The design, synthesis, antioxidant, and antihypoxia activities of two new hydroxydaidzein derivatives

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Abstract
7,8,3',4',5'-Pentahydroxyisoflavone (8,3',5'-trihydroxydaidzein) and 6,7,8,3',4',5'-hexahydroxyisoflavone (6,8,3',5'-tetrahydroxydaidzein) are synthesized via a simple and effective method using commercially available daidzein as a raw material. Their structures are confirmed using spectroscopic analyses (infrared, nuclear magnetic resonance, and mass spectrometry), and their purities are determined by high-performance liquid chromatography. The antioxidant capacities are investigated by utilizing 1,1-diphenyl-2-picryl-hydrazyl radical scavenging, superoxide radical scavenging, nitric oxide radical scavenging, reducing power, and phosphomolybdenum assays. The antihypoxia activity is examined using a hypoxia-induced PC12 cell injury model. The antioxidant and antihypoxia abilities of 8,3',5'-trihydroxydaidzein and 6,8,3',5'-tetrahydroxydaidzein are greatly improved in comparison with daidzein. These results indicate that 8,3',5'-trihydroxydaidzein and 6,8,3',5'-tetrahydroxydaidzein are excellent antioxidant agents and can be used for alleviating injury induced by hypoxia.

Keywords
6,7,8,3',4',5'-hexahydroxyisoflavone, 7,8,3',4',5'-pentahydroxyisoflavone, antihypoxia activity, antioxidant activity, synthesis

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In this study, 8,3',5'-THD and 6,8,3',5'-FHD were synthesized for the first time over four steps from daidzein. As shown in Scheme 1, the first step, that is, selective bromination of daidzein, was the key step for determining the position and number of hydroxy groups introduced on daidzein. It was known that positions C-6 and C-8 of the A ring and C-3' and C-5' of the B ring in daidzein are reactive toward electrophiles and potentially are involved in bromination. We thus used bromine (Br₂) as brominating agent and found that the employed solvent had a strong impact on the bromination of daidzein. When daidzein was treated with 5 equiv. of Br₂ in a mixed solvent of CH₂Cl₂/CH₃CH₂OH (10:1) at 25 °C, 8,3',5'-tribromo-7,4'-dihydroxyisoflavone (1) was obtained as the main product and was easily purified by washing with hot ethyl acetate. In contrast, the bromination of daidzein with 5 equiv. of Br₂ in CH₂OH at reflux yielded 7,8,3',5'-tetramethoxyisoflavone (4) quantitatively. Subsequently, the methanalysis of compounds 1 and 4 was carried out in the presence of CuBr and CH₃ONa/CH₃OH in dimethylformamide (DMF) at 120 °C according to our previous reported method. Unfortunately, we did not obtain the targeted compounds. Our previous study has described the methanalysis of brominated biochanin A, in which the 3'-hydroxy group was replaced by a 3'-methoxy group, and which occurred with a moderate yield. We believed that the 3'-hydroxy group might show a negative effect on the reaction process. Therefore, we used methyl groups for protecting the 7- and 3'-OH groups, respectively. In addition, they possess ortho-dihydroxy (catechol) and/or ortho-trihydroxy (pyrogallol) structures, which have a positive effect on enhancing their antioxidative potential. Theoretically, both compounds should have excellent antioxidant activity. However, they have not been isolated from natural sources or obtained by chemical synthesis or microbial biotransformation. Therefore, it is necessary to develop a practical and economical synthetic pathway toward these compounds. The aim of this study was to provide a simple and effective economical synthetic pathway toward these compounds. The 1H NMR spectrum of 8,3',5'-THD showed the presence of a 1,2,3,4-tetrasubstituted benzene (A ring) with...
signals at $\delta$ H 7.46 (1H, d, $J$=8.8 Hz) and 6.96 (1H, d, $J$=8.8 Hz), a 1,3,4,5-tetrasubstituted benzene (B ring) with a signal at $\delta$ H 6.51 (2H, s), and an isolated olefinic proton (C ring) at $\delta$ H 8.25 (1H, s), as well as three hydroxy protons signals at 10.27 (s, C–OH), 9.40 (s, C–OH) and 8.86 (s, C–OH). Next, the locations of the five hydroxy groups were deduced to be at C-7, C-8, C-3’, C-4’, and C-5’. The $^1$H NMR spectrum of 6,8,3’,5’-FHD showed the presence of a single proton at $\delta$ H 7.00 (1H, s) in the A ring, two protons at $\delta$ H 6.53 (2H, s) in ring B, and an olefinic proton at $\delta$ H 8.22 (1H, s) in C ring. The locations of the six hydroxy groups were deduced to be at C-6, C-7, C-8, C-3’, C-4’, and C-5’, although no hydroxy proton signal was observed. The molecular formulae of 8,3’,5’-THD and 6,8,3’,5’-FHD were further confirmed by $^{13}$C NMR spectroscopy and high-resolution mass spectrometry (HRMS).

**Scheme 1.** Synthetic route to 8,3’,5’-THD and 6,8,3’,5’-FHD.

**The purity of 8,3’,5’-THD and 6,8,3’,5’-FHD**

High-performance liquid chromatography (HPLC) chromatograms of 8,3’,5’-THD and 6,8,3’,5’-FHD are shown in Figure 2, with the retention times being 2.86 and 4.25 min, respectively, indicating that they were pure products.

**Antioxidant activities of 8,3’,5’-THD and 6,8,3’,5’-FHD**

Considering that flavonoids have various antioxidant mechanisms, five different in vitro antioxidant assays were performed to fully elucidate the antioxidant activities of 8,3’,5’-THD and 6,8,3’,5’-FHD.

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free-radical scavenging assay has been widely used for measuring the ability of compounds to act as free-radical scavengers or hydrogen donors. As shown in Figure 3(a), daidzein exhibited very weak DPPH radical scavenging activity. 6,8,3’,5’-FHD presented the best DPPH radical scavenging activity with an IC$_{50}$ value of 0.785 ± 0.070 mmol L$^{-1}$, followed by 8,3’,5’-THD (IC$_{50}$ = 0.816 ± 0.011 mmol L$^{-1}$). The IC$_{50}$ value of the reference compound ascorbic acid (V$_C$) was 1.667 ± 0.009 mmol L$^{-1}$, indicating that 6,8,3’,5’-FHD and 8,3’,5’-THD exhibited significantly higher scavenging activity toward DPPH compared with that of V$_C$.

Superoxide (O$_2$$^-•$) is the primary reactive oxygen species (ROS) in cells and is also responsible for initiating some highly aggressive ROS, such as hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (·OH), that cause oxidative stress and result in cell damage. As shown in Figure 3(b), compared with daidzein (IC$_{50}$ > 2 mmol L$^{-1}$) and V$_C$ (0.487 ± 0.009 mmol L$^{-1}$), 8,3’,5’-THD and 6,8,3’,5’-FHD exhibited higher O$_2$$^-•$ scavenging activity with IC$_{50}$ values of 0.022 ± 0.001 mmol L$^{-1}$ and 0.034 ± 0.001 mmol L$^{-1}$, respectively. Thus, 8,3’,5’-THD and 6,8,3’,5’-FHD present excellent O$_2$$^-•$ scavenging activity, even at low concentration.

Nitric oxide (NO) is an important oxidative biological signaling molecule that participates in diverse physiological processes. Nevertheless, high levels of NO can also lead to
cell damage. As shown in Figure 3(c), 8,3',5'-THD and 6,8,3',5'-FHD exhibited high NO scavenging ratios of 39.66% and 32.93%, respectively, at a low concentration of 0.06125 mmol L\(^{-1}\). While the scavenging ratios of daidzein and VC were only 9.65% and 3.86%, respectively, at the same concentrations. The IC\(_{50}\) value of 6,8,3',5'-FHD (0.042 ± 0.001 mmol L\(^{-1}\)) was slightly lower than that of 8,3',5'-THD (0.077 ± 0.027 mmol L\(^{-1}\)). In addition, 6,8,3',5'-FHD showed the best NO scavenging ratio at a high concentration of 2 mmol L\(^{-1}\).

The electron-donating abilities of 8,3',5'-THD and 6,8,3',5'-FHD were also determined using a reducing power assay and a phosphomolybdenum assay (total antioxidant capacity, TAC). As shown in Figure 4(a), the reducing power of all the samples increased on increasing their concentration. At a dose of 1 mmol L\(^{-1}\), the absorbance values at 700 nm of 8,3',5'-THD, 6,8,3',5'-FHD, daidzein, and VC were 2.537 ± 0.005, 2.633 ± 0.009, 0.314 ± 0.021, and 2.052 ± 0.114, respectively. The order of the potency was 6,8,3',5'-FHD > 8,3',5'-THD > VC > daidzein. As shown in Figure 4(b), the absorbance values at 695 nm of 6,8,3',5'-FHD and 8,3',5'-THD were higher than those of VC and daidzein at the same concentration, indicating that they possessed a stronger TAC than VC and daidzein. The order of the potency was same as in the reducing power assay.

Based on the results of these five antioxidant assays, 6,8,3',5'-FHD and 8,3',5'-THD, which contain six and five phenolic hydroxy substitutions, respectively, were found to exhibit significantly higher antioxidant activity than daidzein, which only possesses two phenolic hydroxy substitutions. This observation agreed nicely with the theory that the antioxidant capacity of flavonoids is proportional to the number of phenolic hydroxy groups. Besides, an ortho-trihydroxy group and an ortho-dihydroxy group also have a positive effect on the antioxidant activity. 6,8,3',5'-FHD exhibited the best antioxidant activity, which may be attributed to the presence of the two ortho-trihydroxy groups on rings A and B. 6,8-DHD with an ortho-trihydroxy group on ring A and an ortho-dihydroxy group on ring B also exhibited excellent antioxidant activity.

**Antihypoxia activities of 8,3',5'-THD and 6,8,3',5'-FHD**

ROS play a key role in hypoxia-induced injury, which can be eliminated by the administration of antioxidants. Therefore, we further evaluated the protective effects of 6,8,3',5'-FHD and 8,3',5'-THD on hypoxia-induced injury in PC12 cells. As shown in Figure 5(a), treatment of PC12 cells with 6,8,3',5'-FHD, 8,3',5'-THD and daidzein at a
**Figure 3.** Antioxidant activities of 8,3',5'-THD, 6,8,3',5'-FHD, and daidzein: (a) DPPH radical scavenging, (b) O$_2^−$ radical scavenging, and (c) NO radical scavenging. Vitamin (VC) is used as the reference compound. Data are expressed as the mean value $\pm$ SD ($n=3$).

**Figure 4.** Reducing activities of 8,3',5'-THD, 6,8,3',5'-FHD, and daidzein: (a) reducing power and (b) total antioxidant capacity. VC is the reference compound. Data are expressed as the mean value $\pm$ SD ($n=3$).
concentration of 1 μmol L⁻¹ had no obvious effect on the survival rate of the PC12 cells under normoxic conditions, indicating that these compounds showed no cytotoxicity or pro-proliferation activities on PC12 cells at the test concentration. In contrast, the viability of the PC12 cells was significantly decreased to 65.17% following hypoxia exposure for 24 h (Figure 5(b)). On pretreatment with 6,8,3',5'-FHD, 8,3',5'-THD, or daidzein, the cell viability significantly increased to 78.22 ± 4.82% (p < 0.01), 75.82 ± 3.80% (p < 0.01) and 72.10 ± 4.30% (p < 0.05), respectively, indicating the positive therapeutic effect of 6,8,3',5'-FHD, 8,3',5'-THD, and daidzein against hypoxia-induced damage. Among them, 6,8,3',5'-FHD had the best cytoprotective activity. 8,3',5'-THD also exhibited better cytoprotective activity than that of daidzein (p < 0.01).

Conclusion

In conclusion, 6,8,3',5'-FHD and 8,3',5'-THD have been synthesized for the first time via a simple and effective method using daidzein as the starting material. 6,8,3',5'-FHD and 8,3',5'-THD showed stronger antioxidant abilities compared with that of VC. In addition, they exhibited better protective effects on hypoxia-induced injury in PC12 cells in comparison with daidzein. These results indicate that 6,8,3',5'-FHD and 8,3',5'-THD are outstanding antioxidant agents and can be used for alleviating injury-induced by hypoxia.

Experimental section

Materials and apparatus

Daidzein (purity >98% by HPLC) was purchased from Ci Yuan Biotechnology Co., Ltd. (Shaanxi, China). Other reagents and solvents were purchased from commercial sources and used without further purification. Melting points were measured using an X-4B melting point apparatus. Flash column chromatography was performed on silica gel (200–300 mesh). Infrared (IR) spectra were recorded on a Bruker ALPHA FTIR spectrophotometer (Billerica, MA, USA). ¹H NMR and ¹³C NMR spectra were recorded on a Bruker Avance III high-definition (HD) spectrometer in CDCl₃ or DMSO-d₆ with tetramethylsilane (TMS) as the internal standard. HRMS was performed on an Apex II instrument by means of the electrospray ionization (ESI). Low-resolution mass spectra were recorded on a liquid chromatography–mass spectrometry LC-MS-API 3200 mass spectrometer in ESI mode. The purity was analyzed using ultra-high-performance liquid chromatography (UHPLC) with a Thermo Acclaim-C18 column (100 × 2.1 mm, 2.2 µm, Waltham, MA, USA).

Chemistry

Synthesis of 8,3',5'-tribromo-7,4'-dihydroxyisoflavone (1). To a stirring solution of daidzein (20 mmol, 5.08 g) in CH₂Cl₂ (200 mL) and ethanol (20 mL) was added dropwise Br₂ (4.1 mL). The reaction mixture was stirred at room temperature (RT, 25 °C) for 2.5 h, followed by treatment with saturated NaHSO₃ solution (5 mL) to remove any remaining traces of Br₂. The organic solvent was removed under reduced pressure to give a precipitate, which was collected by filtration, washed with distilled water, dried under vacuum, and washed with hot ethyl acetate to afford compound 1 as white powdery solid (yield 9.2 g, 92%); m.p. 253–254 °C. IR (KBr) v_max: 3450, 3074, 2927, 1619, 1594, 1434, 1285, 1238, 1216, 1118, 1076, 783 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆): δ 11.70 (s, 1H), 10.11 (s, 1H), 8.62 (s, 1H), 7.97 (d, J=8.8 Hz, 1H, H-5), 7.80 (s, 2H, H-2'-H, H-6'), and 7.14 (d, J=8.8 Hz, 1H, H-5). The ¹H NMR data are in good agreement with the literature.¹⁹ ESI-MS: m/z=488.7 [M+H]+.

Synthesis of 8,3',5'-tribromo-7,4'-dimethoxyisoflavone (2). To a stirring solution of compound 1 (4.90 g, 10 mmol) in dry acetonitrile (200 mL) and ethanol (20 mL) was added dropwise Br₂ (4.1 mL). The reaction mixture was stirred at room temperature (RT, 25 °C) for 2.5 h, followed by treatment with saturated NaHSO₃ solution (5 mL) to remove any remaining traces of Br₂. The organic solvent was removed under reduced pressure to give a precipitate, which was collected by filtration, washed with distilled water, dried under vacuum, and washed with hot ethyl acetate to afford compound 1 as white powdery solid (yield 9.2 g, 92%); m.p. 253–254 °C. IR (KBr) v_max: 3450, 3074, 2927, 1619, 1594, 1434, 1285, 1238, 1216, 1118, 1076, 783 cm⁻¹. ¹H NMR (400 MHz, DMSO-d₆): δ 11.70 (s, 1H), 10.11 (s, 1H), 8.62 (s, 1H), 7.97 (d, J=8.8 Hz, 1H, H-5), 7.80 (s, 2H, H-2'-H, H-6'), and 7.14 (d, J=8.8 Hz, 1H, H-5). The ¹H NMR data are in good agreement with the literature.¹⁹ ESI-MS: m/z=488.7 [M+H]+.
The resulting mixture was cooled to RT, quenched with ammonium hydroxide (2 mL), and then diluted with water (150 mL). The acetone was removed under reduced pressure. The crude residue was filtered, washed with water, and dried under vacuum to afford compound 2 (yield 5.06 g, 98%) as a white powdery solid: m.p. 295–296°C. IR (KBr) \( \nu_{\text{max}} \): 2981, 2946, 1655, 1616, 1597, 1424, 1284, 1273, 1217, 1082, 1051, 784 cm\(^{-1}\). \( ^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) 8.26 (s, J = 8.8 Hz, 1H, H-5), 8.06 (s, 1H, H-2), 7.74 (s, 2H, H-2', H-6'), 7.05 (s, 1H, H-10), 6.82 (s, 3H, –OCH\(_3\)), 3.93 (s, 3H, –OCH\(_3\)). 13C NMR (100 MHz, CDCl\(_3\)): \( \delta \) 175.5 (C-4), 156.5 (C-7), 153.2 (C-2'), 152.7 (C-3'), 152.6 (C-5'), 132.9 (C-5), 128.5 (C-2', C-6'), 126.0 (C-1'), 121.4 (C-3), 117.9 (C-10), 112.0 (C-3', C-5'), 110.4 (C-8), 100.2 (C-6'). ESI-HRMS: \( m/z \) = [M + H]\(^+\) calculated for C\(_{15}\)H\(_{10}\)O\(_4\): 298.0734; found: 298.0731.

Synthesis of 8,3',4',5'-pentahydroxyisoflavone. To a stirred solution of daidzein (10 mmol, 2.50 g) in ethanol (150 mL) was added dropwise Br\(_2\) (3.0 mL) at RT. The resulting mixture was then heated at reflux and stirred for 1 h. Water (200 mL) was added to the resulting mixture under boiling conditions. After cooling to RT, the obtained precipitate was collected by filtration, washed with water, and dried under vacuum to give compound 4 (yield 5.5 g, 96%) as a white powdery solid; m.p. >320°C. IR (KBr) \( \nu_{\text{max}} \): 3439, 3085, 1637, 1615, 1593, 1432, 1293, 1283, 1179, 1075, 855 cm\(^{-1}\). \( ^1\)H NMR (400 MHz, DMSO-d\(_6\)): \( \delta \) 10.14 (s, H, C-1), 8.66 (s, 1H, H-5), 8.18 (s, 1H, H-2), 7.80 (s, 2H, H-2', H-6'). 13C NMR (100 MHz, DMSO-d\(_6\)): \( \delta \) 173.6 (C-4), 156.5 (C-7), 155.3 (C-4'), 153.4 (C-9), 151.1 (C-2), 132.9 (C-5), 128.5 (C-2', C-6'), 126.0 (C-1'), 121.4 (C-3), 118.8 (C-10), 112.0 (C-3', C-5'), 110.4 (C-8), 100.2 (C-6'). ESI-HRMS: \( m/z \) = [M + H]\(^+\) calculated for C\(_{15}\)H\(_{10}\)Br\(_2\)O\(_4\): 570.7041; found: 570.7031.

Synthesis of 8,3',4',5'-tetrabromo-7,4'-dimethoxyisoflavone. To a stirred solution of compound 4 (2.85 g, 5 mmol) in dry acetic acid (100 mL) was added anhydrous K\(_2\)CO\(_3\) (2.07 g, 15 mmol) and dimethyl sulfate (1.5 mL, 15 mmol). The reaction mixture was heated to reflux and stirred for 6 h. The resulting mixture was cooled to RT, quenched with ammonium hydroxide solution (1.5 mL), and then diluted with water (100 mL). The acetone was removed under reduced pressure. The crude residue was filtered, washed with distilled water, and dried under vacuum to give compound 5 (yield 2.48 g, 95%) as a white powdery solid: m.p. 209–210°C. IR (KBr) \( \nu_{\text{max}} \): 2944, 2830, 1653, 1615, 1543, 1471, 1428, 1408, 1268, 1232, 870, 741, 659 cm\(^{-1}\). \( ^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) 8.46 (s, 1H, H-2), 8.10 (s, 1H, H-5), 7.71 (s, 2H, H-2', H-6'), 4.02 (s, 3H, –OCH\(_3\)), 3.92 (s, 3H, –OCH\(_3\)). 13C NMR (100 MHz, CDCl\(_3\)): \( \delta \) 173.7 (C-4), 159.0 (C-7), 154.4 (C-4'), 153.5 (C-9), 153.2 (C-2), 132.9 (C-5), 129.6 (C-2', C-6'), 129.4 (C-1'), 122.9 (C-3'), 122.4 (C-10), 118.3 (C-6), 115.9 (C-3', C-5'), 108.0 (C-8), 61.2 (–OCH\(_3\)), 60.7 (–OCH\(_3\)). ESI-HRMS: \( m/z \) = [M + H]\(^+\) calculated for C\(_{15}\)H\(_{10}\)Br\(_2\)O\(_4\): 598.7345; found: 598.7344.

Synthesis of 6,7,8,3',4',5'-hexamethoxyisoflavone. A solution of 5% sodium methoxide in methanol (50 mL) was added to the suspension of CuBr (1.43 g, 10 mmol) in DMF (4 mL). The mixture was stirred at RT for 1 h and then added to a solution of 6 (1.19 g, 2 mmol) in DMF (3 mL) at 120°C. After stirring for 4 h, the reaction mixture was poured into ice water, adjusted to pH 6 using 2 M HCl (aq), and extracted with ethyl acetate (50 mL × 3). The organic phase was dried over anhydrous Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (petroleum ether/ethyl acetate, v/v, 2/1) to give compound 6 (yield 1.01 g, 68%) as white solid needles: m.p. 142–143°C. IR (KBr) \( \nu_{\text{max}} \): 3385, 1627, 1604, 1511, 1474, 1449, 1407, 1325, 1181, 1023, 798 cm\(^{-1}\). \( ^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta \) 8.43 (s, 1H, H-2), 7.80 (s, 2H, H-2', H-6'), 4.02 (s, 3H, –OCH\(_3\)), 3.92 (s, 3H, –OCH\(_3\)). 13C NMR (100 MHz, CDCl\(_3\)): \( \delta \) 174.5 (C-4), 156.5 (C-7), 153.2 (C-2), 152.7 (C-3'), 152.7 (C-5'), 132.9 (C-5), 128.5 (C-2', C-6'), 126.0 (C-1'), 121.4 (C-3), 118.0 (C-10), 117.9 (C-1'), 115.9 (C-3', C-5'), 108.0 (C-8), 61.2 (–OCH\(_3\)), 60.7 (–OCH\(_3\)). ESI-HRMS: \( m/z \) = [M + H]\(^+\) calculated for C\(_{20}\)H\(_{20}\)O\(_7\): 373.1282; found: 373.1280.
aqueous methanol to afford a precipitate, which was collected by filtration 2 h at RT. The organic solvent was removed under vacuum to give a white solid needles; m.p. 320 °C. IR (KBr) vmax = 3230, 1607, 1540, 1485, 1372, 1335, 1297, 1033, 843 cm−1. 1H NMR (400 MHz, DMSO-d6) δ 8.22 (s, 1H, H-2), 7.00 (s, 1H, H-8), 6.53 (s, 2H, H-2', H-6'), 13C NMR (100 MHz, DMSO-d6): δ 175.1 (C-4), 152.6 (C-2), 146.1 (C-3', C-5'), 144.9 (C-9), 141.8 (C-7), 140.1 (C-6), 134.1 (C-8), 133.4 (C-4'), 123.2 (C-1'), 123.0 (C-3), 116.7 (C-10), 108.5 (C-2', C-6'), 99.0 (C-5), 62.0 (–OCH3), 61.5 (–OCH3), 60.9 (–OCH3), 56.3 (–OCH3), 56.2 (2C, –OCH3). ESI-HRMS: m/z = [M+H]+ calcd for C19H22O6: 403.1387; found: 403.1393.

Synthesis of 6,7,8,3',4',5'-hexahydroxyisoflavone (6,8,3',5'-FHD). Dimethyl sulfide (700 µL) was slowly added drop-wise to a stirred solution of AlCl3 (1.33 g, 10 mmol) in CH2Cl2 (15 mL) at 5°C. Compound 6 (242 mg, 0.6 mmol) was added, and the reaction mixture was stirred at RT for 12 h. After cooling to 0°C, 10% HCl (30 mL) was added, and the resulting mixture was stirred for an additional 2 h at RT. The organic solvent was removed under vacuum to give a precipitate, which was collected by filtration, washed with distilled water, and recrystallized from methanol (A) and H2O (0.01% acetic acid, B) with a gradient elution using methanol (A) and H2O (0.01% acetic acid, B) with the following gradient combination: 30% A for 6,8,3',5'-FHD and 20% A for 6,8,3',4',5'-FHD. The flow rate is 0.2 mL min−1; the injection volume is 10 μL; the wavelength is 254 nm; and the column temperature is 25 °C.

Purity test
The purity was analyzed using a Thermo Scientific UltiMate 3000 UHPLC system with a Thermo Acclaim-C18 column (100 × 2.1 mm, 2.2 μm). Isocratic elution was performed using methanol (A) and H2O (0.01% acetic acid, B) with the following gradient combination: 30% A for 8,3',5'-THD and 20% A for 6,8,3',5'-FHD. The flow rate is 0.2 mL min−1; the injection volume is 10 μL; the wavelength is 254 nm; and the column temperature is 25 °C.

Antioxidant activity
DPPH radical scavenging assay. The DPPH radical scavenging assay was performed according to the reported method with slight modifications. In brief, 100 μL of the sample in DMSO at various concentrations (0.0625–2.0 mmol mL−1) was mixed with 50 μL of sodium nitroprusside (20 mmol L−1 in phosphate buffer, pH 7.4) were added to a well-plate and shaken. After incubation under light for 1.5 h at RT, 50 μL of 0.33% (w/v) sodium nitroprusside (20 mmol L−1 in phosphate buffer, pH 7.4) were added to a well-plate and shaken. After incubation at RT for 30 min at 30 °C. The absorbance of the resulting solution was recorded at 517 nm using a microplate reader (SpectraMax i3, Molecular Devices). The NO radical scavenging rate was calculated according to equation (1), where As is the absorbance of the sample control (containing all reagents except samples), A is the absorbance of the sample control (containing all reagents except samples). The antioxidant activity is expressed as the value IC50 value (mmol L−1), which is calculated as the concentration of sample required to scavenge 50% of the DPPH radicals. A low IC50 value corresponded with higher antioxidant activity.

Superoxide anion (O2−) radical scavenging assay. The O2− scavenging activity was performed using the phenazine methosulfate (PMS)–nicotinamide adenine dinucleotide (NADH)–nitrotetrazolium blue chloride (NBT) system with slight modifications. In brief, 100 μL of the sample in DMSO at various concentrations (0.015625–1.0 mmol mL−1), 50 μL of NBT solution (0.2 mM) in distilled water, and 50 μL of NADH solution (0.5 mmol L−1) in 0.1 M Tris–HCl, pH 8.0 were added to a 96-well plate. PMS solution (50 μL, 25 μM) in distilled water was added to the mixture, which was shaken and then incubated for 10 min at RT. The absorbance of the resulting solution at 570 nm was recorded using a microplate reader (SpectraMax i3, Molecular Devices). The superoxide scavenging rate was calculated using equation (1), where As is the absorbance of the sample control (containing all reagents except samples), and the other parameters are the same as mentioned above.

Reducing power assay. The reducing power assay was performed according to a previous reported method with slight modification. In brief, 100 μL of the sample in DMSO at various concentrations (0.03125–1.0 mmol L−1) were mixed with 2.5 mL of 0.2 mol L−1 sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide and incubated for 30 min at 50 °C. After cooling to RT, 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer fraction (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride and reacted at RT for 30 min. The absorbance was recorded at 700 nm using a UV-Vis spectrophotometer (NanoPhotometer-NP80, Implen, Westlake Village, CA, USA). A higher absorbance indicated a stronger reducing power.
Phosphomolybdenum assay. The TAC of the sample was determined using the phosphomolybdate method. In brief, 100 μL of the sample in DMSO at various concentrations (0.03125–1.0 mmol L⁻¹) was mixed with 1.0 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). After incubation at 95 °C for 90 min and then rapid cooling to RT, the absorbance of the resulting mixture was recorded at 695 nm using UV-Vis spectrophotometry (NanoPhotometer-NP80, Implen). A higher absorbance indicates a higher total antioxidant activity. Five assays were performed using Vitamin (VC) as the positive control. All samples were analyzed in triplicate.

Antihypoxia activity

Cell culture and treatment. Rat pheochromocytoma (PC12) cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). As a normoxic control, cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Bioss, Beijing, China) supplemented with 10% fetal bovine serum (FBS) (Bioss) and 1% streptomycin–penicillin (Bioss) in a humidified incubator with 95% air and 5% CO2 at 37 °C. To simulate hypoxia injury, medium was discarded and then 100 μL of fresh medium containing 1% O2, 94% N2, and 5% CO2 was added to each well. After incubation for 24 h at 37 °C in a hypoxic incubator containing 1% O2, 94% N2, and 5% CO2.

Cell viability assay. Cell survival assays were carried out using the cell counting kit-8 (CCK-8) according to the manufacturer’s instructions. Samples were dissolved in DMSO and subsequently diluted in DMEM with the final concentration of DMSO less than 0.1% (v/v). PC12 cells were cultured in normoxic or hypoxic conditions for 24 h. The absorbance at 450 nm was obtained using a microplate reader (SpectraMax i3, Molecular Devices). The relative cell viability was calculated as the percentage of normoxic control.

Date analysis

The values presented in the figures are mean values ± standard deviation (SD). All experiments were performed at least three times. Statistical analysis was performed using SPSS 19.0 software.

Declaration of conflicting interests

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Supplemental material

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