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Research Article

Synthesis, Radical-Scavenging Activities, and Protective Effects against AAPH-Induced Oxidative Damage in DNA and Erythrocytes of Piperine Derivatives

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Piperine amino acid derivatives containing phenolic hydroxyl groups were synthesized using piperine as the raw material by amide hydrolysis, amidation, ester hydrolysis, and deacetalization. The obtained products were characterized by mass spectrometry and nuclear magnetic resonance. The antioxidant activity of the piperine derivatives was evaluated by the DPPH and ABTS scavenging rates and the total antioxidant capacity. The results showed that the piperine amino acid (**4a**–**4d**) had relatively weak radical-scavenging ability, while the piperine amino acid derivatives (**5a**–**5d**) containing phenolic hydroxyl groups had significant radical-scavenging effects. In addition, the total reducing ability of **5a**–**5d** was better than that of piperine. The study also found that piperine derivatives containing phenolic hydroxyl groups played an important role in inhibiting oxidative damage in DNA and erythrocytes.

1. Introduction

Free radicals are the products of normal metabolism in the body. Production of excess free radicals induces immune dysfunction, which causes lipid peroxidation damage in cell membranes, enzyme deactivation, and oxidative DNA damage, leading to many oxidation-related diseases [1, 2]. Consequently, there has been an increase in research activity focusing on the synthesis and development of free radical scavengers [2–4].

Many studies on the synthesis of antioxidants are based on the structural modification of plant-derived substances, including piperine, an alkaloid derived from plants in the Piperaceae family [5–7], which has received an increasing amount of attention due to its excellent biological and pharmacological activity [6, 8, 9]. Researchers have demonstrated that the antioxidative activity of piperine is manifested through free radical-quenching effects and a reduction in GSH consumption [10]. Therefore, piperine has been widely applied in research on oxidation-related diseases [10–14]. For instance, Khajuria et al. found that piperine can inhibit carcinogen-induced oxidative damage in the intestinal mucosa, increase GSH levels, and restore ATPase activity [15]. Vijayakumar et al. have shown that piperine exerts significant antioxidative effects on the erythrocytes of high-fat diet- and antithyroid drug-induced hyperlipidemic rats [13]. Other researchers have reported that piperine-amino conjugates exhibit enhanced biological activity when compared with piperine [16]. For instance, Koichi et al. have reported the use of piperic acid amides as free radical scavengers and α-glucosidase inhibitors [17], while Inder Pal et al. reported the antileishmanial activity of piperoyl-amino acid conjugates [16]. Furthermore, studies have indicated the antioxidative performance of certain amino acids of food origin is due to characteristics such as the electron-donating and hydrogen bond-donating properties, as well as metal ion chelation and hydrophobic properties [18–20]. On

these grounds, the present study has proposed a novel research approach based on the excellent biological activity of piperic acid amides and the possible antioxidative performance of amino acids, which involves the derivatization of piperine using amino acids followed by the removal of the acetal moiety in the parent structure of piperine to form phenolic hydroxyl groups, thereby obtaining phenolic hydroxyl-containing piperine-amino acid derivatives.

The most common in vitro experimental methods used for the assessment of antioxidant activity can be classified as chemical methods, simulated biological system methods, and cell investigation methods. Chemical methods (such as the DPPH assay [21], ABTS assay [22], and total reductive ability test) mainly assess the free radical-capturing ability and reductive ability of antioxidants. Since such methods are easy to operate and provide rapid, accurate measurements, they are most commonly used in antioxidant prescreening studies. Simulated biological system methods assess the antioxidant performance by measuring the effects of antioxidants on free radical-induced oxidative damage in biomacromolecules (e.g., AAPH-, Cu^{2+}/GSH-, and OH•-induced oxidative DNA damage) [23, 24]. Cell investigation methods provide an assessment of the antioxidant performance by evaluating the effects of antioxidants on free radical-induced oxidative damage and changes in the activity of antioxidant enzymes [20, 25]. To perform a comprehensive assessment of the antioxidant performance of our piperine-amino acid derivatives, we selected the most conventional methods used among these three categories, namely, the DPPH assay, ABTS assay, total reductive ability test, and AAPH-induced oxidative DNA and erythrocyte damage model.

2. Materials and Methods

2.1. Materials. Piperine (98%) was purchased from Shaanxi Sciphar Natural Products Co., Ltd. (China). Oxalyl chloride (95%), boron tribromide (BBr_3) (99%), (2-methylpropionamide)-dihydrochloride (AAPH) (98%), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^+$) (98%), potassium ferricyanide ($K_3[Fe(CN)_6]$) (98%), trichloroacetic acid (TCA) (98%), L-alanine methyl ester hydrochloride (98%), tertiary butylhydroquinone (TBHQ) (98%), L-valine methyl ester hydrochloride (98%), L-leucinate methyl ester hydrochloride (98%), L-methionine methyl ester hydrochloride (98%), 4,6-dihydroxy-2-mercaptopyrimidine (TBA) (98%), and ascorbic acid (VC) (98%) were obtained from Energy Chemical (China). DNA from fish sperm pure was purchased from J&K Scientific (China). Ferric chloride ($FeCl_3$) (98%) was obtained from Tianjin Beilian Fine Chemicals Development Co., Ltd.

2.2. Synthesis of Piperine Derivatives. The synthesis of piperine derivatives **4a**-**4b** and **5a**-**5b** containing different amino acids is shown in Figure 1.

*2.2.1. (2E,4E)-5-(Benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-oic Acid (**1**, $C_{12}H_{10}O_4$).* (2E,4E)-5-(Benzo[d][1,3]dioxol-5-yl) penta-2,4-dien-oic acid (**1**) was prepared by the improved alkaline hydrolysis method according to the method reported by Inder Pal et al. [16]. The method reported in the literature is to directly acidify with hydrochloric acid [16], and the consumption of hydrochloric acid is large. In this experiment, the potassium salt of the compound was separated from the system firstly, followed by the pH adjustment treatment, and the recrystallization operation is omitted. The experimental yield has reached more than 90%. In brief, piperine (10.69 g, 37.51 mmol) was dispersed in a KOH methanol solution (mass fraction 20%, 300 mL) and refluxed at 75°C for 24 h. The suspension was then cooled, and a white solid was obtained after suction filtration. The solid was dispersed in a small amount of methanol. The pH was adjusted to 1 using 6 mol/L hydrochloric acid. The suspension was then filtered through suction and dried to afford compound **2** as a yellow solid (7.61 g, 93.1%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.21 (s, 1H, -COOH), 7.33-7.24 (m, 1H, CH=CH-CO), 7.25-7.24 (d, 1H, Ar-H), 7.03-6.79 (m, 3H, Ar-H, Ar-CH=CH-), 6.94-6.92 (d, 1H, Ar-CH=CH-), 6.06 (s, 2H, -OCH2O-), 5.95-6.91 (d, 1H, CH=CH-CO). 13C NMR (101 MHz, DMSO-d6) δ 168.03, 148.56, 148.45, 145.01, 140.21, 131.00, 125.33, 123.51, 121.63, 108.97, 106.20, 101.82.

*2.2.2. (2E,4E)-5-(Benzo[d][1,3]dioxol-5-yl)penta-2,4-dien-oyl Chloride (**2**, $C_{12}H_9ClO_3$).* Inder Pal et al. [16]. prepared the piperine amino acid methyl ester conjugate from the intermediate product piperonic acid mesylate with a yield of 40–75%. In this experiment, the corresponding acid chloride was prepared by the reaction of compound 1 with oxalyl chloride, and the esterification reaction was carried out. The yield of the reaction was over 79%. Compound **2** (6.91 g, 31.7 mmol) was dispersed in 15 mL of anhydrous dichloromethane (DCM). Oxalyl chloride DCM solution (12 mol/L, 2.70 mL) was then added, and the mixture was stirred at room temperature for 2 h to afford an orange liquid. The oxalyl chloride and DCM were distilled off under reduced pressure to afford an orange acid chloride, which had to be freshly prepared.

*2.2.3. Piperine L-Alanine Methyl Ester Conjugate (**3a**, $C_{16}H_{17}NO_5$).* The above-prepared acid chloride (**2**) was dissolved in 10 mL of anhydrous DCM, and TEA (40 mmol) was added with stirring at room temperature. L-Alanine methyl ester hydrochloride (4.41 g, 31.7 mmol) was dissolved in 20 mL of DCM and was then slowly added into the reaction system using a dropping funnel. After 4 h of reaction at room temperature, the product was washed with 30 mL of 5% $NaHCO_3$ solution, 30 mL of 5% HCl solution, and 30 mL of water. The concentrated crude product was then purified by column chromatography on silica gel with a mixture of EA-PE to afford 8.06 g of the product (**3a**) (yield: 88.0%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42-7.36 (m, 1H, CH=CH-CO), 7.01-7.00 (d, 1H, Ar-CH=), 6.93-6.91 (dd, 1H, Ar-H), 6.82-6.79 (m, 2H, Ar-H, Ar-CH=CH-), 6.74-6.67 (m, 1H, Ar-H), 6.15-6.13 (d, 1H, CH=CH-CO), 6.00 (s, 2H, -OCH2O-), 4.78-4.71 (m, 1H, CONHCH), 3.80 (s, 3H, -OCH_3), 1.49-1.47 (d, 3H, -CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.21, 164.99, 147.82, 147.72, 141.28,

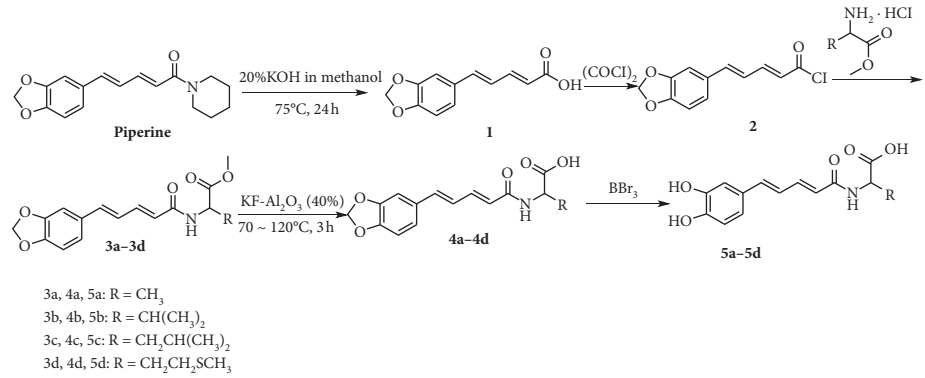

Figure 1: Scheme for the preparation of piperine derivatives. KOH: potassium hydroxide; (COCl)2: oxalyl chloride; KF: potassium fluoride: Al_2O_3: aluminium oxide; BBr3: boron tribromide.

138.86, 130.27, 124.01, 122.20, 121.89, 108.00, 105.28, 100.83, 52.03, 47.61, 18.19. ESI: m/z 304.1 $[M+1]^+$.

*2.2.4. Piperine L-Valine Methyl Ester Conjugate (**3b**, $C_{18}H_{21}NO_5$).* The synthesis process of compound **3b** was similar to that of compound **3a**. The amount of L-valine methyl ester hydrochloride used was 5.30 g (31.7 mmol). The product was a pale yellow solid (8.12 g, 80.3%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43-7.37 (m, 1H, CH=CH-CO), 7.01 (d, 1H, Ar-CH=), 6.93-6.68 (m, 4H, Ar-H, Ar-CH=CH-), 6.03 (s, 1H, CH=CH-CO), 6.00 (s, 2H, -OCH_2O-), 4.74-4.70 (m, 1H, CONHCH), 3.78 (s, 3H, -OCH_3), 2.25-2.20 (m, 1H, CH $(CH_3)_2$), 1.00-0.95 (m, 6H, CH $(CH_3)_2$).^{13}CNMR (101 MHz, $CDCl_3$) δ 172.22, 165.41, 147.82, 147.72, 141.36, 138.86, 130.28, 124.00, 122.22, 121.99, 108.01, 105.27, 100.83, 56.56, 51.71, 31.09, 18.46, 17.41. ESI: m/z 332.1 $[M+1]^+$.

*2.2.5. Piperine L-Leucinate Methyl Ester Conjugate (**3c**, $C_{19}H_{23}NO_5$).* The synthesis process of compound **3c** was similar to that of compound **3a**. The amount of L-leucinate methyl ester hydrochloride used was 5.74 g (31.7 mmol). The product was a pale yellow solid (8.35 g, 79.5%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41–7.35 (m, 1H, CH=CH-CO), 6.98-6.97 (d, 1H, Ar-CH=), 6.90-6.65 (m, 4H, Ar-H, Ar-CH=CH-), 6.16-6.14 (d, 1H, CH=CH-CO), 5.99 (s, 2H, -OCH_2O-), 4.82-4.76 (m, 1H, CONHCH), 3.77 (s, 3H, -OCH_3), 1.73-1.1.70 (m, 2H, CH_2CH $(CH_3)_2$), 1.62-1.58 (m, 1H, CH $(CH_3)_2$), 0.99-0.96 (m, 6H, CH $(CH_3)_2$).^{13}C NMR (101 MHz, CDCl3) δ 173.87, 165.87, 148.29, 148.19, 141.87, 139.33, 130.78, 124.53, 122.69, 122.39, 108.47, 105.78, 101.32, 52.34, 50.77, 41.82, 24.90, 22.84, 21.98. ESI: m/z 346.1 $[M+1]^+$.

*2.2.6. Piperine L-Methionine Methyl Ester Conjugate (**3d**, $C_{18}H_{21}NO_5S$).* The synthesis process of compound **3d** was similar to that of compound **3a**. The amount of L-methionine methyl ester hydrochloride used was 5.14 g (31.7 mmol). The product was a pale yellow solid (8.53 g, 85.6%). ^{1}H NMR (400 MHz, $CDCl_3$) 7.43-7.36 (m, 1H, CH=CH-CO), 7.00 (d, 1H, Ar-CH=), 6.93-6.67 (m, 4H, Ar-H, Ar-CH=CH-), 6.32-6.30 (d, 1H, CH=CH-CO), 6.00 (s, 2H, -OCH2O-), 4.89-4.84 (m, 1H, CONHCH),, 3.80 (s, 3H, -OCH_3), 2.55-2.54 (m, 2H, SCH_2), 2.06-2.05 (m, 5H, $CHCH_2$, SCH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.64, 165.82, 148.37, 148.23, 142.05, 139.57, 130.73, 124.45, 122.76, 122.19, 108.51, 105.79, 101.32, 52.60, 51.63, 31.87, 30.00, 15.51.

*2.2.7. Piperine L-Alanine Conjugate (**4a**, $C_{15}H_{15}NO_5$).* Compounds **4a**–**4d** were synthesized by improving the method reported by Inder Pal et al. [16]. The experiment was carried out by heating hydrolysis with some water in the solid medium. The yield of compounds **4a**–**4d** reached 71–86%.

Compound **3a** (1.99 g, 6.57 mmol) and 9.74 g of KF-Al_2O_3 were thoroughly ground and mixed together. The mixture was then transferred to a 100 mL round-bottomed flask, and 3 mL of water was added. After heating and stirring at 120°C for 1 h, 10 mL of water was added and stirred for another 10 min before suction filtration. The filtrate pH was adjusted to 1 with hydrochloric acid while stirring at room temperature to precipitate solids. The crude product obtained after suction filtration was then purified by column chromatography on silica gel (developing agent DCM : MeOH : HAc = 60 : 3 : 0.1) to afford 1.42 g of the product (**4a**) (yield: 75.1%) as a pale yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.54 (s, 1H, -COOH), 8.38-8.36 (d, 1H, CHNHCO), 7.28-7.27 (d, 1H, CH=CH-CO), 7.17-7.13 (m, 1H, Ar-CH=), 7.01-6.89 (m, 4H, Ar-H, Ar-CH=CH-), 6.16-6.13 (d, 1H, CH=CH-CO), 6.04 (s, 2H, -OCH_2O-), 4.31-4.27 (m, 1H, CONHCH), 1.31-1.29 (d, 3H, -CH_3); ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.24, 164.91, 147.89, 147.71, 139.88, 138.18, 130.77, 125.12, 123.88, 122.73, 108.40, 105.62, 101.23, 47.53, 17.21; ESI: m/z 290.1 $[M+1]^+$.

*2.2.8. Piperine L-Valine Conjugate (**4b**, $C_{17}H_{19}NO_5$).* The synthesis process of compound **4b** was similar to that of compound **4a**. The product was a pale yellow solid (yield: 78.9%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.51 (s, 1H, -COOH), 8.26-8.20 (d, 1H, CHNHCO), 7.29 (d, 1H, CH=CH-CO), 7.20-7.14 (m, 1H, Ar-CH=), 7.02-6.90 (m, 4H, Ar-H, Ar-CH=CH-), 6.31-6.28 (d, 1H, CH=CH-CO), –6.05 (s, 2H, -OCH_2O-), 4.27-4.23 (m, 1H, CONHCH), 2.11-2.04 (m, 1H, CH $(CH_3)_2$), 0.91-0.89 (m, 6H, CH

$(CH_3)_2$). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.10, 165.39, 147.89, 147.70, 139.85, 138.12, 130.78, 125.13, 124.07, 122.69, 108.40, 105.64, 101.23, 57.29, 29.83, 19.17, 18.10. ESI: m/z 318.1 $[M+1]^+$.

*2.2.9. Piperine L-Leucinate Conjugate (**4c**, $C_{18}H_{21}NO_5$).* The synthesis process of compound **4c** was similar to that of compound **4a**. The product was a white solid (yield: 85.7%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.53 (s, 1H, -COOH), 8.31-8.29 (d, 1H, CHNHCO), 7.28 (d, 1H, CH=CH-CO), 7.20-7.14 (m, 1H, Ar-CH=), 7.02-6.86 (m, 4H, Ar-H, Ar-CH=CH-), 6.19-6.15 (d, 1H, CH=CH-CO), 6.05 (s, 2H, -OCH_2O-), 4.35-4.30 (m, 1H, CONHCH), 1.67-1.61 (m, 1H, CH $(CH_3)_2$), 1.58-1.53 (m, 2H, CH_2CH $(CH_3)_2$), 0.92-0.86 (dd, 6H, CH $(CH_3)_2$). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.17, 165.18, 147.89, 147.71, 139.86, 138.13, 130.78, 125.13, 123.94, 122.68, 108.40, 105.64, 101.22, 50.29, 40.00, 24.37, 22.82, 21.27. ESI: m/z 332.1$[M+1]^+$.

*2.2.10. Piperine L-Methionine Conjugate (**4d**, $C_{17}H_{19}NO_5S$).* The synthesis process of compound **4d** was similar to that of compound **4a**. The product was a pale yellow solid (yield: 71.3%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.71 (s, 1H, -COOH), 8.37-8.35 (d, 1H, CHNHCO), 7.29-7.28 (d, 1H, CH=CH-CO), 7.21-7.15 (m, 1H, Ar-CH), 7.02-6.86 (m, 4H, Ar-H, Ar-CH=CH-), 6.18-6.15 (d, 1H, CH=CH-CO), 6.05 (s, 2H, -OCH_2O-), 4.44-4.43 (m, 1H, CONHCH), 2.06-1.86 (m, 5H, SCH_2, SCH_3). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.91, 165.82, 148.39, 148.23, 140.49, 138.75, 131.27, 125.61, 124.32, 123.22, 108.91, 106.13, 101.74, 51.54, 31.23, 30.22, 15.03. ESI: m/z 350.1$[M+1]^+$.

*2.2.11. ((2E,4E)-5-(3,4-Dihydroxyphenyl)penta-2,4-dienoyl) Alanine (**5a**, $C_{14}H_{15}NO_5$).* Compound **4a** (200 mg, 0.69 mmol) was dispersed in 10 mL of anhydrous DCM. A DCM (5 mL) solution of boron tribromide (260.9 μL, 2.76 mmol) was added dropwise to the reaction mixture in an ice-bath condition. After 4 h, the reaction was quenched by adding 10 mL of ice-cold saturated ammonium chloride solution in an ice-bath condition. After stirring at room temperature for 0.5 h, ethyl acetate (3×15 mL) was used for the extraction. The organic phase was dried with anhydrous sodium sulfate for 24 h and then filtered to obtain the concentrated crude product. The crude product was then purified by column chromatography on silica gel (developing agent DCM : MeOH : HAc = 7.5 : 1 : 0.1) to afford 36.52 mg of the product (**5a**) (yield: 19.1%) as an orange solid. ^{1}H NMR (400 MHz, DMSO-d_6) 9.83 (s, 2H, -OH), 8.05-8.03 (d, 1H, CHNHCO), 7.17-7.11 (m, 1H, CH=CH-CO), 6.95 (s, 1H, Ar-CH=), 6.84-6.6.71 (m, 4H, Ar-H, Ar-CH=CH-), 6.18-6.14 (d, 1H, CH=CH-CO), 4.30-4.27 (m, 1H, CONHCH), 1.28-1.26 (d, 3H, -CH_3). ESI: m/z 278.19 $[M+1]^+$.

*2.2.12. ((2E,4E)-5-(3,4-Dihydroxyphenyl)penta-2,4-dienoyl) Valine (**5b**, $C_{16}H_{19}NO_5$).* The synthesis process of compound **5b** was similar to that of compound **5a**. The product was a dark orange solid (yield: 12.3%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.76 (s, 2H, -OH), 7.91 (s, 1H, CHNHCO), 7.17-7.11 (m, 1H, CH=CH-CO), 6.96 (s, 1H, Ar-CH=), 6.84-6.74 (m, 4H, Ar-H, Ar-CH=CH-), 6.29-6.26 (d, 1H, CH=CH-CO), 4.22-4.19 (m, 1H, CONHCH), 2.10 (s, 1H, CH $(CH_3)_2$), 0.88-0.81 (m, 6H, CH $(CH_3)_2$). ESI: m/z 320.1 $[M+1]^+$.

*2.2.13. ((2E,4E)-5-(3,4-Dihydroxyphenyl)penta-2,4-dienoyl) Leucine (**5c**, $C_{17}H_{21}NO_5$).* The synthesis process of compound **5c** was similar to that of compound **5a**. The product was a dark orange solid (yield: 29.3%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.31 (s, 2H, -OH), 8.23-8.21 (d, 1H, CHNHCO), 7.19-7.12 (m, 1H, CH=CH-CO), 6.95 (d, 1H, Ar-CH=), 6.86-6.71 (m, 4H, Ar-H, Ar-CH=CH-), 6.14-6.10 (d, 1H, CH=CH-CO), 4.32-4.30 (m, 1H, CONHCH), 1.65-1.60 (m, 1H, CH $(CH_3)_2$), 1.56-1.52 (m, 2H, CH_2CH $(CH_3)_2$), 0.91-0.85 (dd, 6H, CH $(CH_3)_2$). ESI: m/z 320.2 $[M+1]^+$.

*2.2.14. ((2E,4E)-5-(3,4-Dihydroxyphenyl)penta-2,4-dienoyl) Methionine (**5d**, $C_{16}H_{19}NO_5S$).* The synthesis process of compound **5d** was similar to that of compound **5a**. The product was a dark green solid (yield: 20.4%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.69 (s, 1H, -COOH), 9.31 (s, 1H, -OH), 9.02 (s, 1H, -OH), 8.31-8.26 (m, 1H, CHNHCO), 7.20-7.14 (m, 1H, CH=CH-CO), 6.95 (m, 1H, Ar-CH=), 7.87-6.72 (m, 4H, Ar-H, Ar-CH=CH-), 6.14-6.10 (d, 1H, CH=CH-CO), 4.43-4.37 (m, 1H, CONHCH), 2.07-2.02 (m, 5H, SCH_2, SCH_3). ESI: m/z 338.2$[M+1]^+$.

2.3. ABTS Radical-Scavenging Properties. The ABTS radical-scavenging activities were assayed according to the method reported by Thaipong et al. [22]. (1) $ABTS^+$ aqueous solution (7 mmol/L) preparation: 30 mg $ABTS^+$ was weighed, 8 mL of ultrapure water was added, and it was dissolved by ultrasonication. (2) K2S2O8 solution preparation: 10 mg K2S2O8 was weighed, 15 mL of ultrapure water was added, and it was dissolved by ultrasonication. (3) ABTS working solution preparation: (1) and (2) were mixed at the ratio of 1 : 1 and kept in the dark for 12 to 16 h. The above mixture was diluted four to eight times with 95% ethanol, such that it had an absorbance of ca. 0.7 Abs at 734 nm, to obtain the ABTS working solution. After keeping the 300 μL ABTS working solution and 100 μL ethanol solution of compounds (**4a**–**4d**, **5a**–**5d**, and piperine) with a certain concentration gradient in the dark for 30 min, their absorbances at 734 nm were measured. The experiment was repeated three times in parallel.

2.4. DPPH Radical-Scavenging Properties. The DPPH radical-scavenging activities were assayed according to the method reported by Nimse et al. [26]. The DPPH working solution was prepared by dissolving 20 mg DPPH in 250 mL of 95% ethanol and diluting it until its absorbance was ca. 0.8 at 517 nm. After keeping 200 μL DPPH· working solution and 200 μL ethanol solution of compounds (**4a**–**4d**, **5a**–**5d**,

and piperine) with a certain concentration gradient in the dark for 30 min, their absorbances at 517 nm were measured. The experiment was repeated three times in parallel.

2.5. Measurement of Total Reducing Ability. Total reducing ability of piperine derivatives was detected according to the method reported by Oyaizu [27]. First, 100 μL of PBS (0.2 M, pH = 6.6) solution, 100 μL of 1% potassium ferricyanide solution, and 20 μL of compound solution (1600 μM, 800 μM) were mixed together. The mixed solution was then heated in a 50°C water bath for 20 min and cooled in an ice bath. Subsequently, 10% TCA solution was added to quench the reaction. The mixture was then centrifuged at 3000 r/min for 10 min, and 200 μL of the supernatant was taken to add in 200 μL H_2O and 40 μL 0.1% $FeCl_3$ solution. The solution was allowed to stand for 10 min before measuring its absorbance at 700 nm.

2.6. Inhibiting DNA Oxidation. The ability of piperine derivatives to inhibit DNA oxidation was detected by Gong et al.'s reported methods [23]. A DNA solution (2.24 mg/mL) and an AAPH solution (400 μM) were prepared using PBS1 (NaH_2PO_4 1.9 mM, Na_2HPO_4 8.1 mM, and EDTA 10 μM) as the solvent. First, 8.04 mL of DNA solution and 60 μL of compound solution (24 mM, 12 mM, 0) were mixed together and 900 μL AAPH solution was added. The well-mixed solution was then poured into 18 tubes (400 μL/tube), which were placed in a 37°C water bath for 30 min, 6 h, and 8 h, respectively. After incubation, 200 μL of TBA solution (TBA 0.25 g, NaOH 0.1 g, final volume brought to 25 mL using double-distilled water) and 200 μL of TCA solution (TCA 0.75 g, final volume brought to 25 mL using double-distilled water) were added to the tubes, which were well shaken and heated in a boiling water bath for 15 min. They were then cooled in an ice bath. Subsequently, 300 μL of *n*-butanol was added and a vortex was used to extract the resulting TBARS (thiobarbituric acid reactive material). Finally, the tubes were centrifuged at 1500 r/min for 3 min, and 100 μL of the supernatant was taken out for the absorbance measurement at 535 nm.

2.7. Erythrocyte Hemolysis Assay. The inhibition of piperine derivatives on AAPH-induced erythrocyte hemolysis was evaluated by the method reported by Zheng et al. [20]. SD rats were anesthetized by injecting 20% urethane intraperitoneally. Blood was sampled from the heart and was collected in heparinized tubes. The tubes were then centrifuged at 3500 r/min for 5 min to separate the erythrocytes, which were washed four times using centrifugal washing with PBS (pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). The erythrocytes were dispersed with PBS solution to obtain a 10% erythrocyte suspension. Subsequently, 100 μL of a 10% erythrocyte suspension with 50 μL of compound solution or PBS was incubated at 37°C for 30 min. Then, 100 μL PBS solution of 200 mM AAPH was added, and the mixture was incubated for a certain period of time at 37°C. For the control group, the AAPH solution was substituted with PBS. After incubation, 200 μL of the reaction solution was diluted with 1 mL of PBS and was centrifuged at 3500 r/min for 5 min. The absorbance (A_{PBS}) of the supernatant was measured at 540 nm using a microplate reader. The same volume of the reaction solution was diluted with 1 mL of distilled water to obtain a complete hemolysis solution. The supernatant absorbance (A_{water}) was measured under the same condition. The hemolysis rate was calculated according to the following equation:

$$\text{erythrocyte hemolysis } (\%) = \frac{A_{pbs}}{A_{water}} \times 100. \quad (1)$$

2.8. Measurement of Hemoglobin Oxidation. The inhibition of piperine derivatives on hemoglobin oxidation was also evaluated by the method reported by Zheng et al. [20]. First, 200 μL of erythrocyte suspension was diluted with 1 mL of distilled water and centrifuged at 3500 r/min for 5 min. The supernatant absorbance was measured at 630 nm and 700 nm and recorded as A_{630} and A_{700}, respectively. In addition, the supernatant was treated with 10 μL of 5% potassium ferricyanide and the absorbances at 630 nm and 700 nm were measured, which were recorded as A_{hb630} and A_{hb700}, respectively. The hemoglobin oxidation rate was calculated according to the following equation:

$$\text{Hemoglobin oxidation } (\%) = \left[\frac{A_{630} - A_{700}}{A_{hb630} - A_{hb700}}\right] \times 100. \quad (2)$$

2.9. Influence of Piperine Derivatives on the Antioxidant Enzyme System of AAPH-Treated Rat Erythrocytes. The compound solution (50 μL) was added to 100 μL of a 10% erythrocyte suspension and incubated at 37°C for 30 min. Then, 100 μL of 200 mmol/L AAPH was added, and the mixture was further incubated at 37°C for 1–4 h. After incubation, ultrapure water precooled to 4°C was added to lyse all the erythrocytes. The resultant mixture was centrifuged, and the sediment was removed and washed three times in PBS. 990 μL of double-distilled water, precooled to 4°C, was then added to 10 μL of the erythrocyte sediment, and the mixture was stored in a −80°C freezer before use. The glutathione peroxidase (GSH-Px) content and the total superoxide dismutase and catalase (CAT) activities were determined using assay kits in accordance with the manufacturer's instructions.

2.10. Statistical Analysis. Each experiment was repeated at least three times in parallel. The results were reported as average ± standard deviation (SD). SPSS version 19.0 (SPSS Inc., Chicago, IL) was used for the statistical analysis. The data were analyzed using one-way ANOVA. The significance of difference was determined by the LSD range test ($p < 0.05$).

3. Results and Discussion

*3.1. Radical-Scavenging Activities of **4a–4d** and **5a–5d** Determined in DPPH and ABTS Assays.* The radical-scavenging

ability of piperine derivatives (**4a**–**4d** and **5a**–**5d**) and piperine was evaluated using the DPPH and ABTS assays. The results are shown in Figure 2. It can be seen from the figure that compounds **4a**–**4d** had no significant scavenging effect on the DPPH radicals below the concentration of 160 μM, and the scavenging rate was below 20%. Among them, **4a**, **4b**, and **4c** showed a stronger scavenging ability than the prototype compound (piperine) at a high concentration ($p < 0.05$). It can be seen that, after the amino acid derivatization of piperine, its DPPH radical-scavenging ability improved; however, the effect was not obvious. Compounds containing phenolic hydroxyl groups (**5a**–**5d**) were obtained by removing the acetal from compounds **4a**–**4d**. It can be observed from Figure 2 that the phenolic hydroxyl groups played a significant role in improving the DPPH radical-scavenging ability of the compounds. At a concentration of 160 μM, the scavenging rates of DPPH of the four compounds, namely, **5a**, **5b**, **5c**, **5d**, and VC, were not significantly different, at $80.0 \pm 1.07\%$, $78.1 \pm 0.36\%$, $76.6 \pm 6.09\%$, $78.7 \pm 0.78\%$ ($p < 0.05$), and $93.34 \pm 1.42\%$, respectively. The values of IC_{50} for **5a**, **5b**, **5c**, **5d**, and VC were 57.5 μM, 65.3 μM, 66.4 μM, 80.9 μM, and 59.40 μM, respectively. It can be seen that the DPPH radical-scavenging effect of **5a** is similar to that of VC in the concentration range of 10–160 μm.

The scavenging effect of piperine derivatives on ABTS radicals was also evaluated in this experiment. It can be seen from Figure 3 that, at concentrations of 2.5 to 80 μM, the compounds **4a**–**4d** showed a slightly stronger ABTS radical-scavenging effect than the prototype compound piperine, but the improvement was not significant. It can also be seen that the ABTS radical-scavenging rates of compounds **5a**, **5b**, **5c**, **5d**, and VC at 80 μM concentration were $88.2 \pm 3.04\%$, $79.44 \pm 2.75\%$, $76.36 \pm 2.89\%$, $54.26 \pm 5.08\%$, and $78.50 \pm 5.49\%$ ($p < 0.05$), respectively, and the values of IC_{50} were 42.7 μM, 49.0 μM, 51.8 μM, 51.8 μM, and 49.3 μM, respectively. Among them, compounds **5a** and **5b** showed the most significant ABTS radical-scavenging abilities, which were slightly stronger than that of VC. It can be seen that the presence of phenolic hydroxyl groups in the structure of piperine derivatives had a significant effect on improving the DPPH and ABTS radical-scavenging abilities of the compounds.

3.2. Reducing Ability of Piperine Derivatives. The reducing ability is an important indicator to evaluate the antioxidant ability of compounds. In this experiment, the method described by Oyaizu [27] was used to test the reducing ability of the compound. The principle of reducing ability determination is that a reducing substance reacts with potassium ferricyanide ($K_3Fe(CN)_6$) to form potassium ferrocyanide ($K_4Fe(CN)_6$), which then reacts with ferric chloride to form Prunbull's Blue ($KFe[Fe(CN)_6]$) that has the maximum absorbance at 700 nm. Therefore, the larger the absorbance at 700 nm, the stronger the reducing ability of the sample. It can be seen from Figure 4 that all compounds were significantly concentration-dependent such that the reducing ability at a high concentration (160 μM) was stronger than

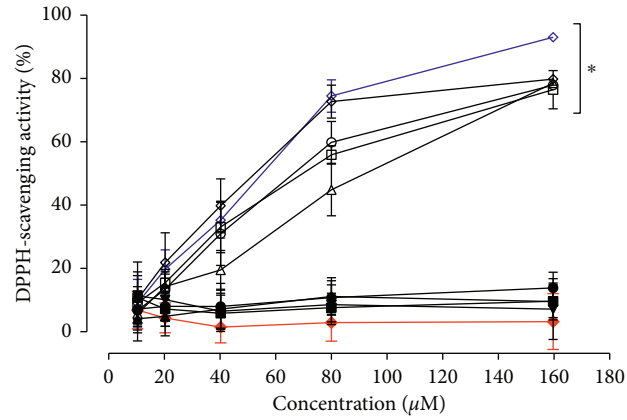

Figure 2: DPPH radical-scavenging activity of **4a** (●), **4b** (■), **4c** (▲), **4d** (▼), **5a** (◇), **5b** (○), **5c** (□), **5d** (△), piperine (◆), and VC (◆). *indicates a significant difference from the piperine group ($p < 0.05$).

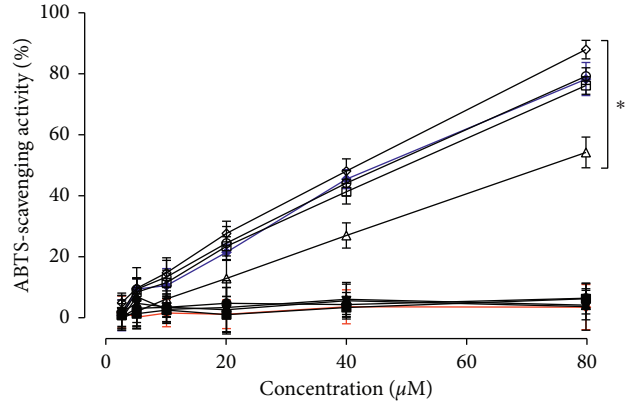

Figure 3: ABTS radical-scavenging activity of **4a** (●), **4b** (■), **4c** (▲), **4d** (▼), **5a** (◇), **5b** (○), **5c** (□), **5d** (△), piperine (◆), and VC (◆). *indicates a significant difference from the piperine group ($p < 0.05$).

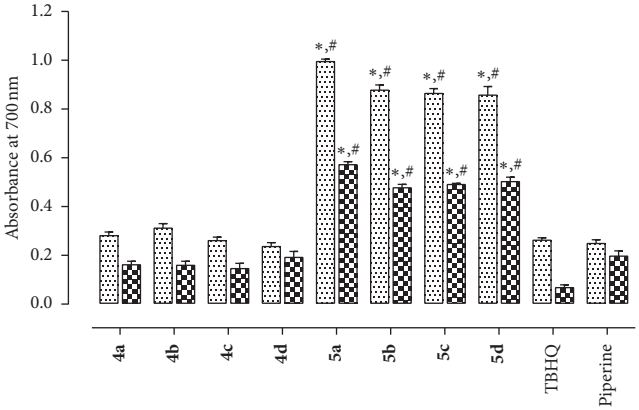

Figure 4: Measurement of total reducing ability. 80 μM (▩); 160 μM (▩). *indicates a significant difference from the piperine group ($p < 0.05$). #indicates a significant difference from the TBHQ group ($p < 0.05$).

that at a low concentration (80 μM). The experimental results showed that the reductive ability of compounds **4a**, **4b**, **4c**, and **4d** was similar to that of piperine. The reducing strength of the compound has a certain correlation with the number of active hydrogen atoms supplied by the compound. Compounds **5a**, **5b**, **5c**, and **5d** all had three active hydrogen atoms, TBHQ had two active hydrogen atoms, and piperine had no active hydrogen atom. From Figure 4, it can be seen that, at a concentration of 160 μM, the absorbances of **5a**, **5b**, **5c**, and **5d** at 700 nm were 0.999 ± 0.010, 0.880 ± 0.022, 0.866 ± 0.021, and 0.860 ± 0.036, respectively. The absorbances of piperine and TBHQ were 0.247 ± 0.015 and 0.261 ± 0.010, respectively. This showed that the reducing ability of piperine was comparable to that of TBHQ, which was not strong. However, compounds **5a**–**5d** had a relatively strong reducing ability, which was related to their electron-donating ability (hydrogen-donating ability).

*3.3. Protective Effects of **5a**–**5d** on AAPH-Induced DNA Oxidation.* AAPH can react with oxygen in the air to produce peroxy radicals (ROO·) at a steady rate. ROO· can capture the hydrogen atom supplied by the C-4′ atom in DNA, which could result in the DNA molecule unwinding and generating a variety of carbonyl-containing small molecular substances [23, 28]. These substances can react with TBA to produce thiobarbituric acid reactive species (TBARS, $\lambda_{max} = 535$ nm) under acidic conditions. Therefore, in this study, the content of carbonyl-containing small-molecule compounds produced by AAPH-oxidized DNA was quantitatively calculated by testing the absorbance of TBARS, thereby tracking the extent of AAPH-induced oxidative damage in DNA. As shown in the control experiment in Figure 5, in the process of DNA oxidation induced by AAPH, the absorbance of TBARS increased with time, indicating that as the reaction time increased, more carbonyl-containing small-molecule compounds were produced as a result of AAPH-induced oxidative damage in DNA. It can be seen from Figure 6 that the addition of piperine did not affect the increase in absorbance of TBARS in the above-mentioned system. This indicated that neither the potential antioxidant units N-H nor the conjugated double bonds in the piperine molecule could significantly inhibit AAPH-induced oxidative damage in DNA. However, when the other five derivatives were added to the above system, the increase in the absorbance of TBARS was significantly reduced. This showed preliminarily that phenolic hydroxyl groups could protect DNA from AAPH-induced oxidative damage. The inhibition effect of compounds on AAPH-induced oxidative damage in DNA is shown in Figure 5, from which it can be seen that the inhibition effect of compounds **5a**-**5b** on AAPH-induced DNA oxidative damage was concentration-dependent such that the inhibition rate at 160 μM was higher than that at 80 μM. In addition, as AAPH could generate ROO· continuously in the oxidative damage process, the inhibition rate of compounds **5a**-**5b** on DNA oxidative damage first increased and then decreased with time and was the highest at 360 min.

*3.4. Protective Effects of **5a**–**5d** on AAPH-Induced Hemolysis in Erythrocytes.* In this study, compounds **5a**–**5d** with strong radical-scavenging ability were studied. The protective effect of derivatives on the AAPH-induced hemolysis of rat erythrocytes was discussed. It can be seen from Figure 7(a) that the erythrocyte suspension was stable and the hemolysis rate was 14.45 in the PBS solution (pH = 7.4) (control group) after incubation at 37°C for 3 h. No obvious hemolysis was observed. After adding AAPH under the same condition, the hemolysis of erythrocytes increased to 62.08% (AAPH group). The significant increase in hemolysis showed that AAPH could induce damage in erythrocytes, thereby inducing hemolysis. When a low concentration (20 μM) of piperine derivatives was added to the erythrocytes, the hemolysis rates all decreased significantly ($p < 0.05$) and showed significant concentration dependency such that the higher the compound concentration, the lower the hemolysis rate. All four types of piperine derivatives reduced the hemolysis rate of erythrocytes to less than 50%. Among them, compound **5d** showed the most prominent effect and reduced the hemolysis rate to 25.90% after 3 h of incubation.

The protection time of **5a**-**5d** on the AAPH-induced hemolysis of erythrocytes is shown in Figure 7(b). It can be seen that the erythrocytes were stable in the PBS solution, and no significant change in the hemolysis rate was observed with time. The hemolysis of the erythrocyte suspension caused by AAPH-induced oxidative damage showed a significant time effect such that the hemolysis rate increased with increasing damage time. The hemolysis rate increased from 36.64% to 74.75%, from 2 h to 4 h. This was because the antioxidant system of erythrocytes (GSH, SOD, CAT, etc.) could inhibit the oxidative damage from ROO· in the initial stage. However, due to the consumption of antioxidants and the increase of free radical chain reactions over time, the erythrocyte membrane became damaged and the hemolysis occurred rapidly. The addition of piperine derivatives could effectively suppress hemolysis. As shown in the figure, the hemolysis rate of the AAPH group was 36.64% after 2 h of incubation, while the hemolysis rates of **5a**, **5b**, **5c**, and **5d** groups were reduced to 28.08%, 33.71%, 18.99%, and 14.92%, respectively. Therefore, the piperine derivatives had a certain protective effect on hemolysis caused by oxidative damage. Among all groups, compound **5d** showed the best effect before 3 h, while compound **5a** had the most significant effect at 4 h.

*3.5. Effects of **5a**–**5d** on AAPH-Induced Hemoglobin Oxidation in Erythrocytes.* Hemoglobin is the main protein in erythrocytes. Excessive free radicals can cause hemoglobin to oxidize and form methemoglobin, thereby leading to the disorder of erythrocyte function [20, 29]. This study further demonstrated the inhibitory effect of derivatives on oxidative damage in erythrocytes by examining the content of methemoglobin in erythrocytes. The effect of piperine derivatives against AAPH-induced hemoglobin oxidation in erythrocytes is shown in Figure 8. After 3 h of incubation (Figure 8(a)), the methemoglobin content in the normal control group was low (1.74%), whereas it was relatively high (25.65%) after oxidation by AAPH. In addition, as shown in Figure 8(b), the methemoglobin content in the normal

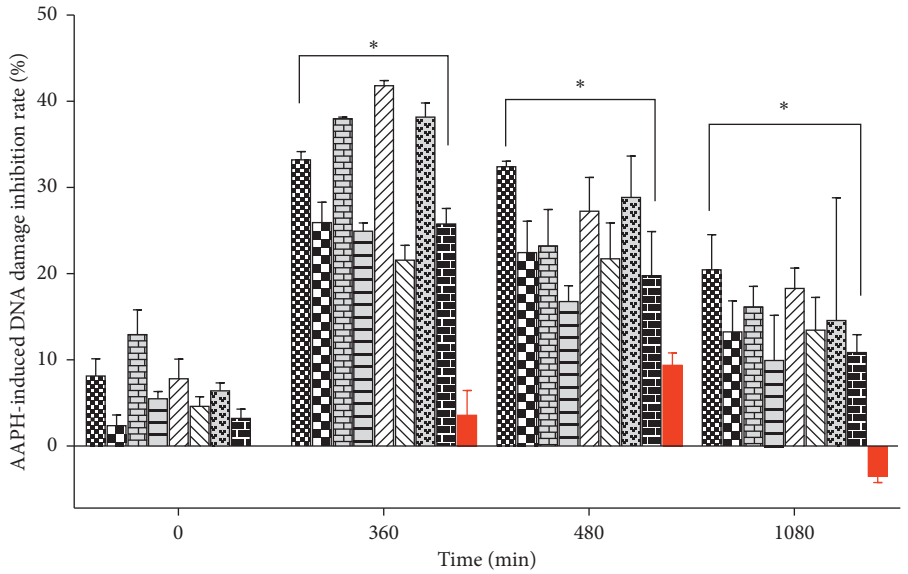

Figure 5: Inhibition effect of piperine derivatives on AAPH-induced oxidative damage in DNA: **5a** 160 μM (▩); **5a** 80 μM (▩); **5b** 160 μM (▤); **5b** 80 μM (▤); **5c** 160 μM (▨); **5c** 80 μM (▨); **5d** 160 μM (▩); **5d** 80 μM (▩); piperine 80 μM (▬). *indicates a significant difference from the piperine group ($p < 0.05$).

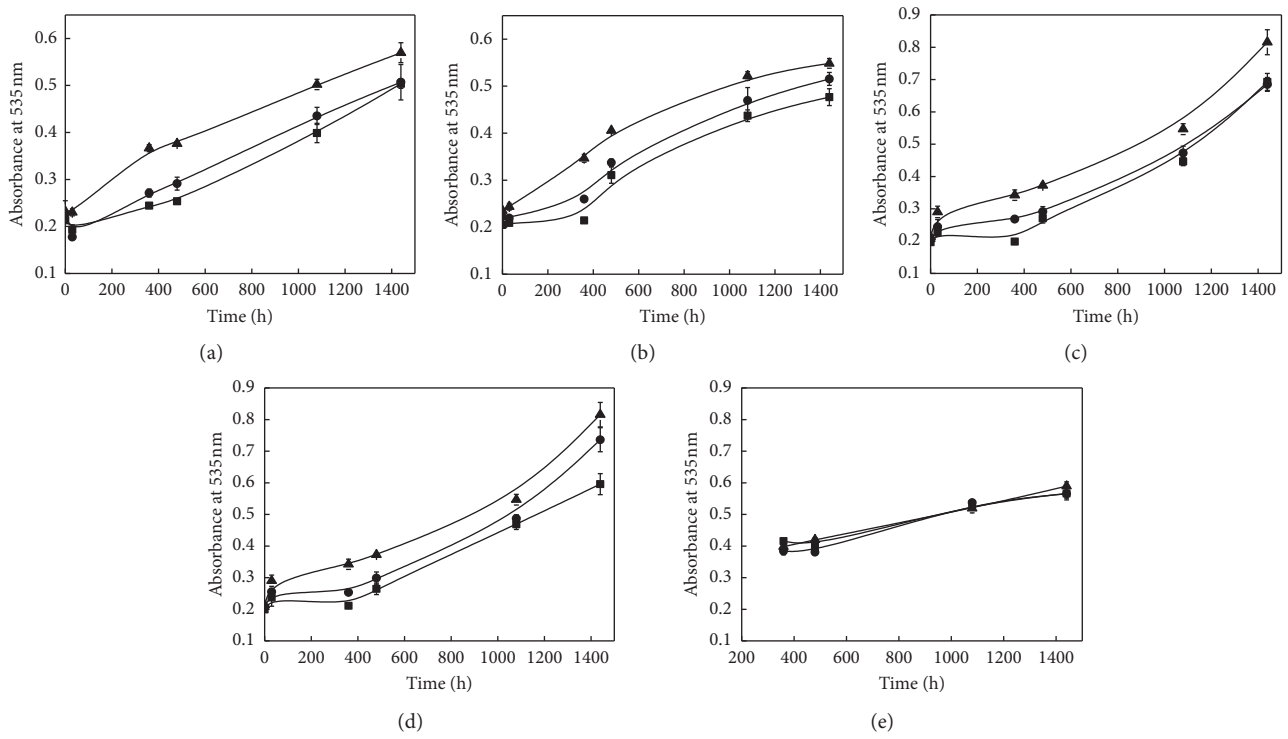

Figure 6: Variation in the absorbance of piperine derivatives in AAPH-induced oxidative damage in DNA over time. 0 μM (▲); 80 μM (●); 160 μM (■). (a) **5a**. (b) **5b**. (c) **5c**. (d) **5d**. (e) Piperine.

group was relatively stable, while that in the AAPH group continued to increase with time. Thus, it can be seen that AAPH could induce the continuous oxidation of hemoglobin.

It can be seen from Figure 8(a) that, after 3 h of incubation, the compounds showed some inhibitory effect on the production of methemoglobin. Among them, compounds **5c** and **5d** showed the most significant effects. A certain concentration effect was also observed such that the inhibitory effect increased with concentration. In addition, as shown in Figure 8(b), the inhibitory effect of the compounds on hemoglobin oxidation weakened with time. This was because AAPH could continuously release ROO˙ over time.

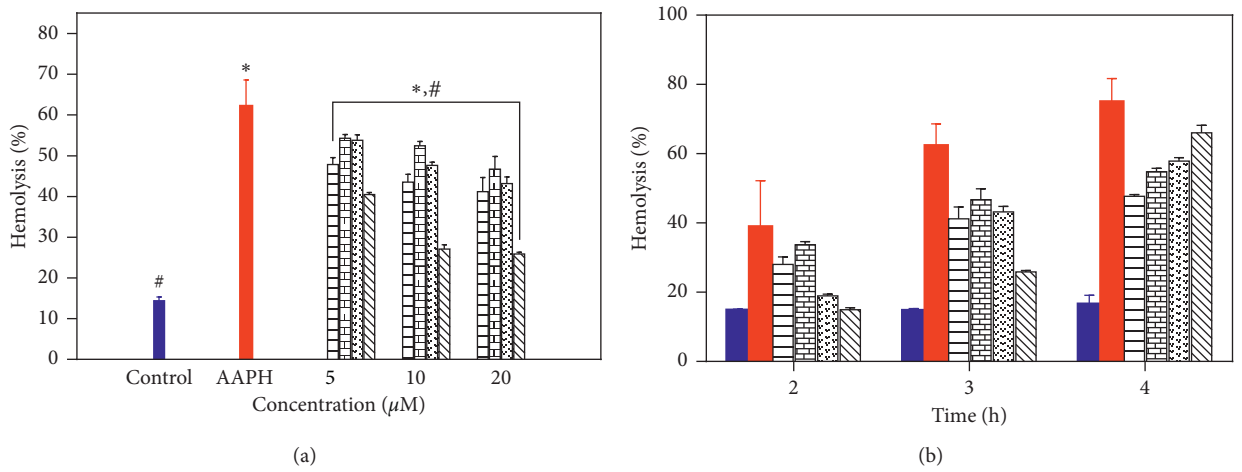

Figure 7: (a) Protective effect of piperine derivatives on AAPH-induced hemolysis of erythrocytes (3 h). (b) Kinetic curve of the protective effect of piperine derivatives on AAPH-induced hemolysis of erythrocytes at 20 μM. Control (■); AAPH (■); AAPH + **5a** (▭); AAPH + **5b** (▭); AAPH + **5c** (▭); AAPH + **5d** (▭). *indicates a significant difference from the control group ($p < 0.05$); indicates a significant difference from the AAPH group.

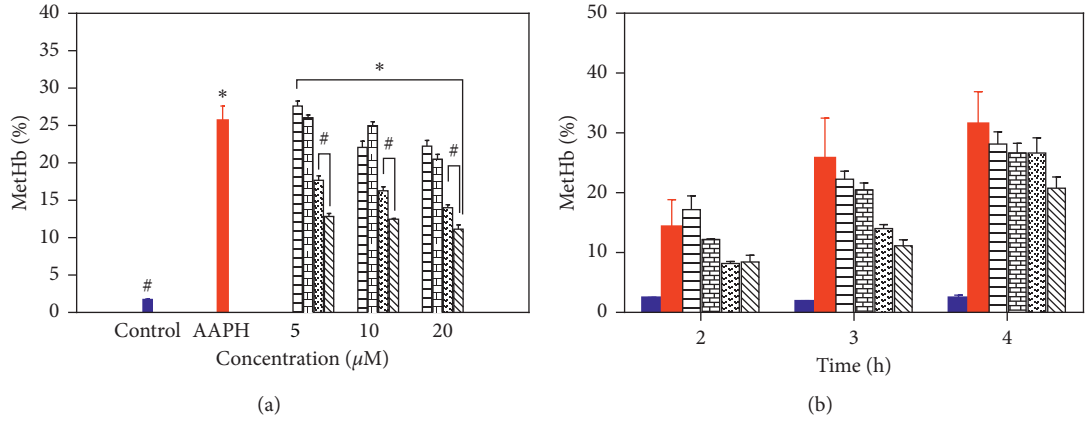

Figure 8: (a) Protective effects of piperine derivatives on AAPH-induced hemoglobin oxidation (3 h). (b) Kinetic curve of the protective effect of 20 μM piperine derivatives on AAPH-induced hemoglobin oxidation. Control (■); AAPH (■); AAPH + **5a** (▭); AAPH + **5b** (▭); AAPH + **5c** (▭); AAPH + **5d** (▭). *indicates a significant difference from the control group ($p < 0.05$); #indicates a significant difference from the control group ($p < 0.05$).

3.6. Influence of Piperine Derivatives on GSH-Px, T-SOD, and CAT Activities in AAPH-Treated Erythrocytes. GSH-Px is an antioxidant enzyme present in cells that catalyzes the reduction of glutathione (GSH) and the removal of peroxides in the body, such as H_2O_2 in cells, causing antilipid oxidation [30]. As shown in Figure 9(a), the GSH-Px content in erythrocytes belonging to the control group was stable, while that in erythrocytes of the AAPH group showed a statistically significant gradual decrease with increasing incubation time. Following the addition of 50 μM of piperine derivatives to erythrocytes prior to the induction of oxidative damage by AAPH, the piperine derivatives did not significantly influence the erythrocyte GSH-Px content after 1 h of incubation, compared with the AAPH group. After 2 h of incubation, compounds **5a** and **5b** significantly increased the GSH-Px content ($p < 0.05$); after 4 h of incubation, only the GSH-Px content in the **5b** and **5d** groups was increased, compared with that in the AAPH group. These results indicate that the amino acid derivatives of piperine can increase GSH-Px activity in erythrocytes. Particularly, compounds **5b** and **5d** produced the strongest effects, with the differences being statistically insignificant compared with the positive drug, i.e., vitamin C (VC) ($p > 0.05$).

SOD is a key enzyme for removing free radicals within the body. The SOD activity level serves as an indicator of the body's ability to remove free radicals [30, 31]. Figure 9(b) shows the influence of amino acid derivatives of piperine on T-SOD activity in AAPH-treated erythrocytes. In the AAPH group, with increasing incubation time, T-SOD activity decreased gradually. Following the addition of 50 μM of piperine derivatives, T-SOD activity increased after 1 h of incubation, albeit insignificantly compared with the AAPH group ($p > 0.05$). After 2 h of incubation, compounds **5a**, **5b**, **5c**, and **5d** increased T-SOD activity significantly ($p < 0.05$),

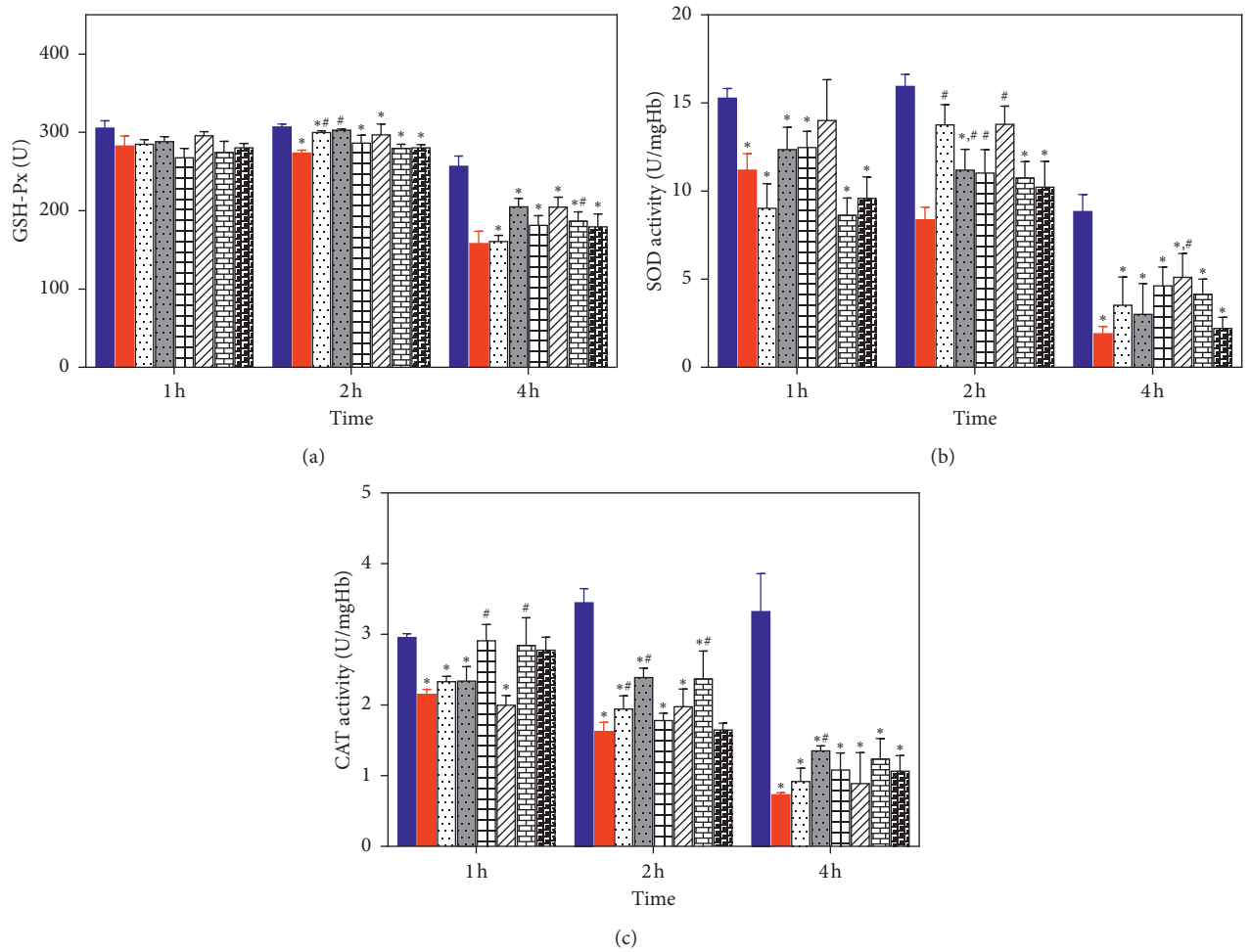

FIGURE 9: Influence of piperine derivatives on the (a) GSH-Px content, (b) T-SOD activity, and (c) CAT activity in AAPH-treated rat erythrocytes. *indicates a significant difference from the control group ($p < 0.05$). Control (■); AAPH (■); AAPH + **5a** (▦); AAPH + **5b** (▦); AAPH + **5c** (▦); AAPH + **5d** (▨); AAPH + piperine (▦); AAPH + VC (▦). #indicates a significant difference from the AAPH group ($p < 0.05$).

while the positive control and prototype drug (piperine) had no significant influence on erythrocyte T-SOD activity ($p > 0.05$). After 4 h of incubation, compound **5d** increased T-SOD activity significantly ($p > 0.05$), while other compounds had no significant influence on T-SOD activity ($p > 0.05$). These results highlight the significant enhancement, caused by piperine derivatives, of T-SOD activity in erythrocytes with AAPH-induced oxidative damage, compared to the relatively inferior effect of the prototype compound and of the positive drug VC.

CAT, an abundant enzyme in erythrocytes, catalyzes H_2O_2 decomposition, which reduces oxidative damage caused by OH•, an oxidation product formed from H_2O_2 under catalysis by metal ions [30, 31]. As shown in Figure 9(c), CAT activity in the AAPH group decreases gradually relative to that in the control group, with increasing incubation time. Following the addition of 50 μM of piperine derivatives, compound **5c** and piperine increased erythrocyte CAT activity after 1 h of incubation. After 2 h of incubation, compounds **5a** and **5b** and piperine increased CAT activity in erythrocytes significantly ($p < 0.05$), while the positive control had no significant influence on CAT activity ($p > 0.05$). After 4 h of incubation, compound **5b** increased CAT activity significantly ($p > 0.05$), while other compounds had no significant influence ($p > 0.05$). These results indicate that piperine derivatives produce significant differences in CAT activity in AAPH-treated rat erythrocytes.

4. Conclusion

In the present study, piperine-amino acid derivatives were synthesized, and the results indicate that the free radical removal ability and total reductive ability of the phenolic hydroxyl-containing piperine-amino acid derivatives were higher than those of the parent compound. Specifically, compounds **5a** and **5b** have free radical removal performances comparable to that of VC. The derivatives have good inhibitory effects on AAPH-induced oxidative DNA damage, while certain compounds also show inhibitory effects on AAPH-induced hemolysis in rat erythrocytes. Particularly, compound **5b** can significantly inhibit AAPH-induced erythrocyte hemolysis and hemoglobin oxidation. In addition, the experiments further verified that the protection

provided by piperine derivatives against AAPH-induced oxidative damage can be achieved by preserving the activity of the antioxidant enzyme system (GSH-Px, T-SOD, and CAT) within cells. The protection by piperine derivatives was also found to be superior to that by piperine and vitamin C. This paper described a structural modification method that could effectively improve the antioxidant ability of piperine. This type of compound could be a candidate for the development and research of oxidative damage-related diseases (hyperlipidemia, diabetes, cancer, whitening, etc.)

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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