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SYNTHESIS AND ANTIOXIDANT PROPERTIES OF 5,6,7,8-TETRAHYDROXYFLAVONE

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Flavones are a group of plant secondary metabolites with multiple biological properties. In the present study, 5,6,7,8-tetrahydroxyflavone (5,6,7,8-THF) was synthesized and characterized. In vitro *antioxidant study, the effect of 5,6,7,8-THF on total antioxidant activity, reducing power, DPPH radical scavenging, ABTS radical scavenging, superoxide radical scavenging, hydroxyl radical scavenging, nitric oxide radical scavenging, and ferrous chelating activities was examined. According to the results, 5,6,7,8-THF showed excellent free radical scavenging effect and reducing power but weak the ferrous chelating activity. In conclusion, 5,6,7,8-THF can be regarded as an excellent source of antioxidants.*

Keywords: flavonoid, 5,6,7,8-tetrahydroxyflavone, synthesis, antioxidant activity, chrysin.

Flavonoids are the most widely distributed natural antioxidants in the plant kingdom, especially in fruits and vegetables, and present lots of biological activities, such as antidiabetic, anti-atherogenic, antitumoral, antimutagenic, antihypertensive, and neuroprotective [1]. Most of these potential health-promoting properties are attributed to their antioxidant activities [2]. Flavonoids can exert their antioxidant activity by various mechanisms, such as scavenging radicals, binding metal ions, and inhibiting enzymatic systems responsible for free radical generation [3].

The basic structure of flavonoids is composed of fused phenyl and pyranyl rings (ring A and C) and a phenyl moiety (ring B) attached to the ring C. The antioxidant activities of various flavonoids have been suggested to be dependent on the number and positions of the hydroxyl (OH) groups on their skeletal carbons [4]. It is reported that the catechol or pyrogallol moiety in the A or B ring is required for flavones to have excellent antioxidant activity [5]. A 2,3-double bond combined with a 4-keto group in the C ring [6] and hydroxyl groups on either positions 3, 7, or 8 can enhance antioxidant activity [7]. It was therefore hypothesized that 5,6,7,8-tetrahydroxyflavone (5,6,7,8-THF), which has four consecutive hydrogen group in the A ring, might exhibit strong antioxidant activity.

The objective of this study was to synthesize and evaluate the antioxidant activity of 5,6,7,8-THF. Firstly, 5,6,7,8-THF was synthesized via three steps using chrysin as starting material. The synthetic procedures are shown in Scheme 1. Chrysin, a simple and commercially available flavone, was directly halogenated by bromination to form 6,8-dibromochrysin in 93% yield. Then the methanolysis of 6,8-dibromochrysin promoted by the system MeO^-/CuBr gave the 5,7-dihydroxy-6,8-dimethoxyflavone in 85% yield. The 5,6,7,8-THF was obtained by the demethylation reaction of 5,7-dihydroxy-6,8-dimethoxyflavone with BBr_3 in anhydrous CH_2Cl_2 in 83% yield. The structures of the synthesized compounds were characterized by ^1H NMR, ^{13}C NMR, and ESI-MS spectra. This synthetic route was simple, effective, and suitable for industrial production.

The antioxidant scavenging capability of the compound *in vitro* has also revealed its strong antioxidant potential in quenching radicals that cause oxidative trauma *in vivo*. There are many methods with different reaction mechanisms that have been developed to evaluate the antioxidant capacity of a sample *in vitro.* As flavonoids have various antioxidant mechanisms, several different antioxidant assays are required to elucidate the full antioxidant activity profile of 5,6,7,8-THF.

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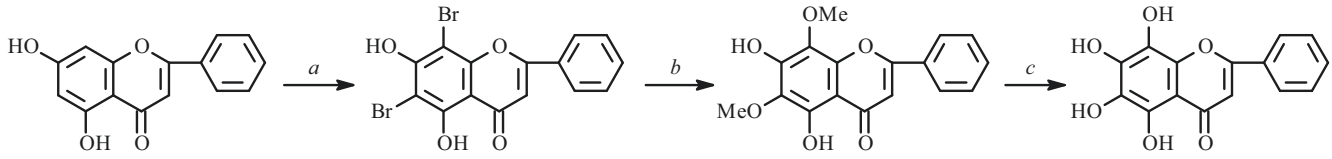

a. Br_2; *b*. CuBr, DMF, CH_3ONa, CH_3OH; *c*. BBr_3, CH_2Cl_2, –15°C – r.t.

Scheme 1

The antioxidant capacity of 5,6,7,8-THF was determined using eight methods, including DPPH, ABTS, hydroxyl radical, superoxide anion, nitric oxide radical scavenging activity, ferrous chelating activity, reducing power, and phosphomolybdenum assay.

The DPPH scavenging activities of antioxidants are attributed to their hydrogen donating abilities. As shown in Fig. 1, *a*, the scavenging activity of 5,6,7,8-THF and vitamin C (Vc) on DPPH radical increased in a dose-dependent manner. At the concentration of 0.5 mmol/mL, the DPPH scavenging activity of 5,6,7,8-THF and Vc was 91.2 and 82.6%. The IC_{50} values for 5,6,7,8-THF and Vc were 0.15 ± 0.01 and 0.30 ± 0.02 mmol/mL, respectively.

In the ABTS assay, 5,6,7,8-THF showed prominent $ABTS^{\cdot+}$ radical scavenging activities, which was higher than that of Vc (Fig. 1, *b*). At the concentration of 0.5 mmol/mL, the inhibition of $ABTS^{\cdot+}$ radical for 5,6,7,8-THF was more than 90%. The IC_{50} values for 5,6,7,8-THF and Vc were 0.24 ± 0.01 and 1.88 ± 0.02 mmol/mL, respectively.

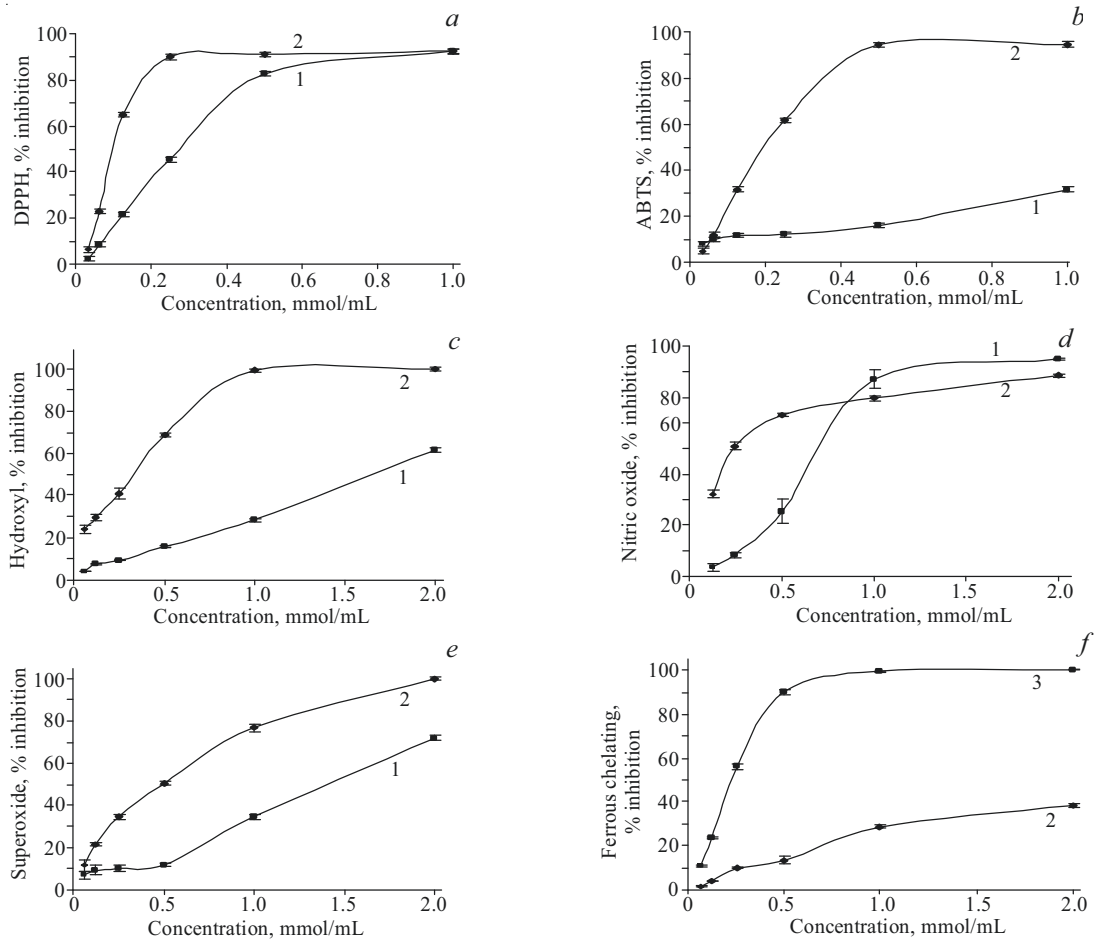

Fig. 1. (*a*) DPPH radical scavenging effect of 5,6,7,8-THF and vitamin C. (*b*) ABTS radical scavenging effect of 5,6,7,8-THF and vitamin C. (*c*) The ·OH scavenging effect of 5,6,7,8-THF and vitamin C. (*d*) Nitric oxide radical scavenging effect of 5,6,7,8-THF and vitamin C. (*e*) Superoxide radical scavenging effect of 5,6,7,8-THF and vitamin C. (*f*) Ferrous chelating activity of 5,6,7,8-THF and EDTA. Each value represents the mean ±SD of triplicate experiments. Vitamin C (1); 5,6,7,8-THF (2); EDTA (3).

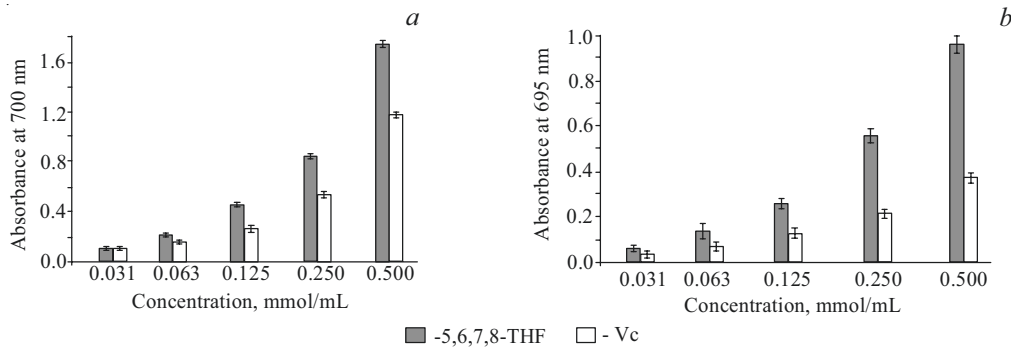

Fig. 2. (*a*) Reductive ability of 5,6,7,8-THF and vitamin C (Vc). (*b*) Total antioxidant activity of 5,6,7,8-THF and vitamin C. Each value represents the mean ± SD of triplicate experiments.

Hydroxyl radicals are regarded as the most damaging radicals in the body and can nonspecifically damage almost all classes of biomacromolecule in living cells. The OH radical scavenging activity of 5,6,7,8-THF and Vc is shown in Fig. 1, *c*. The scavenging activity correlates well with increase in concentration. 5,6,7,8-THF exhibited higher hydroxyl radical scavenging ability, with IC_{50} value 0.36 ± 0.01 mmol/mL, compared to positive control Vc (IC_{50} = 1.66 ± 0.03 mmol/mL).

Superoxide radical is a weak radical *in vivo*, but it can interact with other molecules to generate more powerful and dangerous species, including $^1O_2$, H_2O_2, and OH, causing damage to vital biological macromoleculars such as lipids, proteins, and DNA [8]. Thus, decreasing the concentration of superoxide anions under conditions of oxidative stress is very important for preventing diseases. According to the results (Fig. 1, *d*), inhibition by 5,6,7,8-THF of superoxide radical generation is higher than by Vc. The IC_{50} values for 5,6,7,8-THF and Vc are 0.20 ± 0.01 and 1.38 ± 0.02 mmol/mL, respectively.

Nitric oxide (NO) is an essential bioregulatory molecule with many physiological functions. However, excess NO can react with superoxide anion and form more oxidative and active molecules such as peroxynitrite ion ($ONOO^-$), which causes oxidative damage to lipids, proteins, and DNA [9]. The NO scavenging effect of the samples was determined using Griess reagent. As shown in Fig. 1, *e*, at low concentrations, 5,6,7,8-THF shows higher NO scavenging ability than Vc, but as the concentration increases, the inhibition of NO radical by Vc increases sharply. At a concentration of 1.0 mmol/mL, the NO scavenging activity of Vc was 77.1%, which is higher than that of 5,6,7,8-THF (69.6%). The IC_{50} value for 5,6,7,8-THF was 0.29 ± 0.01 mmol/mL, which is lower than that of Vc (IC_{50} = 0.72 ± 0.04 mmol/mL).

Iron is required for oxygen transport, respiration, and activity of many enzymes in the body. However, iron can catalyze the conversion of hydrogen peroxide to hydroxyl radical via the Fenton reaction, which induces lipid peroxidation and oxidative damage of protein and DNA [10]. Thus, compounds with ferrous chelating ability can inhibit radical generation and protect the body against oxidative damage. The chelating effects of EDTA and 5,6,7,8-THF on ferrous ions increase with increasing concentration (Fig. 1, *f*). EDTA shows very strong chelating capacity with IC_{50} value 0.26 ± 0.01 mmol/mL. At a concentration of 2 mmol/mL, 5,6,7,8-THF exhibited chelating effects of 38.37%. The above-mentioned results suggest that 5,6,7,8-THF exhibits weaker chelating activity on ferrous ions than EDTA.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Results presented in Fig. 2, *a* indicate that the reducing power increases with increasing concentration of 5,6,7,8-THF and Vc. At a concentration of 0.25 mmol/mL, the reducing powers of 5,6,7,8-THF and Vc were 0.84 and 0.53, respectively. The IC_{50} values for 5,6,7,8-THF and Vc are 0.14 ± 0.01 and 0.22 ± 0.02 mmol/mL, respectively. This result indicates that 5,6,7,8-THF has higher reducing power than Vc.

The total antioxidant capacity of the sample was measured by the phosphomolybdenum method, based on the reduction of molybdenum (VI) to molybdenum (V) by the test sample and the subsequent formation of green phosphomolybdenum (V) complex with a maximum absorption at 765 nm under acidic pH conditions [11]. The present study demonstrates that 5,6,7,8-THF exhibits a higher antioxidant capacity for phosphomolybdate reduction than standard Vc. As shown in Fig. 2, *b*, the total antioxidant activity of 5,6,7,8-THF and Vc increased in a dose-dependent manner. The IC_{50} values for 5,6,7,8-THF and Vc are 0.25 ± 0.02 and 0.68 ± 0.02 mmol/mL, respectively.

In the present study, 5,6,7,8-THF, a flavone with four consecutive hydrogen groups in the A ring, was synthesized via a simple and effective method using chrysin as starting material. 5,6,7,8-THF showed excellent antioxidant activity against various antioxidant systems *in vitro*, which was comparable or even better than Vc. The obtained results suggest that the 5,6,7,8-THF can be regarded as an excellent source of antioxidants.

EXPERIMENTAL

Reagents and Materials. Chrysin was purchased from Ci Yuan Biotechology Co., Ltd. (Shaanxi, China). CuBr, BBr_3, sodium nitroprusside, sulfanilamide, naphthylethylenediamine hydrochloride, ammonium molybdate, and potassium persulfate ($K_2S_2O_8$) were obtained from Aladdin Industrial Co. 2,2-Diphenyl-1-picrylhydrazyl radicals (DPPH), nitroblue tetrazolium (NBT), 2,2′-azino-*bis*-(3-ethylbenzothiozoline-6-sulfonic acid) disodium salt (ABTS), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), thiobarbituric acid (TBA), and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co.

Physical Measurements. Melting points (uncorrected) were determined on a micro-melting point apparatus X-4A (Shanghai Cany Precision Instrument Company, China). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 spectrometer with DMSO-d_6 as solvent. Chemical shifts are reported in δ values relative to TMS as internal standard. Coupling constants were in units of Hz. Low-resolution mass spectra (ESI-MS) were obtained on an Applied Biosystems LCMS-API 3200 spectrometer.

Procedure for the Preparation of 6,8-Dibromo-5,7-dihydroxyflavone. The synthesis of 6,8-dibromo-5,7-dihydroxyflavone was carried out according to the Park method with some modifications [12]. To a suspension of chrysin (2.54 g 10 mmol) in 100 mL of CH_2Cl_2, a solution of 1.3 mL of bromine in 20 mL of CH_2Cl_2 was added slowly with magnetic stirring over 30 min at 0°C. The mixture was left under stirring for another 2 h. At the end of reaction, the solvent was removed by evaporation in vacuum, followed by treatment with saturated $NaHCO_3$ solution to remove traces of HBr. The resulting solid was collected and purified by washing with water to give the title compound as a yellow powder. Yield 93%, mp 283.1–284.7°C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 13.70 (1H, s, 5-OH), 11.34 (1H, s, 7-OH), 8.10 (2H, d, J = 7.2, H-2′, 6′), 7.58–7.62 (3H, m, H-3′, 4′, 5′), 7.16 (1H, s, H-3). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 181.5 (C-4), 162.4 (C-7), 157.3 (C-2), 157.0 (C-10), 152.2 (C-5), 132.4 (C-1′), 130.2 (C-3′, 5′), 129.2 (C-4′), 126.4 (C-2′, 6′), 105.1 (C-9), 94.5 (C-6), 88.4 (C-8). ESI-MS *m/z* 412.8 $[M + 1]^+$.

Procedure for the Preparation of 5,7-Dihydroxy-6,8-dimethoxyflavone. The synthesis of 5,7-dihydroxy-6,8-dimethoxyflavone was carried out according to the Bovicelli method with some modications [13]. To a suspension of CuBr (0.7 g, 5 mmol) in DMF (15 mL) a 25% solution of sodium methoxide in methanol (35 mL, 0.15 mol) was added at room temperature and left under stirring for 1 h. The mixture was added to a solution of 6,8-dibromo-5,7-dihydroxyflavone (2.06 g, 5 mmol) in DMF (25 mL) at 120°C in portions. The mixture was left under stirring for 40 min, then cooled to room temperature and carefully poured onto a cold 2 M solution of HCl in water (500 mL). The precipitate was collected and recrystallized from aqueous methanol (5:1) to give the title compound as yellow crystals. Yield 85%, mp 228.7–229.5°C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 12.68 (1H, s, 5-OH), 10.52 (1H, s, 7-OH), 8.07 (2H, d, J = 8.0, H-2′, 6′), 7.59–7.62 (3H, m, H-3′, 4′, 5′), 7.00 (1H, s, H-3), 3.89 (3H, s, CH_3O), 3.79 (3H, s, CH_3O). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 182.4 (C-4), 163.0 (C-2), 151.1 (C-9), 148.3 (C-5), 145.5 (C-7), 132.1 (C-1′), 131.6 (C-6), 130.8 (C-8), 129.2 (C-3′, 5′), 128.0 (C-4′), 126.2 (C-2′, 6′), 104.7 (C-10), 103.2 (C-3), 61.2 (OCH_3), 60.2 (OCH_3). ESI-MS *m/z* 315.2 $[M + 1]^+$.

Procedure for the Preparation of 5,6,7,8-Tetrahydroxyflavone. The demethylation reaction of 5,7-dihydroxy-6,8-dimethoxyflavone was carried out according to the McOmie method with some modications [14]. To a stirred solution of 5,7-dihydroxy-6,8-dimethoxyflavone (314 mg, 1 mmol) in 10 mL anhydrous CH_2Cl_2, 4 mL of 1 M BBr_3 in dichloromethane was added dropwise at –15°C. The reaction mixture was stirred at room temperature for another 12 h. The mixture was cooled to 0°C and quenched with water. Then the CH_2Cl_2 was removed under vacuum. The residue was poured into water. The resulting solid was collected and dried under vacuum and then recrystallized from aqueous methanol (2:1) to give the title compound as an orange powder. Yield 83%, mp 221.3–222.5°C. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 12.22 (1H, s, 5-OH), 10.02 (1H, s, 7-OH), 8.99 (2H, s, 6, 8-OH), 8.18 (2H, d, J = 8.0, H-2′, 6′), 7.59–7.61 (3H, m, H-3′, 4′, 5′), 6.96 (1H, s, H-3). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 182.5 (C-4), 162.7 (C-2), 143.8 (C-9), 139.7 (C-5), 139.0 (C-7), 131.8 (C-1′), 129.7 (C-6, 8), 129.6 (C-3′, 5′), 126.5 (C-4′), 125.6 (C-2′, 6′), 104.1 (C-10), 103.0 (C-3). ESI-MS *m/z* 287.3 $[M + 1]^+$.

DPPH Radical Scavenging Assay. The DPPH radical scavenging activity was carried out in a 96-well microplate using a Spectramax i3 reader according to the Vaz method with some modifications [15]. For this, 150 μL of various concentrations (0.03125–1 mmol/mL) of 5,6,7,8-THF was added to 150 μL of 0.1 mM DPPH radical solution in ethanol and the resulting mixture incubated for 30 min in the dark at room temperature. The absorbance of the mixed solution was measured at 517 nm using a microplate reader (Spectramax i3, Molecular Devices). Ascorbic acid (Vc) was used as positive control. DPPH radical scavenging activity was calculated using the equation

$$\text{DPPH scavenging effect (\%)} = [(A_1 - A_0)/A_1] \times 100, \quad (1)$$

where A_1 is the absorbance of control (DPPH solution without sample) at 517 nm; A_0 is the absorbance at 517 nm of sample at different concentrations with DPPH. The antioxidant activity is expressed as IC_{50} (mmol/mL), the dose of the sample required to cause a 50% decrease in absorbance at 517 nm. A lower IC_{50} value corresponds to a higher antioxidant activity.

ABTS Radical Scavenging Assay. The ABTS radical scavenging capacity was performed according to the method of Re and Pellegrini [16]. ABTS radical cation ($ABTS^{\cdot+}$) was obtained by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) solution and keeping the reaction mixture for 16 h in the dark at room temperature. Prior to use, the stock solution was diluted with methanol in order to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Then 100 μL of various concentrations (0.03125–1 mmol/mL) of 5,6,7,8-THF was mixed with 3.9 mL $ABTS^{\cdot+}$ solution. The reaction mixture was homogenized and incubated for 10 min at room temperature in the dark. The absorbance of the resulting solution at 734 nm was measured, and ascorbic acid (Vc) was used as positive control. Inhibition of ABTS radical was calculated using Eq. (1).

Hydroxyl Radical Scavenging Assay. The hydroxyl radical scavenging assay was performed according to the method of Liu with some modifications [17]. For this, 100 μL of various concentration of 5,6,7,8-THF (0.0625–2 mmol/mL), 50 μL of 9 mM $FeSO_4$, and 50 μL 9 mM salicylic acid–ethanol mixture was mixed. Then 50 μL 60 mM H_2O_2 was added to the mixture and the whole shaken vigorously. After incubation at 37°C for 30 min, the absorbance of the resulting solution was determined at 510 nm. Ascorbic acid was used as a positive control. In the control, the sample was replaced by DMSO. The hydroxyl radical scavenging activity was calculated according to Eq. (1).

Superoxide Radical Scavenging Assay. The superoxide scavenging activity was determined by the PMS–NADH–NBT system with slightly modifications [18]. For this, 50 μL of NBT solution (0.2 mM in distilled water), 50 μL of NADH solution (0.5 mmol/L in 0.1M Tris-HCl, pH 8.0), and 100 μL of 5,6,7,8-THF with different concentrations (0.0625–2 mmol/mL) were mixed and treated with 50 μL of PMS solution (25 μM PMS in distilled water). The reaction mixture was incubated at room temperature for 10 min, and the absorbance at 570 nm was measured. Ascorbic acid was used as positive control. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The percentage of scavenging was calculated according to Eq. (1).

Nitric Oxide Radical Scavenging Assay. The nitric oxide radical scavenging assay was measured by the Griess reaction with some modifications [19]. In brief, 50 μL of various concentrations (0.125–2 mmol/mL) of 5,6,7,8-THF was added to 50 μL of sodium nitroprusside (10 mmol/L in phosphate buffer, pH 7.4). The reaction mixture was incubated under light at room temperature for 150 min. After incubation, 50 μL of 0.33% (w/v) sulfanilamide (in 20% glacial acetic acid) was added and the whole kept standing for 10 min. Then 50 μL 0.1% (w/v) naphthylethylenediamine hydrochloride was added, and the resulting solution was further incubated for 30 min. The absorbance was measured at 540 nm in a microplate reader. Ascorbic acid was used as reference standard. The nitric oxide radical scavenging activity was calculated according to Eq. (1).

Ferrous (Fe^{2+}) Chelating Activity. The chelating ability of ferrous ion by various fractions was measured by the method of [20]. In brief, 1 mL of various concentrations of 5,6,7,8-THF (0.0625–2 mmol/mL), 50 μL of $FeCl_2 \cdot 4H_2O$ solution (2 mM), and 200 μL of ferrozine solution (5 mM) were mixed and shaken vigorously. Then the mixture was allowed to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. EDTA (0.625–2 mmol/mL) served as the positive control. The chelating activity was calculated using Eq. (1).

Reducing Power Assay. The reducing power assay was performed according to the method of Oyaizu [21]. For this, 100 μL of various concentrations (0.03125–1 mmol/mL) of 5,6,7,8-THF was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide. The mixture was incubated for 30 min at 50°C in a water bath, follow by addition of 2.5 mL of 10% TCA. The mixture was 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. The absorbance was read at 700 nm after 10 min. Ascorbic acid was used as positive control. A higher absorbance indicates a higher reducing power. The IC_{50} value (mmol/mL) is the effective concentration giving an absorbance of 0.5 at 595 nm and is obtained by linear regression analysis.

Phosphomolybdenum Assay (Total Antioxidant Activity). Total antioxidant activity of 5,6,7,8-THF was determined by the phosphomolybdate method according to Prieto [11]. For this, 100 μmL of various concentrations (0.03125–1 mmol/mL) of 5,6,7,8-THF was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The reaction mixture was incubated for 90 min at 95°C in a water bath. Then the resulting solution was rapidly cooled to room temperature. The absorbance of the resulting solution was measured at 695 nm. Ascorbic acid was used as positive control. A higher absorbance indicates a higher total antioxidant activity. The IC_{50} value (mmol/mL) is the effective concentration giving an absorbance of 0.5 at 695 nm and is obtained by linear regression analysis.

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