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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis of *N*-hydroxycinnamoyl amino acid ester analogues and their free radical scavenging and antioxidative activities

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Abstract Hydroxycinnamic acids have a variety of biological activities, including antioxidant activity. To find more active antioxidants with hydroxycinnamoyl moiety, we synthesized a series of *N*-hydroxycinnamoyl amino acid esters and evaluated their antioxidative activities by 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging and human red blood cells (RBCs) haemolysis methods. It was found that *N*-caffeoyl amides exhibited the highest DPPH-scavenging activities, whereas *N*-feruloyl amides demonstrated the highest antihaemolysis activities among the three different hydroxycinnamamides (caffeoyl, feruloyl, and *p*-coumaroyl), and that hydroxycinnamoyl amides were more effective than their corresponding free acid and ester compounds in both DPPH and RBC haemolysis tests.

Keywords Hydroxycinnamic acid · Antioxidant · DPPH · Haemolysis · Amide · Amino acid

Introduction

A large body of clinical and experimental evidences accumulated in the past decade suggested that free radical–mediated peroxidation of membrane lipids and oxidative damage of DNA are associated with a variety of chronic health problems, such as cancer, atherosclerosis, and ageing (Anderson *et al.*, 2001; Lass and Sohal, 1998; Wei *et al.*, 2006a; Cheng *et al.*, 2006). Therefore, inhibition of free radical–induced oxidative damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of these diseases (Wei *et al.*, 2006b; Tsuda *et al.*, 2004).

Phenolic acids, especially hydroxycinnamic acid derivatives, are widely distributed in plants and found in considerable amounts in propolis, fruits, vegetables, and beverages of human diet (Scalbert and Williamson, 2000; Robbins, 2003). As one important kind of these derivatives, hydroxycinnamamides are also widely spread in plants (Tanguy *et al.*, 1978; Tanguy, 1985). The plant hydroxycinnamamides are known to have antioxidant, HIV-1 integrase, antimicrobial, antiplatelet, antituberculosis, and tyrosinase properties (Lee *et al.*, 2003, 2004; Tebayashi *et al.*, 2000; Charvat *et al.*, 2006; Narasimhan *et al.*, 2004; Lai *et al.*, 2002; Yoya *et al.*, 2009; Kang *et al.*, 2009). Because of the limitation of availability of hydroxycinnamic acid derivatives from nature, the knowledge to compare their antioxidative activities is still limited. Therefore, we synthesized a series of *N*-hydroxycinnamoyl amino acid esters and conducted a structure–activity relationship study of them in this study. In this study, three series of *N*-hydroxycinnamoyl amino acid esters (*p*-coumaroyl-, feruloyl- and caffeic and their structure were shown in Scheme 1) were synthesized. Their efficiency as radical scavengers was evaluated by their activity toward DPPH-scavenging and their potency as antioxidants was evaluated by antihaemolysis of human red blood cells (RBCs).

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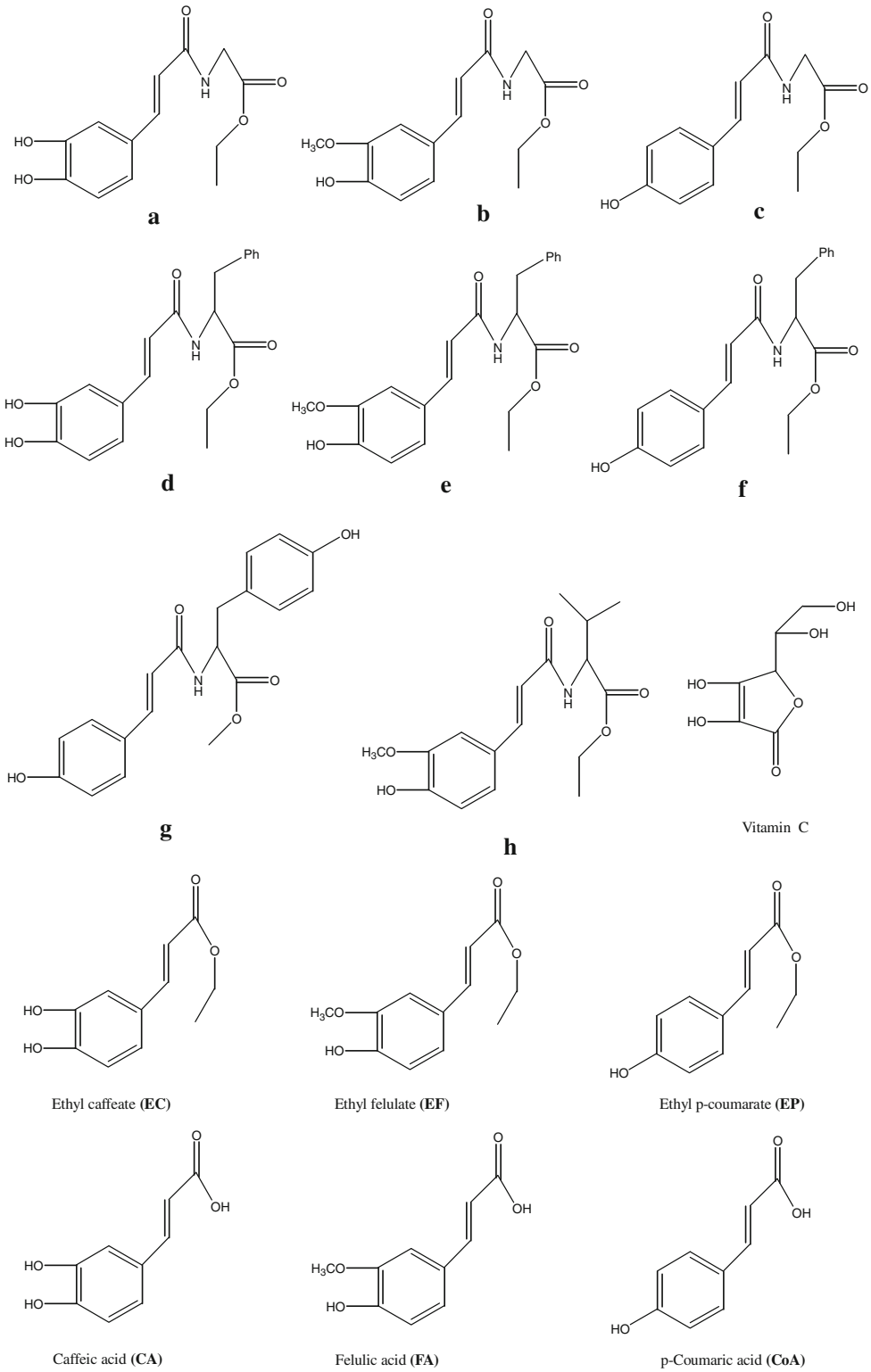
Scheme 1 The tested compounds structure



Results and discussion

Scavenging effect on DPPH radicals

The DPPH method was carried out in a homogeneous phase to measure the ability of antioxidants to intercept free radicals. This assay is based on a decolorization technique by UV–vis spectroscopy at 517 nm in which the radical is generated directly in a stable form before reaction with putative antioxidants. DPPH method has been widely used to assess radical-scavenging activity of phenolic compounds (Goupy *et al.*, 2003). On addition of

hydroxycinnamic acids analogues to an ethanol solution of DPPH, the absorption at 517 nm decreased immediately. The concentrations giving 50% reduction in the absorbance of DPPH solution (IC_{50}) were shown in Table 1. Their scavenging activity of DPPH radicals decreased in the following order: **d** > **a** > caffeic acid (CA) > Vitamin C > ethyl caffeate (EC) > ferulic acid (FA) > **e** > **b** > **h** > ethyl ferulate (EP) > **c**, **f**, **g**, ethyl *p*-coumarate (EP). It can be seen from Table 1 that the activity of these compounds depends significantly on the introduction of electron-donating groups (hydroxyl and methoxyl). Therefore, the scavenging activity has the following sequence: CA derivatives > FA derivatives > *p*-coumaric acid (CoA) derivatives (as we can see from IC_{50} values, for amide: **a** < **b** < **c**; **d** < **e** < **f**; for ester: EC < EF < EP; and for free acid: CA < FA < CoA, respectively). The scavenging activity for the same parent molecule has the following order: amide > ester > free acid. It is also noticeable that free CA and its amide derivative are more effective than Vitamin C (a famous antioxidant, positive control) in DPPH-scavenging activity.

Inhibition of RBC haemolysis by hydroxycinnamic acid derivatives

Although DPPH assay has been widely used to conveniently test the free radical scavenging activity of phenolic compounds, the method is only chemical relevance and the system used is a homogenous solution. It has been recognized that the antioxidant activity in homogenous solutions may not parallel that in heterogeneous media (Zhou *et al.*, 2005). Human RBCs are heterogeneous media. Therefore, the antioxidative effect of hydroxycinnamoyl amide and ester analogues was investigated in RBC model to evaluate the influence of microenvironment on the antioxidative activity.

Thermal decomposition of AAPH produces an initiating radical that can attack the polyunsaturated lipids (LH) in RBC membranes to induce lipid peroxidation. Then, the RBC membrane is quickly damaged, leading to haemolysis. Antioxidants (ArOHs) present or added to RBCs react with the chain-propagating peroxyl radicals to stop the peroxidation and hence inhibit haemolysis.

Figure 1 shows the AAPH-induced RBC haemolysis in an aerobic atmosphere. Addition of AAPH induced, after an inhibition period, fast haemolysis. As the blank (1) experiment shows, haemolysis does not occur at the beginning of the reaction because the endogenous antioxidants, e.g., vitamin E and/or ubiquinol-10, present in biomembranes defense the attack from radicals. The inhibitory time of haemolysis was 108 min in the absence of added antioxidants when the concentration of AAPH was 50 mM.

Addition of hydroxycinnamic acid and its analogues (ArOHs) to the 5% RBC suspension significantly increased the inhibition time of the RBCs. The inhibition time produced by ArOHs was illustrated in Fig. 1. The inhibitory times produced by 10-μM test compounds in decreasing order were **e** > **b** > **a** > **d** > **h** > EF > EA > **c** > Vitamin C, with the inhibition times, being 198, 193, 180, 160, 158, 150, 148, 138, and 133 min, respectively. It is clearly seen that the activity of these compounds depends significantly on the number of phenolic groups and methoxyl group in the molecules. Interestingly, *N*-feruloyl amino acid esters that possess a methoxyl groups demonstrated better inhibitory activities towards RBCs haemolysis than corresponding *N*-caffeoyl amides of corresponding amino

Table 1 Scavenging activity of antioxidants for DPPH radical

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
a	12.80	Ethyl caffeate (EC)	48.98
b	64.58	Ethyl ferulate (EF)	150.02
c	>200	Ethyl *p*-Coumarate (EP)	>200
d	1.45	Caffeic acid (CA)	18.25
e	52.35	Ferulic acid (FA)	50.13
f	>200	*p*-Coumaric acid (CoA)	>200
g	>200	Vitamin C	29.61
h	87.24		

Note: Vitamin C was used for positive control. IC_{50} value was determined to be the effective concentration at which DPPH radical was scavenged by 50%. The IC_{50} value was obtained by interpolation from linear regression analysis

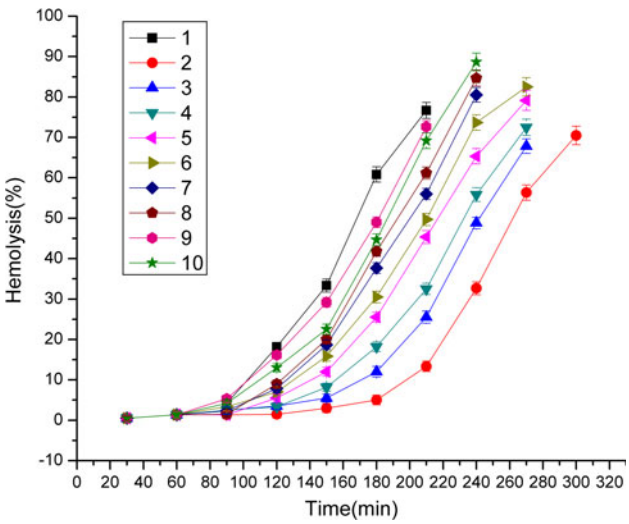


Fig. 1 Inhibition of AAPH-induced haemolysis of 5% human RBCs in PBS (0.15 M, pH 7.4) in an aerobic atmosphere at 37°C by hydroxycinnamic derivatives (ArOH). $[ArOH]_0 = 10$ μM and $[AAPH]_0 = 50$ mM. The numbers on the *lines* represent different compounds: (1) native RBCs, (2) **e**, (3) **b**, (4) **a**, (5) **d**, (6) **h**, (7) **EF**, (8) **EA**, (9) **c**, and (10) Vitamin C. Lines for compound **g**, **f**, CA, FA, EP, and CoA are not shown for clarity. Data are expressed as means ± SD

acid. Therefore, compound **e**, the most active one, is followed by compound **b**.

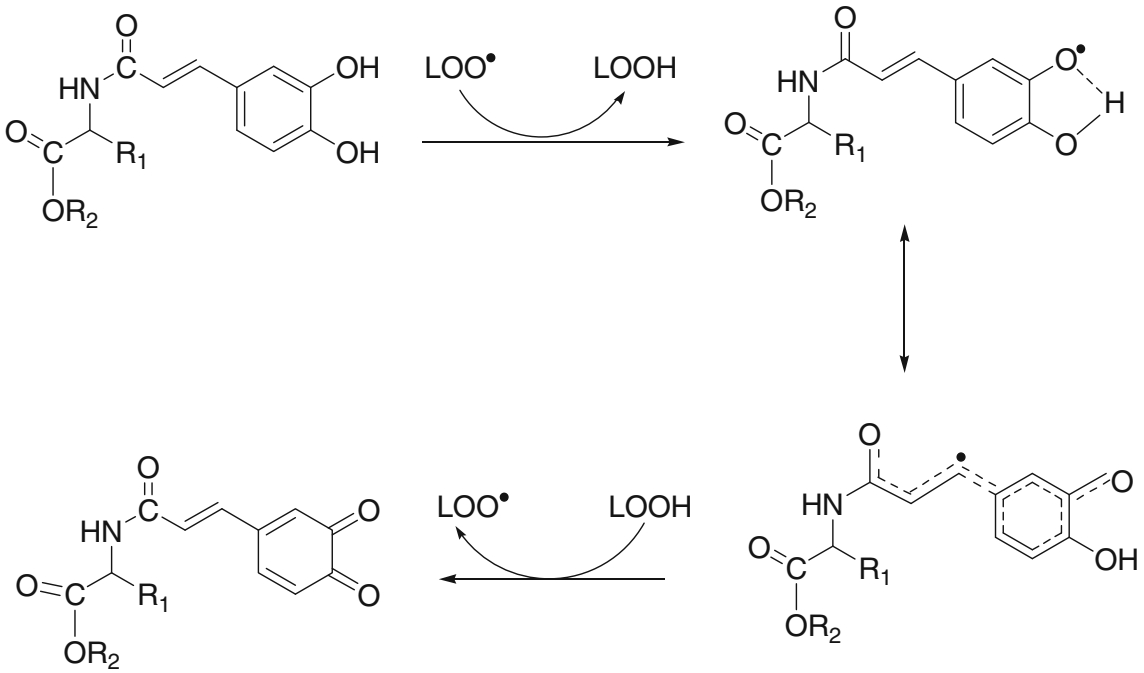
The antioxidant mechanism

It is proposed nowadays that the use of more than one marker of oxidation is required to evaluate the efficacy of an antioxidant. Hence, we evaluated the antioxidant effects of hydroxycinnamic acid analogues to scavenge DPPH and protection of human red blood cells (RBCs) from oxidative haemolysis.

The structural feature responsible for the better DPPH free radical scavenging activity of **d**, **a**, and CA is the *ortho*-dihydroxyl functionality in the catechol ring; therefore, the phenolic group plays a major role in the activity of hydroxycinnamic acid analogues. The presence of the electron-donating hydroxyl group at the *ortho*-position makes H-atom transfer to peroxyl radicals easier (Lucarini *et al.*, 2002), resulting in the formation of a phenoxy radical. It was also known that the *ortho*-hydroxyl substitution on phenol would make the oxidation intermediate, *ortho*-hydroxyphenoxyl radical, more stable because of the intramolecular hydrogen bonding interaction as reported recently from both experimental (Foti and Ruberto, 2001) and theoretical calculations (Wright *et al.*, 2001). In addition, *ortho*-OH phenoxyl radical and/or *ortho*-semiquinone radical anion shall be easier to further oxidize to form the final product *ortho*-quinone (Scheme 2). Although the *ortho*-methoxyl group can also form intramolecular hydrogen bond with the phenolic hydrogen, methoxylation of one hydroxyl group makes it impossible to form quinone oxidation products, so they are inefficient in quenching radicals in DPPH systems. So CA and its derivatives are more effective than FA corresponding derivatives in DPPH system. Accordingly, it is understandable for the DPPH-scavenging ability in the following order **d** > **e** > **f**. Compound **g**, with two hydroxyl groups on different phenyl ring, cannot form intramolecular hydrogen bond, so its antiradical and antihaemolysis activities were lower than CA and FA analogues.

In the biomembrane systems, antioxidative activity depends not only on the number of hydroxyl groups but also on the solubility, hydrophobicity (or partition coefficient, log P) (Rice-Evans *et al.*, 1996). It has been recognized that peroxyl radicals produced by AAPH in the aqueous phase attack phospholipids at the membrane surface (Niki, 1990), suggesting that antioxidants need to be located near the surface of the membrane to act, so the lipophilicity of the antioxidants makes great effects in inhibiting the lipid peroxidation. It is reasonable for the highly active antihaemolysis of *N*-feruloyl amino acid esters, especially for compounds **e**, for its high log P values. For the same reason, amide and ester have better activity compared with their corresponding free acid.

In conclusion, our results show that the synthetic amide analogues of hydroxycinnamic acid exhibit stronger antioxidative activity than their corresponding free acids and esters in the both assay systems. The observation that the feruloyl amides bearing *ortho*-methoxyl group vicinal to the hydroxyl functionality exhibit markedly higher antihaemolytic activities gives us useful information for antioxidant drug design.



Scheme 2 The proposed antioxidative mechanism of *N*-hydroxycinnamoyl amino acid ester analogues

Experiment

Materials and methods

CA, FA, and CoA were purchased from Sigma Chemical (St. Louis, MO, USA) and DPPH (1,1-Diphenyl-2-picrylhydrazyl) and AAPH (2,2′-Azobis(2-amidinopropane hydrochloride)) were purchased from Aldrich (St. Louis, MO, USA). All other chemicals were of the highest quality available.

Synthesized compounds were identified by IR, ^{1}HNMR, and MS analysis. Melting points were determined on a RY-2 melting apparatus; IR spectra were recorded on a Avatar 330 infrared spectrophotometer (KBr pellet); ^{1}H NMR data were acquired on a Bruker AV 400-MHz operating at 400 MHz. $CDCl_3$ or DMSO-d6 was used as solvent; mass is performed on a Saturn 2000 mass spectrometer. A Spectronic Genesys 8 UV/VIS spectrophotometer was used in the DPPH and RBC haemolysis assays.

Synthetic procedure for *N*-Hydroxycinnamoyl amino acid esters

N-hydroxycinnamoyl amino acid esters **a**–**h** were synthesized from hydroxycinnamic acid and the corresponding amino acid esters according to literature with some modifications (Orlandi *et al.*, 2001). Generally, to a solution of 3,4-dihydroxycinnamic acid (3.0 g, 16.5 mmol), ethyl glycinate hydrochloride (2.2 g, 16.5 mmol) and 1-hydroxybenzotriazole (2.3 g, 17.0 mmol) in *N*,*N*-dimethylformamide (50 ml) were added to triethylamine (6.9 ml, 49.5 mmol) at 0°C. After 10 min, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimidehydrochloride (3.2 g, 16.7 mmol) was added at 0°C and the resultant reaction mixture was stirred for 18 h at room temperature. The mixture was poured into water (200 ml), extracted with ethyl acetate (4 × 200 ml), washed with water (2 × 100 ml) and brine (100 ml), and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the residual paste was purified by column chromatography (silica gel, Hexane/EtOAc [1:1]) to give **a**–**h**.

*Ethyl N-[3-(3,4-dihydroxyphenyl)-1-oxo-2-propeny-yl] glycinate (**a**)*

Compound **a**, pale yellow solid, m.p. 170–171°C, ^{1}H NMR (400 MHz, DMSO-d6), 9.415 (S, 1H, OH), 9.170 (S, 1H, OH), 8.414 (br s, 1H, NH), 7.282 (d, 1H, $J = 15.6$, HC=C), 6.964 (S, 1H, ArH), 6.870 (d, 1H, $J = 8.0$, ArH), 6.757(d, 1H, $J = 8.0$, ArH), 6.419 (d, 1H, $J = 15.6$, C=CH), 4.113 (d, 2H, $J = 6.8$, CH_2), 3.930–3.946 (q, 2H, $J = 6.8$, $–OCH_2$), 1.217–1.181 (t, 3H, $J = 7.6$, CH_3). IR (KBr) υ, cm^{-1}: 3,503 (HO–), 3,365 (N–H), 3,046 (Ar–H), 3,027 (C=C–H), 1,728 (C=O), 1,654 (O=C–N), 1,605 (C=C). MS (*m*/*z*, %): 264.7 (M^+, 75.0), 219 (76.5), 55 (100).

*Ethyl N-[3-(4-hydroxy-3-methoxyphenyl)-1-oxo-2-propen-1-yl] glycinate (**b**)*

Compound **b**, pale yellow solid, m.p. 154–155°C, ^{1}H NMR (400 MHz, $CDCl_3$), 7.595 (d, 1H, $J = 15.6$, HC=C), 7.083 (d, 1H, $J = 8.0$, ArH), 7.012 (S, 1H, ArH), 6.924 (d, 1H, $J = 8.0$, ArH), 6.329 (d, 1H, $J = 15.6$, C=CH), 6.118 (br s, 1H, NH), 5.877 (br s, 1H, OH), 4.270–4.252 (q, 2H, $J = 7.2$, $–OCH_2$), 4.181 (d, 2H, $J = 5.2$, $–CH_2$), 3.9725 (s, 3H, OCH_3), 1.328–1293 (t, 3H, $J = 7.2$, $–CH_3$). IR (KBr) υ, cm^{-1}: 3,503 (HO–), 3,365 (N–H), 3,046 (Ar–H), 3,027 (C=C–H), 1,728 (C=O), 1,654 (O=C–N), 1,605 (C=C). MS (*m*/*z*, %): 278 (M^+, 100.0).

*Ethyl N-[3-(4-hydroxyphenyl)-1-oxo-2-propen-1-yl] glycinate (**c**)*

Compound **c**, white solid, m.p. 169–170°C, ^{1}H NMR (400 MHz, $CDCl_3$), 7.609 (d, 1H, $J = 15.6$, HC=C), 7.410 (d, 2H, $J = 8.4$, ArH), 6.849 (d, 2H, $J = 8.4$, ArH), 6.329 (d, 1H, $J = 15.6$, C=CH), 6.118 (br s, 1H, NH), 4.270–4.252 (q, 2H, $J = 7.2$, $–OCH_2$), 4.181 (d, 2H, $J = 5.2$, CH_2), 1.328–1.292 (t, 3H, $J = 7.2$, $–CH_3$). IR (KBr) υ, cm^{-1}: 3,503 (HO–), 3,365 (N–H), 3,046 (Ar–H), 3,027 (C=C–H), 1,728 (C=O), 1,654 (O=C–N), 1,605 (C=C), 1,210. MS (*m*/*z*, %): 249 (M^+, 100.0).

*Ethyl N-[3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1-yl]-L-phenylalaninate (**d**)*

Compound **d**, pale yellow solid, m.p. 167–168°C, ^{1}H NMR (400 MHz, DMSO-d6): 9.430 (br s, 1H, OH), 9.208 (br s, 1H, OH), 8.414 (d, 1H, $J = 7.6$, NH), 7.288 (d, 1H, $J = 15.6$, HC=C), 7.270–7.187 (m, 5H HC=C, ArH), 6.939 (S, 1H, ArH), 6.844 (d, 1H, $J = 8.0$, ArH), 6.748 (d, 1H, $J = 8.0$, ArH), 6.402 (d, 1H, $J = 15.6$, C=CH), 4.580–4.524 (m, 1H, CH), 4.089–4.070 (q, 2H, $J = 7.6$, CH_2), 3.073–2.924 (m, 2H, CH_2), 1.128–1.093 (t, 3H, $J = 7.6$, CH_2CH_3). IR (KBr) υ, cm^{-1}: 3,503 (HO–), 3,365 (N–H), 3,046 (Ar–H), 3,027 (C=C–H), 1,728 (C=O), 1,654 (O=C–N), 1,605 (C=C). MS (*m*/*z*, %): 355 (M^+, 81.2), 284.0 (28.3), 149.0 (65.7), 73 (100.0).

*Ethyl N-[3-(4-hydroxy-3-methoxyphenyl)-1-oxo-2-propen-1-yl]-L-phenylalaninate (**e**)*

Compound **e**, pale yellow solid, m.p. 154–155°C, ^{1}H NMR (400 MHz, DMSO-d6): 9.483 (S, 1H, OH), 8.411 (d, 1H, NH), 7.314–7.273 (m, 6H, HC=C, ArH), 7.119 (S, 1H,

ArH), 6.996 (d, 1H, $J = 8.0$, ArH), 6.795 (d, 1H, $J = 8.0$, ArH), 6.521 (d, 1H, $J = 15.6$, C=CH), 4.600–4.554 (m, 1H, CH), 4.085–4.032 (q, 2H, $J = 7.2$, OCH_2), 3.801 (S, 3H, OCH_3), 3.065–2.929 (m, 2H, CH_2), 1.135–1.099 (t, 3H, $J = 7.2$, CH_3). IR (KBr) υ, cm^{-1}: 3,503 (HO–), 3,365 (N–H), 3,046 (Ar–H), 3,027 (C=C–H), 1,728 (C=O), 1,654 (O=C–N), 1,605 (C=C). MS (*m/z*, %): 369.0 (M^+, 43.0), 295.0 (80.0), 221.0 (100.0), 73 (95.3).

*Ethyl N-[3-(4-hydroxyphenyl)-1-oxo-2-propen-1-yl]-L-phenylalaninate (**f**)*

Compound **f**, white solid, m.p. 129–130°C, ^{1}H NMR (400 MHz, DMSO-d6): 9.879 (S, 1H, OH), 8.444 (d, 1H, $J = 7.6$, NH), 7.395 (d, 2H, $J = 8.4$, ArH), 7.314 (d, 1H, $J = 15.6$, CH=C), 7.269–7.210 (d, 5H, ArH), 6.794 (d, 2H, $J = 8.4$, ArH), 6.478 (d, 1H, $J = 15.6$, C=CH), 4.568 (d, 1H, $J = 4.8$, CH), 4.081–4.028 (q, 2H, $J = 7.2$, OCH_2), 3.075–2.927 (m, 2H, CH_2), 1.130–1.094 (t, 3H, $J = 7.2$, CH_3). IR (KBr) υ, cm^{-1}: 3,503 (HO–), 3,365 (N–H), 3,046 (Ar–H), 3,027 (C=C–H), 1,728 (C=O), 1,654 (O=C–N), 1,605 (C=C). MS (*m/z*, %): 339.8 (M^+, 32.0), 129.9 (100.0).

*Methyl N-[3-(4-hydroxyphenyl)-1-oxo-2-propen-1-yl]-L-tyrosinate (**g**)*

Compound **g**, white solid, m.p. 173–175°C, ^{1}H NMR (400 MHz, DMSO-d6): 9.885 (S, 1H, OH), 9.249 (S, 1H, OH), 8.388 (d, 1H, NH), 7.396 (d, 2H, $J = 8.4$, ArH), 7.309 (d, 1H, $J = 15.6$, HC=C), 7.025 (d, 2H, $J = 8.4$, ArH), 6.797 (d, 2H, $J = 8.4$, ArH), 6.666 (d, 2H, $J = 8.4$, ArH), 6.482 (d, 1H, $J = 15.6$, C=CH), 4.523–4.468 (q, 1H, $J = 8.0$, CH), 3.602 (S, 3H, OCH_3), 2.964–2.794 (m, 2H, CH_2). IR (KBr) υ, cm^{-1}: 3,503 (HO–), 3,365 (N–H), 3,046 (Ar–H), 3,027 (C=C–H), 1,728 (C=O), 1,654 (O=C–N), 1,605 (C=C). MS (*m/z*, %): 341.9 (M^+, 100.0).

*Ethyl N-[3-(4-hydroxy-3-methoxyphenyl)-1-oxo-2-propen-1-yl]-L-valinate (**h**)*

Compound **h**, white solid, m.p. 79–80°C, ^{1}H NMR (600 MHz, $CDCl_3$), 7.573 (d, 1H, $J = 15.6$, HC=C), 7.068 (d, 1H, $J = 7.8$, ArH), 7.009 (S, 1H, ArH), 6.915 (d, 1H, $J = 7.8$, ArH), 6.347 (d, 1H, $J = 15.6$, C=CH), 6.136 (br s, 1H, NH), 4.728–4.706 (m, 1H, CH), 4.239 (q, 2H, $J = 6.0$, CH_2 CH_3), 3.927 (S, 3H, OCH_3), 2.241–2.230 (m, 1H, CH), 1.316–1.292 (t, 3H, $J = 4.2$, CH_3), 0.995–0.950 (m, 6H, CH_3). IR (KBr) υ, cm^{-1}: 3,503 (HO–), 3,365 (N–H), 3,046 (Ar–H), 3,027 (C=C–H), 1,728 (C=O), 1,654 (O=C–N), 1,605 (C=C). MS (*m/z*, %): 322.0 (M^+, 100.0), 177 (47.0).

Determination of partition coefficient (Log P)

The logarithm of the partition coefficient for *n*-octanol/water was computed using CS ChemPropPro software, an add-on program to ChemDraw Ultra (CambridgeSoft). The log P values of our test compounds were as follows: **a** (0.61), **b** (0.87), **c** (1), **d** (2.77), **e** (3.04), **f** (3.16), **g** (2.77), **h** (2.25), VC (−3.36), EC (1.75), EF (2.02), EP (2.14), CA (1.15), FA (1.42), and CoA (1.54).

Assay of DPPH radical scavenging activity

The DPPH free radical scavenging activity was determined by the method in literature with some modifications (Anselmi *et al.*, 2004). Each sample at different concentration in ethanol (0.5 ml) was mixed with 2.5 ml of ethanolic solution containing 0.1-mM DPPH. The mixture was shaken vigorously, and then left to stand for 30 min in the dark. The absorbance was measured at 517 nm. The absorbance of the control was obtained by replacing the sample with ethanol. The radical-scavenging activity of the samples (antioxidants) was expressed in terms of IC_{50} (concentration in μM required for a 50% decrease in absorbance of DPPH radical).

Preparation of RBC

Human red blood cells were separated from heparinized blood from a healthy volunteer. Erythrocytes were separated from blood plasma by centrifugation (10 min at 2,000 rpm at 4°C), and then the RBCs were washed three times with phosphate-buffered saline (PBS) at pH 7.4. During the last washing, the cells were centrifuged at exactly 2,000 rpm for 10 min to obtain a constantly packed cell volume.

Assay for haemolysis

The 5% suspension of RBCs in PBS (pH 7.4) was incubated under air atmosphere at 37°C for 5 min, and haemolysis was initiated by introducing a PBS solution of AAPH. This reaction mixture was incubated at 37°C with gentle shaking. The extent of haemolysis was determined spectrophotometrically by measuring the absorbance of haemolysate at 540 nm. Briefly, aliquots of the reaction mixture were taken at appropriate time intervals, diluted with 0.15-M NaCl, and centrifuged at 2,000 rpm for 10 min to separate the RBCs. The percentage haemolysis was determined by measuring the absorbance of the supernatant at 540 nm and compared with that of complete haemolysis by treating the same RBC suspension with distilled water. In the case

of antihaemolysis experiments, hydroxycinnamic acid derivatives dissolved in dimethyl sulfoxide (DMSO) was added and incubated before addition of AAPH. The final concentration of DMSO was 0.1% (v/v) and did not interfere with the determination. Every experiment was repeated three times and the results were reproducible within 10% deviation.

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