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Article

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Synthesis, electrochemical and biological studies on novel coumarin-chalcone hybrid compounds

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Key words: coumarin, chalcone, Knoevenagel reaction, electrochemical study, antioxidant activity, cytotoxicity, cytoprotection, ADME.

Abstract

A series of novel hydroxy-coumarin-chalcone hybrid compounds **2a-i** has been synthesized employing a simple and efficient methodology. An electrochemical characterization using cyclic voltammetry and ESR spectroscopy were carried out in order to characterize the oxidation mechanism for the target compounds. The antioxidant capacity and reactivity were determined by ORAC and ESR assays, respectively. Biological assays were assessed in order to evaluate the cytotoxicity and cytoprotection capacity against ROS/RNS on BAEC. The results revealed that all tested compounds present ORAC values much higher than other well-known antioxidant compounds such as quercetin and catechin. Compound **2e** showed the highest ORAC value (14.1) and also presented low oxidation potential, good scavenging capacity against hydroxyl radicals, low cytotoxicity and high cytoprotective activity.

Introduction

Free radicals and reactive oxygen and nitrogen species (ROS and RNS) are atoms, molecules or ions possessing at least one unpaired electron and therefore, highly reactive with a wide range of other different molecules. They are constantly generated and maintained in balance on biological systems through metabolic processes and play

important roles in a variety of normal biochemical functions, such as cell signaling apoptosis, gene expression, ion transport and pathological processes.[1,2]

It is accepted that ROS/RNS play different roles in vivo. Some are positive and are related to their involvement in energy production, phagocytosis, regulation of cell growth and intercellular signaling, and synthesis of biologically important compounds.[3] However, the overproduction of ROS/RNS can have damaging effects on many molecules. ROS/RNS are capable of oxidizing cellular proteins, nucleic acids and lipids, since they are very small and highly reactive. For example, lipid peroxidation is a free radical-mediated propagation of oxidative damage to polyunsaturated fatty acids involving several types of free radicals, and termination occurs through enzymatic means or by free radical scavenging by antioxidants.[4,5] Therefore, the excess of ROS/RNS causes a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage, known as oxidative stress.[1,6,7] Oxidative stress considerably contributes to the pathogenesis of inflammatory and cardiovascular diseases, cancer, diabetes, Alzheimer's disease, autism or aging among others.[8-13]

The consumption of natural antioxidants, such as flavonoids, supports the endogenous antioxidant defense system. Both dietary and enzymatic antioxidants are components of interrelated systems that interact with each other to control ROS production, thereby ensuring adequate defenses against oxidative stress.[14,15]

In this sense, many studies concerning antioxidant capacity of phenolic compounds of different types (e.g. flavonoids, coumarins or tanins) have been established and their physico-chemical properties have been correlated with their antioxidant properties.[16] In

general, free radical scavenging, redox potential and the antioxidant activity of these classes of compounds mainly depends on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules, and is also affected by other factors.[17] Therefore, low oxidation potential, number of present hydroxyl groups, existence of electron-donating groups and/or stabilization of a secondary radical via delocalization through conjugated systems play a key role in the antioxidant activity.[18-21]

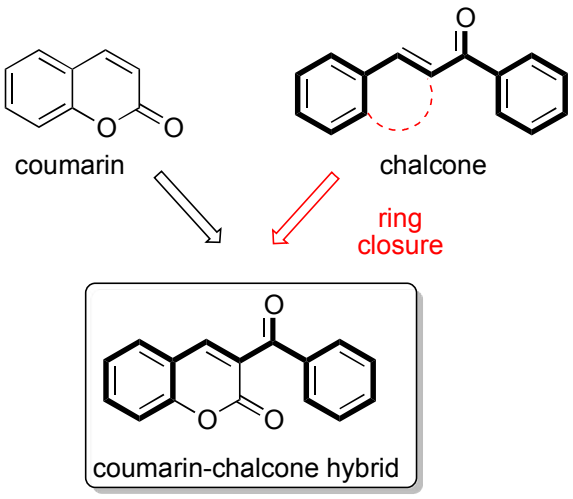
Most of the beneficial health effects of flavonoids are attributed to their antioxidant and chelating abilities. Due to their ability to inhibit low-density lipoprotein (LDL) oxidation, flavonoids have demonstrated unique cardioprotective effects.[22,23] The protective effects of flavonoids in biological systems are attributed to their capacity for transfer electrons to radicals, chelate metal catalysts,[24] activate antioxidant enzymes,[25] reduce alpha-tocopherol radicals,[26] and inhibit oxidases.[27]

Chalcones (α-phenyl-β-benzoylethylene) are prominent secondary metabolites precursors of flavonoids and isoflavonoids in plants. Chemically, they consist of two aromatic rings linked by a three-carbon α,β- unsaturated system that are known to exhibit an impressive array of biological properties.[28-30] Chalcones attract much research attention because of their pharmaceutical potential due to the radical-scavenging properties,[31,32] antitumour,[33,34] anti-inflammatory[35] or neuroprotective properties.[36] Thus, it is reasonable to explore, among others, the radical-scavenging properties of hydroxyl derivatives of chalcones.

On the other hand, coumarins (*2H*-chromen-2-one) are a large family of compounds, of both natural and synthetic origin, important because of the pharmacological activities that

these kind of compounds display,[37] such as antimicrobial,[38] monoamine oxidase (MAO) inhibitors,[39-41] antitumor,[42] adenosine receptors[43] or antioxidant[17] among others.

Coumarin and chalcones represent, therefore, promising scaffolds in the medicinal chemistry realm. Taking into account the previously mentioned features,[18,19] and with the aim of finding new chemical structures with antioxidant activity, we have design a new scaffold in which the chalcone fragment is partially incorporated in the coumarin moiety. The resulting coumarin-chalcone hybrid fixes the *trans*-conformation of the chalcone scaffold by sharing the double bond of the pyrane ring of the coumarin nucleus (Scheme 1).

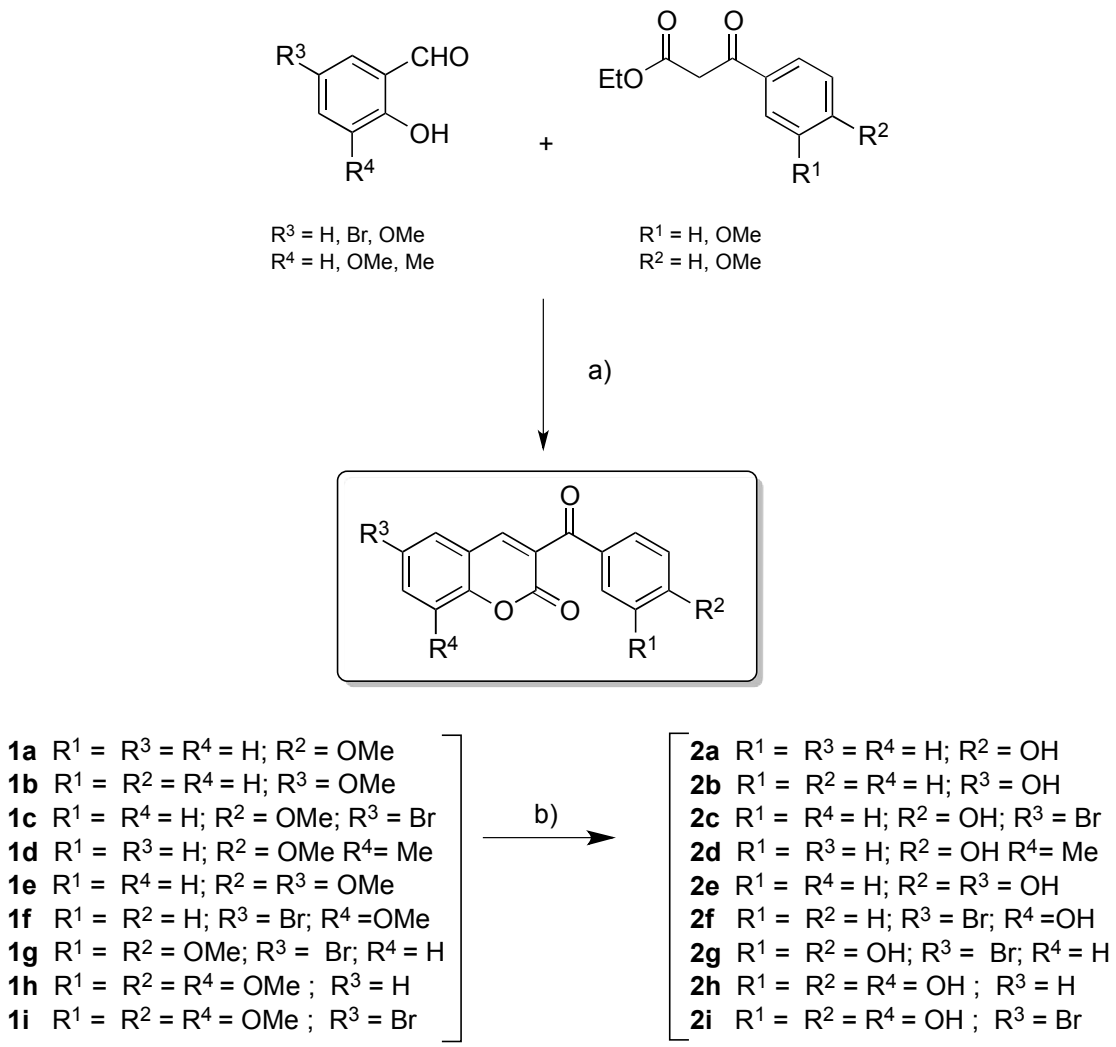


Scheme 1. Structural rational design of coumarin-chalcone hybrid compounds

Chemistry

The coumarin derivatives **2a-i** were efficiently synthesized in two steps[40] briefly described as follows: i) synthesis of methoxy-3-benzoylcoumarins **1a-i** and ii) synthesis of the hydroxy-3-benzoylcoumarins (**2a-i**). The above steps are outlined in Scheme 2.

Based on the widely used Knoevenagel condensation reaction for the preparation of coumarin derivatives,[37,44] we used an efficient one-step synthesis to generate the methoxy-3-benzoylcoumarin precursors **1a-i**. These compounds were prepared in good yields (74-98%) using the appropriately substituted salicylaldehyde and the corresponding β-ketoester in presence of piperidine in ethanol at reflux for 2-5 hours, obtaining the desired compound as a precipitate that was separated by filtration and further purified by recrystallization in MeOH/DCM. The final compounds **2a-i** were then synthesized by hydrolysis of the corresponding methoxy precursors **1a-i** employing an excess of a Lewis acid, BBr_3, in DCM at 80 ºC in a Schlenk tube for 48 hours followed by treatment of MeOH and purification by flash chromatography of the crude product (69-98%).



1a $R^1 = R^3 = R^4 = H$; $R^2 = OMe$
1b $R^1 = R^2 = R^4 = H$; $R^3 = OMe$
1c $R^1 = R^4 = H$; $R^2 = OMe$; $R^3 = Br$
1d $R^1 = R^3 = H$; $R^2 = OMe$ $R^4 = Me$
1e $R^1 = R^4 = H$; $R^2 = R^3 = OMe$
1f $R^1 = R^2 = H$; $R^3 = Br$; $R^4 = OMe$
1g $R^1 = R^2 = OMe$; $R^3 = Br$; $R^4 = H$
1h $R^1 = R^2 = R^4 = OMe$; $R^3 = H$
1i $R^1 = R^2 = R^4 = OMe$; $R^3 = Br$

b)

2a $R^1 = R^3 = R^4 = H$; $R^2 = OH$
2b $R^1 = R^2 = R^4 = H$; $R^3 = OH$
2c $R^1 = R^4 = H$; $R^2 = OH$; $R^3 = Br$
2d $R^1 = R^3 = H$; $R^2 = OH$ $R^4 = Me$
2e $R^1 = R^4 = H$; $R^2 = R^3 = OH$
2f $R^1 = R^2 = H$; $R^3 = Br$; $R^4 = OH$
2g $R^1 = R^2 = OH$; $R^3 = Br$; $R^4 = H$
2h $R^1 = R^2 = R^4 = OH$; $R^3 = H$
2i $R^1 = R^2 = R^4 = OH$; $R^3 = Br$

Scheme 2. Synthesis of methoxylated (**1a-i**) and hydroxylated (**2a-i**) 3-benzoylcoumarin derivatives. *Reagents and conditions:* a) piperidine, EtOH, reflux, 2-5 h; b) BBr_3, DCM, 80 ºC, 48 h.

Electrochemical Study

Cyclic Voltammetry

The electrochemical properties of hydroxy-coumarin-chalcone derivatives (**2a-i**) were studied by cyclic voltammetry, employing an electrochemical cell with three electrodes and different scan rates between 0.050-0.75 V*s^{-1}. Under these conditions, all derivatives exhibited one (compounds **2a-g**) or two (compounds **2h** and **2i**) well anodic peaks without the corresponding cathodic peak. The anodic peak potentials (Epa I and Epa II) obtained at a 0.75 V* s-^{1} scan rate are listed in Table 1.

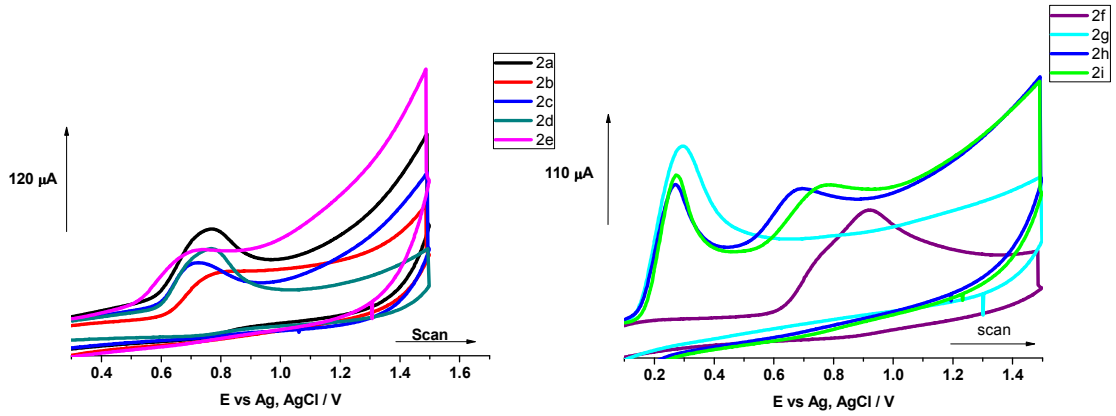


Figure 1. Cyclic voltammograms of the hydroxy-coumarin-chalcone derivatives **2a-i** in phosphate buffer pH 7.4 at 0.75 V*s^{-1}.

Table 1. Oxidation potential for compounds **2a-i** in study to 0.75 $V*s^{-1}$

Compound	Epa I (V)	Epa II (V)
2a		0.77
2b		0.79
2c		0.72
2d		0.77
2e		0.74
2f		0.92
2g	0.30	-
2h	0.27	0.69
2i	0.27	0.77

Characterization of coumarin-chalcone radicals by ESR spectroscopy

In order to confirm the formation of semiquinone radical from compounds **2a-i**, an electron spin resonance (ESR) spectroscopy study was performed. For compounds **2a-d** and **2g** the spin trap PBN was used. This nitrone derivative is a diamagnetic compound which in presence of free radicals produce a spin adduct (paramagnetic species) increasing the half life of radicals species.[45] The experiment was carried out by electrolysis of derivatives to control potential *in situ* by using DMSO as solvent and TBAP as supporting electrolyte at

room temperature. Then PBN spin trap was added. In absence of PBN spin trap, for derivatives **2e**, **2f**, **2h** and **2i**, an ESR spectrum was observed. Compound **2e** with two hydroxyl groups, evidenced a symmetric ESR spectrum with five lines (Figure 2a). Compound **2f** showed a hyperfine pattern with two lines (Figure 2b) and was simulated and characterized by two doublets due to two non-equivalent hydrogen atoms.

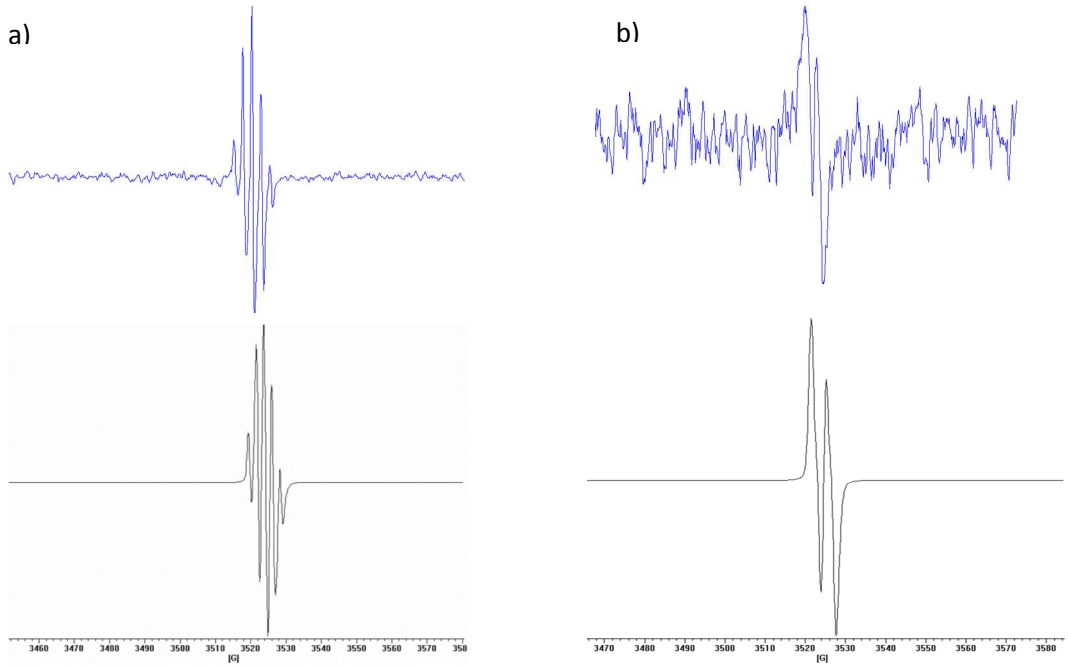


Figure 2. a) Experimental (blue) and simulated (black) ESR spectra for compound **2e** and b) for compound **2f**. Simulated hyperfine splitting constants (hfscs) were A_H=3.57 G, A_H=1.34 G for compound **2e**; A_H=2.35 G, A_H=2.35 G (two equivalent hydrogen atom) and A_H=1.75 G for compound **2f**.

ORAC-FL assay

The capacity for peroxyl radical scavenging by coumarin-chalcone hybrids was assessed using the oxygen radical absorbance capacity (ORAC) assay. This methodology gives a relative index to a standard molecule (Trolox), a hydrosoluble analog of vitamin E. The ORAC values obtained for all derivatives are showed in Table 2. All derivatives presented an ORAC index higher than the well-known antioxidants quercetin and catechin.[46] Compound **2e** presented the highest ORAC value of the studied series (14.1).

Table 2. ORAC values and percentage of radicals scavenging for compounds **2a-i** as measured by ESR spin trapping in competition with DMPO.

Compound	ORAC value	% radical scavenging
2a	9.2 ± 0.4	26
2b	11.5 ± 0.4	39
2c	9.5 ± 0.3	28
2d	10.9 ± 0.3	45
2e	14.1 ± 0.3	58
2f	8.3 ± 0.3	65
2g	13.0 ± 0.4	100
2h	8.1 ± 0.6	75
2i	8.8 ± 0.5	100

Trolox	1.0 ± 0.2	31
Quercetion	7.3 ± 0.2*	
Catechin	6.8 ± 0.2*	

* Data obtained from reference 46

Antioxidant reactivity by ESR Assay

The reactivity against mixture of radical species for all the studied coumarin derivatives was carried out adapting the H_2O_2/NaOH/DMSO/DMPO system[47] a non-catalytic and competitive fenton reaction, in which the spin trap DMPO and the antioxidant molecule competes for hydroxyl, superoxide anion and methyl radicals.

Four wide hyperfine lines were observed in the control measure (namely as blank, absence of the tested or antioxidant molecule, Figure 3) due to the mixture of DMPO-OH, DMPO-CH_3 and DMPO-$O_2^{\bullet-}$ adducts formation.

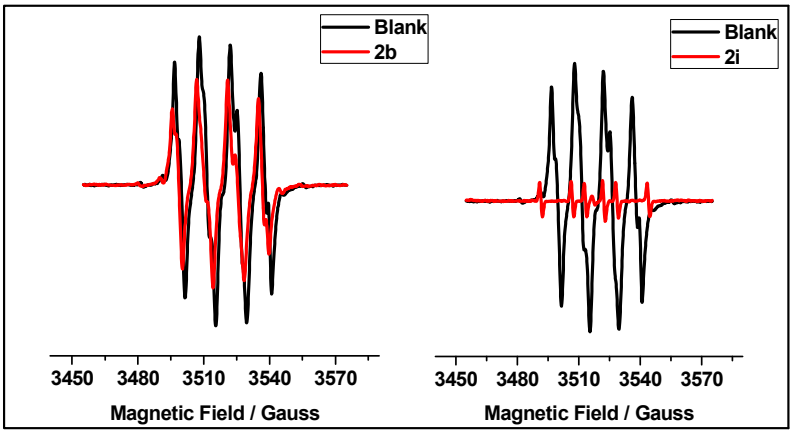


Figure 3. ESR spectra for reactivity assays of compounds **2b** and **2i**.

Biology

Cytotoxicity assays

Cytotoxicity of compounds **2a-i** on bovine aortic endothelial cells (BAEC) was assessed and evaluated over a concentration range of 0-50 μM employing the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Bromide (MTT) assay.[47] Results are shown on Table 3 for concentrations of 10, 20 and 50 μM for each studied compound.

Table 3. Cytotoxicity of compounds **2a-i** tested at three different concentrations on BAEC. Results are expressed on percentage (%) of cell viability (n = 3)

Compound	10 μM	20 μM	50 μM
2a	76 ± 7	60 ± 6	44 ± 5
2b	88 ± 2	95 ± 2	86 ± 1
2c	95 ± 3	93 ± 9	104 ± 6
2d	87 ± 7	82 ± 5	101 ± 8
2e	68 ± 1	94 ± 5	93 ± 10
2f	96 ± 3	101 ± 1	93 ± 4
2g	102 ± 4	100 ± 5	92 ± 5
2h	49 ± 2	68 ± 2	63 ± 2
2i	64 ± 2	23 ± 2	71 ± 6

Cytoprotection assays: ROS/RNS scavenging activity in cell systems

Hydrogen peroxide

In order to evaluate the cytoprotection against H_2O_2, derivatives presenting high cell viability values (compounds **2b-g**) were selected. These compounds also presented high antioxidant capacity in ORAC assays and showed notable hydroxyl radical scavenging activity.

BAEC were incubated with two different concentrations of each derivative (10 and 20 μM) for 24 h in a 24 well plate, followed by treatment with 1 mM of H_2O_2 for 2h. Cell viability was then quantified by MTT reduction assay (Figure 4a).

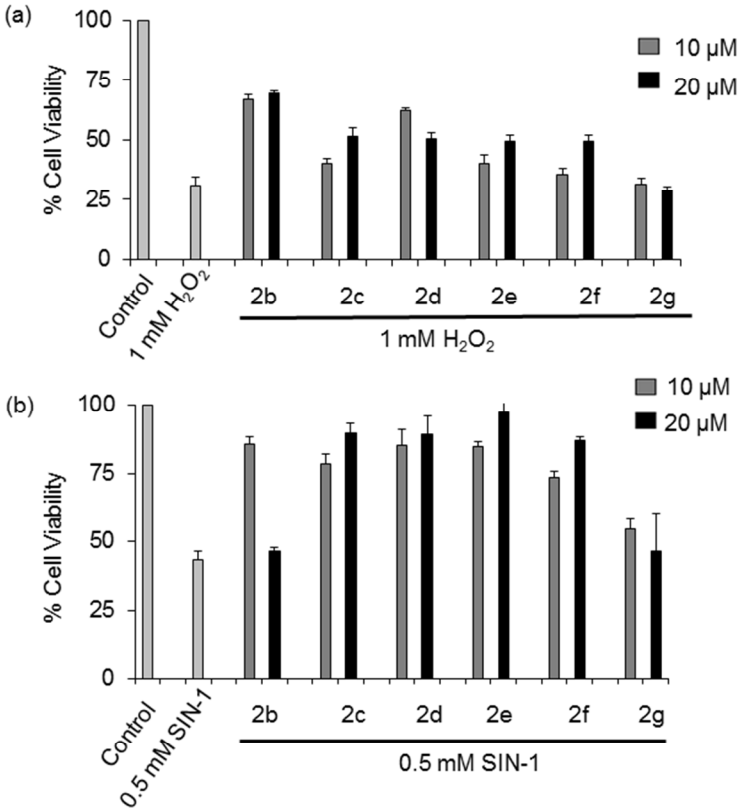


Figure 4. Cytoprotection of compounds **2b-g** at 10 and 20 μM against (a) 1 mM H_2O_2 and (b) 0.5 mM SIN-1 induced cytotoxicity on bovine aortic endothelial cells using MTT assay.

Peroxynitrite

Cytoprotective property of the compounds against peroxynitrite in BAEC was investigated by MTT assay. The peroxynitrite was generated in cell using 3-morpholino-sydnonimine (SIN-1).[48] Figure 4b shows the results for cytoprotection assays against peroxynitrite for compounds **2b**-**g**.

Theoretical evaluation of ADME properties.

To better correlate the drug-like properties of the coumarin-chalcone hybrid compounds the lipophilicity, expressed as the octanol/water partition coefficient and herein called logP, as well as other theoretical calculations such as the topological polar surface area (TPSA), the number of hydrogen acceptors and the number of hydrogen bond donors were calculated using the Molinspiration property program.[49] Theoretical prediction of absorption, distribution, metabolism and excretion (ADME) properties of all compounds is summarized in table 4.

Table 4. Theoretical prediction[a] of the ADME properties of compounds **2a-i**.

Compound	log*P*	Molecular Weight	TPSA (Å^2)	*n*-OH acceptors	*n*-OHNH donors	Volume (Å^3)
2a	2.92	266.25	67.51	4	1	227.00
2b	2.89	266.25	67.51	4	1	227.00
2c	3.70	345.15	67.51	4	1	244.88
2d	3.32	280.28	67.51	4	1	243.56
2e	2.42	282.25	87.74	5	2	235.01
2f	3.89	345.15	67.51	4	1	244.88
2g	3.21	361.15	87.74	5	2	252.90
2h	2.16	298.25	107.97	6	3	243.03
2i	2.92	377.15	107.97	6	3	260.92

[a] TPSA, topological polar surface area; *n*-OH, number of hydrogen acceptors; *n*-OHNH, number of hydrogen bond donors. The data was determined with Molinspiration calculation software.

Results and Discussion

Taking into account the importance of the hydroxyl substituents in the antioxidant properties,[17,50] we have focused the study in hydroxy-coumarin-chalcone hybrids. All coumarin derivatives described in this report (compounds **1a-i** and **2a-i**) were efficiently synthesized according to the simple and generalized experimental procedure outlined in Scheme 2. Electrochemical, biological and theoretical studies were carried out for the final tested compounds **2a-i**.

From the experimental results obtained for the cyclic voltammetry, we observed that under these conditions, all derivatives exhibited one (compounds **2a-g**) or two (compounds **2h** and **2i**) characteristic anodic peaks without the corresponding cathodic peak (Figure 1), indicating an irreversible oxidation process as a result of the oxidation of one hydroxyl group in any of the aromatic rings, forming the semiquinone radical.

The anodic peak potentials (Epa I and Epa II) obtained at a 0.75 V* s^{-1} scan rate are listed in Table 1. Monohydroxyl derivatives **2a**, **2c** and **2d**, which contain a hydroxyl substituent at the *para* position of the benzoyl moiety, presented an anodic peak potential in the same positive potential range (Epa II = 0.77, 0.72 and 0.77 V, respectively). The same positive potential in derivatives **2a** and **2d** indicates that the methyl substituent at the position 8 of the coumarin core in **2d** is not relevant in the oxidation process. In contrast, the bromine group in derivative **2c** seems to provoke a slight decrease in the anodic peak potential (Epa II = 0.72 V). The same effect is observed for compound **2e** (Epa II = 0.74 V), which in addition to the hydroxyl group present in the benzoyl moiety also bears an additional hydroxyl group in the coumarin core. Compounds **2b** and **2f** with a hydroxyl group in the coumarin moiety, presented the oxidation potential shifted to more positive values (0.79 V and 0.92 V, respectively) disfavoring the oxidation process. This effect is more pronounced for compound **2f** due to the interaction through intramolecular hydrogen bonding between the OH group in position 8 and the oxygen atom of the pyrone ring, as well as due to the electronwithdrawing properties of this pyrone system.[51]

Compounds **2g**, **2h** and **2i**, showed a characteristic peak (Epa I), attributed to catechol moiety. This oxidation occurred at low potential (0.30 V, 0.27 V and 0.27 V, respectively) due to the electron donating effect of the hydroxyl group at the 3' position of the benzoyl moiety and to the favorable quinone formation as final product in these derivatives. In

addition to this low potential oxidation peak, compounds **2h** and **2i** showed an additional second peak, assigned to the hydroxyl group at position 8 of the coumarin core.

Data obtained for the characterization of coumarin-chalcone radicals by ESR spectroscopy showed that in the absence of the spin trap *N*-*tert*-butyl-α-phenylnitrone (PBN), the oxidation of compounds **2a-d** and **2g** gave no ESR signal. However, in the presence of PBN, a hyperfine pattern with six lines was observed corresponding to the formation of a carbon-centered semiquinone spin adduct ($a_H \approx 13.5$ and $a_H \approx 2.3$ G were found for all compounds).[52] For compound **2e** with two hydroxyl groups, evidenced a symmetric ESR spectrum with five lines (Figure 2a) and it was interpreted as a semiquinone radical which hyperfine pattern corresponds to one triplet assigned to two equivalent hydrogen atoms with $a_H = 2.37$ G and two doublets corresponding to one non equivalent hydrogen atom with hyperfine constant $a_H = 2.35$, $a_H = 1.75$ G.

Compound **2f** showed a hyperfine pattern with two lines (Figure 2b) and was simulated and characterized by two doublets due to two non-equivalent hydrogen atoms with $a_H = 1.34$ and $a_H = 3.57$ G. For radical species of compound **2h**, the ESR spectrum (data not shown) was simulated by two doublets attributed to two non-equivalent hydrogen atoms with $a_H = 4.35$ and $a_H = 2.37$ G. Finally, for the radical species of compound **2i**, the hyperfine pattern was interpreted and simulated by four doublets due to four non-equivalent hydrogen atoms with hyperfine coupling constants $a_H = 2.82$, $a_H = 2.93$, $a_H = 2.06$ and $a_H = 1.83$ G and also one triplet with $a_H = 3.37$ G due to two equivalent hydrogen atoms.

The antioxidant capacity of compounds **2a-i** was studied by ORAC-FL assays. From the data obtained, summarized in Table 2, we observed that the difference between the ORAC values could be related with the substituents present in these derivatives. Derivatives **2a** and **2b** with one hydroxyl group at the *para* position of the benzoyl moiety, and at the 6

position of the coumarin scaffold, respectively, presented ORAC values of 9.2 and 11.5, correspondingly. Compound **2c** and **2d** with a hydroxyl group in the same position as in compound **2a** but with an additional bromine substituent in position 6 of the coumarin or a methyl substituent at the position 8, respectively, presented ORAC values of 9.5 and 10.9. Derivative **2e**, which combines the substitution pattern of compounds **2a** and **2b**, resulted to be the compound with the highest ORAC value (14.1).

On the other hand, structurally comparing compounds **2c** and **2g**, we observe that the only difference is the presence in compound **2g** of an additional hydroxyl group at the 3' position of the benzoyl moiety, forming a catechol function. This modification increases the antioxidant capacity in compound **2g** (ORAC = 13.0). ORAC values around 8 were observed for compound **2f**, **2h** and **2i**. In addition to the catechol function, compounds **2h** and **2i** present an additional hydroxyl group at position 8 of the coumarin ring comparing to the structurally related compound **2g**. This additional hydroxyl group could form a hydrogen bond with the oxygen atom of the pyrone ring, disfavoring therefore the transfer of the hydrogen atom.

The antioxidant reactivity of compounds **2a-i** was studied by ESR assay. With this method it was observed that the intensity of the spectrum decreased in the presence of the tested coumarin-chalcone hybrid compounds added to the system (red line, Figure 3). This behavior was observed in all derivatives **2a-i** and the percent of the radicals ($CH_3^{\bullet}$, $OH^{\bullet}$, $O_2^{\bullet-}$) scavenging activity is represented in table 2. The scavenging values obtained could be related with the number and position of the hydroxyl substituents. Compounds **2g** and **2i** showed a different hyperfine pattern, which were attributed to carbon-centered spin adducts formation (Figure 3), meaning that the secondary semiquinone radical for each compound was trapped by DMPO spin trap and the radical scavenging percentage were close to 100%

In general, MTT assay only provides qualitative trends in the cytotoxicity of the compounds and the cytoprotective properties. Cytotoxicity of compounds **2a-i** on BAEC using different concentrations (10, 20 and 50 μM) is shown in Table 3**.** Derivatives **2b-g** showed cell viability between 86-100% up to the maximum concentration of 50 μM. Compounds **2a**, **2h** and **2i** showed significant toxicity at 50 μM, where compound **2a** being the most toxic exhibiting only 44% viability. The varying degrees in the toxicity of the compounds can be accounted to several factors such differences in their compartmentalization properties, nature and concentration of ROS produced, ability to scavenge radicals, and/or initiate pro-apoptotic signaling pathways.

Because compounds **2a, 2i** and **2h** have significant toxicity, they were not used for cytoprotection assays. However, compounds **2b-g**, due to their low toxicity, were then examined at lower doses of 10 and 20 μM for cytoprotection against hydrogen peroxide (H_2O_2) or peroxynitrite ($ONOO^-$) generated from SIN-1, and are shown in Figures 4a and 4b, respectively. Compounds **2b** and **2d** showed the highest cytoprotection against H_2O_2 compared to all compounds, which presented around 40% cytoprotection at 10 μM. Compounds **2c-2f** at 20 μM were cytoprotective against $ONOO^-$ by ~90-95%, whereas compound **2b** imparted no protection at this concentration. Compound **2g** was not cytoprotective against both H_2O_2 and $ONOO^-$ at any of the concentrations tested. All the studied compounds showed observable cytoprotection, with remarkable results for compounds **2b-f**. In our previous work,[53] it was shown that lypophilic spin trap such as the PBN protect BAEC from $H_2O_2^-$ induced toxicity while the more polar DMPO protects cells more efficiently from $ONOO^-$. The studied compounds show a better cytoprotective effect against $ONOO^-$. The studied compounds show a better cytoprotective effect against $ONOO^-$. However, due to the complexity of the mechanisms that are directly and

indirectly involved in this assay, it is difficult to find correlation between the cytoprotection and partition coefficients since these latter values were theoretically calculated. Moreover, the oxidation potentials may not show direct correlation with cytotoxicity/cytoprotection data due to other cellular events that are involved such as variations in the ability of the compounds to redox cycle, subcellular compartmentalization, degradation by cellular oxido-reductants, or the ability to induce (or suppress) non-radical mediated pro-apoptotic pathways. Therefore, although we would like to find direct correlation of cytotoxicity with the physical data such as partition coefficient and redox potentials, the complexity of the mechanisms involved in cell viability makes this correlation difficult to realize.[19,54,55]

From the data obtained for the theoretical evaluation of the ADME properties, one can notice that all the hybrid compounds **2a-i** do not break any point of the Lipinski's rule of five, making them promising leads for drug candidates.[56] TPSA and logP values are compatible with those described as a predictive indicator of the drug capacity of membrane penetration.[57]

Conclusions

In this paper, we describe the synthesis of coumarin-chalcone hybrid compounds employing the Knoevenagel reaction as a key step for the efficient preparation of a selected series. The electrochemical properties of the studied compounds resulted to be excellent, presenting low oxidation potentials, very high ORAC values (all of them higher than the reference compounds quercetin and catechin) and high percentage of radical scavenging. In most cases, compounds presented low cytotoxicity for BAEC culture even at the highest concentration tested and good cytoprotective effects against H_2O_2 and $ONOO^-$ induced cell death. In addition, the whole series of compounds present good theoretical ADME

properties. Therefore, based on these results, the synthesized coumarin-chalcone hybrid compounds can be considered as promising scaffolds in the studied area.

It is worth noting that compound **2e** presented the highest ORAC value (14.1), good scavenging capacity, low cytotoxicity and high cytoprotection values, especially against $ONOO^-$ induced cell death (almost 100% of cell viability at 20 μM), and so can be considered as a potential candidate as lead compound for more thorough studies of its antioxidant properties.

Experimental Section

Chemistry

Melting points were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX spectrometer at 300 and 75.47 MHz, respectively, using TMS as internal standard (chemical shifts in δ values, J in Hz). Mass spectra were obtained using a Hewlett-Packard 5988A spectrometer. Elemental analyses were performed using a Perkin-Elmer 240B microanalyser and were within ± 0.4% of calculated values in all cases. Silica gel (Merck 60, 230–00 mesh) was used for flash chromatography (FC). Analytical thin layer chromatography (TLC) was performed on plates precoated with silica gel (Merck 60 F254, 0.25 mm). The purity of compounds **1a-i** and **2a-i** was assessed by HPLC and was found to be higher than 95%. All the chemical reagents employed in the synthetic process were obtained from Aldrich Chemical Company, Fluka, Across or Merck (analytical reagent grade). All reactions were carried out under deoxygenated and dry Argon atmosphere

otherwise indicated. Argon was dried flowing it through $CaCl_2$ columns, NaOH stones and P_2O_5.

General procedure for the synthesis of methoxy-3-benzoylcoumarins 1a-i.[40] To a solution of the appropriate β-ketoester (1 mmol) and the corresponding salicylaldehyde (1 mmol) in ethanol (3 mL) was added piperidine in catalytic amount. The mixture was refluxed for 2–5 h and after completion (followed by TLC), the reaction was cooled and the precipitated was filtered and washed with cold ethanol and ether to afford the desired compound. Compounds were further recrystallized from $MeOH/CH_2Cl_2$.

6-Bromo-3-(4'-methoxybenzoyl)coumarin (1c). Yellow solid; yield: 92%; mp 222-224 ºC. ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ ppm 8.25 (s, 1H, H-4), 8.05 (s, 1H, H-5), 7.92 (d, *J* = 8.5 Hz, 2H, H-2', H-6'), 7.84 (d, *J* = 8.7 Hz, 1H, H-7), 7.44 (d, *J* = 8.7 Hz, 1H, H-8), 7.05 (d, *J* = 8.5 Hz, 2H, H-3', H-5'), 3.85 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ ppm 190.1, 164.5, 158.1, 153.5, 143.3, 135.9, 132.7, 131.9, 129.0, 128.3, 120.6, 119.0, 116.7, 114.6, 56.2. MS (EI) *m/z* (%): 359/361 (M^+, 28), 135 (100), 92 (19), 77 (20). Anal. Calcd for $C_{17}H_{11}BrO_4$: C, 56.85; H, 3.09. Found: C, 56.82; H, 3.07.

8-Methyl-3-(4'-methoxybenzoyl)coumarin (1d). White solid; yield 93%; mp 250-252 ºC. ^{1}H-NMR (300 MHz, DMSO-*d*$_6$) *δ* ppm 8.34 (s, 1H, H-4), 7.91 (d, J= 8.7 Hz, 2H, H-2', H-4'), 7.73-7.24 m, 3H, H-5, H-6, H-7), 7.06 (d, *J* = 8.7 Hz, 2H, H-3', H-5'), 3.86 (s, 3H, OCH_3), 2.41 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) *δ* ppm 190.2, 164.4, 158.4, 152.9, 152.8, 144.7, 134.7, 132.3, 129.6, 127.7, 125.8, 124.7, 118.5, 114.6, 56.1, 15.0. MS (EI) *m/z* (%): 295 ($[M+1]^+$, 19), 294 (M^+, 100), 135 (88), 77 (31). Anal. Calcd for $C_{18}H_{14}O_4$: C, 73.46; H, 4.79; Found: C, 73.39; H, 4.57.

6-Methoxy-3-(4'-methoxybenzoyl)coumarin (1e). Yellow solid; yield 80%; mp 182-184 ºC. ^{1}H-NMR (300 MHz, DMSO-d_6) δ ppm 7.84 (s, 1H, H-4), 7.71 (d, J = 8.9 Hz, 2H, H-2', H-6'), 7.13-6.94 (m, 3H, H-5, H-7, H-8) 6.77 (d, J = 8.9 Hz, 2H, H-3', H-5'), 3.83 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm 190.2, 164.0, 158.3, 155.9, 148.6, 144.2, 132.3, 128.9, 127.3, 121.1, 118.9, 117.6, 114.2, 111.6, 55.9, 55.8. MS (EI) *m/z* (%): 311 ([M+1]$^+$, 29), 310 (M$^+$, 92), 135 (100), 92 (22), 77 (27). Anal. Calcd for $C_{18}H_{14}O_5$: C, 69.67; H, 4.55. Found: C, 69.45; H, 4.33.

6-Bromo-3-(3',4'-dimethoxybenzoyl)coumarin (1g). Yellow solid; yield 98%; mp 223-224 ºC. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.23 (s, 1H, H-4), 8.05 (d, J = 2.4 Hz, 1H, H-5), 7.84 (dd, J = 8.8, 2.4 Hz, 1H, H-7), 7.56 (dd, J = 8.4, 2.0 Hz, 1H, H-6'), 7.52-7.38 (m, 2H, H-8, H-2'), 7.05 (d, J = 8.4 Hz, 1H, H-5'), 3.86 (s, 3H, 4'-OCH_3), 3.81 (s, 3H, 3'-OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm 189.8, 157.7, 154.3, 153.2, 149.0, 142.8, 135.6, 131.6, 128.7, 127.9, 125.8, 120.4, 118.8, 116.4, 111.3, 111.1, 56.1, 55.9. MS (EI) *m/z* (%): 389/391 (M$^+$, 59), 165 (100), 71 (31). Anal. Calcd for $C_{18}H_{13}BrO_5$: C, 55.55; H, 3.37. Found: C, 55.54; H, 3.39.

8-Methoxy-3-(3',4'-dimethoxybenzoyl)coumarin (1h). Pale yellow solid; yield: 95%; mp 238-240 ºC. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.28 (s, 1H, H-4), 7.54 (dd, J = 8.2, 2.1 Hz, 1H, H-6'), 7.46 (d, J = 2.1 Hz, 1H, H-2'), 7.43-7.27 (m, 3H, H-5, H-6, H-7), 7.04 (d, J = 8.2 Hz, 1H, H-5'), 3.93 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ ppm 190.2, 157.9, 154.1, 148.9, 146.6, 144.6, 143.5, 128.8, 127.1, 125.7, 124.9, 120.7, 119.0, 115.5, 111.1, 111.0, 56.3, 56.0, 55.8. MS (EI) *m/z* (%): 341 ([M+1]$^+$, 25), 340 (M$^+$, 97), 165 (100), 137 (9), 77 (10). Anal. Calcd for $C_{19}H_{16}O_6$: C, 67.05; H, 4.74. Found: C, 67.06; H, 4.74.

6-Bromo-8-methoxy-3-(3',4'-dimethoxybenzoyl)coumarin (1i). Pale yellow solid; yield 90%; mp 276-277 ºC. ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ ppm 8.21 (s, 1H, H-4), 7.67-7.49 (m, 3H, H-5, H-7, H-6'), 7.46 (s, 1H, H-2'), 7.04 (d, *J* = 8.5 Hz, 1H, H-5'), 3.96 (s, 3H, 4'-OCH_3), 3.85 (s, 3H, 8-OCH_3), 3.80 (s, 3H, 3'-OCH_3). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ ppm 195.3, 155.1, 149.9, 149.8, 148.2, 143.6, 138.1, 129.4, 128.9, 128.7, 126.0, 123.4, 119.0, 116.8, 112.6, 112.0, 59.0, 57.6, 56.7. MS (EI) *m/z* (%): 418/420 (M^+, 98), 165 (100), 77 (18). Anal. Calcd for $C_{19}H_{15}BrO_6$: C, 54.43; H, 3.61. Found: C, 54.43; H, 3.62.

General procedure to the synthesis of hydroxy-3-benzoylcoumarins (2a-i).[40] To the corresponding methoxy-3-benzoylcoumarin (1 mmol), BBr_3 in DCM (20 mmol, 1M) was added in a Schlenk tube. Tube was sealed, and the reaction mixture was heated at 80 ºC for 48 h. The resulted crude product was treated with MeOH and rotated to dryness. The obtained precipitate was recrystallized from MeOH or purified by flash chromatography, using hexane/ethyl acetate mixtures as eluent, to afford the desired hydroxy derivative.

3-(4'-Hydroxybenzoyl)coumarin (2a). White solid; yield: 78%; mp 242-243 ºC. (mp literature 239 ºC)[58] ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ ppm 10.58 (bs, 1H, OH), 8.30 (s, 1H, H-4), 7.89-7.75 (m, 3H, H-5, H-2', H-6'), 7.78-7.64 (m, 1H, H-7), 7.51-7.37 (m, 2H, H-6, H-8), 6.86 (d, *J* = 8.8, 2H, H-3',H-5'). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ ppm 190.1, 163.4, 158.5, 154.3, 144.2, 133.6, 132.9, 129.9, 127.8, 127.5, 125.3, 118.7, 116.7, 115.8. MS (EI) *m/z* (%): 267 ($[M+1]^+$, 20), 266 (M^+, 93), 237 (25), 121 (100), 93 (22), 65 (25). Anal Calcd for $C_{16}H_{10}O_4$: C, 72.18; H, 3.79. Found: C, 71.83; H, 3.53.

6-Bromo-3-(4'-hydroxybenzoyl)coumarin (2c). Pale yellow solid; yield: 73%; mp 295-298 ºC. ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ ppm 10.61 (bs, 1H, OH), 8.22 (s, 1H, H-4), 8.05 (d, *J* = 2.4 Hz, 1H, H-5), 7.93-7.73 (m, 3H, H-7, H-2', H-6'), 7.45 (d, *J* = 8.8 Hz, 1H, H-8), 6.86 (d, *J* = 8.7 Hz, 2H, H-3', H-5'). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ ppm 190.0, 163.6,

158.1, 153.4, 142.8, 136.2, 133.0, 131.8, 128.5, 127.6, 120.7, 119.0, 116.7, 115.9. MS (EI) *m/z* (%): 343/345 (M^+, 49), 121 (100). Anal. Calcd for $C_{16}H_9BrO_4$: C, 55.68; H, 2.63. Found: C, 55.30; H, 2.45.

3-(4'-Hydroxybenzoyl)-8-methylcoumarin (2d). White solid; yield: 56%; mp 252-253 ºC. ^{1}H NMR (300 MHz DMSO-*d*$_6$) δ ppm 10.56 (bs, 1H, OH), 8.27 (s, 1H, H-4), 7.80 (d, *J* = 8.4 Hz, 2H, H-2', H-6'), 7.63 (d, *J* = 7.5 Hz, 1H, H-5), 7.57 (d, *J* = 7.5 Hz, 1H, H-7), 7.30 (t, *J* = 7.5 Hz, 1H, H-6), 6.86 (d, *J* = 8.4 Hz, 2H, H-3', H-5'), 2.40 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ ppm 190.2, 163.4, 158.6, 152.6, 144.6, 134.6, 132.9, 127.8, 127.7, 127.2, 125.6, 124.8, 118.5, 115.9, 15.3. MS (EI) *m/z* (%): 281 ($[M+1]^+$, 54), 280 (M+, 96), 252 (46), 251 (51), 120 (100), 93 (56), 65 (67). Anal. Calcd for $C_{17}H_{12}O_4$: C, 72.85; H, 4.32. Found: C, 72.67; H, 4.12.

6-Hydroxy-3-(4'-hydroxybenzoyl)coumarin (2e). Brown crystals; yield: 65%; mp 301-303 ºC. ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ ppm 10.58 (bs, 1H, 4'-OH), 9.86 (bs, 1H, 8-OH,), 8.21 (s, 1H, H-4), 7.79 (d, *J* = 8.8 Hz, 2H, H-2', H-6'), 7.37-7.24 (m, 1H, H-5), 7.19-7.01 (m, 2H, H-7, H-8), 6.86 (d, *J* = 8.8 Hz, 2H, H-3', H-5'). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ ppm 190.3, 163.4, 158.8, 154.4, 147.7, 144.0, 132.9, 127.8, 127.6, 121.6, 119.2, 117.6, 115.9, 113.7. MS (EI) *m/z* (%): 283 ($[M+1]^+$, 9), 282 (M^+, 51), 121 (100), 93 (15), 65 (18). Anal. Calcd for $C_{16}H_{10}O_5$: C, 68.09; H, 3.57. Found: C, 68.23; H, 3.33.

3-Benzoyl-6-bromo-8-hydroxycoumarin (2f). Pale yellow solid; yield: 98%; mp 293-295 ºC. ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ ppm 10.92 (s, 1H, OH), 8.30 (s, 1H, H-4), 7.93 (dd, *J* = 8.1, 1.4 Hz, 2H, H-2', H-6'), 7.79-7.63 (m, 1H, H-5), 7.63-7.44 (m, 3H, H-3', H-4', H-5'), 7.34-7.24 (m, 1H, H-8). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ ppm 191.9, 157.9, 146.3, 144.8, 142.7, 136.3, 134.5, 130.0, 129.2, 127.8, 121.9, 121.8, 120.9, 116.2. MS (EI) *m/z*

(%): 344/346 (M^+, 46), 105 (100), 77 (59). Anal. Calcd for $C_{16}H_9BrO_5$: C, 53.21; H, 2.51. Found: C, 52.97; H, 2.41.

6-Bromo-3-(3',4'-dihydroxybenzoyl)coumarin (2g). Yellow solid; yield: 72%; mp 264-266 ºC. ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ ppm 10.14 (s, 1H, OH), 9.47 (s, 1H, OH), 8.19 (s, 1H, H-4), 8.05 (d, *J* = 2.1 Hz, 1H, H-5), 7.84 (dd, *J* = 8.8, 2.2 Hz, 1H, H-7), 7.45 (d, *J* = 8.8 Hz, 1H, H-8), 7.39-7.20 (m, 2H, H-2', H-6'), 6.82 (d, *J* = 8.1 Hz, 1H, H-5'). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ ppm 189.6, 157.9, 153.3, 152.5, 145.9, 142.3, 135.7, 131.7, 128.4, 127.8, 124.5, 120.5, 119.0, 116.7, 116.3, 115.7. MS (EI) *m/z* (%): 360/362 (M^+, 87), 253 (47), 251 (45), 227 (14), 225 (18), 137 (100), 109 (44). Anal. Calcd for $C_{16}H_9BrO_5$: C, 53.21; H, 2.51. Found: C, 53.23; H, 2.52.

8-Hydroxy-3-(3',4'-dihydroxybenzoyl)coumarin (2h). Bright yellow solid; yield: 81%; mp 277-279 ºC. ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ ppm 10.35 (s, 1H, OH), 10.10 (s, 1H, OH), 9.47 (s, 1H, OH), 8.20 (s, 1H, H-4), 7.36-7.06 (m, 5H, H-5, H-6, H-7, H-2', H-6'), 6.82 (d, *J* = 8.2 Hz, 1H, H-5'). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ ppm 190.2, 158.4, 152.3, 145.8, 145.0, 144.2, 142.8, 128.0, 127.4, 125.3, 124.2, 119.7, 119.5, 116.4, 115.7. MS (EI) *m/z* (%): 299 ($[M+1]^+$, 8), 298 (M^+, 41), 155 (16), 137 (100), 91 (31). Anal. Calcd for $C_{16}H_{10}O_6$: C, 64.43; H, 3.38. Found: 64.43; H, 3.39.

6-Bromo-8-hydroxy-3-(3',4'-dihydroxybenzoyl)coumarin (2i). Pale yellow solid; yield 69%; mp 300-302 ºC. ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ ppm 10.89 (s, 1H, OH), 10.14 (s, 1H, OH), 9.48 (s, 1H, OH), 8.14 (s, 1H, H-4), 7.45 (d, *J* = 2.2 Hz, 1H, H-5), 7.38-7.12 (m, 3H, H-7, H-2', H-5'), 6.81 (dd, *J* = 8.1, 3.0 Hz, 1H, H-6'). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ ppm 189.3, 157.4, 152.0, 145.8, 145.5, 142.3, 141.9, 128.0, 127.4, 124.0, 121.1, 121.0, 120.5, 115.9, 115.8, 115.2. MS (EI) *m/z* (%): 376/378 (M^+, 100), 269 (30), 267 (24), 137 (80), 109 (74). Anal. Calcd for $C_{16}H_9BrO_6$: C, 50.95; H, 2.41. Found: C, 50.92; H, 2. 43.

Cyclic voltammetry

Cyclic Voltammetry was carried out using a potentiostat/galvanostat VersaSTAT 3 provided with a V3-Studio electrochemistry software package, in 50 mM sodium phosphate buffer (pH 7.4), room temperature, using a three electrode cell, a glassy carbon electrode was used as working electrode, a platinum wire as auxiliary electrode and Ag, AgCl/KCl (ca. 3.5 M) as reference electrode. All coumarin-chalcone hybrids were studied to 1 mM in 20% methanol as final concentration.

Characterization of coumarin-chalcone radicals by ESR spectroscopy. ESR measurements were carried out on an ESR spectrometer equipped with a high-sensitivity resonator at room temperature. Unless otherwise indicated, the instrument settings used for general spectral acquisition are microwave power 10 mW, modulation amplitude 1 G, receiver gain 1.0×10^4 or 1.0×10^5, scan time 21.5 s, time constant 40.0 s, and sweep width 120 G. The oxidation process was carried out by using 30% H_2O_2 and NaOH (25 mM) in presence of chalcone-coumarins hybrid. Scans were integrated using the Bruker WINEPR v.2.11b software. Sample cells used were 50 μL glass capillary tubes. An automatic fitting program carried out the spectrum simulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G.

Radical generating system to test coumarin-chalcone hybrids. To the corresponding coumarin-chalcone hybrid compound (1.2 mmol) in dimethyl sulfoxide (20 µL), a hydrogen peroxide solution (50 µL, 30%) and a sodium hydroxide solution (50 µL, 25 mM)

were added. The total volume of the resulting solution (300 μL) was completed with DMSO in order to achieve a final 4 mM solution of the tested compounds.

ORAC-FL assay. The ORAC-FL assays were carried out on a Synergy HT multidetection microplate reader, from Bio-Tek Instruments Inc. (Winooski, USA), using white polystyrene 96-well plates. Fluorescence was read from the top, with an excitation wavelength of 485/20 nm and an emission filter of 528/20 nm. The plate reader was controlled by Gen 5 software. The oxygen radical absorbance capacity was determined as previously described[46] with slight modifications. The reaction was carried out in 75 mM sodium phosphate buffer (pH 7.4), and 200 μL final volume, FL (70 nM, final concentration) and coumarin-chalcone solutions in methanol with a range of concentration between 0.3 μM to 2 μM and 0.5 μM to 7 μM for monohydroxy and dihydroxy derivatives, respectively, were placed in each well of 96-well plate. This range of concentration was selected empirically in order to obtain a good separation between the fluorescence curves. This separation was crucial for a more accurate data treatment.[59,60] The mixture was pre-incubated for 15 min at 37 °C, before rapidly adding the AAPH solution (18 mM, final concentration). The microplate was immediately placed in the reader and automatically shaken prior to each reading. The fluorescence was recorded every 1 min for 120 min. A blank with FL and AAPH using methanol instead of the antioxidant solution and five calibration solutions using Trolox (0.5 μM to 2.5 μM) as standard molecule were also used in each assay. The inhibition capacity was expressed as ORAC values, and it was quantified employing the equation 1. All reaction mixtures were prepared in triplicate and at least three independent assays were performed for each sample.

Equation 1:

$$\text{Relative ORAC value} = \frac{[\text{AUC}-\text{AUC}^0]}{[\text{AUC}_{\text{trolox}}-\text{AUC}^0]} * \frac{[\text{trolox}]}{[\text{derivative}]}$$

Where,

AUC^0= Area under the curve blank; AUC= Area under the curve derivative; AUC_{trolox}= Area under the curve trolox (standart); [trolox] = Trolox concentration, Molar; [Derivative]= Derivative concentration, Molar

The AUC was calculated by integrating the decay of the fluorescence where F_0 is the initial fluorescence read at 0 min and F is the fluorescence read at a particular time. The AUC_{NET} corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. Data processing was performed using Origin Pro 8 SR2 (Origin Lab Corporation, USA).

Antioxidant reactivity by ESR Assay. The reactivity of all coumarin-chalcone hybrids against hydroxyl radical was assessed using the non-catalytic Fenton method described by Yoshimura Y. et al.[56] In a typical experiment, 150 µL of dimethyl sulfoxide was mixed with 50 µL of NaOH (3 mM) followed by addition of 50 µL of DMPO (30 mM) spin trap and finally with 50 µL of hydrogen peroxide 30 %. All derivatives were studied at a 4 mM final concentration and 300 µL of final volume. The mixture was positioned in ESR Cell and the spectra were recorded after five minutes of reaction.

Biological studies.

All chemicals and solvents used were of the highest analytical grade. For cell culture, all reagents or materials were purchased and used without further purification. Bovine aortic endothelial cells were purchased from Cell Systems (Kirkland, WA), non-essential amino acid and Fetal Bovine Serum (FBS) were purchased from Gibco and Invitrogen, respectively.

Cell Culture. Cells were cultured in 75 cm^2 tissue culture flasks using Dulbecco's modified Eagle's medium with 1 g/L D-glucose and 4 mM L-glutamine, and supplemented with 10% fetal bovine serum, 2.5 mg/L endothelial cell growth supplement, and 1% nonessential amino acids in the absence of antibiotics at 37 °C in a humidified atmosphere of 5% CO_2 and 20% O_2. The medium was changed every 2-3 days, and cells were subcultured once they reached 80-85% confluence. [51]

Cytotoxicity and cytoprotection. Cytotoxicity assays in BAEC cells were performed for all coumarin-chalcone hybrids, using the MTT reduction assay. BAEC were seeded in 24-well plates at a density ranging between 10^4-10^5 cells/mL, per well. After the cells reached 60-70% confluency, cells were incubated in the presence of 10 to 50 μM of the corresponding coumarin-chalcone derivatives, for 24 hours. After incubation, the medium was changed (2 mL) and 45 μL of a solution of MTT (5 mg/mL in DMEM supplemented with 0.5% FBS) was added to each well. Cells were incubated for another 2 h at 37 °C. The medium was then removed, and wells were rinsed once with PBS and 0.6 mL of DMSO were added to each well at room temperature to solubilize the formazan crystals.

The dissolved formazan was then transferred into 96-well culture plates, and the absorbance was measured at 570 nm using a UV-vis spectrophotometer. [57,58]

The (%) of inhibition of the cell viability was calculated using the equation 2:

Equation 2: % Inhibition = $(1 - A_t/A_s) \times 100\%$

where A_t and A_s are absorbances of the sample solution and the solvent alone, respectively.

To assess the cytoprotective properties of the compounds against H_2O_2 and SIN-1, BAEC (10^4-10^5 cells/mL) were grown to 60-70% confluency using 24-well plate, and each incubated with the selected derivative at 10 μM and 20 μM concentrations, for 24 hours After incubation, cells were washed with 1X PBS twice to remove the residual drug and exposed to 1 mM of H_2O_2 and 500 μM of SIN-1 for 2 h and 4h, respectively. This time periods were chosen based on the observed cell death (> 50%) using the H_2O_2 and SIN-1 concetrations mentioned above. Finally, cell viability was evaluated by the MTT reduction assay and calculated through equation 2.

Statistical analysis. Experimental results are expressed as the mean ± standard error of the mean (SEM) and are accompanied by the number (n) of observations. Differences between groups were analyzed by student's t-test. A *p* value lower than 0.05 was considered statistically significant.

Theoretical evaluation of ADME properties. Theoretical properties of logP, TPSA, *n*-OH and *n*-OHNH were calculated by the methodology developed by Molinspiration using their software available on http://www.molinspiration.com/services/properties.html.

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Abbreviations

AAPH, 2,2'-azobis(2-methylpropionamidine)dihydrochloride; BAEC, bovine aortic endothelial cells; DCM, dichloromethane; DMEM, Dulbecco's modified Eagle's medium; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; Epa, anodic peak potential; FL, fluorescein; MAO, monoamine oxidase; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Bromide; ORAC, oxygen radical absorbance capacity; PBN, α-Phenyl-N-tert-butylnitrone; RNS, reactive nitrogen species; SIN-1, 3-morpholinosydnonimine; TBAP, tetrabutylammonium perchlorate; TLC, thin layer chromatography; TPSA, topological polar surface area; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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