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SYNTHESIS, CHARACTERIZATION, AND ANTIRADICAL ACTIVITY OF 6-HYDROXYGENISTEIN

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A convergent synthesis route of 6-hydroxygenistein (6-OHG) was reported, starting from cheap and readily available biochanin A, via methylation, bromination, methoxylation, and demethylation. The structure of the products was confirmed by MS, IR, ^{1}H NMR, and ^{13}C NMR analysis. The antiradical activity of 6-OHG was determined using six different methods, namely, DPPH assay, ABTS assay, nitric oxide assay, superoxide assay, reducing power assay, and phosphomolybdenum assay using ascorbic acid (V_C) as positive control. The results show that 6-OHG possesses similar or greater antiradical activity than V_C. In conclusion, 6-OHG can be useful as an antioxidant agent.

Keywords: genistein, 6-hydroxygenistein, synthesis, antiradical activity.

Genistein is biosynthetically the simplest isoflavonoid compound of the Leguminosae [1] and exhibits a wide range of biological activities, such as antidiabetic effect [2], neuroprotective effect [3], antioxidant [4], anti-cancer [5], and antimicrobial activity [6]. However, there are some drawbacks, such as low fat solubility, low water solubility [7], low bioavailability [8], and multiple targets, which significantly limit its clinical and therapeutic applications. In order to obtain more pharmacologically active and more selective drugs with less adverse reactions for clinical use, many studies have been performed on the synthesis of genistein derivatives.

Genistein derivatives are mainly modified by glycosylation, alkylation, esterification, and hydroxylation [9, 10]. Among those mentioned above, hydroxylation, an ordinary modification of isoflavones which happens in nature, can produce more complicated isoflavones with stronger bioactivity than their precursors. For example, 3′-hydroxygenistein was demonstrated to be a potent melanogenesis inhibitor from the biotransformation of genistein by recombinant *Pichia pastoris* [11]. In structure–activity relationships, the functions of the isoflavones are influenced by the number and position of hydroxyl groups in the chemical structures. In addition, the bioactivity can be enhanced by growing number of phenolic hydroxyl groups.

6-Hydroxygenistein (**1**, 6-OHG, 4′,5,6,7-tetrahydroxyisoflavone), which has three consecutive hydrogen groups in the A ring, may exhibit strong antioxidant activity. Previous studies have reported that 6-OHG can be isolated from fermented soybean or microbial fermentation broth feeding with soybean meal. But these methods have some disadvantages, including high enzyme requirements, low yield, and complicated process [11, 12]. In addition, the chemical synthesis methods to access **1** are rare. Therefore, new practical and economical methods for the synthesis of **1** are required to be developed.

The purpose of this study was to synthesize and evaluate the antiradical activity of **1**. As shown in Scheme 1, 6-OHG was synthesized via four steps using biochanin A as raw material. Firstly, biochanin A (**2**) was selectively methylated to get 5-hydroxy-4′,7-dimethoxyisoflavone (**3**) using iodomethane (CH_3I) as a methylation agent in 90% yield via controlling the amount of CH_3I and reaction time. Secondly, NBS was used to brominate the C-6 and C-8 positions of compound **3** in good yield (> 80%).

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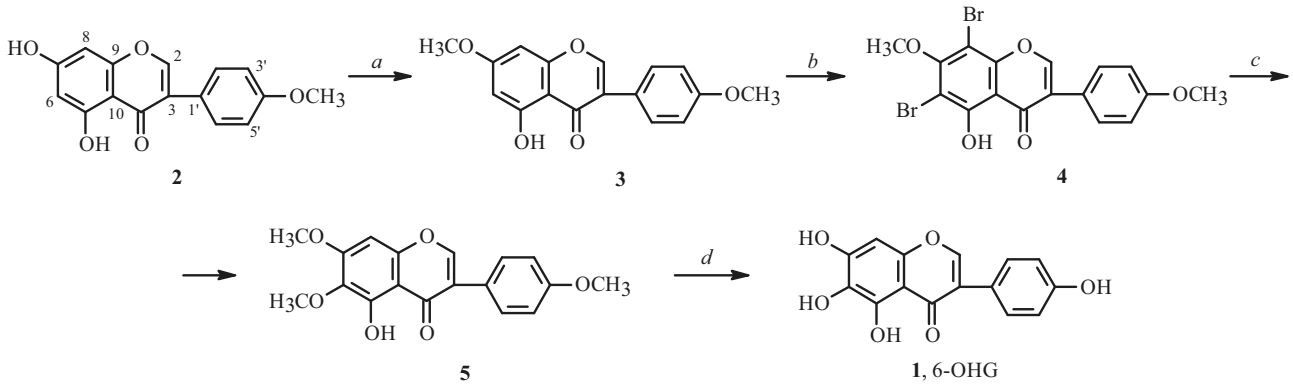

a. CH_3I, K_2CO_3; *b*. 2.2 times NBS, DMF; *c*. CuBr, DMF, CH_3OH, CH_3ONa, *d*. BBr_3, CH_2Cl_2

Scheme 1.

Brominated isoflavone **4** was a key intermediate throughout the synthetic route. Thirdly, the bromo group was replaced with methoxyl groups according to a Ullmann-type reaction with slight modification [13]. In the reaction, the sodium methoxide used to bring about the nucleophilic substitution should be freshly prepared and a light blue-green reaction mixture was got by reacting with copper bromide. Surprisingly, the methanolysis of **4** caused a methoxylation reaction only at the C-6 position. It was speculated that the copper bromide ion formed at the C-8 position in the methoxylation reaction was unstable. We also found that the reaction temperature had great influence on the yield of compound **5**. When the reaction temperature was either too high or too low, the yield decreased significantly. Finally, 6-OHG (**1**) was obtained by the demethylation reaction of **5** with BBr_3 in anhydrous CH_2Cl_2 in 81% yield.

6-OHG was obtained in a total yield of 38% (calculated as biochanin A (**2**)). The purity of **1** was greater than 95% as determined by UHPLC.

In the present study, four common free radicals (DPPH radicals, ABTS radicals, NO radicals, and $O_2^{-\cdot}$ radicals scavenging) assay, reducing power, and the phosphomolybdenum assay were selected to determine and confirm the antioxidant property of 6-OHG (**1**).

The DPPH and ABTS assays, which reflect the hydrogen donating or proton radical scavenging capacities of the target compounds, have been widely used to evaluate the antioxidant capacities of natural compounds. The DPPH radical scavenging activity of **1** and V_C at different concentrations is shown in Fig. 1*A*. Obviously, 6-OHG showed the excellent antioxidant activity on scavenging DPPH radical, being nearly identical to that of V_C. For example, at 1.0 mmol/L, the scavenging rates of **1** and V_C are 64.54 and 76.45%, respectively. On the other hand, the IC_{50} values were calculated as 0.32 mmol/L for 6-OHG and 0.27 mmol/L for V_C. The ABTS radical scavenging activity of 6-OHG (**1**) and V_C at different concentrations is shown in Fig. 1*B*. 6-OHG possesses stronger scavenging activity on ABTS radical than V_C. For example, at 1.0 mmol/L, the scavenging rates of **1** and V_C are 72.08 and 50.30%, respectively. On the other hand, the IC_{50} values are calculated as 0.26 mmol/L for 6-OHG and 0.79 mmol/L for V_C.

Nitric oxide (NO) is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation, and immune regulation. However, excess NO could react with superoxide radical to form a second reactive compound, peroxynitrite anion ($ONOO^-$), which is directly toxic to tissues, resulting in vascular damage and other ailments. The NO radical scavenging activity of **1** and V_C at different concentrations is shown in Fig. 1*C*. At 1.0 mmol/L, the scavenging rates of **1** and V_C were 76.48 and 51.01%, respectively. When the concentration is increased, the NO scavenging rate changes slowly. The IC_{50} values are calculated as 0.25 mmol/L for 6-OHG and 1.14 mmol/L for V_C. The results indicated that **1** had significantly higher NO radical scavenging effect than V_C.

Superoxide ($O_2^{-\cdot}$) is known to be a primary ROS and is widespread in the human body. $O_2^{-\cdot}$ is a weak free radical, but it is the main source of other highly reactive ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and hydroxyl anions (OH^-) [14]. Compounds with the ability to remove superoxide can prevent oxidative stress induced injury. As shown in Fig. 1*D*, the superoxide radical scavenging ability of 6-OHG increases with increasing concentration. The IC_{50} values is 0.48 mmol/L for 6-OHG, which is significantly lower than V_C (0.69 mmol/L).

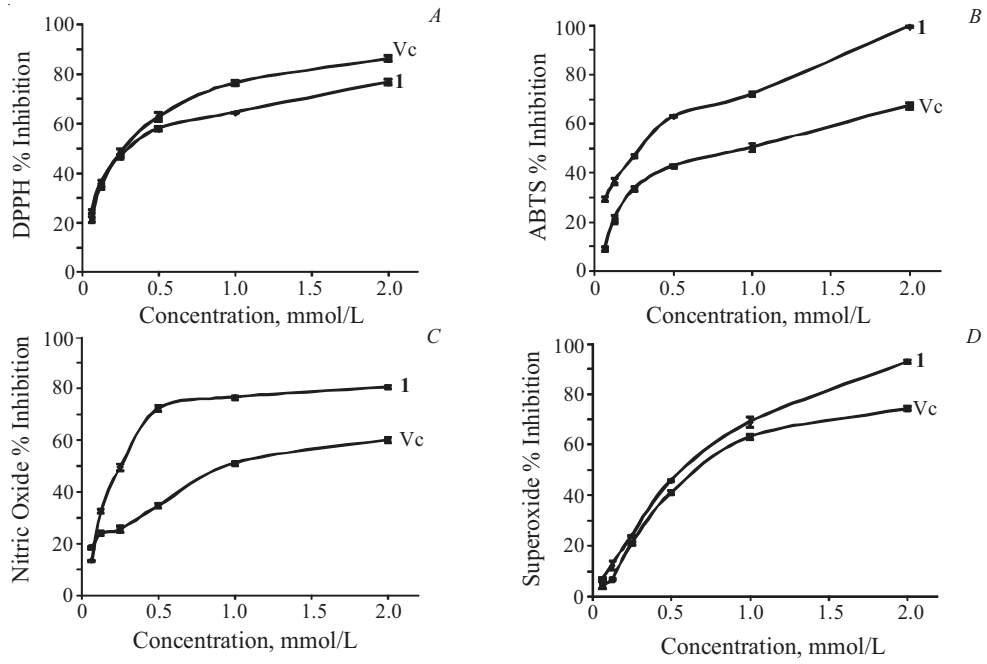

Fig. 1. Antiradical activity of 6-OHG: DPPH radical scavenging assay (*A*); ABTS radical scavenging assay (*B*); NO radical scavenging assay (*C*); Superoxide radical scavenging effect assay (*D*). Each value represents the mean ±SD of triplicate experiments.

The antioxidant activities of 6-OHG (**1**) and V_C were also evaluated using the reducing power assay and the phosphomolybdenum assay (total antioxidant capacity). The reducing power assay is a convenient and rapid screening method for measuring the antioxidant potential. As shown in Fig. 2*A*, the reducing power of **1** increases with increase in concentration but is lower than V_C. At the concentration of 0.5 mmol/L, the absorbance values of **1** and V_C are 0.443 and 1.422, respectively. The phosphomolybdenum assay is based on the reduction of molybdenum(VI) phosphate to molybdenum (V) phosphate, which will increase the absorbance at 695 nm. As shown in Fig. 2*B*, 6-OHG exhibits higher total antioxidant capacity, as evidenced by the fact that the absorbance value of **1** is always higher than V_C at the same concentration. These results show that **1** has effective reducing capability.

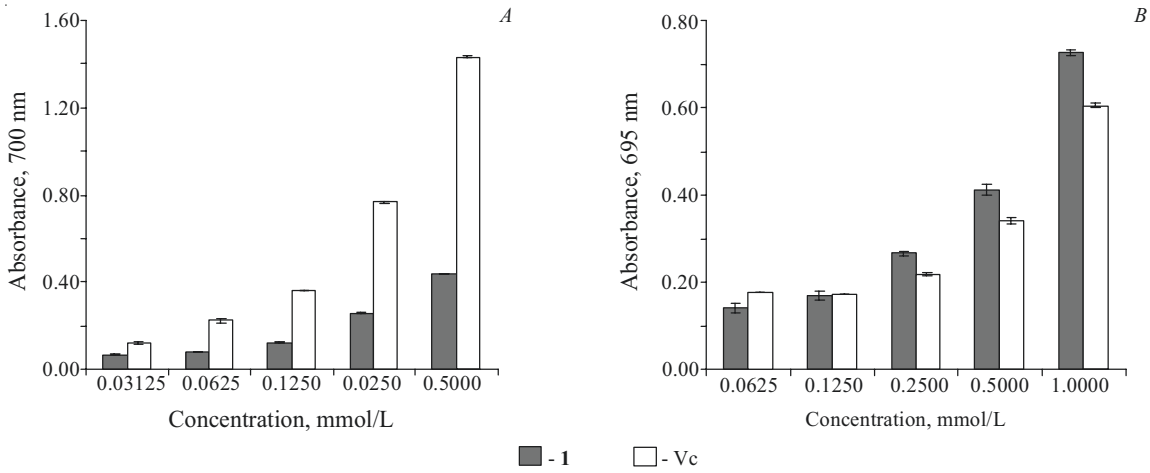

Fig. 2. The reducing capability of 6-OHG: Fe^{3+} reducing capacity (*A*); total antioxidant capacity (*B*). Each value represents the mean ±SD of triplicate experiments.

EXPERIMENTAL

General. Melting points (uncorrected) were obtained using an X-4B micro melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III HD spectrometer at 400 MHz and 100 MHz, respectively. Infrared spectra were obtained on a Bruker ALPHA FT/IR spectrometer. Absorptions are reported on the wave number (cm^{-1}) scale, in the range 400–4000 cm^{-1}. High-resolution mass spectra were recorded on the Apex II by means of the electrospray ionization (ESI) technique. Low-resolution mass spectra were recorded on an Agilent 6460 triple-quadrupole mass spectrometer with ESI. Data are quoted as *m/z* values (relative abundance). Purity was analyzed by UHPLC using a Thermo instrument with a Thermo Acclaim-C18 column (100 × 2.1 mm, 2.2 μm, USA).

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

5-Hydroxy-4′,7-dimethoxyisoflavone (3). A solution of biochanin A (**2**, 2.84 g, 10 mmol) in acetonitrile (30 mL) was supplemented by K_2CO_3 (2.69 g, 10 mmol) and CH_3I (930 μL, 15 mmol). The mixture was refluxed for 6 h at 85°C (course of reaction monitored by TLC). Then the mixture was allowed to stand at room temperature and 10% HCl solution (50 mL) was added. The white deposit that precipitated was separated from the solvents by filtration, washed twice with water, and dried in air to give **1a** as pale yellow needles. Yield 90%, mp 132.3–133.7°C (138–139°C [15]). IR (KBr, ν, cm^{-1}): 3434, 2958, 2837, 1666, 1608, 1572, 1516, 1441, 1287, 1252, 1161, 1048, 835, 826. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 12.86 (1H, s, 5-OH), 7.86 (1H, s, H-2), 7.44 (2H, d, J = 8.8, H-2′, 6′), 6.98 (2H, d, J = 8.8, H-3′, 5′), 6.39 (1H, d, J = 2.0, H-8), 6.37 (1H, d, J = 2.4, H-6), 3.86 (3H, s, CH_3O), 3.84 (3H, s, CH_3O). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 180.9 (C-4, C=O), 165.5 (C-7), 162.7 (C-5), 159.8 (C-4′), 158.0 (C-9), 152.7 (C-2), 130.1 (C-2′, 6′), 123.6 (C-1′), 123.0 (C-3), 114.1 (C-3′, 5′), 106.3 (C-10), 98.2 (C-6), 92.4 (C-8), 55.8 (CH_3O-7), 55.4 (CH_3O-4′). MS *m/z* 299.1 $[M + H]^+$.

6,8-Dibromo-5-hydroxy-4′,7-dimethoxyisoflavone (4). NBS (3.92 g, 22 mmol) was added to a solution of compound **3** (3 g, 10 mmol) in DMF (60 mL) in portions. The reaction mixture was stirred at room temperature for 2.5 h; then the mixture was poured into cold 2M HCl (20 mL). The DMF was removed under vacuum. The precipitate was filtered off, washed with H_2O, dried, and recrystallized from methanol to give compound **2** as a white powder, yield 81%, mp 185.6–187.7°C. IR (KBr, ν, cm^{-1}): 3412, 2958, 1637, 1611, 1580, 1515, 1412, 1246, 1187, 1104, 1024, 824, 693. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 13.71 (1H, s, 5-OH), 8.07 (1H, s, H-2), 7.46 (2H, d, J = 8.6, H-2′, 6′), 7.00 (2H, d, J = 8.6, H-3′, 5′), 4.00 (3H, s, CH_3O), 3.85 (3H, s, CH_3O). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 180.8 (C-4), 160.2 (C-7), 158.5 (C-5), 153.4 (C-4′), 153.1 (C-9), 152.8 (C-2), 130.1 (C-2′, 6′), 124.1 (C-1′), 121.8 (C-3), 114.3 (C-3′, 5′), 109.3 (C-10), 101.4 (C-6), 95.2 (C-8), 61.2 (CH_3O-7), 55.4 (CH_3O-4′). HR-MS *m/z* 456.9109 $[M + H]^+$ (calcd for $C_{17}H_{12}Br_2O_5$, 456.9104).

5-Hydroxy-4′,6,7-trimethoxyisoflavone (5). To a solution of 25% sodium methoxide in methanol (35 mL, 0.15 mol) was added a suspension of CuBr (0.7 g, 5 mmol) in DMF (15 mL), and the whole left to stand under stirring at room temperature for 1 h. The mixture was added in one portion to a solution of **4** (1.49 g, 5 mmol) in DMF (25 mL) at 120°C and the whole stirred for 40 min. Then the reaction mixture was poured into ice-water and acidified with 20% HCl (aq.) to pH 6. The resulting solution was extracted by ethyl acetate (20 mL × 3). The organic layer was dried over anhydrous Na_2SO_4 and filtered off. After evaporating the solvent under vacuum, the residue was purified by silica gel column chromatography (petroleum ether–ethyl acetate, 3:1) to give **5** as a white powder. Yield 65%, mp 192.0–192.7°C (192.5–194°C [16]). IR (KBr, ν, cm^{-1}): 3060, 2933, 2831, 1660, 1614, 1576, 1515, 1455, 1293, 1271, 1240, 1140, 1108, 1043, 826, 814. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 12.83 (1H, s, 5-OH), 7.89 (1H, s, H-2), 7.46 (2H, d, J = 8.8, H-2′, 6′), 6.98 (2H, d, J = 8.6, H-3′, 5′), 6.46 (1H, s, H-8), 3.95 (3H, s, CH_3O), 3.92 (3H, s, CH_3O), 3.84 (3H, s, CH_3O). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 181.1 (C-4), 159.8 (C-4′), 158.9 (C-7), 153.6 (C-2), 153.5 (C-5), 152.7 (C-9), 132.6 (C-6), 130.1 (C-2′, 6′), 123.3 (C-1′), 122.8 (C-3), 114.1 (C-3′, 5′), 106.8 (C-10), 90.3 (C-8), 60.8 (CH_3O-6), 56.3 (CH_3O-7), 55.3 (CH_3O-4′). MS *m/z* 329.1$[M + H]^+$.

6-Hydroxygenistein (1, 6-OHG). Compound **5** (328 mg, 1 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL); then 1 M BBr_3 (2 mL) in dichloromethane was added dropwise at –15°C. The reaction mixture was stirred at room temperature for another 12 h (course of reaction monitored by TLC). Then the mixture was quenched with cold water, and the dichloromethane was removed under vacuum. The residue was poured into water. The obtained solid was collected, dried under vacuum, and then recrystallized from aqueous methanol (2:1) to give 6-OHG as yellow crystals. Yield 81%,

mp 276.4–277.1°C (275–276°C [17]). IR (KBr, ν, cm^{-1}): 3397, 1663, 1616, 1579, 1518, 1474, 1370, 1280, 1234, 1056, 827, 762. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 12.81 (1H, s, 5-OH), 10.52 (1H, s, 4′-OH), 9.57 (1H, s, 7-OH), 8.30 (1H, s, H-2), 7.39 (2H, d, J = 8.6, H-2′, 6′), 6.83 (2H, d, J = 8.6, H-3′, 5′), 6.50 (1H, s, H-8). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 180.1 (C-4), 157.7 (C-4′), 154.31 (C-2), 154.01 (C-7), 150.5 (C-9), 147.8 (C-5), 130.6 (C-2′, 6′), 129.6 (C-6), 121.9 (C-1′), 121.9 (C-3), 115.5 (C-3′, 5′), 105.2 (C-10), 93.9 (C-8). MS *m/z* 287.1 $[M + H]^+$.

Purity Test. The purity of 6-OHG was analyzed using UHPLC with a Thermo Acclaim-C18 column (100 × 2.1 mm, 2.2 μm, USA). Isocratic elution was performed using H_2O (A) and methanol (B) with the following gradient combination: 40% B (0–10 min). The column temperature was 25°C. The flow rate was 0.2 mL/min, and 10 μL of the sample was injected. The detection wavelength was 254 nm.

DPPH Radical Scavenging Assay. The DPPH radical scavenging activity was determined according to the previous reported method with some modifications [18, 19]. In brief, 50 μL of samples of various concentrations (0.0625–2.0 mmol/L) was added to 150 μL of 0.2 mM DPPH radical solution in ethanol, and the resulting mixture was incubated for 0.5 h in the dark at room temperature. The absorbance of the mixture was measured at 517 nm using a microplate reader (Spectramax i3, Molecular Devices). The absorbance of the positive control (ascorbic acid, V_C) and control (DPPH radical without sample) was also measured. The percentage of DPPH free radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = (1 - A_1/A_0) \times 100, \quad (1)$$

where A_1 and A_0 are the absorbance of sample and control. respectively.

The antioxidant activity is expressed as IC_{50} (mmol/L) values. Here, the IC_{50} values is the concentration of sample with a 50% DPPH radical scavenging rate. The IC_{50} is calculated using the linear equation obtained based on the concentration and inhibition percentage. A lower IC_{50} value corresponded to a higher antioxidant activity. All samples were analyzed in triplicate.

ABTS Radical Scavenging Assay. The ABTS radical scavenging activity was measured based on the following method [20]. In brief, the ABTS-mixture solution was prepared by mixing equal amounts of 7 mM ABTS and 2.45 mM potassium peroxodisulfate solution for 16 h with rotation in the dark at room temperature. Thereafter, the ABTS-working solution was prepared after dilution of the ABTS-mixture solution (1 mL) in methanol (3.9 mL) before use. The reaction was started by the addition of the ABTS-working solution (200 μL) to varying concentrations of the test sample (50 μL) and then allowing the reaction to proceed at room temperature for 10 min in the dark. The absorbance of the resulting solution was measured at 734 nm in a microplate reader (Spectramax i3, Molecular Devices); V_C was used as reference standard.

NO Radical Scavenging Assay. The nitric oxide radical scavenging assay was measured by the Griess reaction with some modifications [21]. In brief, 50 μL of various concentrations (0.0625–2.0 mmol/L) of samples was added to 50 μL of sodium nitroprusside (20 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) naphthyl ethylenediamine hydrochloride was added, and the resulting solution was further incubated for 30 min. The absorbance was measured at 540 nm in a microplate reader (Spectramax i3, Molecular Devices; V_C was used as reference standard. The nitric oxide 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 [22]. In brief, 50 μL of NBT solution (0.2 mM in distilled water), 50 μL of NADH solution (0.5 mmol/L in 0.1 M Tris-HCl, pH 8.0), and 100 μL of samples with different concentrations (0.0625–2.0 mmol/L) 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 in a microplate reader (Spectramax i3, Molecular Devices); V_C 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).

Reducing Power Assay. The reducing power assay was performed according to the method of Oztaskin [23]. For this, 100 μL of various concentrations (0.03125–0.5 mmol/L) of samples was mixed with 2.5 mL of 0.2 mol/L sodium phosphate buffer (PBS, pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated for 30 min at 50°C and then 2.5 mL of 10% TCA was added. Subsequently, 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 ($FeCl_3$). The absorbance was measured at 700 nm after 10 min (Spectramax i3, Molecular Devices); V_C was used as positive control. A higher absorbance indicates a higher reducing power. All samples were tested in triplicate.

Phosphomolybdenum Assay (Total Antioxidant Capacity). The total antioxidant capacity of samples was determined by the phosphomolybdate method according to Albayrak [24]. In brief, 100 μL of various concentrations (0.0625–1.0 mmol/L) of samples was mixed with 1.0 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; V_C was used as positive control. A higher absorbance indicates a higher total antioxidant activity. All samples were tested in triplicate.

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