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Synthesis of coumarin–chalcone hybrids and evaluation of their antioxidant and trypanocidal properties†

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Based on the observed biological activities of coumarins and chalcones, we have synthesized coumarin–chalcone hybrids with the aim of evaluating their antioxidant properties and trypanocidal activity against *Trypanosoma cruzi*, the parasite responsible for Chagas disease. All derivatives have been proved to be good antioxidants in spite of their moderate trypanocidal activity in the epimastigote stage (clone Dm28c). Based on these results, we can conclude that compounds **4** and **5** are potential candidates for *in vitro* studies of their antioxidant activity. These preliminary findings encourage us to the future structural optimization of these kinds of compounds.

1 Introduction

Chalcones (1,3-diaryl-2-propen-1-ones) are one of the major classes of naturally occurring compounds with widespread distribution in fruits, vegetables, spices, tea and soy based foodstuff and they have been subject of great interest for their remarkable pharmacological activities.[1,2] Chemically, they are open chain precursors of flavonoids and isoflavonoids, in which the two aromatic rings are linked by a three-carbon α,β-unsaturated carbonyl system. Chalcones have been reported to possess many useful properties, including antibacterial,[3,4] antimalarial,[5–7] antifungal,[8,9] antiviral[10,11] and anti-inflammatory[12–14] properties.

On the other hand, coumarins (2*H*-1-benzopyran-2-ones) are another family of naturally occurring compounds widely distributed in plants. These kinds of molecules of both natural and synthetic origin, have also attracted considerable interest due to their pharmacological activities,[15] for example, their enzyme inhibitory[16,17] antitumor[18,19] and antiviral[20,21] activities as well as their anti-inflammatory[22,23] and antioxidant[22,24–26] properties.

Chagas disease is caused due to the *Trypanosoma cruzi* (*T. cruzi*) parasite and it represents a major health problem in several countries of Latin America and in the southern United States. The only two available drugs (nifurtimox and benznidazole) are limited and suffer from several drawbacks such as poor efficacy, toxicity, resistance, and multiple side effects.[27,28] During the course of *T. cruzi* infection and disease development, ROS can be produced as a consequence of tissue destruction caused by toxic secretions of the parasite, immune-mediated cytotoxic reactions, and secondary damage in the myocardium. Therefore, interventions with antioxidant compounds, that reduce the generation or the effects of ROS, may exert beneficial effects in preventing or arresting oxidative damage.[29,30] Therefore, there is an urgent need for the discovery of new, more effective, and safer drugs for human use.

Antioxidants are a class of substances presenting a wide range of different chemical structures. They are able to decrease or prevent the oxidation of other sensitive molecules through different mechanisms like chelation of active metal ions, free radicals scavenging or inhibition of pro-oxidant enzymes.[31] Free radicals and reactive oxygen species (ROS) are atoms, molecules or ions possessing unpaired electrons, which are highly reactive with a wide range of other different molecules. They are constantly generated and eliminated in the biological system through metabolic processes and play important roles in a variety of normal biochemical functions, cell signaling apoptosis, gene expression, ion transport and abnormal pathological processes.[32,33] The overproduction of ROS can have damaging effects on many molecules. For example, ROS producing oxidative stress may contribute to Chagas disease progression.[34]

It is also known that the antioxidant properties of chalcones are quite dependent on the two aryl structures,[25,35] that is, the substituents on the two aryl rings of the chalcone moiety and their substitution patterns. Especially, the hydroxyl substituent

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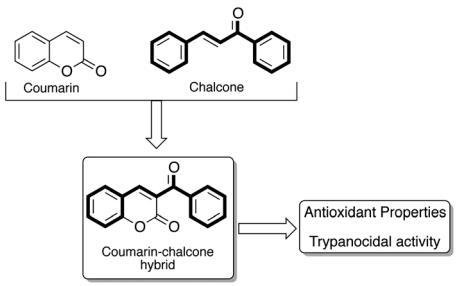


Fig. 1 Structural approach employed for the design of new coumarin–chalcone hybrid compounds.

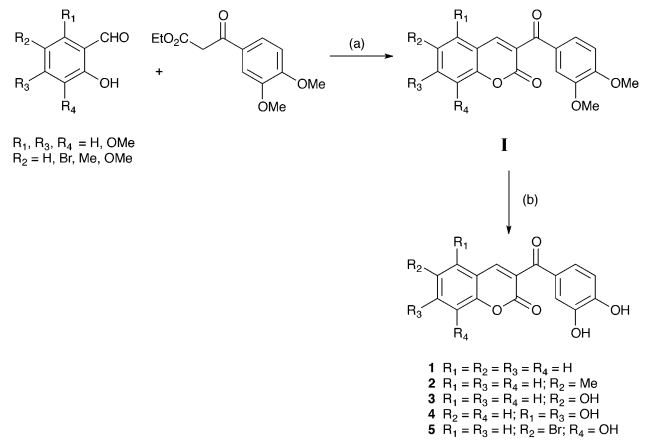
is one of the key groups that enhance the antioxidant activity of chalcone mainly due to its easy conversion to phenoxy radicals through the hydrogen atom transfer (HAT) mechanism. This phenoxy radical formation may be essential for the antioxidant properties, which are assessed primarily as radical scavenging potential of phenolic chalcones. In fact, the hydroxyl substituent is widespread among chalcones from natural sources.

Due to the potential antioxidant property and trypanocidal activity of the chalcone and coumarin moieties,[36,37] in the present work a series of coumarin–chalcone hybrids (Fig. 1) were synthesized and their antioxidant capacity and trypanocidal activity were evaluated. The chalcone moiety in the prepared molecules is included in the coumarin scaffold so that the geometry of the chalcone remains as a *trans* isomer due to the double bond position of the pyrone ring.

2 Chemistry

In order to achieve the final products, the synthesis of the hydroxy-3-benzoylcoumarins was carried out in two steps, shown in Scheme 1 and briefly described as follows: (a) synthesis of methoxy-3-benzoylcoumarins and (b) hydrolysis of the previous compounds to afford the final hydroxyl-3-benzoylcoumarins (**1–5**).

The methoxy-3-benzoylcoumarins, with a general structure **I** were efficiently synthesized with 78–94% yield by a Knoevenagel reaction treating the appropriate salicylaldehyde and ethyl 3,4-dimethoxybenzoylacetate with piperidine in ethanol at reflux for 2–6 h. The resulting methoxy derivatives were treated with boron tribromide in DCM at 80 °C in a Schlenk tube for 48 h to give the corresponding hydroxy derivatives **1–5** with 69–94% yield.



Scheme 1 Preparation of coumarin–chalcone hybrids. *Reagents and conditions*: (a) piperidine, EtOH, reflux, 2–6 h and (b) BBr_3, DCM, 80 °C, 48 h.

3 Electrochemical study

In order to evaluate the antioxidant behavior of the five compounds of this family, cyclic voltammetry, the Oxygen Radical Absorbance Capacity (ORAC FL-PGR) and the Electron Spin Resonance (ESR) studies were carried out.

3.1 Cyclic voltammetry

The electrochemical properties of hydroxyl coumarin–chalcone derivatives were studied by cyclic voltammetry. Fig. 2 shows a cyclic voltammogram at a glassy carbon electrode (GCE) immersed in DMSO/75 mmol L^{-1} phosphate (pH 7.4) buffer 30/70 media containing 1 mmol L^{-1} of compound **3** for several scan rates (v). Under these experimental conditions, two oxidation processes (peaks I and II) were observed. In general, it has been proposed that the charge transfer process at peak I corresponds to the oxidation of the catechol substituent, while the process at peak II comprises oxidation reaction involving the hydroxyl groups present in the coumarinic ring.

Results of the cyclic voltammetry, reported in Table 1 show that coumarin–chalcone hybrids present low oxidation potentials.

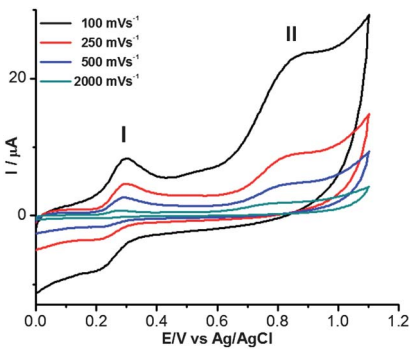


Fig. 2 Cyclic voltammogram for 1 mmol L^{-1} compound **3** in DMSO/75 mmol L^{-1} phosphate (pH 7.4) buffer 30/70 media at a GCE for v = 100, 250, 500, and 2000 mV s^{-1}.

Table 1 Oxidation potential peaks obtained by cyclic voltammetry

Compounds	E_{pa}[a] (mV)
1	322
2	449
3	312
4	337
5	247

[a] First oxidation peak potential at a scan rate of 2000 mV s^{-1}.

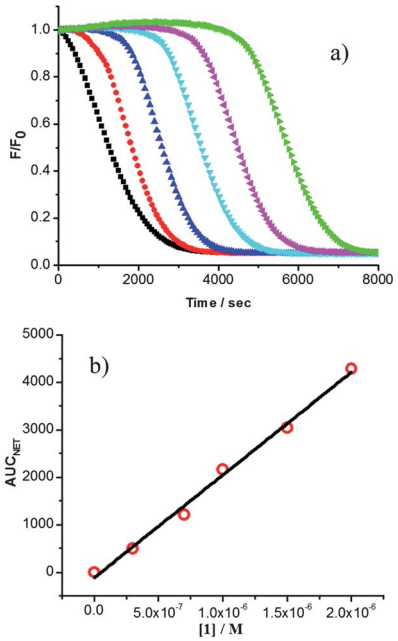


Fig. 3 (a) Kinetic profile of fluorescein consumption, AAPH mediated, in the presence of compound **5**. F_0 is the fluorescence in the absence of the compound and F the fluorescence in the presence of compound **5** and (b) graph of AUC_{NET} (net area under the curve) *versus* concentration of compound **5**.

3.2 ORAC assays

The capacity of scavenging peroxyl radicals was studied employing the ORAC method. In this assay, Trolox and 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) were used as a reference antioxidant and a peroxyl radical source, respectively.[38] This assay evaluates the capacity of antioxidants (or of their complex mixtures) to inhibit the bleaching of a target molecule (probe) induced by peroxyl radicals.

The oxidation occurs due to exposition of the target molecule, fluorescein (FL)[39] or pyrogallol red (PGR),[40] to the peroxyl radical, leading to decay of fluorescence emission or decrease of PGR absorbance with time.

The consumption of FL is commonly inhibited, even by antioxidants of low reactivity, throughout kinetic profiles characterized by the presence of induction times. Fig. 3a shows the results of AAPH mediated fluorescein oxidation in the absence and presence of increasing concentration of compound **5** in phosphate pH 7.4. Therefore, ORAC-FL values would be more influenced by the stoichiometry of the reaction (defined as the number of radicals that each additive molecule can remove) than by the reactivity of the additives. Fig. 3b shows that the area under the curve (AUC_{NET}) of the kinetic profiles for compound **5** was linearly related to the concentration of the additive.

This behavior was observed for all compounds studied. However, the consumption of PGR by reactive antioxidants is inhibited without generating induction times.[41,42] This characteristic profile is observed for all the studied compounds, as well as for Trolox (Fig. 4).

The absence of induction times in the protective kinetic profiles of PGR would imply that the ORAC-PGR index is more related to the reactivity of the antioxidants than to stoichiometric factors. Taking into account the characteristics of the ORAC-FL and ORAC-PGR assays, it has been recently proposed that both methods could be considered as rendering complementary indexes.[42] Results of this study are expressed as ORAC-FL and ORAC-PGR values and are tabulated in Table 2.

All experiments were carried out in triplicate, and the data represent the mean values (±S.D.).

Table 2 ORAC-FL and ORAC-PGR values and the scavenging hydroxyl radical (%)

Compound	ORAC-FL	ORAC-PGR	% Scavenging
1	4.37 ± 0.22	[a]	84.6 ± 5.3
2	4.95 ± 0.14	[a]	82.1 ± 7.1
3	6.02 ± 0.12	[a]	84.8 ± 7.3
4	8.32 ± 0.27	0.52 ± 0.05	84.2 ± 6.4
5	8.51 ± 0.32	1.17 ± 0.10	90.9 ± 8.2
Quercetin	7.28 ± 0.22[c]	—	—
Catechin	6.76 ± 0.22[c]	—	—
DHMcoumarin[b]	3.3[d]		
Helichrysetin	4.4[e]		

[a] Inactive compound. [b] 6,7-Dihydroxy-4-methylcoumarin. [c] Data collected from ref. 39. [d] Data collected from ref. 49. [e] Data collected from ref. 50.

3.3 ESR assays

In order to study the antioxidant reactivity of all the hydroxylated coumarin–chalcone hybrids, we have adopted a

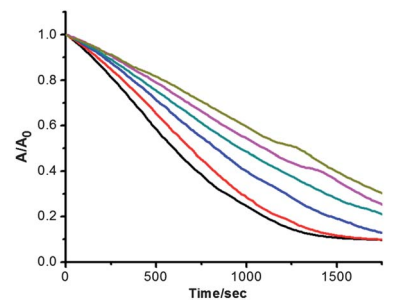


Fig. 4 Time-course of the consumption of PGR, AAPH mediated, in the presence of increasing concentration of Trolox.

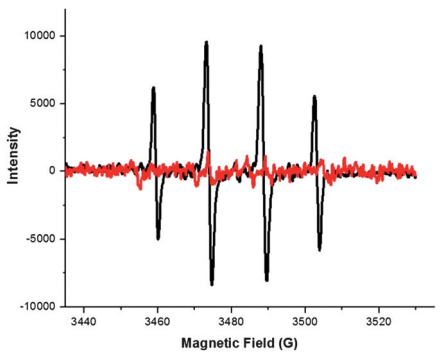


Fig. 5 ESR spectra for adduct DMPO–OH without an antioxidant molecule (black) and in the presence of compound **5** (red).

non-catalytic and competitive Fenton system.[23] Electron spin resonance (ESR) in combination with spin trapping techniques was employed to further verify that the tested compounds possess the ability to scavenge hydroxyl radicals, measuring thus, the percentage of scavenging of the compounds, summarized in Table 2. DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) reacts with hydroxyl radicals to generate the spin resonance signal, and ESR can quantify this spin resonance signal. The ESR spectrum shows four hyperfine lines due to the DMPO–OH adduct formation. The coumarin–chalcone hybrids compete with DMPO for hydroxyl radicals. Thus, the ESR signal will diminish as presented in Fig. 5 for compound **5**.

4 Pharmacology

4.1 Trypanocidal activity

Studies on trypanocidal activitywere carried out in order to investigate a possible relationship between the antioxidant capacity of the compounds and their antiparasitic activity. Trypanocidal activity was evaluated against the *T. cruzi* epimastigote stage (clone Dm28c). It was measured through the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay,[43] widely used for *in vitro* measurement of the metabolic activity or viability of cell cultures. After incubation time, we determined the number of viable parasites by measuring absorbance at 570 nm in a multiwell reader (Asys Expert Plus©, Austria). Untreated parasites were used as controls (100% of viability). Results are reported as the percentage of non-viable epimastigotes regarding the control.

Results of two different concentrations of the synthesized compounds (10 and 100 μmol L^{-1}) are reported in Table 3.

5 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 log *P*, as well as other theoretical calculations such as the TPSA, the number of hydrogen acceptors and the number of hydrogen bond donors were calculated using the Molinspiration property program. The ADME properties of the studied compounds were calculated using the Molinspiration property program, and results are summarized in Table 4.[44]

Table 3 Trypanocidal activity results for the coumarin–chalcone hybrids and nifurtimox (Nfx)

Cmpd	% Trypanocidal activity at 10 μmol L^{-1}	% Trypanocidal activity at 100 μmol L^{-1}
1	23.8 ± 0.4	38.7 ± 2.1
2	27.9 ± 0.3	47.9 ± 1.0
3	18.9 ± 0.4	46.4 ± 0.8
4	[a]	[a]
5	9.5 ± 0.2	24.1 ± 0.7
Nfx	52.5 ± 2.2	100 ± 3.2

[a] Inactive at concentration tested.

Table 4 Theoretical structural properties of the coumarin–chalcone hybrids[a]

Compd	log *P*	TPSA (Å^2)	*n*-OH acceptors	*n*-OHNH donors	Volume (Å^3)
1	2.43	87.74	5	2	235.01
2	2.85	87.74	5	2	251.58
3	1.93	107.97	6	3	243.03
4	1.63	128.20	7	4	251.05
5	2.92	107.97	6	3	260.92

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

6 Results and discussion

Taking into account the importance of the hydroxyl substituents in the antioxidant properties,[26,45] we have focused the study on hydroxylated derivatives of the coumarin–chalcone hybrid compounds, which contain a catechol fragment in the benzoyl ring at the 3 position of the coumarin.

According to the data obtained in the cyclic voltammetry, all derivatives exhibited two anodic peaks without the corresponding cathodic peak, 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. We have thus firstly investigated the influence of the scan rate (ν) in order to access in more detail the characteristics of electrochemical and chemical reactions that take place at the electrode/solution interface. The current ratio I_{pc}/I_{pa} progressively increases as a function of ν, corroborating an EC-type mechanism[46] associated with peak I. It is known that the antioxidant capacity is conceivably related to the electrochemical behavior, being indicative that a low oxidation potential corresponds to a high antioxidant power.[47] In our case, this fact could be explained as mainly due to the strong electron donating effect of the catechol group, present in all the synthesized derivatives, which can form stable radicals in which the remaining *o*-OH group stabilizes the radical as a result of the intramolecular hydrogen bonding.[48]

Considering the ORAC-FL assays, we observed that the highest ORAC-FL values were found for compounds **4** and **5** (8.32 and 8.51 respectively). The results obtained are better than for quercetin (7.28) and catechin (6.76), flavonoids currently used as reference compounds,[39] as well as other simple coumarins, such as the 6,7-dihydroxy-4-methylcoumarin (DHMcoumarin) studied by our group[49] or chalcones collected from bibliographic sources such as helichrystein.[50] It is also worth mentioning the activity of compound **3** (6.02), comparable to catechin.

The different ORAC-FL values can be related to the substituents existing in the coumarin moiety and their pattern of substitution. In addition, the presence of a benzoyl substituent improves the electron delocalization with respect to the coumarin skeleton, influencing ORAC values.

Compound **1** is the structurally simplest compound, presenting a catechol group in the benzoyl ring, with no substituents on the coumarin skeleton. This feature could explain why

compound **1** presents the lowest ORAC value (4.37) of all the studied compounds, but still being 4 times more active than the employed reference compound Trolox. If we introduce a methyl group at position 6 (compound **2**), we observe a slight increase in the ORAC-FL index (4.95) compared to compound **1**. When analyzed the obtained ORAC-FL values of compounds **1–3** based on the chemical structure and pattern of substitution, we observed that, among other features, the higher the electron donating effect of the substituent, the more active the compound. Compound **3** containing a hydroxyl at position 6, increases its ORAC value to 6.02 compared to the simplest synthesized compound **1**.

Compounds with the highest activities (**3–5**), with ORAC values 6.02, 8.51 and 8.32 respectively, all present, in addition to the catechol group in the benzoyl ring, at least one hydroxyl group that increases the electronic density, favoring the hydrogen atom transfer (HAT) mechanism. Compound **4**, with two hydroxyl groups in the coumarin moiety, showed a considerable increase in the ORAC-FL value (8.32) compared to compound **3**, in which only one hydroxyl substituent is present in the coumarin ring. In any case, the highest ORAC-FL value was found for compound **5**, in which one hydroxyl group is present at position 8 and a bromide atom is present at position 6 of the coumarin. Therefore, it has been found that the presence, the number and the position of substituents with different electronic effects on the coumarin skeleton are important structural factors that contribute to increase the antioxidant capacity of these coumarin–chalcone hybrids.

Regarding the ESR assays, whose results are reported in Table 2 we observed accuracy with the obtained ORAC values and the radical scavenging activity, as high as 80% for all tested compounds.

On the other hand, results reported in Table 3 have shown that all compounds present moderate trypanocidal activity with exception of compound **4** that resulted to be inactive at the concentration tested. However, these compounds have less activity than the positive control nifurtimox, the drug currently used against *T. cruzi* in the treatment of Chagas disease.

From the data obtained for the theoretical evaluation of the ADME properties, one can notice that all the hybrid compounds **1–5** do not break any point of the Lipinski's rule of five, making them promising leads for drug candidates.[51] Topological polar surface area (TPSA) and log *P* values are compatible with those described as a predictive indicator of the drug capacity of membrane penetration.[52]

It is noteworthy that compounds with the higher trypanocidal activities were those with lower ORAC values. These data can be taken into account for future studies on the mechanism of action of this kind of drug against *T. cruzi*.

7 Experimental

7.1 General methods

Starting materials and reagents were obtained from commercial suppliers and were used without purification. Melting points (mp) are uncorrected and were determined with a Reichert Kofler thermopan or in capillary tubes in a Buchi 510 apparatus. ^{1}H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra were recorded with a Bruker AMX spectrometer using DMSO-d_6 or $CDCl_3$ as solvent. Chemical shifts (δ) are expressed in parts per million (ppm) using TMS as an internal standard. Coupling constants *J* are expressed in hertz (Hz). Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets) and m (multiplet). Mass spectrometry was carried out with a Kratos MS-50 or a Varian MAT-711 spectrometer. Elemental analyses were performed by a Perkin-Elmer 240B microanalyzer and were within $\pm$0.4% of calculated values in all cases. The analytical results were $\geq$95% purity for all compounds. Flash Chromatography (FC) was performed on silica gel (Merck 60, 230–400 mesh) and analytical TLC was performed on precoated silica gel plates (Merck 60 F254). Organic solutions were dried over anhydrous sodium sulfate. Concentration and evaporation of the solvent after reaction or extraction was carried out on a rotary evaporator (Büchi Rotavapor) operating at reduced pressure.

7.2 Synthesis

7.2.1 General procedure for the synthesis of hydroxy-3-benzoylcoumarins (1–5). To a solution of the appropriate β-ketoester (1 mmol) and the corresponding salicylaldehyde (1 mmol) in ethanol (5 mL) was added piperidine in a catalytic amount. The reaction mixture was refluxed for 2–6 h and after completion (followed by TLC), the reaction mixture was cooled and the precipitate was filtered and washed with cold ethanol and ether to afford the corresponding methoxy-3-benzoylcoumarin compounds with a general structure **I**. The appropriate methoxy derivative compound (1 mmol) was dissolved in DCM (1 mL) and BBr_3 in DCM (20 mmol, 1 M) was added in a Schlenk tube. The tube was sealed, and the reaction mixture was heated at 80 °C for 48 h. The resulting crude product was treated with MeOH and rotated to dryness. The obtained precipitate was recrystallized in MeOH or purified by flash chromatography using hexane–ethyl acetate mixtures as an eluent, to afford the desired hydroxy derivatives (Scheme 1).

7.3 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), at room temperature, using a three electrode cell, a glassy carbon electrode was used as a working electrode, a platinum wire as an auxiliary electrode and Ag, AgCl/KCl (*ca.* 3.5 M) as a reference electrode. All coumarin–chalcone hybrids were studied to 1 mM in 20% methanol as final concentration.

7.4 Determination of ORAC

The ORAC analyses were carried out on a Synergy HT multi detection microplate reader, from Bio-Tek Instruments, Inc. (Winooski, USA), using white polystyrene 96-well plates, purchased from Nunc (Denmark). The consumption of the probe molecules, FL or PGR, associated with its incubation in the presence of AAPH, was estimated from fluorescence (*F*) and absorbance (*A*) measurements, respectively. PGR consumption was evaluated from its absorption intensity (*A*) decrease at

540 nm, while FL consumption was evaluated from its decrease in the fluorescence intensity (F, excitation: 485/20 nm; emission: 528/20 nm). The plate reader was controlled by Gen 5 software. The reaction was carried out in 75 mM sodium phosphate buffer (pH 7.4), and a final volume of 200 μL. FL (40 nM, final concentration) or PGR (70 μM, final concentration) and coumarin–chalcone hybrid solutions in methanol with a range of concentrations between 0.3 μM and 2 μM were placed in each well of 96-well plate. The mixture was preincubated for 15 min at 37 °C, before rapidly adding the AAPH solution (18 mM and 0.1 M for FL and PGR final concentration, respectively). The microplate was immediately placed in the reader and automatically shaken prior to each reading. The fluorescence and absorbance were recorded every 1 min for 120 min. A blank with FL or PGR and AAPH using methanol instead of the antioxidant solution and five calibration solutions using Trolox (0.5 μM to 2.5 μM for Fl and 50 μM to 250 μM for PGR) as antioxidant were also used in each assay. The inhibition capacity was expressed as ORAC-FL and ORAC-PGR values, and is quantified by integration of the area under the curve (AUC-NET). All reaction mixtures were prepared in triplicate and at least three independent assays were performed for each sample. The area under the fluorescence decay curve (AUC) was calculated integrating the decay of the fluorescence where F_0 is the initial fluorescence read at 0 min and F is the fluorescence read at time. The net AUC corresponding to the sample was calculated by subtracting the AUC corresponding to the blank. The ORAC-PGR values were calculated like we indicated previously. Data processing was performed using Origin Pro 8 SR2 (Origin Lab Corporation, USA).

7.5 ESR spectroscopy

To a solution of 100 μL of NaOH (50 μL, 4 mM), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (50 μL, 30 mM) and hydrogen peroxide (50 μL, 30%) was added the antioxidant compound (50 μL, 3 mM in DMF). The resulting mixture was placed in an EPR cell, at room temperature (25 °C) and the spectrum was recorded after five minutes of reaction in the X band (9.68 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation.

7.6 Determination of trypanocidal activity

Trypanocidal activity was evaluated against the *T. cruzi* epimastigote stage (clone Dm28c) and it was measured through the MTT assay,[43] using 0.22 mg mL^{-1} phenazine metosulfate (as electron carrier). The *T. cruzi* epimastigotes Dm28c strain, from our own collection (*Programa de Farmacología Molecular y Clínica, Facultad de Medicina, Universidad de Chile*) were grown at 28 °C in the Diamond's monophasic medium as reported earlier[53] but replacing blood by 4 μM hemin and adding fetal calf serum (5%). The coumarin–chalcone hybrids (10 and 100 μM in DMSO) were added to 3×10^6 parasites per mL in the RPMI 1640 culture medium (5% bovine fetal serum) for 24 h at 28 °C. The DMSO final concentration was less than 0.1% v/v. The same procedure was followed with nifurtimox as the positive control. The tetrazolium salt was added at a final concentration of 0.5 mg mL^{-1}, incubated at 28 °C for 4 h and then solubilized with sodium dodecyl sulfate (10%) in HCl (0.1 mmol L^{-1}) and incubated overnight. After incubation time, the number of viable parasites was determined by measuring the absorbance at 570 nm in a multiwell reader (Asys Expert Plus©, Austria). Untreated parasites were used as controls (100% of viability). Results are reported as the percentage of non-viable epimastigotes regarding the control. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc. San Diego, CA). The data are expressed as mean ± S.D. The experimental data were analyzed by one-way analysis of variance (ANOVA), and differences between groups were assessed using the Tukey's post-test. The level of significance was set at $p < 0.05$, and all experiments were replicated three times.

7.7 Theoretical evaluation of ADME properties

The ADME properties of the studied compounds were calculated using the Molinspiration property program.[44] log P is calculated by the methodology developed by Molinspiration as a sum of fragment-based contributions and correction factors. Topological Polar Surface Area (TPSA) is calculated based on the methodology published by Ertl *et al.* as a sum of fragment contributions.[52] Oxygen and nitrogen centered polar fragments are considered. PSA has been shown to be a very good descriptor characterizing drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability and blood–brain barrier penetration. The method for calculation of molecular volume developed by Molinspiration is based on group contributions. These have been obtained by fitting the sum of fragment contributions to "real" 3D volume for a training set of about twelve thousand, mostly drug-like molecules. 3D molecular geometries for a training set were fully optimized by the semiempirical AM1 method.

8 Conclusions

In conclusion, we have confirmed the good antioxidant activity of the hydroxyl coumarin–chalcone hybrids **1–5**, two of them (compounds **4** and **5**) presenting better ORAC-FL values than the reference compounds quercetin and catechin. The use of a catechol group in the benzoyl ring and the presence of hydroxyl and bromine groups in the coumarin moiety make a big improvement in the antioxidant activity, compound **5** being the most active of the series under study. The results of the antioxidant assay using ESR showed significant reactivity against the hydroxyl radical for all tested compounds. A very interesting finding is that compounds **4** and **5** resulted to be very reactive and present good antioxidant capacity and scavenging percentage against hydroxyl and peroxyl radicals, as well as low oxidation potentials. Based on these results, we can conclude that compounds **4** and **5** are potential candidates for deeper studies of antioxidant activity. On the other hand, it is worth mentioning that molecules with higher trypanocidal activities showed a lower ORAC-FL index, which can be a key in the understanding of the mechanism of action of the compounds on the *T. cruzi* parasite.

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