹³C NMR data of compounds 1-4 (DMSO-d₆, ¹H NMR 400 MHz, and ¹³C NMR 100 MHz) and compound 5 (CD₃OD, ¹H NMR 400 MHz, and ¹³C NMR 100 MHz).

Table S2. In vitro anti-oxidative effects of the isolated compounds at concentration of 50 µM against H₂O₂-induced cytotoxicity in cultured H9c2 cells.

Figure S1. Key HMBC and ROESY correlations for Compounds 1 to 4

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Figure S39. HR-ESI-MS spectrum Compound 5

Figure S40. Inhibition rates of the isolated compounds at concentration of 50 µM against H$_2$O$_2$-induced cytotoxicity in cultured H9c2 cells *in vitro*.

Experimental
Table S1. The $^1$H and $^{13}$C NMR data of compounds 1-4 (DMSO-$d_6$, $^1$H NMR 400 MHz, and $^{13}$C NMR 100 MHz) and compound 5 (CD$_2$OD, $^1$H NMR 400 MHz, and $^{13}$C NMR 100 MHz).

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3'-OCH$_3$ 56.3 3.83, s
4'-OCH$_3$ 60.5 3.76, s

Table S2. In vitro anti-oxidative effects of the isolated compounds at concentration of 50 µM against H$_2$O$_2$-induced cytotoxicity in cultured H9c2 cells.

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</table>
**Figure S1.** Key HMBC and ROESY correlations for Compounds 1 to 4

**Figure S2.** $^1$H NMR spectrum of Compound 1 (DMSO-$d_6$, 400MHz)
Figure S3. $^{13}$C NMR spectrum of Compound 1 (DMSO-$d_6$, 400MHz)

Figure S4. HSQC correlations of Compound 1 (DMSO-$d_6$, 400MHz)
Figure S5. HMBC correlations of Compound 1 (DMSO-d$_6$, 400MHz)

Figure S6. $^1$H-$^1$H COSY correlations of Compound 1 (DMSO-d$_6$, 400MHz)
Figure S7. ROESY correlations of Compound 1 (DMSO-d$_6$, 400MHz)

Figure S8. TOCSY correlations of Compound 1 (DMSO-d$_6$, 400MHz)
Figure S9. HR-ESI-MS spectrum Compound 1

Figure S10. $^1$H NMR spectrum of Compound 2 (DMSO-d$_6$, 400MHz)
Figure S11. $^{13}$C NMR spectrum of Compound 2 (DMSO-d$_6$, 400MHz)

Figure S12. HSQC correlations of Compound 2 (DMSO-d$_6$, 400MHz)
Figure S13. HMBC correlations of Compound 2 (DMSO-d$_6$, 400MHz)

Figure S14. $^1$H-$^1$H COSY correlations of Compound 2 (DMSO-d$_6$, 400MHz)
Figure S15. ROESY correlations of Compound 2 (DMSO-d$_6$, 400MHz)

Figure S16. HR-ESI-MS spectrum Compound 2
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Figure S20. HMBC correlations of Compound 3 (DMSO-d$_6$, 400MHz)
Figure S21. $^1$H-$^1$H COSY correlations of Compound 3 (DMSO-d$_6$, 400MHz)

Figure S22. ROESY correlations of Compound 3 (DMSO-d$_6$, 400MHz)
Figure S23. TOCSY correlations of Compound 3 (DMSO-\textit{d}_6, 400MHz)

Figure S24. HR-ESI-MS spectrum Compound 3
Figure S25. $^1$H NMR spectrum of Compound 4 (DMSO-d$_6$, 400MHz)

Figure S26. $^{13}$C NMR spectrum of Compound 4 (DMSO-d$_6$, 100MHz)
**Figure S27.** HSQC correlations of Compound 4 (DMSO-d<sub>6</sub>, 400MHz)

**Figure S28.** HMBC correlations of Compound 4 (DMSO-d<sub>6</sub>, 400MHz)
Figure S29. $^1$H-$^1$H COSY correlations of Compound 4 (DMSO-d$_6$, 400MHz)

Figure S30. ROESY correlations of Compound 4 (DMSO-d$_6$, 400MHz)
Figure S31. TOCSY correlations of Compound 4 (DMSO-\textsubscript{d6}, 400MHz)

Figure S32. HR-ESI-MS spectrum Compound 4
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Figure S34. $^{13}$C NMR spectrum of Compound 5 (MeOD, 400MHz)
Figure S35. HSQC correlations of Compound 5 (MeOD, 400MHz)

Figure S36. HMBC correlations of Compound 5 (MeOD, 400MHz)
Figure S37. $^1$H-$^1$H COSY correlations of Compound 5 (MeOD, 400MHz)

Figure S38. NOESY correlations of Compound 5 (MeOD, 400MHz)
Figure S39. HR-ESI-MS spectrum Compound 5

Figure S40. Inhibition rates of the isolated compounds at concentration of 50 μM against H$_2$O$_2$-induced cytotoxicity in cultured H9c2 cells \textit{in vitro}.

\textbf{Experimental}

\textit{1 General experimental procedures}

NMR spectra were recorded on a Bruker AV-400 instrument using TMS as an internal
standard (Bruker Scientific Technology Co., Ltd., Zurich, Switzerland). HR-ESI-MS spectra were measured on a LCT Premier XE (Waters) mass spectrometer (Waters Technology Ltd., Milford, USA). Industrial chromatography was put on a dynamic axial compression-HB 80 system (Hanbon Sci. & Tech. Co., Ltd., Jiangsu, China). Preparative HPLC was performed on an Agilent 1260 system using an Ultimate Polar-RP column (250 mm × 21.2 mm, 5 μm, Welch Materials, Shanghai, Inc., China). Sephadex LH-20 (Pharmacia, Stockholm, Sweden) and C_{18} reverse-phased silica gel (60μm, Acchrom Technologies Co., Ltd., Dalian, China). Thin-layer chromatography (TLC) was conducted on plates pre-coated with silica gel GF_{254} (Qingdao Marine Chemical Ltd., Qingdao, China). All solvents were of analytical grade (Nanjing Chemical Reagent Co., Ltd., Nanjing, China), and the solvents used in HPLC were of HPLC grade (J&K Scientific Ltd., Beijing, China).

2 Plant material

The leaves of *Ginkgo biloba* L. were collected from Pizhou, Jiangsu Province, People’s Republic of China. The plant material was identified by Zhou Wu, an employee of Jiangsu Kanion Pharmaceutical Co. Ltd., Lianyungang, People’s Republic of China, where a voucher specimen (NO. KY20161002-1) was deposited.

3 Extraction and isolation

The dried leaves of *Ginkgo biloba* (2.2 kg) were powdered and extracted with 70% ethyl alcohol (EtOH) twice for 2 h with water under reflux, and the solvent was evaporated in vacuo at 60 °C. The 70% EtOH extract (720 g) was successively partitioned with petroleum ether (4 L × 3), ethyl acetate (EtOAc) (4 L × 3), and *n*-butyl alcohol (*n*-BuOH) (4 L × 3) to give petroleum ether-(35.2 g), EtOAc-(98.3 g), *n*-BuOH-(125.0 g), and water-soluble (103.0 g) extracts, respectively. The *n*-BuOH-soluble
fraction (120.0 g) was dissolved in water and passed through a reverse-phased industrial chromatography of dynamic axial compression (DAC) column sequentially eluted with H₂O and MeOH to give 6 fractions, B1-B6. Fr. B2 was purified by recrystallization (MeOH) to obtain compound 6 (265 mg). Fr. B3 (4.5g) was further chromatographed on a sephadex LH-20 column with 50% MeOH-H₂O to furnish 5 fractions. The preparative HPLC separation of Fr. B3-2 with CH₃CN-H₂O (18:82) yielded compounds 1 (8 mg) and 10 (31 mg). Fr. B3-3 was further separated by preparative HPLC with CH₃CN-H₂O (14:86) to afford compounds 2 (11 mg), 3 (10 mg) and 8 (32 mg). Fr. B4 was purified by preparative HPLC eluted with MeOH-H₂O (12:88) to give compounds 4 (6 mg), 7 (15 mg) and 9 (18 mg). Compounds 5 (11 mg), 11 (22 mg) and 12 (17 mg) were isolated from Fr. B5 by Sephadex LH-20 column chromatography with 50% MeOH-H₂O, followed by purification via preparative HPLC with CH₃CN-H₂O (13:87). Fr. B6 was chromatographed over Sephadex LH-20 column with 50% MeOH-H₂O, then separated to preparative HPLC with CH₃CN-H₂O (13:87) to yield compounds 13 (13 mg), 14 (279 mg) and 15 (13 mg).

4 Acid hydrolysis

A solution of each compounds 1-5 (2 mg each) was refluxed with concentrated AcOH (3.5 mL), concentrated HCl (1 mL), and H₂O (5.5 mL) at 100 °C for 3 h. The reaction mixture was extracted with EtOAc, and the remaining aqueous layer was evaporated to dryness. And then analyzed by TLC over silica gel with n-BuOH-MeOH-CHCl₃-AcOH-H₂O (12.5:5:4.5:1.5:1.5) (Yuan et al. 2018) as mobile phase and the result consistent with authentic samples of D-glucose and L-rhamnose.

5 Spectroscopic data

5, 7, 5’-trihydroxy-3’, 4’-dimethoxyflavonol-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-
glucopyranoside (1): pale yellow amorphous powder; negative HR-ESI-MS (found m/z 653.1733[M−H]) and positive HR-ESI-MS (found m/z 677.1704 [M+Na]+); 1H NMR (400 MHz, DMSO-d6): δ 12.51 (1H, s, 5-OH), 6.21 (1H, d, J = 1.8 Hz, H-6), 6.40 (1H, d, J = 1.8 Hz, H-8), 7.45 (1H, d, J = 2.0 Hz, H-2'), 7.12 (1H, d, J = 2.0 Hz, H-6'), 5.52 (1H, d, J = 7.4 Hz, H-1''), 3.24 (1H, m, H-2''), 3.24 (1H, m, H-3''), 3.26 (1H, m, H-4''), 3.30 (1H, m, H-5''), 3.72 (1H, m, H-6''a), 3.39 (1H, m, H-6''b), 4.45 (1H, d, J = 0.8 Hz, H-1''), 3.41 (1H, m, H-2''), 3.06 (1H, m, H-3''), 3.05 (1H, m, H-4''), 3.26 (1H, m, H-5''), 0.98 (3H, d, J = 6.2 Hz, H-6''), 3.83 (3H, s, 3'-OCH3), 3.76 (3H, s, 4'-OCH3). 13C NMR (100 MHz, DMSO-d6): δ 156.1 (C-2), 134.1 (C-3), 177.8 (C-4), 161.7 (C-5), 99.4 (C-6), 165.4 (C-7), 94.2 (C-8), 157.0 (C-9), 104.4 (C-10), 125.7 (C-1'), 105.7 (C-2'), 153.0 (C-3'), 139.1 (C-4'), 150.8 (C-5'), 110.2 (C-6'), 101.4 (C-1''), 74.6 (C-2''), 76.8 (C-3''), 71.0 (C-4''), 76.5 (C-5''), 67.2 (C-6''), 101.3 (C-1''), 70.7 (C-2''), 70.5 (C-3''), 72.2 (C-4''), 68.7 (C-5''), 18.1 (C-6''), 56.3 (3'-OCH3), 60.5 (4'-OCH3).

Quercetin 3-O-(6-trans-feruloyl)-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside (2): pale yellow amorphous powder; negative HR-ESI-MS (found m/z 785.1944[M−H]) and positive HR-ESI-MS (found m/z 809.1912 [M+Na]+). 1H NMR (400 MHz, DMSO-d6): δ 6.18 (1H, s, H-6), 6.33 (1H, s, H-8), 7.38 (H, d, J = 2.0 Hz, H-2'), 6.90 (1H, d, J = 8.4 Hz, H-5'), 7.26 (1H, d, J = 8.4, 2.0 Hz, H-6'), 5.52 (1H, s, H-1''), 4.16 (1H, d, J = 2.7 Hz, H-2''), 3.61 (1H,dd,J = 9.6, 2.7 Hz, H-3''), 3.13 (1H, t, J = 9.6 Hz, H-4''), 3.56 (1H, dd, J = 9.6, 6.2 Hz, H-5''), 0.92 (3H, d, J = 6.2 Hz, H-6''), 4.29 (1H, d, J = 7.8 Hz, H-1''' ), 3.05 (1H, t, J = 8.2 Hz, H-2'''), 3.20 (1H, m, H-3''), 3.21 (1H, m, H-4''), 3.27 (1H, m, H-5''), 4.04 (1H, d, J = 11.8 Hz), 4.22 (1H, dd, J = 11.8, 3.8 Hz, H-6''), 6.35 (1H, d, J = 15.9 Hz, H-2''''), 7.45 (1H, d, J = 15.9 Hz, H-3''''), 7.21 (1H, d, J = 1.7 Hz, H-5''''), 6.72 (1H, d, J = 8.2 Hz, H-8''''), 6.99 (1H,dd, J = 8.2, 1.7 Hz, H-9'''), 3.75 (3H, s, 6''-OCH3). 13C NMR (100 MHz, DMSO-d6): δ 157.0 (C-2),
Kaempferol 3-O-(6-trans-caffeoyl)-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside (3): pale yellow amorphous powder; negative HR-ESI-MS (found m/z 755.1897 [M−H]−) and positive HR-ESI-MS (found m/z 779.1835 [M+Na]+).1H NMR (400 MHz, DMSO-d6): δ 6.19 (1H, s, H-6), 6.37 (1H, s, H-8), 7.38 (2H, d, J = 8.8 Hz, H-2′, 6′), 6.93 (2H, d, J = 8.8 Hz, H-3′, 5′), 5.60 (1H, s, H-1′), 4.13 (1H, d, J = 3.1 Hz, H-2″), 3.55 (1H, dd, J = 9.5, 3.1 Hz, H-3″), 3.13 (1H, t, J = 9.5 Hz, H-4″), 3.32 (1H, m, H-5″), 0.89 (3H, d, J = 6.1 Hz, H-6″), 4.33 (1H, d, J = 7.8 Hz, H-1″), 3.06 (1H, t, J = 8.0 Hz, H-2″), 3.20 (1H, m, H-3″), 3.19 (1H, m, H-4″), 3.32 (1H, m, H-5″), 4.18 (2H, m, H-6″), 6.11 (1H, d, J = 15.9 Hz, H-2‴), 7.39 (1H, d, J = 15.9 Hz, H-3‴), 6.96 (1H, d, J = 1.7 Hz, H-5‴), 6.69 (1H, d, J = 8.2 Hz, H-8‴), 6.87 (1H, dd, J = 8.2, 1.7 Hz, H-9‴).13C NMR (100 MHz, DMSO-d6): δ 157.1 (C-2), 134.8 (C-3), 178.1 (C-4), 161.7 (C-5), 99.2 (C-6), 164.8 (C-7), 94.0 (C-8), 156.9 (C-9), 104.5 (C-10), 120.8 (C-1′), 130.9 (C-2′, 6′), 115.9 (3′, 5′), 160.6 (C-4′), 101.0 (C-1″), 82.0 (C-2″), 70.6 (C-3″), 72.1 (C-4″), 70.9 (C-5″), 17.8 (C-6″), 106.5 (C-1‴), 74.2 (C-2‴), 76.5 (C-3‴), 70.1 (C-4‴), 74.2 (C-5‴), 63.4 (C-6‴), 166.8 (C-1‴′), 114.1 (C-2‴′), 145.6 (C-3‴′), 125.9 (C-4‴′), 115.5 (C-5‴′), 145.9 (C-6‴′), 148.8 (C-7‴′), 116.1 (C-8‴′), 121.4 (C-9‴′).

Myricetin 3-O-(6-trans-p-coumaroyl)-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranoside (4): pale yellow amorphous powder; negative HR-ESI-MS (found m/z 771.1788 [M−H]−) and positive HR-ESI-MS (found m/z 795.1759 [M+Na]+).1H
NMR (400 MHz, DMSO-\textit{d}_6): \(\delta \) 6.17 (1H, d, \(J = 1.7 \text{ Hz, H-6}\)), 6.32 (1H, d, \(J = 1.7 \text{ Hz, H-8}\)), 6.96 (2H, s, H-2', 6'), 5.46 (1H, s, H-1''), 4.19 (1H, d, \(J = 2.9 \text{ Hz, H-2''}\)), 3.65 (1H, dd, \(J = 9.7, 2.9 \text{ Hz, H-3''}\)), 3.12 (1H, t, \(J = 9.7 \text{ Hz, H-4''}\)), 3.73 (1H, dd, \(J = 9.7, 6.2 \text{ Hz, H-5''}\)), 0.94 (3H, d, \(J = 6.2 \text{ Hz, H-6''}\)), 4.27 (1H, d, \(J = 7.8 \text{ Hz, H-1''}\)), 3.03 (1H, m, H-2'''), 3.17 (1H, m, H-3''''), 3.19 (1H, m, H-4''''), 3.22 (1H, m, H-5''''), 3.96 (1H, d, \(J = 11.5 \text{ Hz}\)), 4.22 (1H, d, \(J = 11.5, 3.7 \text{ Hz, H-6''''}\)), 6.27 (1H, d, \(J = 15.9 \text{ Hz, H-2''''}\)), 7.46 (1H, d, \(J = 15.9 \text{ Hz, H-3''''}\)), 7.44 (2H, d, \(J = 8.6 \text{ Hz, H-5''''}, 9''''\)), 6.72 (2H, d, \(J = 8.6 \text{ Hz, H-6''''}, 8''''\)).\textsuperscript{13}C NMR (100 MHz, DMSO-\textit{d}_6): \(\delta \) 155.5 (C-2), 133.3 (C-3), 176.6 (C-4), 160.1 (C-5), 97.5 (C-6), 163.1 (C-7), 92.3 (C-8), 155.1 (C-9), 102.7 (C-10), 118.2 (C-1'), 106.5 (C-2', 6'), 144.7 (C-3', 5'), 135.5 (C-4'), 99.6 (C-1''), 80.8 (C-2'''), 69.0 (C-3''), 70.7 (C-4''), 69.2 (C-5''), 16.3 (C-6''), 105.2 (C-1''''), 72.6 (C-2''''), 74.7 (C-3''''), 68.0 (C-4''''), 72.4 (C-5''''), 61.5 (C-6''''), 165.3 (C-1''''''), 112.8 (C-2''''''), 143.5 (C-3'''''''), 123.8 (C-4'''''''), 129.0 (C-5'''''', 9'''''), 114.5 (C-6'''''', 8'''''), 158.6(C-7''''''').

Kaempferol 3-O-(6-cis-p-coumaroyl)-\(\beta\)-D-glucopyranosyl-(1→2)-\(\alpha\)-L-rhamnopyranoside (5): yellow amorphous powder; HR-ESI-MS \textit{m/z}: 739.1889 [M−H]−, calcd for C\textsubscript{36}H\textsubscript{35}O\textsubscript{17}, 739.1874. \textsuperscript{1}H NMR (400 MHz, MeOD): \(\delta \) 6.19 (1H, d, \(J = 1.6 \text{ Hz, H-6}\)), 6.35 (1H, d, \(J = 1.6 \text{ Hz, H-8}\)), 7.66 (2H, d, \(J = 8.7 \text{ Hz, H-2',6'}\)), 6.90 (2H, d, \(J = 8.7 \text{ Hz, H-3', 5'}\)), 5.61 (1H, d, \(J = 1.0 \text{ Hz, H-1''}\)), 4.42 (1H, m, H-2''), 3.88 (1H, m, H-3''), 3.39 (1H, m, H-4''), 3.59 (1H, m, H-5''), 1.02 (3H, d, \(J = 6.2 \text{ Hz, H-6''}\)), 4.43 (1H, d, \(J = 7.8 \text{ Hz, H-1'''\}'), 3.29 (1H, m, H-2''''), 3.42 (1H, m, H-3'''''), 3.25 (1H, m, H-4''''''), 3.45 (1H, m, H-5''''''), 4.28 (2H, m, H-6'''''), 5.34 (1H, d, \(J = 12.8 \text{ Hz, H-2'''\})), 6.39 (1H, d, \(J = 12.8 \text{ Hz, H-3'''\})), 7.48 (2H, d, \(J = 8.7 \text{ Hz, H-5'''}, 9'''\)), 6.69 (2H, d, \(J = 8.7 \text{ Hz, H-6''''}, 8'''\)). \textsuperscript{13}C NMR (100 MHz, MeOD): \(\delta \) 158.9 (C-2), 137.4 (C-3), 179.6 (C-4), 163.2 (C-5), 99.9 (C-6), 165.7 (C-7), 94.9 (C-8), 158.5 (C-9), 106.3 (C-10), 122.6 (C-1'), 132.0 (C-2', 6'), 116.4 (C-3', 5'), 161.3 (C-4'), 103.3 (C-1''), 83.8 (C-2'''), 71.8 (C-3''''), 73.6 (C-
Biological assay

6.1 Cell Culture

H9c2 cardiomyocytes were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a water-saturated 5% CO₂ incubator. Reagents for cell cultures were purchased from Sigma (St. Louis, MO, USA).

6.2 Analysis of Cell viability

The cell viability was determined colorimetrically by CCK-8 assay. Briefly, H9c2 cells (8000 cells/well) were seeded into 96-well plates for overnight. After 1 h of treatment with compounds 1–15 (final concentration 50 µM) followed by incubation with 80 µM H₂O₂ for 1 h, 10 µL of CCK-8 solution was added to each well and incubated for 3 h. Cell viability was measured at 450 nm by Flex Station 3 microplate spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA). The percentage cell viability was calculated as a ratio of the optical density (OD) value of the sample to the OD value of the control. Each test was performed six times. The content was calculated in all three sets using the following equation:

\[
\% \text{ Inhibition} = \frac{A_s - A_m}{A_c - A_m} \times 100\%
\]

Where \(A_s\) is the absorbance of sample, \(A_m\) is the absorbance of model and \(A_c\) is the
absorbance of control.

6.3 Statistical analysis

All data were expressed as means ± standard deviation (SD). Statistical analyses were performed with one-way analysis of variance (ANOVA) (SPSS version 17.0: Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

References