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Antioxidant properties of 3-hydroxycoumarin derivatives

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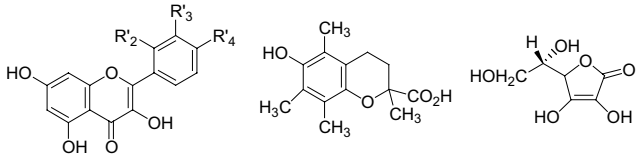
Abstract—A series of hydroxylated 3-hydroxycoumarins was synthesised by the reaction of 3-aryl-2-hydroxypropenoic derivatives with boron tribromide. They were evaluated for their ability to scavenge the 2,2-diphenyl-1-picrylhydrazyl radical, the superoxide anion radical, the hydroxyl radical and the peroxynitrite anion and to inhibit copper-induced human LDL peroxidation. The physicochemical results were in accordance to establish the compounds hydroxylated on C-6 and C-7 positions as the most active of the series with antioxidant potencies comparable to those of quercetin and vitamin C. These compounds form *o*- and *p*-quinonoid derivatives upon radical scavenging and may serve as new lead compounds for pharmacological investigations.

1. Introduction

Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods. They may play an important contributory role in the treatment of many degenerative or chronic diseases, such as atherosclerosis, brain dysfunction, immune system decline and cancer, since considerable experimental evidence links the production of reactive oxygen species to the initiation and/or progression of those pathologies.[1–5] Catechols and related compounds, which are widely distributed in nature, offer a great number of pharmacologically interesting molecules such as flavonoids (quercetin, myricetin, rutin, kaempferol, etc.),[6,7] chlorogenic and caffeic acid derivatives (rosmarinic acid, salvianolic acids)[8–10] and coumarin derivatives (esculetin, umbelliferon).[11–15] Among the tested coumarin-related compounds, 3-hydroxycoumarins recently emerged as promising antioxidant candidates. Known 3-hydroxycoumarins in nature are quite rare[16] and their formation generally results from hydroxylation of coumarins at the 3-position by liver microsomes. Their first direct chemical preparation from coumarins was described by Aihara et al.[17] This motif was isolated from *Melilotus messanensis*[18] and is also present in the structure of the antioxidant sagecoumarin, a novel coumarin compound linked via an ether bond on its C-3 position to a rosmarinic acid moiety, which was recently isolated from sage.[19,20] 5-Lipoxygenase and α-D-glucosidase inhibitory activities of coumarins greatly increased through 3-hydroxylation.[17] To our knowledge, the antioxidant properties of 3-hydroxycoumarins have never been investigated.

In this report, we studied the antioxidant activity of five 3-hydroxycoumarins using five different test methods, namely superoxide anion radical generation from the xanthine/xanthine oxidase system, hydroxyl radical production from a Fenton system, copper-induced oxidation of human LDL, chemical generation of peroxynitrite and the 2,2-diphenyl-1-picrylhydrazyl radical. Quercetin, trolox, ascorbic acid (Scheme 1) and



Galangin (R'_2=R'_3=R'_4=H)
Quercetin (R'_2 =H, R'_3=OH, R'_4=OH)
Morin (R'_2 =OH, R'_3=H, R'_4=OH)

Trolox

Vitamin C

Scheme 1.

Keywords: 3-Hydroxycoumarins; Antioxidant; Semiquinonic radicals; LDL peroxidation.
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coumarin were used as reference compounds. EPR spectroscopy of the radicals formed during the aerobic alkaline oxidation of the most active compounds was finally investigated.

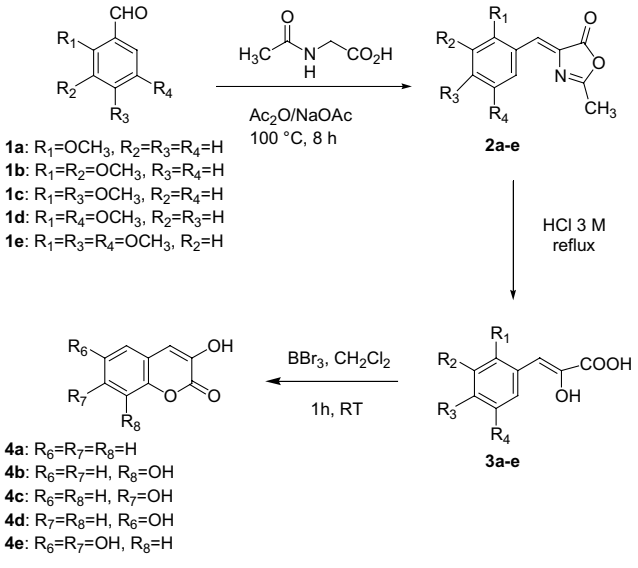
2. Results and discussion

2.1. Chemistry

Hydroxycoumarins **4a**–**e** were obtained in three steps from benzaldehydes **1a**–**e**. As can be seen in Scheme 2, the Erlenmeyer synthesis of 4-benzylideneoxazol-5(*4H*)-ones **2a**–**e** from benzaldehydes **1a**–**e** and *N*-acetylglycine was employed as the first step.[21] Then hydrolysis by HCl 3 N gave the corresponding (*Z*)-3-aryl-2-hydroxypropenoic acids **3a**–**e**. Compounds **3a**–**e** were treated by boron tribromide to give, as expected,[22] a mixture of benzofuran-2-carboxylic acids and 3-hydroxycoumarins **4a**–**e** separated by column chromatography. Compound **4e**, namely 3-hydroxyesculetin, was obtained more conveniently from the commercially available 2,4,5-trihydroxybenzaldehyde in two steps in 45% overall yield. 2,4,5-Trihydroxybenzaldehyde was converted into 3-acetamido-6,7-diacetoxycoumarin by a modified Erlenmeyer method[21] that was hydrolysed by an acid aqueous solution to give **4e**.[23]

2.2. Antioxidant studies

The superoxide scavenging activity of coumarins was determined by monitoring the competition of these compounds with the superoxide radical generated by the xanthine–xanthine oxidase system for the reduction of cytochrome *c*. The addition of **4a**, **4b** or **4d** did not significantly decrease the absorbance of reduced cytochrome *c* (Table 1). On the contrary, **4c** and **4e** were much more effective with scavenging potencies greater than those of trolox and ascorbic acid. Compound **4e** was almost as potent as quercetin (% scavenging around 60%).



Scheme 2.

Produced by a Fenton reaction, the hydroxyl radical was detected by the chemiluminescence method, using luminol as a probe.[24,25] All the hydroxycoumarin derivatives inhibited the hydroxyl radical-induced chemiluminescence of luminol (Table 1). Here again the most active compounds were **4c** and **4e** with a scavenging activity of around 80%, in the same range as quercetin. Trolox, **4b** and **4d** were 1.5 times less effective (around 50%) whereas vitamin C was far less potent (11%) and **4a** was inactive.

Hydroxycoumarins were also evaluated for the inhibition of human LDL oxidation by copper according to a previously reported method.[26] We defined the 50% efficacious dose (ED_{50}) of a test compound as the concentration that increased the control lag time of oxidised LDL by 1.5. Coumarin derivatives effectively inhibited the lipid peroxidation in a dose-dependent manner (Fig. 1). Weakly active compounds were **4a** and **4d** (ED_{50} around 30.0 μM; Table 1) whereas **4b** and **4e** were three times more active (ED_{50} 10.0 μM). The most interesting coumarin, **4c**, with an ED_{50} of 4.53 μM, was slightly less effective than quercetin and ascorbic acid (Table 1).

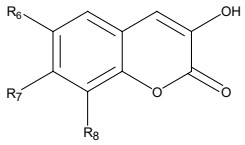
Peroxynitrite synthesis was carried out as previously described.[27] The protective effects of the compounds against peroxynitrite damage were expressed as the concentration giving 50% inhibition of the peroxynitrite-induced oxidation of dihydrorhodamine 123 (IC_{50}). Reference compounds, that is, trolox, quercetin, vitamin C, and **4c** rapidly inhibited DHR 123 oxidation with half maximal inhibitory concentrations around 1.0 μM. Compounds **4e** (ED_{50} of 1.94 μM), **4b** (ED_{50} of 3.44 μM) and **4d** (ED_{50} of 5.49 μM) were two, three and five times less inhibitory than quercetin (Fig. 2). Compound **4a** was almost unreactive.

Finally we used the stable free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH) to investigate the radical scavenging features of the hydroxycoumarins in terms of antioxidant reactivity (rate constants) and stoichiometry (number of radicals trapped per antioxidant molecule). The results obtained are given in Table 1. Coumarins **4a**, **4b** and **4d** were the less potent DPPH radical scavengers of the series with ECR_{50} values greater than 0.50. A large excess of antioxidant compounds over DPPH was necessary to induce a noticeable extinction of DPPH. In contrast, hydroxycoumarin **4c** and quercetin were the most powerful DPPH radical scavengers with comparable scavenging parameters (ECR_{50} around 0.25 and $\log Z$ around 2.60). Hydroxycoumarin **4e** was a bit less reactive towards DPPH radical ($ECR_{50} = 0.55$; $\log Z = 2.20$).

2.3. EPR spectroscopy

Using similar conditions to those previously described by us[23] in the case of **4e**, EPR spectra of 1 N NaOH solution of **4c** were recorded as a function of time. After a

Table 1. Antioxidant activities of test compounds and 3-hydroxycoumarins



Entry	DPPH $\log Z$	Scavenging ECR_{50}	% Superoxide radical scavenging	% Hydroxyl radical scavenging	LDL oxidation ED_{50} (μM)	Peroxynitrite scavenging IC_{50} (μM)
4a	1.34 ± 0.08	>19.00	Inactive	Inactive	27.2 ± 1.7	>50.0
4b	1.59 ± 0.07	2.85 ± 0.10	7.0 ± 0.3	46.6 ± 3.1	10.0 ± 0.6	3.44 ± 0.14
4c	2.55 ± 0.13	0.24 ± 0.01	40.7 ± 3.0	82.4 ± 4.4	4.53 ± 0.09	1.24 ± 0.04
4d	1.60 ± 0.08	7.61 ± 0.39	4.2 ± 0.2	51.0 ± 2.4	36.3 ± 2.5	5.49 ± 0.32
4e	2.18 ± 0.10	0.55 ± 0.03	57.2 ± 3.5	81.6 ± 4.3	8.92 ± 0.30	1.94 ± 0.05
Quercetin	2.43 ± 0.12	0.25 ± 0.01	65.2 ± 2.6	78.0 ± 3.9	3.50 ± 0.07	1.10 ± 0.04
Trolox	—	—	27.5 ± 1.4	48.8 ± 2.2	10.0[35] ± 0.7	1.02 ± 0.03
Vitamin C	—	—	30.4 ± 1.6	11.0 ± 0.8	2.50[36] ± 0.05	1.17 ± 0.03
Coumarin	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive

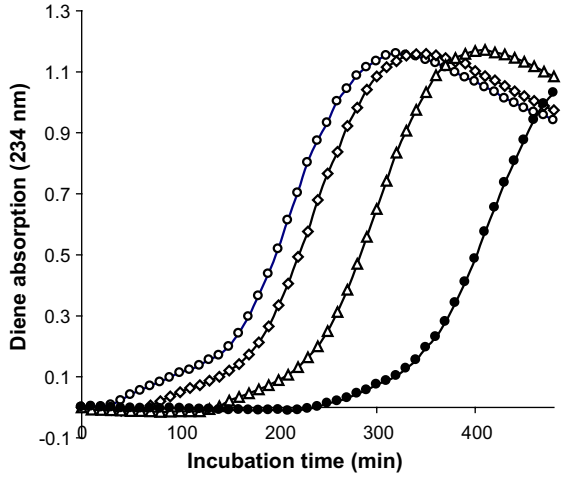


Figure 1. Effect of **4e** on the lag-phase of LDL oxidation by copper II by increase of the diene absorption. LDL (0.1 mg/mL) incubated with 1.66 μM Cu^{2+} in phosphate buffer with varying concentrations of **4e**: 0 (○), 2.0 (◇), 5.0 (△) and 10.0 μM (●).

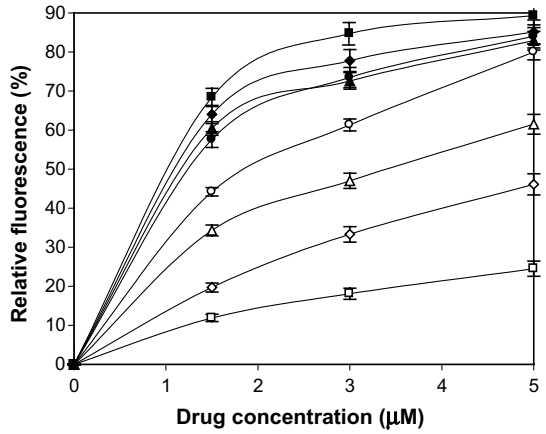


Figure 2. Effect of antioxidants on peroxynitrite-(1.5 μM) mediated DHR 123 (10.0 μM) oxidation. Trolox (■), quercetin (◆), vitamin C (▲), compound **4c** (●), compound **4e** (○), compound **2** (△), compound **4d** (◇), compound **4a** (□).

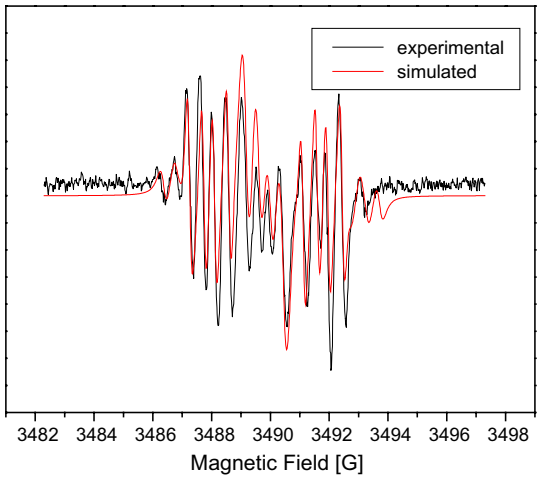
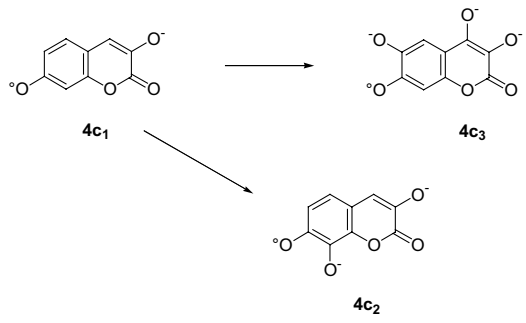


Figure 3. Experimental and simulated spectra of $\mathbf{4c_1}$ radical species.



Scheme 3.

few minutes, a 16-line spectrum was obtained (Fig. 3) attributed to the primary radical $\mathbf{4c_1}$ (Scheme 3). The signal rapidly evolved to a more complex spectrum (Fig. 4) consisting of a mixture of two radicals. The eight-line radical (+) slowly decayed leaving after several hours a clean four-line spectrum (*) (Fig. 5). The eight-line spectrum was attributed to the secondary radical $\mathbf{4c_2}$ and the four-line one to the tertiary radical $\mathbf{4c_3}$. All the

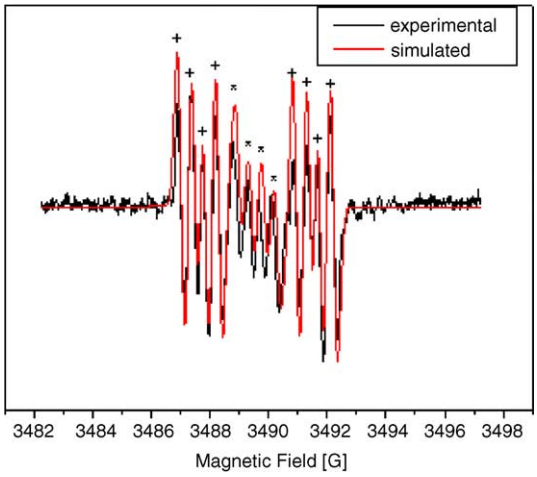


Figure 4. Experimental and simulated spectra of mixture of **4c$_2$** (+) and **4c$_3$** (*) radical species.

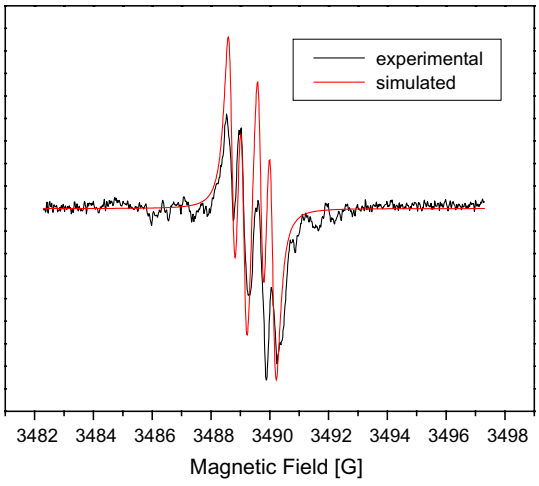


Figure 5. Experimental and simulated spectra of **4c$_3$** radical species.

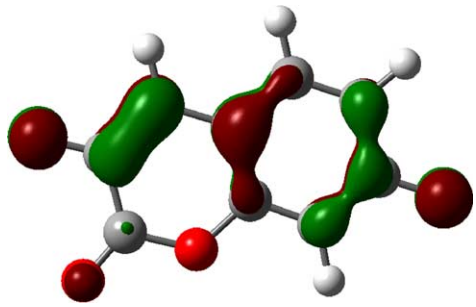


Figure 6. SOMO of radical **4c$_1$**.

attributions were made on the basis of previous experimental observations[23] and theoretical calculations. The SOMO of **4c$_1$** (Fig. 6) gives position 4 as the most reactive site followed by positions 6 and 8. The hyperfine splitting coupling constants of radical **4c$_2$** are quite different from those of the primary radical generated from an alkaline solution of **4e**[23] indicating that radical **4c$_1$** was not hydroxylated at position 6 but more likely at positions 4 and 8. The structure of radical **4c$_2$** was established on the basis of calculated hyperfine constants. In the case of a first hydroxylation of **4c** at position 4, the secondary radical would have the following calculated hyperfine constants: $a_{H5} = -1.04$ G; $a_{H6} = 0.02$ G and $a_{H8} = 0.03$ G that are very far from the experimental ones. Conversely a first hydroxylation of **4c** at position 8 gives a good fit between experimental and calculated hyperfine constants (Table 2). The tertiary radical is probably due to a double hydroxylation of **4c**. Calculations of the hyperfine constants for a radical issued from 3,4,7,8-tetrahydroxycoumarin give the following values: $a_{H5} = 3.28$ G and $a_{H6} = 0.22$ G whereas, in the case of 3,4,6,7-tetrahydroxycoumarin, the calculated values are in accordance with the experimental ones (Table 2).

3-Hydroxycoumarins exhibited various antioxidant activities. The introduction of a hydroxyl group on the C-3 position is sufficient to enhance quenching properties since coumarin was unreactive in all four tests whereas **4a** was moderately protecting. Substitution of the phenyl ring by another hydroxyl group brought a significant increase in the antioxidant properties. A precise analysis of the results showed that the most effective compounds of the series were **4c** and **4e**. This is particularly evident for the superoxide anion and hydroxyl radical scavenging tests. In these studies, **4c** and **4e** were largely more potent than the rest of the series of coumarin derivatives. The importance of a catechol group was already established for the prominent superoxide scavenging activities of polyphenols. Polyphenols like pyrogallol and gallic acid, which have phenolic OH groups at *ortho*-position, had stronger superoxide scavenging activities than pyrocatechol and resorcinol.[28] In the same way, the flavonoid quercetin, which contains a catechol moiety, was seven times more potent than its analogue, morin.[29] The presence of this catechol moiety in **4e** must explain the first position of this molecule as superoxide scavenger. Indeed it has been shown that this compound forms a stable semiquinonic primary radical upon aerobic alkaline oxidation.[23] **4c**, which lacks a catechol moiety, ranks second. For this compound, the hydroxyl group on C-7 position is conjugated with the other hydroxyl group on C-3 position in a manner similar to a hydroquinone system. The radical formed upon reaction with the superoxide anion is expected to be particularly stabilised by delocalisation over the cyclic structure. A similar suggestion was advanced in a recent report about the structure-activity relationships of flavonoids as peroxynitrite scavengers.[30] The 3-OH group of flavonoids was established as an important pharmacophore, whose activity is influenced by the substituents of positions 5 and 7. This was particularly evident for galangin (Scheme 1). In this compound, not only the presence of the 5-OH group may lead by tautomery to a catechol-like structure in ring C, but also an electronic effect of the 7-OH group may enhance the peroxynitrite scavenging. The 3-OH and 7-OH groups of galangin form a hydroquinone system, which was also shown to be a better peroxynitrite scavenger than catechol.[30] The contribution of this hydroquinone is essential to explain the position of **4c** as the best hydroxyl and peroxy-

Table 2. Proton hyperfine splitting constants obtained from simulation of experimental EPR spectra and calculated using DFT formalism with B3LYP hybrid function

Radicals	Experimental and calculated (in parentheses) B3LYP/6-31G(2d, 2p)//B3LYP/EPR-II ^{1}H hyperfine splitting constants			
	a_{H4}	a_{H5}	a_{H6}	a_{H8}
4c$_1$	3.50 (−3.63)	0.42 (−0.42)	2.81 (−2.79)	0.80 (−2.25)
4c$_2$	0.48 (0.31)	3.86 (−4.29)	0.84 (−1.19)	
4c$_3$		0.42 (0.44)		0.89 (−0.88)

nitrite scavenger and inhibitor of LDL oxidation. In the LDL oxidation study, compounds ranked in the same order as for the other tests. Compounds **4c** and **4e**, whose effects mainly resulted from their radical scavenging properties, were the best protecting compounds. But one may also suggest that **4e** may inhibit LDL peroxidation by chelating copper via its catechol moiety. Compound **4c** showed antioxidant properties similar to those of quercetin. This was confirmed by the DPPH study. Compound **4c** and quercetin were the best DPPH scavengers with ECR_{50} parameters around 0.25. This parameter describes the global reaction of antioxidants with DPPH and its low value suggests that both compounds are able to scavenge more than one DPPH radical. In terms of reactivity, the kinetic parameter $\log Z$ derived from electron transfer from DPPH describes initial reaction kinetics and is thus related to H-atom abstraction. The reactivity kinetics displayed by **4c** and quercetin were similar, and were the highest of all compounds tested. Finally, EPR experiments showed a propensity of **4c** to be oxidised under alkaline conditions into a primary semiquinonic radical, which rapidly evolved into stable secondary and tertiary polyhydroxylated radicals. So we can identify two pharmacophores in these coumarins, namely the hydroxyl group at the 3-position and the hydroxyl groups at 6- and 7-positions. This leads to compounds, which are almost as antioxidant as quercetin or vitamin C and which are certainly converted upon radical scavenging to *o*- and *p*-quinonoid derivatives via an electron transfer mechanism. This was already demonstrated for the catecholates, caffeic acid and chlorogenic acid.[31,32]

3. Conclusion

In this paper, the 3,7-dihydroxycoumarin derivatives **4c** and **4e** were found to be potent antioxidant compounds comparable to quercetin or vitamin C. Their antioxidant activities can be related to their abilities to give stable semiquinonic radicals (for **4e**)[23] or nonclassical radicals (for **4c**, this paper). These 3-hydroxycoumarins are worth being evaluated more accurately for their pharmacological properties.

4. Experimental

4.1. Chemistry

All reagents and solvents were purchased from Aldrich-Chimie (Saint-Quentin-Fallavier, France) of ACS reagent grade and were used as provided. TLC analyses were performed on plastic sheets precoated with silica gel 60F254 (Merck). SiO_2, 200–400 mesh (Merck) was used for column chromatography. NMR spectra were obtained on an AC 200 Bruker spectrometer in the appropriate solvent with TMS as internal reference. Melting points were obtained on a Reichert Thermopan melting point apparatus, equipped with a microscope and are uncorrected. Elemental analyses were performed by CNRS laboratories (Vernaison).

4.1.1. Synthesis of (*Z*)-4-benzylideneoxazol-5(4H)-ones 2; general procedure. Aromatic aldehyde **1** (100 mmol), N-acetylglycine (14.04 g; 120 mmol), anhydrous NaOAc (10.66 g; 130 mmol) and Ac_2O (51 g; 500 mmol) were mixed and stirred at 100 °C for 8 h. The mixture was allowed to cool at room temperature. Then ice water (50 mL) was added. The resulting precipitate was filtered, washed with 50% aq EtOH (4 × 20 mL) and dried at 60 °C to give **2**.

Compound **2a**: 63% yield; brown powder; mp 150–152 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) 2.38 (s, 3H), 3.88 (s, 3H), 6.90 (d, 1H, ^3J = 7.95 Hz), 7.02 (t, 1H, ^3J = 7.65 Hz), 7.39 (m, 1H), 7.74 (s, 1H), 8.61 (dd, 1H, ^3J = 7.95 Hz, ^4J = 1.60 Hz); **2b**: 58% yield; brown powder; mp 181–183 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) 2.40 (s, 3H), 3.88 (s, 3H), 3.90 (s, 3H), 6.99 (dd, 1H, ^3J = 7.95 Hz, ^4J = 1.60 Hz), 7.13 (t, 1H, ^3J = 7.95 Hz), 7.65 (s, 1H), 8.27 (dd, 1H, ^3J = 7.95 Hz, ^4J = 1.60 Hz); **2c**: 65% yield; brown powder; mp 164–165 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) 2.37 (s, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 6.42 (d, 1H, ^4J = 2.20 Hz), 6.58 (dd, 1H, ^3J = 8.90 Hz, ^4J = 2.20 Hz), 7.67 (s, 1H), 8.64 (d, 1H, ^3J = 8.90 Hz); **2d**: 76% yield; reddish brown powder; mp 125–126 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) 2.38 (s, 3H), 3.82 (s, 3H), 3.84 (s, 3H), 6.83 (d, 1H, ^3J = 8.90 Hz), 6.97 (dd, 1H, ^3J = 8.90 Hz, ^4J = 3.20 Hz), 7.69 (s, 1H), 8.27 (d, 1H, ^4J = 3.20 Hz); **2e**: 40% yield; red powder; mp 195–196 °C; ^{1}H NMR ($CDCl_3$, 200 MHz) 2.37 (s, 3H), 3.89 (s, 3H), 3.92 (s, 3H), 3.96 (s, 3H), 6.47 (s, 1H), 7.68 (s, 1H), 8.38 (s, 1H).

4.1.2. Synthesis of (*Z*)-3-aryl-2-hydroxypropenoic acids 3; general procedure. A mixture of the 4-benzylideneoxazol-5-one **2** (16 mmol) in 3 N HCl (25 mL) was refluxed at 100 °C for 4 h. The mixture was allowed to cool at room temperature and was filtered to give **3** as dark brown powders.

Compound **3a**: 45% yield; black powder; mp 144–146 °C (dec); ^{1}H NMR (DMSO-d_6, 200 MHz) 3.81 (s, 3H), 6.83

(s, 1H), 6.91–7.01 (m, 2H), 7.23 (m, 1H), 8.20 (dd, 1H, $^3J = 7.75$ Hz, $^4J = 1.50$ Hz). Compound **3b**: 23% yield; black powder; mp 135–137 °C (dec); ^{1}H NMR (DMSO-d_6, 200 MHz) 3.70 (s, 3H), 3.79 (s, 3H), 6.70 (s, 1H), 6.90 (dd, 1H, $^3J = 8.05$ Hz, $^4J = 1.60$ Hz), 7.05 (dd, 1H, $^3J = 8.05$ Hz, $^3J = 7.75$ Hz), 7.78 (dd, 1H, $^3J = 7.75$ Hz, $^4J = 1.60$ Hz). Compound **3c**: 29% yield; brown powder; mp 156–158 °C (dec); ^{1}H NMR (DMSO-d_6, 200 MHz) 3.79 (s, 3H), 3.82 (s, 3H), 6.44 (d, 1H, $^4J = 2.25$ Hz), 6.53 (dd, 1H, $^3J = 8.90$ Hz, $^4J = 2.25$ Hz), 7.07 (s, 1H), 8.10 (d, 1H, $^3J = 8.90$ Hz). Compound **3d**: 40% yield; black powder; mp 157–159 °C (dec); ^{1}H NMR (DMSO-d_6, 200 MHz) 3.70 (s, 3H), 3.76 (s, 3H), 6.76 (s, 1H), 6.79–6.94 (m, 2H), 7.79 (d, 1H, $^4J = 2.10$ Hz). Compound **3e**: 55% yield; black powder; mp 174–176 °C (dec); ^{1}H NMR (DMSO-d_6, 200 MHz) 3.68 (s, 3H), 3.81 (2s, 6H), 6.68 (s, 1H), 6.75 (s, 1H), 7.86 (s, 1H).

4.1.3. Synthesis of 3-hydroxycoumarins 4; general procedure. A solution of (*Z*)-3-aryl-2-hydroxypropenoic acid **3** (*x* mol) in CH_2Cl_2 (25 mL) was treated by a 1.0 M solution of BBr_3 in CH_2Cl_2 ([*n* + 4] equivalents, *n* being the number of methoxy groups of **3**). The solution was stirred at room temperature for 1 h and then hydrolysed by water (30 mL). After 30 min stirring, the formed precipitate was filtered. The organic layer was separated, dried over Na_2SO_4 and the organic solvent was evaporated in vacuo to give a residue. Column chromatography on grouped precipitate and residue (typical solvent system: hexane/ethyl acetate, 50/50) gave benzofuran-2-carboxylic acids and the desired 3-hydroxycoumarins.

Compound **4a**: 15% yield; brown powder; mp 150–152 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) 7.11 (s, 1H, H-4), 7.25 (td, 1H, $^3J = 7.05$ Hz, $^4J = 1.45$ Hz, H-6), 7.27 (dd, 1H, $^3J = 7.05$ Hz, $^4J = 1.45$ Hz, H-8), 7.38 (td, 1H, $^3J = 7.05$ Hz, $^4J = 1.45$ Hz, H-7), 7.42 (dd, 1H, $^3J = 7.05$ Hz, $^4J = 1.45$ Hz, H-5), 10.15 (b s, 1H, OH); ^{13}C NMR (DMSO-d_6, 50 MHz) 115.0 (C-4), 115.6 (C-8), 120.7 (C-10), 124.5 (C-6), 126.3 (C-5), 127.5 (C-7), 141.8 (C-3), 149.2 (C-9), 158.5 (C-2); Anal. Calcd for $C_9H_6O_3$: C, 66.67; H, 3.73. Found: C, 66.49; H, 3.84.

Compound **4b**: 12% yield; black powder; mp 214–216 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) 6.90 (m, 2H), 7.04 (m, 1H), 7.05 (s, 1H, H-4), 10.05 (b s, 1H, OH), 10.29 (b s, 1H, OH); ^{13}C NMR (DMSO-d_6, 50 MHz) 115.2 (C-4), 118.4 (C-7), 118.5 (C-5), 119.9 (C-10), 124.5 (C-6), 141.5 (C-3), 142.6 (C-9), 144.8 (C-8), 159.9 (C-2); Anal. Calcd for $C_9H_6O_4$: C, 60.68; H, 3.39. Found: C, 60.39; H, 3.46.

Compound **4c**: 10% yield; black powder; mp 284–286 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) 6.75 (d, 1H, $^4J = 1.50$ Hz, H-8), 6.80 (dd, 1H, $^3J = 9.50$ Hz, $^4J = 1.50$ Hz, H-6), 7.15 (s, 1H, H-4), 7.40 (d, 1H, $^3J = 9.50$ Hz, H-5), 10.0 (b s, 2H, 2 OH); ^{13}C NMR (DMSO-d_6, 50 MHz) 102.5 (C-8), 111.7 (C-10), 113.7 (C-6), 115.9 (C-4), 129.8 (C-5), 142.2 (C-3), 154.9 (C-9), 160.8 (C-2), 161.3 (C-7); Anal. Calcd for $C_9H_6O_4$: C, 60.68; H, 3.39. Found: C, 60.81; H, 3.51.

Compound **4d**: 11% yield; reddish brown powder; mp 229–231 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) 6.78 (dd, 1H, $^3J = 8.80$ Hz, $^4J = 2.60$ Hz, H-7), 6.84 (d, 1H, $^4J = 2.60$ Hz, H-5), 7.02 (s, 1H, H-4), 7.15 (d, 1H, $^3J = 8.80$ Hz, H-8), 9.55 (b s, 1H, OH), 10.18 (b s, 1H, OH); ^{13}C NMR (DMSO-d_6, 50 MHz) 112.6 (C-5), 115.8 (C-4), 117.3 (C-8), 120.1 (C-10), 120.4 (C-7), 142.4 (C-3), 147.2 (C-9), 154.1 (C-6), 160.4 (C-2); Anal. Calcd for $C_9H_6O_4$: C, 60.68; H, 3.39. Found: C, 60.79; H, 3.36.

Compound **4e**: 8% yield; black powder; mp 273 °C; ^{1}H NMR (DMSO-d_6, 200 MHz) 6.70 (s, 1H, H-8), 6.81 (s, 1H, H-5), 6.99 (s, 1H, H-4), 9.19 (b s, 1H, OH), 9.67 (b s, 1H, OH), 10.21 (b s, 1H, OH); ^{13}C NMR (DMSO-d_6, 50 MHz) 102.7 (C-8), 110.9 (C-10), 111.8 (C-5), 117.4 (C-4), 142.6 (C-3), 143.4 (C-6), 148.3 (C-9), 150.5 (C-7), 161.7 (C-2); Anal. Calcd for $C_9H_6O_5$: C, 55.68; H, 3.12. Found: C, 55.44; H, 3.30.

4.1.4. 3-Acetamido-6,7-diacetoxycoumarin. 2,4,5-Trihydroxybenzaldehyde (2.31 g, 15 mmol), 2.10 g of acetylglycine (23 mmol) and 1.59 g of sodium acetate (20 mmol) in 7.5 g of acetic anhydride were refluxed for 4 h. The solution was cooled to room temperature and iced-water (10 mL) was added. The precipitate was filtered, washed with ethanol/water 50/50 (4 × 10 mL) and dried to give 3.26 g (68%) of 3-acetamido-6,7-diacetoxycoumarin; mp 235–236 °C (EtOH); ^{1}H NMR ($CDCl_3$): 2.23 (s, 3H), 2.313 (s, 3H), 2.316 (s, 3H), 7.22 (s, 1H), 7.33 (s, 1H), 8.09 (b s, 1H), 8.62 (s, 1H).

4.1.5. 3,6,7-Trihydroxycoumarin 4e. A solution of 1.59 g of 3-acetamido-6,7-diacetoxycoumarin (5 mmol) in 50 mL of HCl 3 M and 2 mL of acetic acid was refluxed for 1 h. The solution was cooled to room temperature and the precipitate was filtered, washed with water and dried to give 0.65 g (67%) of **4e**.

4.2. Superoxide anion assay

Reaction mixtures contained xanthine (100 μM), bovine heart cytochrome *c* (25 μM) and test compound (100 μM) in air-saturated sodium-phosphate buffer (50 mM, pH 7.4) supplemented with 0.1 mM EDTA. The reaction was started by the addition at 37 °C of xanthine oxidase (0.07 U/mL) and reduced cytochrome *c* was spectrophotometrically monitored at 550 nm after 8 min using an Uvikon 932 spectrophotometer.

4.3. Hydroxyl radical assay

Buffer solutions (3 mL, borate 50 mM, pH 9.2) containing Cu^+ (500 μM CuCl) were freshly prepared and vortexed in a test tube with 2.4 μL of the test compound stock solution (0.01 M in DMF, 8.0 μM final concentration) and 150 μL of the luminol stock solution (5.0 mM in 0.05 M NaOH; 250 μM final concentration). Then the reaction was initiated by the addition of 13.1 μL of H_2O_2 aqueous solution (500 μM final concentration) and chemiluminescence was recorded continuously for 1 min at room temperature on a luminometer analyser (Packard Pico-lite).

4.4. LDL peroxidation

LDL were isolated from plasma of normolipidemic donors by low speed ultracentrifugation in the density range of 1.019–1.063 g/mL. Then, LDL were dialysed against 0.01 M PBS (phosphate buffer saline: 0.15 M NaCl, 0.01 M Na-phosphate, pH 7.4) containing 0.01% EDTA and sterilised by filtration through a 0.22 μM pore-size filter and stored at 4 °C before use. The protein concentration was determined by Peterson's method[33] using bovine serum albumin as standard. To initiate peroxidation, an aliquot of a freshly prepared stock aqueous solution of $CuSO_4$ was added to LDL (100 μg/mL) in PBS to yield 1.66 μM final copper concentration. Where indicated, prior to copper addition, aliquots of freshly prepared stock solutions of test compounds in ethanol were immediately added and mixed to yield final concentrations of 0–100 μM. The final ethanol concentration never exceeded 1% (v/v) and the same amount of ethanol was added in the corresponding blanks. LDL was incubated at 30 °C for 8 h. Conjugated dienes (O.D. 234 nm) were measured every 10 min to assess the degree of LDL oxidation. The 50% efficacious dose (ED_{50}) of a test compound defined as the concentration that increased the control lag time of oxidised LDL by 1.5 times was calculated from semilogarithmic plots (log of drug dose vs lag time expressed as percent of control).

4.5. Peroxynitrite synthesis and assay

Acidified hydrogen peroxide (8.2 M in 1.85 M HNO_3, 6.6 mL) and sodium nitrite (2 M, 6 mL) solutions were drawn into two separate syringes. The contents of both syringes were simultaneously injected into an ice-cooled beaker containing 4.2 M sodium hydroxide (6 mL) through a 'Y'-shaped junction. Excess hydrogen peroxide was removed by MnO_2 treatment. The concentration of the resultant stock was measured spectrophotometrically at 302 nm ($\varepsilon = 1670\ M^{-1}cm^{-1}$). The typical yield ranged from 70 to 80 mM.

Experiments were conducted at room temperature in a 50 mM phosphate-buffered saline containing 0.1 mM diethylenetriaminepentaacetic acid, 90 mM NaCl and 5 mM KCl, pH 7.4. Blanks using DMF and degraded peroxynitrite were run. Test compound, dihydrorhodamine 123 (DHR 123, 10.0 μM) and peroxynitrite (1.5 μM) were rapidly mixed and fluorescence measurements were performed on a Perkin Elmer LS50 spectrophotometer with excitation and emission wavelengths of 500 and 536 nm, respectively. The effects are expressed as the concentration giving 50% inhibition of the oxidation of DHR 123 (IC_{50}).

4.6. Reaction with DPPH

All spectrophotometric measurements were performed with a Kontron Uvikon 932 spectrophotometer. Calibration curves for 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical were carried out in triplicate and the DPPH concentration (micromolar) was calculated from the following equation:

$$A_{515} = 0.011 \times C_{DPPH} - 0.0031$$

Stock solutions (0.01 M) of antioxidants were prepared in ethanol and rapidly mixed (volumes from 1.5 μL to 270 μL) with an ethanol solution of DPPH (final volume 3.0 mL). Initial concentrations of DPPH were around 50 μM. The decrease in absorbance at 515 nm was recorded every 0.02 min; 10 measurements per potential antioxidant were recorded with [antioxidant]/[DPPH] ratios varying from 0.09 to 19. The effective concentration ratio (ECR_{50}) is the ratio of antioxidant concentration to DPPH concentration producing a 50% decrease in DPPH after 5 min of reaction. The initial reaction followed a second-order kinetics; the rate constants k (obtained from plots of 1/[C] vs time), were plotted against the ratio [antioxidant]/[DPPH]. Linear regression ($r^2 > 0.95$) gave the parameter Z (slope of the line in $L\,mol^{-1}\,s^{-1}$), which was examined as a quantification of radical scavenging activity.

4.7. EPR spectroscopy

EPR spectra were recorded using a Bruker ESP300 spectrometer operating at 9.5 GHz with a 100 kHz high frequency modulation and a modulation amplitude of 0.07 G. The sample solutions were examined in a flat quartz cell inserted in standard rectangular cavity for the EPR spectra recorded at room temperature. The spectra were simulated with WinSim program using Simplex method for parameter optimisations. The hyperfine coupling constants have been computed from the nuclear spin density $\rho(r_x)$ using the following equation: $a(X) = 8\pi/3(g_e/g_0)g_x\beta_x\rho(r_x)$. Spin density was calculated using Gaussian-98 suite program (Fermi contact term) using spin density function. Results were obtained using B3LYP density functional method and 6-31G (2d, 2p) basis set for geometry optimisation followed by a single point energy calculation with EPR-II basis set of Barone.[34] This well describes the core region of electron density and allowed us to compare directly the experimental and theoretical hyperfine coupling constants.

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