Synthesis, Antioxidant, and Antihypoxia Activities of 6,7,8,4’-Tetrahydroxyisoflavone and 6,7,8,3’,4’-Pentahydroxyisoflavone

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Abstract
In the present study, 6,8-dihydroxydaidzein (6,8-DHD or 6,8,4’-tetrahydroxyisoflavone) and 6,8,3’-trihydroxydaidzein (6,8,3’-THD or 6,7,8,3’,4’-pentahydroxyisoflavone) were synthesized via a facile and efficient way using commercially available formononetin as starting material. Their structures were confirmed using spectroscopic analyses (infrared, nuclear magnetic resonance, and mass spectrometry). The purity was checked by ultra-high performance liquid chromatography. Their antioxidant activities were evaluated via 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay and reducing power assay using ascorbic acid (vitamin C) as a reference compound. The antihypoxia capacity was determined by a hypoxia injury model in PC12 cells. Our study revealed that 6,8-DHD and 6,8,3’-THD exhibited higher antioxidant activities than that of vitamin C and could protect PC12 cells against hypoxia-induced damage. These results indicate that 6,8-DHD and 6,8,3’-THD are excellent antioxidant agents and could be used for alleviating injury induced by hypoxia.

Keywords
formononetin, hydroxydaidzein, synthesis, antioxidant activity, antihypoxia activity

Introduction
Daidzein (7,4’-dihydroxyisoflavone; Figure 1), abundantly present in legumes, especially in soybeans, is a naturally occurring isoflavone with a variety of pharmacological activities, such as antioxidant, anti-inflammatory, anti-diabetic, neuroprotective, and cardioprotective activities. The antioxidant activities of daidzein are considered to be responsible in part for its beneficial effects. After absorption, daidzein can be readily metabolized by liver microsomes and converted to hydroxylated metabolites, mainly including 7,3’,4’, 6,7,4’, and 7,8,4’-trihydroxyisoflavones. Recently, these hydroxydaidzein derivatives have attracted more attention due to their higher antioxidant, anti-inflammatory, antitumor, and antibacterial activities than that of daidzein.

Generally, the hydroxyl groups are the main active groups of isoflavone, and increasing the number of hydroxyl groups can enhance the antioxidant capacity. As shown in Figure 1, 6,8-dihydroxydaidzein (6,8-DHD or 6,7,8,4’-tetrahydroxyisoflavone) and 6,8,3’-trihydroxydaidzein (6,8,3’-THD or 6,7,8,3’,4’-pentahydroxyisoflavone) were 2 hydroxylated daidzein derivatives with 4 and 5 hydroxyl groups, respectively, in the structures. 6,8-DHD was first isolated from biotransformation of daidzein by Aspergillus oryzae and exhibited excellent 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity. However, the content (1.5 mg/3 L of culture filtrate) was not very high. While 6,8,3’-THD is a novel compound. Therefore, it is hard or impossible to get enough quantity of 6,8-DHD and 6,8,3’-THD for biological analysis from natural products.

Results and Discussion
The aim of this study was to synthesize and assess the antioxidant and antihypoxia activities of 6,8-DHD and 6,8,3’-THD. Formononetin (7-hydroxy-4’-methoxyisoflavone, 4), which was widely presented in a greater quantity in legumes and could easily be purchased, was used as starting material in this study. As shown in Figure 2, the reaction of formononetin with N-bromosuccinimide (NBS) under various conditions afforded 2 different brominated products. When the formononetin was treated with 3 equivalents of NBS in dimethylformamide (DMF) at 25 °C, 6,8-dibromo-7-hydroxy-4’-methoxyisoflavone (5) was obtained exclusively in 95% yield. While the bromination...
reaction of formononetin with 4 equivalents of NBS in DMF at 80 °C could yield 6,8,3′-tribromo-7-hydroxy-4′-methoxyisoavone (6) in 94% yield. The structures of compounds 5 and 6 were confirmed using 1H-nuclear magnetic resonance (1H-NMR) spectroscopy (Figures S2 and S6). The 1H-NMR spectrum of 5 was suggestive of an isoavone nucleus (H-2, δ 8.20). Two doublets at δ 7.52 and 7.00 were attributable to H-2′, H-6′ and H-3′, H-5′, respectively. A proton signal at δ 8.54 was assigned to H-5. The proton signals of H-6 and H-8 disappeared, indicating that they were replaced by Br. As shown in the 1H-NMR spectrum of 6 (Figure S6), 2 proton signals at δ 8.62 and 8.19 were assigned to H-5 and H-2. Signals for 4 aromatic protons appearing as an ABX spin system at δ 7.84 (d, J = 2.0, H-2′), 7.60 (dd, J = 8.8, 2.0 Hz, H-6′), 7.18 (d, J = 8.4 Hz, H-5′) for the B-ring protons were displayed. The disappearance of proton signals of H-6, H-8, and H-3′ indicated that they were replaced by Br. The molecular formula of 5 and 6 was further confirmed by their 13C-NMR (Figures S3 and S7) and high-resolution mass spectrum (HRMS) (Figures S4 and S8). Subsequently, the methanolysis of compounds 5 and 6 was carried out in the CuBr/CH3ONa/CH3OH system in DMF at 120 °C based on our previously reported method.14 7-Hydroxy-6,8,4′-trimethoxyisoavone (7) and 7-hydroxy-6,8,3′,4′-tetramethoxyisoavone (8) were obtained in yields of 70% and 70%, respectively. The structures of compounds 7 and 8 were readily confirmed by the number of methoxyl signals in 1H-NMR. Three methoxyl signals were observed at δ 4.07, 3.99, and 3.84 for compound 7 (Figure S10). While 4 methoxyl signals occurred at δ 4.07, 3.99, 3.93, and 3.91 for compound 8 (Figure S14). Data of 13C-NMR and HRMS were consistent with the molecular formula. Finally, removal of the methyl groups from compounds 7 and 8 in the AlCl3/CH3SCH3 system provided 6,8-DHD and 6,8,3′-THD in yields of 93% and 92%, respectively. A comparison of the NMR spectrum of compounds 7 and 8, 6,8-DHD and 6,8,3′-THD exhibited no methoxyl unit. The 1H-NMR spectrum of 6,8-DHD showed 4 hydroxyl proton signals at δ 9.53-9.43 (m, 3H) and 9.76 (s, 1H) (Figure S18). While 6,8,3′-THD exhibited 5 hydroxyl proton signals at δ 8.95 (s, 2H), 9.44 (s, 1H), 9.54 (s, 1H), and 9.78 (s, 1H) (Figure S22). The molecular formula of 6,8-DHD and 6,8,3′-THD was further confirmed by their mass spectrometry (MS) or HRMS. Thus, 6,8-DHD and 6,8,3′-THD were synthesized for the first time over 3 steps starting from formononetin in 62% and 61% yields, respectively.

As shown in Figure 3, the purities of 6,8-DHD and 6,8,3′-THD were determined by ultra-high performance liquid chromatography (UHPLC) to be >97%.

In this study, the antiradical activity of 6,8-DHD and 6,8,3′-THD was evaluated using a DPPH scavenging assay, which is a facile method and has been widely applicable for measuring the capacity of samples to act as free radical scavengers.15 As described in Figure 4(A), the DPPH radical scavenging capacity of daidzein was weak and negligible (IC50 = 2 mmol/L). 6,8,3′-THD showed the best DPPH radical scavenging capacity with the IC50 = 0.911 ± 0.033 mmol/L, while...
6,8-DHD also showed good scavenging capacity with the IC\textsubscript{50} = 1.203 ± 0.115 mmol/L. The reference compound vitamin C exhibited moderate potency with the IC\textsubscript{50} = 1.667 ± 0.009 mmol/L. 6,8,3\textsuperscript{′}-THD and 6,8-DHD showed significantly higher scavenging capacity on DPPH than that of vitamin C.

The antioxidant activity of 6,8,3\textsuperscript{′}-THD and 6,8-DHD expressed as the capacity of the donating electrons was determined by reducing power assay. As described in Figure 4(B), the reducing power of all compounds increased with increasing concentrations. At a concentration of 1 mmol/L, these compounds exhibited the highest reducing power. The absorbance values at 700 nm of 6,8,3\textsuperscript{′}-THD, 6,8-DHD, daidzein, and vitamin C were 2.501 ± 0.002, 2.296 ± 0.025, 0.314 ± 0.021, and 2.052 ± 0.114, respectively. The order of reduced power was ranked as 6,8,3\textsuperscript{′}-THD > 6,8-DHD > vitamin C > daidzein.

Based on the results of antioxidant assays, 6,8,3\textsuperscript{′}-THD and 6,8-DHD, which had 5 and 4 phenolic hydroxyl groups, were found to exhibit significantly higher antioxidant activity than that of daidzein, which only had 2 phenolic hydroxyl groups. This observation agreed nicely with the theory that the antioxidant capacity of flavonoids was proportional to the number of phenolic hydroxyl groups.\textsuperscript{16} In addition the position of the hydroxyl group also affected the antioxidant activity. It has been proved that the ortho-trihydroxyl group and ortho-dihydroxy group have a positive effect on antioxidant activity.\textsuperscript{17} Accordingly, 6,8,3\textsuperscript{′}-THD exhibited the best antioxidant capacity, which might ascribe to the presentence of the ortho-trihydroxyl group at ring A ring and ortho-dihydroxy group at ring B. Compared with 6,8,3\textsuperscript{′}-THD, 6,8-DHD exhibited a little weaker antioxidant activity because of the absence of the ortho-dihydroxy group at ring B.

Hypoxia-induced reactive oxygen species overproduction,\textsuperscript{18} and administration of antioxidants may exert protection against hypoxia-induced cell death.\textsuperscript{19} Therefore, we further evaluate the protective effect of 6,8-DHD and 6,8,3\textsuperscript{′}-THD on hypoxia-treated PC12 cells. As shown in Figure 5(A), treatment with 6,8-DHD, 6,8,3\textsuperscript{′}-THD, or daidzein at a concentration of 1 μmol/L did not exert any cytotoxic or proliferative effects on the PC12 cells under normoxic conditions. However, hypoxia exposure for 24 h significantly reduced the viability of the

Figure 3. UHPLC spectra of 6,8-DHD (A) and 6,8,3\textsuperscript{′}-THD (B).

Abbreviations: UHPLC, ultra-high performance liquid chromatography; 6,8-DHD, 6,8-dihydroxydaidzein; 6,8,3\textsuperscript{′}-THD, 6,8,3\textsuperscript{′}-trihydroxydaidzein.
PC12 cells (Figure 5(B)). Compared to the hypoxia group, pre-treated with 6,8-DHD, 6,8,3′-THD or daidzein significantly improved the cell viability (\(P < .01\) or \(P < .05\)), indicating the therapeutic effect of 6,8-DHD, 6,8,3′-THD, and daidzein against hypoxia-induced damage. The cytoprotective activity of daidzein was similar to that of 6,8-DHD \((P > .05)\), but was lower than 6,8,3′-THD \((P < .05)\). Nevertheless, no significant differences were observed between the 6,8-DHD and 6,8,3′-THD groups.

**Conclusions**

In conclusion, 6,8-DHD and 6,8,3′-THD were synthesized for the first time in a facile and efficient way utilizing formononetin as starting material. 6,8-DHD and 6,8,3′-THD showed stronger antioxidant capacities than that of daidzein and vitamin C. In addition, they also exhibited excellent protective activities against damage induced by hypoxia in PC12 cells. These results indicate that 6,8-DHD and 6,8,3′-THD are outstanding antioxidant agents and can be used for alleviating injury induced by hypoxia.

**Experimental**

**General**

Flash column chromatography was performed on silica gel (200-300 mesh). The melting points were measured on an
X-4B melting point equipment without correction. Infrared (IR) spectra were recorded using Bruker ALPHA II Fourier transform infrared spectroscopy. The $^1$H-NMR and $^{13}$C-NMR spectra were recorded using a Bruker Avance III HD spectrometer using CDCl$_3$ or dimethyl sulfoxide-d$_6$ (DMSO-d$_6$) as solvents. The chemical shifts (δ) were expressed in parts per million (ppm) and were referenced to the signals of tetramethylsilane. A high-resolution mass spectrum (HRMS) was acquired on the Bruker Apex Fourier transform ion cyclotron resonance mass spectrometer. A low-resolution mass spectrum was acquired on an LCMS-API 3200 mass spectrometer.

Reagents and Materials

Formononetin (purity >98% by high-performance liquid chromatography) was purchased from Ci Yuan Biotech Co., Ltd. Other commonly used reagents were purchased from commercial sources, and no further purification was carried out.

6,8-Dibromo-7-Hydroxy-4'-Methoxyisoflavone (5)

To a stirring solution of formononetin (2.68 g, 10 mmol) in DMF (40 mL) at 25 °C was added NBS (3.60 g, 25 mmol). The resulting mixture was stirred for 1.5 h and then poured into 100 mL of cold 2 M hydrochloric acid (HCl). Then 250 mL of distilled water was added. The precipitate was filtered, washed with distilled water, and dried under reduced pressure to afford compound 5 (4.04 g, 95%) as a white powdery solid, which was purified by recrystallization from petroleum ether-EtOAc (1:1) and then used for the following characterization. Melting point (MP): 278.0 °C-278.8 °C. IR(KBr): 3411, 1637, 1606, 1517, 1494, 1376, 1345, 1299, 1231, 1103, 1026, and 834 cm$^{-1}$. Please see Figure S9. $^1$H-NMR (400 MHz, CDCl$_3$) δ: 8.00 (s, 1H, H-2'), 7.52 (d, J = 8.8 Hz, 2H, H-3', H-5'), 7.47 (s, 1H, 7-OH), 7.18 (d, J = 8.4 Hz, 1H, H-6'), 6.93 (d, J = 8.4 Hz, 1H, H-5), 4.07 (s, 3H, −OCH$_3$). Please see Figure S10. $^{13}$C-NMR (100 MHz, CDCl$_3$) δ: 175.58 (C-4'), 159.58 (C-4'), 151.75 (C-2'), 146.18 (C-6'), 145.92 (C-9'), 143.83 (C-3'), 134.74 (C-8), 130.17 (C-2', C-6'), 124.25 (C-1'), 124.21 (C-3), 117.38 (C-10), 113.97 (C-3', C-5'), 99.85 (C-5), 61.64 (−OCH$_3$), 55.34 (−OCH$_3$). Please see Figure S11. ESI-HRMS m/z [M+H]$^+$ for C$_{16}$H$_{10}$Br$_2$O$_4$: 541.0002; found: 541.0004. Please see Figure S12.

7-Hydroxy-6,8,4'-Trimethoxyisoflavone (7)

CaBr (1.75 g, 12.5 mmol) was suspended in 4 mL of DMF and stirred in the dark for 0.5 h at 25 °C. A solution of 25% sodium methoxide in methanol (60 mL) was added to the mixture, which was stirred in the dark for another 1 h. The resulting mixture was added to a stirring solution of compound 5 (2.13 g, 5 mmol) in DMF (5 mL) at 120 °C. The reaction mixture was stirred for 3 h and then cooled to 25 °C, poured into ice water, adjusted to pH 6 using 2 M HCl (aq), and extracted with EtOAc (70 mL×3). The combined organic phase was dried with anhydrous Na$_2$SO$_4$, filtered, and evaporated in vacuo. The crude product was purified by silica gel chromatography (petroleum ether-CH$_2$Cl$_2$-EtOAc, 4:1:2) to afford compound 7 (1.15 g, 70%) as a white powdery solid. MP: 199.4 °C-200.1 °C. IR(KBr): 3092, 2939, 1610, 1512, 1470, 1427, 1299, 1231, 1103, 1026, and 834 cm$^{-1}$. Please see Figure S9. $^1$H-NMR (400 MHz, CDCl$_3$) δ: 8.00 (s, 1H, H-2), 7.50 (d, J = 8.8 Hz, 2H, H-2', H-6'), 7.47 (s, 1H, 7-OH), 7.18 (d, J = 8.4 Hz, 1H, H-6'), 6.93 (d, J = 8.4 Hz, 1H, H-5), 4.07 (s, 3H, −OCH$_3$), 3.99 (s, 3H, −OCH$_3$), 3.84 (s, 3H, −OCH$_3$). Please see Figure S10. $^{13}$C-NMR (100 MHz, CDCl$_3$) δ: 175.58 (C-4'), 159.58 (C-4'), 151.75 (C-2'), 146.18 (C-6'), 145.92 (C-9'), 143.83 (C-3'), 134.74 (C-8), 130.17 (C-2', C-6'), 124.25 (C-1'), 124.21 (C-3), 117.38 (C-10), 113.97 (C-3', C-5'), 99.85 (C-5), 61.64 (−OCH$_3$), 55.34 (−OCH$_3$). Please see Figure S11. ESI-HRMS m/z [M+H]$^+$ for C$_{16}$H$_{14}$O$_6$: 329.1021; found: 329.1020. Please see Figure S12.

7-Hydroxy-6,8,3'-Tetramethoxyisoflavone (8)

Compound 8 was synthesized via a similar procedure as compound 3 employing CaBr (2.80 g, 20 mmol) and compound 6 (2.52 g, 5 mmol). The crude product was purified by silica gel chromatography (petroleum ether-CH$_2$Cl$_2$-EtOAc, 3:1:2) to obtain compound 8 (1.25 g, 70%) as a white powdery solid. MP: 184.2 °C-184.8 °C. IR(KBr): 3411, 1637, 1606, 1517, 1474, 1318, 1255, 1217, 1022, 849, and 809 cm$^{-1}$. Please see Figure S13. $^1$H-NMR (400 MHz, CDCl$_3$) δ: 8.03 (s, 1H, H-2), 7.47 (s, 1H, H-5), 7.24 (d, J = 2.0 Hz, 1H, H-2'), 7.05 (dd, J = 8.0, 2.0 Hz, 1H, H-6'), 6.93 (d, J = 8.4 Hz, 1H, H-5'), 4.07 (s, 3H, −OCH$_3$), 3.99 (s, 3H, −OCH$_3$), 3.95 (s, 3H, −OCH$_3$), 3.91 (s, 3H, −OCH$_3$). Please see Figure S14. $^{13}$C-NMR
(101 MHz, CDC13) δ 175.61 (C-4), 151.92 (C-2), 149.10 (C-3′), 148.75 (C-4′), 146.15 (C-6), 145.98 (C-9), 143.89 (C-7), 134.76 (C-8), 124.66 (C-1′), 124.21 (C-3), 121.00 (C-6′), 117.35 (C-10), 112.57 (C-2′), 111.16 (C-5′), 99.76 (C-5), 61.64 (−OCH3), 56.51 (−OCH3), 55.94 (2−OCH3). Please see Figure S15. ESI-HRMS m/z [M+H]⁺ calcd for C15H11O7 303.0499, found 303.0502. Please see Figure S16.

6,7,8,4′-Tetrahydroxyslawonol (6,8-DHD, 2)

To a stirred solution of AlCl3 (269 mg, 2.25 mmol) in CH2Cl2 (12 mL) at 5 °C, 30 was added dropwise 150 µL of dimethyl sulfoxide (DMS, CH3SCH3). Then compound 7 (98 mg, 0.3 mmol) was added to the reaction mixture and stirred at 25 °C for 6 h. After cooling to 0 °C, 10% HCl (15 mL) was then added to the mixture, which was then stirred for 2 h at 25 °C. The organic solvent was concentrated in vacuo to form the precipitate, which was washed with distilled water to obtain 6,8-DHD (80 mg, 93%) as a white needle solid. MP: >320 °C. IR(KBr): 3460, 3131, 1601, 1480, 1349, 1283, 1215, 1187, 1117, and 851 cm−1. Please see Figure S17. 1H-NMR (400 MHz, DMSO-d6) δ: 9.76 (s, 1H, 4′-OH), 9.53-9.43 (m, 3H, 6-OH, 7-OH, 8-OH), 8.29 (s, 1H, H-2), 7.39 (d, J = 8.8 Hz, 2H, H-3′, H-6′), 7.01 (s, 1H, H-5), 6.81 (d, J = 8.6 Hz, 2H, H-3′, H-5′). Please see Figure S18. 13C-NMR (101 MHz, DMSO-d6) δ: 175.19 (C-4), 157.44 (C-4′), 152.68 (C-2), 144.97 (C-9), 141.95 (C-8), 140.15 (C-7), 134.17 (C-6′), 130.58 (C-2′, C-6′), 123.50 (C-3), 122.87 (C-1′), 116.70 (C-5), 115.34 (C-3′, C-5′), 99.04 (C-10). Please see Figure S19. MS (ESI) m/z: 287.2 ([M+H]⁺). Please see Figure S20. The MS and NMR data mentioned above were consistent with those that were previously reported.12

6,7,8,3′,4′-Pentahydroxyisoflavone (6,8,3′-THD, 3)

6,8,3′-THD was synthesized by a similar procedure as for 6,8-DHD employing 300 µL of DMS, AlCl3 (533 mg, 4 mmol), and compound 8 (143 mg, 0.4 mmol) for 8 h.

6,8,3′-THD was obtained as a white needle solid (111 mg, 92%). MP: >320 °C. IR(KBr): 3460, 3131, 1601, 1480, 1349, 1283, 1215, 1187, 1117, and 851 cm−1. Please see Figure S21. 1H-NMR (400 MHz, DMSO-d6) δ: 9.78 (s, 1H, −OH), 9.34 (s, 1H, −OH), 9.44 (s, 1H, −OH), 8.95 (s, 2H, −OH), 8.25 (s, 1H, H-2), 7.03 (d, J = 2.0 Hz, 1H, H-2′), 6.99 (s, 1H, H-5), 6.81 (dd, J = 8.0, 2.0 Hz, 1H, H-6′), 6.76 (d, J = 8.4 Hz, 1H, H-5′). Please see Figure S22. 13C-NMR (100 MHz, DMSO-d6) δ: 175.15 (C-4), 152.62 (C-2), 145.52 (C-3′), 145.17 (C-4′), 144.94 (C-9), 141.88 (C-7), 140.12 (C-6), 134.15 (C-8), 123.96 (C-1′), 123.01 (C-3′), 120.34 (C-6′), 117.14 (C-10), 116.70 (C-2′), 115.70 (C-5′), and 99.04 (C-5). Please see Figure S23. ESI-HRMS m/z [M+H]⁺ calcd for C13H11O7 303.0499, found 303.0502. Please see Figure S24.

Purity Test

Purity was analyzed using a Thermo Ultimate 3000 UHPLC system equipped with an Acclain-C18 column (particle size = 2.2 µm, dimensions = 100 mm, and pore size = 2.1 nm) maintained at 25 °C. The mobile phase was composed of water (0.01% acetic acid)/methanol (30:60, v/v). Then 10 µL of the sample dissolved in methanol was injected. The ultraviolet detection wavelength was 254 nm and the flow rate was 0.2 mL/min.

DPPH Radical Scavenging Assay

The DPPH radical scavenging activity of 6,8-DHD and 6,8,3′-THD was evaluated according to the reported method with slight modifications.20 Then 100 µL of different concentrations (0.0625-2.0 mmol/L) of samples in DMSO and 100 µL of DPPH solution (100 µM) in methanol were added to a 96-well plate, shaken vigorously, and reacted in the dark for 0.5 h at 25 °C. The absorbance of the resulting solution was recorded on a microplate reader (Spectramax i3 Multi-Mode Detection Platform, Molecular Devices) at a wavelength of 517 nm. Vitamin C was used as the reference compound. The DPPH radical inhibition ratio was assessed using the following equation:

DPPH inhibition ratio (%) = (1 − (A2 − A1)/A0) × 100%

where A0 was the absorbance of the control blank (containing all reagents without samples), and A1 was the absorbance of the sample control (containing all reagents except DPPH), A2 was the absorbance of the sample (containing all reagents with the samples). The assay was performed in triplicate and the results were expressed as IC50 (mmol/L) values.

Reducing Power Assay

The reducing power of 6,8-DHD and 6,8,3′-THD was determined according to the previously reported method with slight modification.21 Then 100 µL of different concentrations (0.03125-1.0 mmol/L) of samples in DMSO were mixed with 2 mol/L sodium phosphate buffer (pH = 6.6, 2.5 mL) and 1% (w/v) potassium ferricyanide (2.5 mL). The resulting mixture was incubated at 50 °C for 0.5 h. After cooling to 25 °C, 10% trichloroacetic acid (2.5 mL) was added to the mixture, which was then centrifuged at 3000 r/min for 10 min. The upper layer (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% FeCl3 solution (0.5 mL) at 25 °C. After 0.5 h, the absorbance was recorded on an ultraviolet-visible spectrophotometer (NanoPhotometer-NP80, Implen) at a wavelength of 700 nm. Vitamin C was used as the reference compound. The assay was performed in triplicate.

Cell Culture and Treatment

The PC12 cells were obtained from the Cell Bank of the Chinese Academy of Sciences and cultured in Dulbecco’s
modified Eagle’s medium (DMEM; Bioss) with 10% (v/v) fetal bovine serum (Bioss), and 1% penicillin-streptomycin (Bioss) in a humidified atmosphere containing 95% N₂ and 5% CO₂ at 37 °C. To establish a hypoxia damage model, the PC12 cells (passage 4-6) were subject to hypoxic conditions (94% N₂, 1% O₂, and 5% CO₂) at 37 °C for 24 h. To evaluate the protective effects of hydroxydaidzein derivatives, PC12 cells were cultured. Following treatment as described above, 90 µL of media and 10% CCK-8 solution (Bioss) was added to each well via media exchange modes. Then, the plates were incubated for an additional 2 h at 37 °C. The optical density was recorded on a microplate reader (Spectramax i3 Multi-Mode Detection Platform, Molecular Devices) at a wavelength of 450 nm. Cell viability was assessed by a cell counting kit-8 (CCK-8).

Cell Viability Assay

The cell viability was assessed by a cell counting kit-8 (CCK-8). PC12 cells were added to 96-well plates (1 × 10⁴ cells/well) and cultured. Following treatment as described above, 90 µL of media and 10% CCK-8 solution (Bioss) was added to each well via media exchange modes. Then, the plates were incubated for an additional 2 h at 37 °C. The optical density was recorded on a microplate reader (Spectramax i3 Multi-Mode Detection Platform, Molecular Devices) at a wavelength of 450 nm. Cell viability was expressed as a percentage of the normoxic control.

Declaration of Conflicting Interests

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Ethical Approval

Ethical Approval is not applicable for this article.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

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Supplemental Material

Supplemental material for this article is available online.

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