Coumarin-chalcone derivatives as potential antitrypanosomal and antioxidant compounds

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Trypanosoma cruzi, the causative agent of Chagas' disease, which is widely disseminated in Central and South America, represents an endemic neglected disease located in this geographic region. Therefore, there is an urgent need for the discovery of new, more effective, and safer drugs for human use.

Natural products have played a major role in the chemotherapy of parasitic diseases. Based on the observed biological activities of two kind of natural compounds, coumarins and chalcones, we have synthesized coumarin-chalcone hybrids with the aim of evaluating their activity against *Trypanosoma cruzi*, and their antioxidant properties. All derivates have shown moderate trypanocidal activity in the epimastigote stage (clone Dm28c) being compound **4** the one with the highest activity, aproximately half of the Nifurtimox, the comercial standard. These preliminary findings encourage us to future structural optimization of this compound. In spite of the moderate trypanocidal activity of coumarin-chalcone hybrids, they have been proved to be very good antioxidants. Based on these results, we can conclude that compounds **2** and **3** are potential candidates for *in vitro* studies of their antioxidant activity.

Keywords: Coumarin; Chalcone; Antitrypanosomal; Antioxidant; Reactive Oxygen Species (ROS).

1. Introduction

Despite the fact that Carlos Chagas described, near to a century ago[1] the vector, microorganism and clinical signs, American Trypanosomiasis (or Chagas disease) remains the largest parasitic disease burden on the American continent. This disease is widely disseminated in Central and South America and represents an endemic disease in 21 countries located in this geographic region. It has been estimated that this disease affects 9.8 to 11.0 million people, and 60.0 million are at risk.[2] Like other neglected diseases, it is an important health problem due to inadequate therapy and the lack of an effective vaccine.[3] Nufurtimox (Nfx) and benznidazole (Bz) are the only licensed drugs for Chagas' disease which have shown clinical efficacy, but they are far for being optimal due to their low effectiveness in chronic phase and they adverse effects.[4] Currently, optimal curative treatments for Chagas disease do not exist and therefore, all the possible efforts are necessary in the search of effective treatment for this disease.

Therefore, there is an urgent need for the discovery of new, more effective, and safer drugs for human use. One of such important drug targets receiving considerable attention is the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), a key protein in the glycolytic pathway of trypanosomatids. Several studies have been carried out, observing the inhibitory properties of many coumarins for this enzyme[4],[5],[6] During the course of *T. cruzi* infection and disease development, reactive oxygen species (ROS) can be produced as a consequence of tissue destruction caused by toxic secretions of 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 the oxidative damage.[7]

The use of natural products and synthetic derivatives is widely extended against protozoal infection and they have been studied and evaluated against *T. cruzi.*[8] Chalcones and coumarins show a wide variety of pharmacological activities, including anticancer, anti-inflammatory, immunomodulatory, antibacterial, and immunosuppressive, as well as antiprotozoan activity, including trypanocidal, leishmanicidal, and antimalarial.[9],[10]

Chalcones, one of the major classes of natural products with widespread distribution in fruits, vegetables, spices, tea and soy based foodstuff have been subject of great interest for their interesting pharmacological activities.[11] Chalcones (1,3-diaryl-2-propen-1-ones) belong to the flavonoid family. Chemically they can be considered open-chain flavonoids in which the two aromatic rings are joined by a three-carbon α,β-unsaturated carbonyl system. Chalcones have been reported to possess many useful properties, including antibacterial,[12],[13] antimalarial,[14],[15]

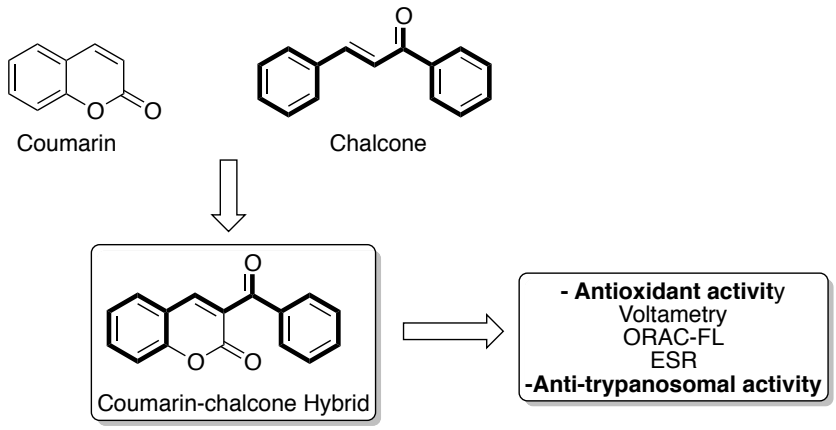
antifungal,[16] antiviral[17],[18] and anti-inflammatory[19],[20] properties.

It is known that the antioxidant properties of chalcones are quite dependant on the two aryl structures, that is, the substitution pattern on the two aryl rings of the chalcone moiety. Especially, the hydroxyl substituent is one of the key groups that enhance greatly the antioxidant activity of chalcone mainly due to its easy conversion to phenoxy radicals through the hydrogen atom transfer mechanism. This phenoxy radical formation may be crucial to the antioxidant properties, which are assessed primarily as radical scavenging potential of phenolic chalcones. In fact, the hydroxyl substituent is common among chalcones from natural sources.[21]

On the other hand, coumarins are a large family of compounds, of both natural and synthetic origin, important because of the pharmacological activities that these kind of compounds display, such as antimicrobial, [22] monoamine oxidase (MAO) inhibitors,[23,24] antitumor[25] or antioxidant [26] among others.

Due to the potential antioxidant and trypanocidal activity of the chalcone and coumarin moieties, in the present work a series of coumarin-chalcone hybrids have been synthesized and it has been studied their antioxidant and anti-trypanosomal activity (Scheme 1).

Scheme 1. Rational design of coumarin-chalcone hybrid compounds



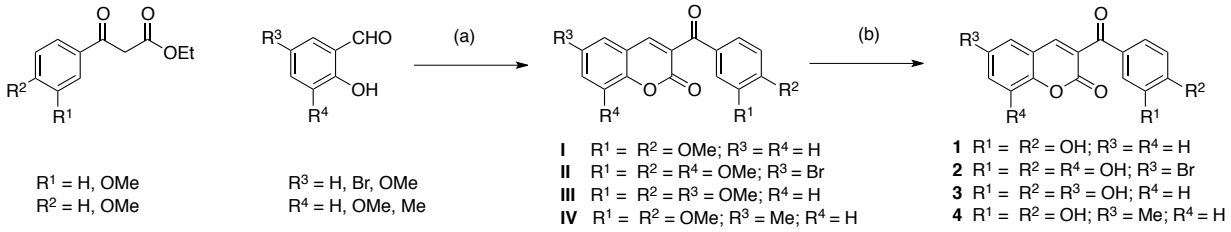
2. Results and Discussion

2.1. Chemistry

The synthesis of the final tested compound (**1-4**) was carried out in two steps briefly described as follows: i) synthesis of methoxy-3-benzoylcoumarins (or methoxy coumarin-chalcone

hybrids) **I-IV** and ii) synthesis of the hydroxyl-3-benzoylcoumarins (**1-4**). The above steps are shown in Scheme 2.

Scheme 2. Synthesis of coumarin-chalcone hybrid compounds. *Reagents and conditions:* (a) EtOH, piperidine, relux, 2-6 h; (b) BBr_3, DCM, 80 ºC, 48 h.



Based on the widely used Knoevenagel condensation reaction for the preparation of coumarin derivatives,[10,27] we used an efficient one-step synthesis to generate the methoxy 3-benzoylcoumarin precursors **I-IV**. These compounds were prepared in good yields (78-94%) using the appropriate salicylaldehyde and the corresponding β-ketoester in presence of piperidine in ethanol, obtaining the desired compound as a precipitate that was separated by filtration and further purified by recrystallization in MeOH/DCM. The final compounds **1-4** were then synthesized in good yields (69-94%) by hydrolysis of the corresponding methoxy precursors 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 of the crude chromatography using mixtures of hexane/ethyl acetate as eluent and/or recrystallization in MeOH.

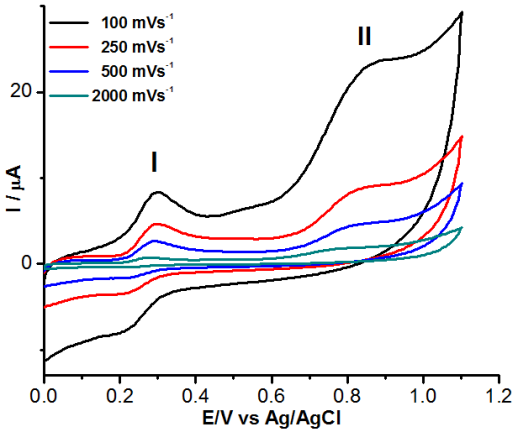
2.2. Electrochemical study

It is known that the antioxidant capacity is possibly related to the electrochemical behaviour, being indicative that the lower oxidation potential the higher antioxidant capacity. [28]

Figure 1 shows a cyclic voltammogram at a glassy carbon electrode (GCE) immersed in DMSO/75 mmol L^{-1} fosfate (pH 7.4) buffer 30/70 media containing 1 mmol L^{-1} of compound **3** for several scan rates (v). In these experimental conditions, two oxidation processes (peaks I, 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 others peaks (II) comprise oxidation reactions involving the hydroxyl groups present in the coumarinic ring, respectively.

We have thus firstly investigated the influence of the scan rate (v) in order to access in more detail to the features of electrochemical and chemical reactions taking place at the electrode/solution interface. The current ratio Ipc/Ipa progressively increases as a function of v, corroborating an EC-type mechanism[29] associated to peak I.

Figure 1. Cyclic voltammogram for 1 mmol L^{-1} of compound **3** in DMSO/75 mmol L^{-1} fosfate (pH 7.4) buffer 30/70 media at a GCE for v = 100, 250, 500, 2000 mVs^{-1}.



Results of the oxidation potential of compounds **1-4** are summarized in Table 1. We can observe that coumarin-chalcone hybrids present low oxidation potential. This fact can be explained principally by the strong electron donating effect of catechol group, present in all the tested compounds.

Table 1. Oxidation peak potential by cyclic voltammetry

Compounds	Epa* (mV)
1	322
2	247
3	312
4	449

* First oxidation peak potential at scan rate of 2000 mVs^{-1}.

The capacity of scavenging peroxyl radicals was studied through the oxygen radical absorbance capacity (ORAC) method. In this assay, Trolox and 2,2'-Azo-bis(2-amidinopropane) dihydrochloride (AAPH) are employed as reference antioxidant and peroxyl radical source,

respectively.[30] This assay evaluates the capacity of antioxidants (or their complex mixtures) to inhibit the bleaching of a target molecule (probe) induced by peroxyl radicals.

The highest ORAC-FL values are found for compounds are shown in Table 2. Compounds **2** and **3** present the highest values. The results are comparable to quercetin (7.28) and catechin (6.76), used as reference compounds.[31] The different ORAC-FL values are related to the substituents present in the benzoyl-coumarin skeleton. The presence of benzoyl ring improves electron delocalization respect coumarin skeleton influencing ORAC values. The catechol group was present in all tested compounds. This substituent is an important structural factor that improves the antioxidant capacity. Compound **1** presents only a catechol group without electron donating group (EDG) on the coumarin skeleton. For this reason presents the lowest ORAC value.

In order to study the antioxidant reactivity of all coumarin-chalcone hybrids, we have adapted a non-catalytic and competitive Fenton system.[32] ESR in combination with spin trapping techniques was employed to further verify that the tested compounds possess the ability to scavenge hydroxyl radicals (Table 2)

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

Compound	ORAC-FL	% scavenging
1	4.37 ± 0.22	84.6± 5.3
2	8.51 ± 0.32	90.9 ± 8.2
3	6.02 ± 0.12	84.8 ± 7.3
4	4.95 ± 0.14	82.1 ± 7.1

2.3. Trypanosomal activity

The antitrypanosomal activity for all synthesized compounds was evaluated using MTT assays.[33] The results reported in Table 3 have shown that all compounds present weak antitrypanosomal activity being less activity than the positive control nifurtimox.

Table 3. % Trypanocidal activity results for compounds **1-4** and nifurtimox (Nfx).

Compounds	% trypanocidal activity at 10 mol L^{-1}	% trypanocidal activity at 100 mol L^{-1}
1	23.8 ± 0.4	38.7 ± 2.1
2	9.5 ± 0.2	24.1 ± 0.7
3	18.9 ± 0.4	46.4 ± 0.8
4	27.9 ± 0.3	47.9 ± 1.0
Nfx	52.5 ± 2.2	100 ± 3.2

Compounds **1** and **4** present the higher values of trypanocidal activity of the synthesized compounds, but still not comparable with Nfx. Comparing the trypanocidal activity versus the antioxidant activity, we observe that the higher ORAC-FL index, the lower trypanocidal activity. Thus, compounds **2** and **3**, with higher ORAC-FL values (8.51 and 6.02 respectively), present the lowest trypanocidal activities (24.1 and 46.4 % at 100 mol L^{-1} respectively). On the contrary, compounds **1** and **4**, with lower ORAC-FL values (4.37 and 4.95 respectively) than **2** and **3**. Compound **4** presents the highest trypanocidal activity (47.9% at 100 mol L^{-1}).

3. Conclusions

In conclusion, we have confirmed the considerable antioxidant activity of new hydroxylated coumarin-chalcone hybrid compounds **1-4**. Their antioxidant activity is affected by the introduction of a benzoyl moiety at the C3 position regarding to the coumarin ring. A very interesting finding is that compound **1** is very reactive and presents good antioxidant capacity against hydroxyl and peroxyl radicals as well as low oxidation potential.

In spite of the moderate trypanocidal activity of coumarin-chalcone hybrids, they have been proved to be very good antioxidants. Based on these results, we can conclude that compounds **2** and **3** are potential candidates for *in vitro* studies of their antioxidant activity.

4. Experimental

Melting points were determined using a Reichert Kofler thermopan or in capillary tubes on a Büchi 510 apparatus and are uncorrected. ^{1}H 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). 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 unless indicated. Argon was dried flowing it through $CaCl_2$ columns, NaOH stones and P_2O_5.

4.1. General procedure for the synthesis of 3-benzoylcoumarins **I-IV**: To a solution of the appropriate *β*-ketoester (1 equivalent) and the corresponding salicylaldehyde (1 equivalent) in ethanol was added piperidine in catalytic amount. The reaction 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 in methanol/CH_2Cl_2.

4.1.1. 3-(3',4'-Dimethoxybenzoyl)coumarin (**I**): Yellow solid; Yield 78%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.01 (s, 1H, H-4), 7.71 – 7.52 (m, 3H, H-5, H-7, H-6'), 7.50 – 7.29 (m, 3H, H-6, H-8,

H-2'), 6.87 (d, *J* = 8.4 Hz, 1H, H-5'), 3.95 (s, 6H, 2xOMe); MS (EI) *m/z* (%): 311 ([M+1]$^+$, 59), 310 ([M]$^+$, 100), 173 (41), 166 (25), 165 (99), 79 (22), 77 (22).

4.1.2. 6-Bromo-3-(3',4'-dimethoxybenzoyl)-8-methoxycoumarin (**II**): Pale yellow solid; Yield 90%; ^{1}H NMR (300 MHz, DMSO) δ 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'-OMe)), 3.85 (s, 3H, (8-OMe)), 3.80 (s, 3H, 3'-OMe); MS (EI) *m/z* (%): 420 ([M+2]$^+$, 95), 418 ([M]$^+$, 98), 165 (100), 77 (18).

4.1.3. 6-Methoxy-3-(3',4'-dimethoxybenzoyl)coumarin (**III**): Bright yellow; Yield 94%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 (s, 1H, H-4), 7.38 (d, *J* = 1.9 Hz, 1H, H-6'), 7.26 (dd, *J* = 8.4, 2.0 Hz, 1H, H-5), 7.16 (d, *J* = 9.1 Hz, 1H, H-2'), 7.04 (dd, *J* = 9.1, 2.9 Hz, 1H, H-7), 6.82 (d, *J* = 2.9 Hz, 1H, H-5'), 6.70 (d, *J* = 8.4 Hz, 1H, H-8), 3.78 (d, *J* = 0.8 Hz, 6H, 2xOMe), 3.69 (s, 3H, OMe); MS (EI) *m/z* (%): 341 ([M+1]$^+$, 58), 340 ([M]$^+$, 94), 165 (100), 77 (22).

4.1.4. 6-Methyl-3-(3',4'-dimethoxybenzoyl)coumarin (**IV**): White solid; Yield: 82%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 (s, 1H), 7.38 (d, *J* = 1.8 Hz, 1H), 7.33 – 7.03 (m, 4H), 6.70 (d, *J* = 8.4 Hz, 1H), 3.78 (d, *J* = 0.8 Hz, 6H), 2.26 (s, 3H); MS (EI) *m/z* (%): 325 ([M+1]+, 36), 324 ([M]+, 99), 165 (100), 77 (20).

*4.2. General procedure to the synthesis of the hydroxylated 3-benzoylcoumarins (***1-4***):*

To the corresponding methoxy-3-benzoylcoumarin (1 mmol) in DCM, BBr_3 in DCM (20 mmol, 1M) was added in a Schlenk tube. Tube was sealed, and the reaction mixture was heated at 80ºC for 48 h. The resulting crude was treated with MeOH and rotated to dryness. The obtained precipitated was recrystallized in MeOH or purified by flash chromatography using hexane/ ethyl acetate as mixtures as eluent, to afford the desired hydroxy derivative.

4.2.1. 3-(3',4'-Dihydroxybenzoyl)coumarin (**1**): White solid; Yield: 89%; ^{1}H NMR (300 MHz, DMSO) δ 10.08 (s, 1H, OH), 9.41 (s, 1H, OH), 8.26 (s, 1H, H-4), 7.81 (d, *J* = 7.7 Hz, 1H, H-6'), 7.76 – 7.62 (m, 1H, H-7), 7.56 – 7.20 (m, 4H, H-5, H-6, H-2', H-5'), 6.82 (d, *J* = 8.2 Hz,

1H, H-8); MS (EI) *m/z* (%): 283 ([M+1]$^+$, 18), 282 ([M]$^+$, 98), 173 (64), 147 (42), 137 (100), 109 (29)

4.2.2. 6-Bromo-3-(3',4'-dihydroxybenxoyl)-8-hydroxycoumarin (**2**): Pale yellow solid; Yield 69%; ^{1}H NMR (300 MHz, DMSO) δ 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-6'), 7.38 – 7.12 (m, 3H, H-5, H-7, H-2'), 6.81 (dd, *J* = 8.1, 3.0 Hz, 1H, H-5'); MS (EI) *m/z* (%): 378 ([M+2]$^+$, 100), 376 ([M]$^+$, 99), 269 (30), 267 (24), 137 (80), 109 (74).

4.2.3. 6-Hydroxy3-(3',4'-dihydroxybenzoyl)coumarin (**3**): Bright yellow solid; Yield: 94%; ^{1}H NMR (300 MHz, DMSO) δ 10.09 (s, 1H-OH), 9.85 (s, 1H, OH), 9.47 (s, 1H, OH), 8.17 (s, 1H, H-4), 7.38 – 7.18 (m, 3H, H-5, H-7, H-6'), 7.18 – 7.02 (m, 2H, H-5', H-2'), 6.81 (d, *J* = 8.2 Hz, 1H, H-8); MS (EI) *m/z* (%): 299 ([M+1]$^+$, 11), 298 ([M]$^+$, 43), 189 (20), 163 (62), 137 (100), 109 (25).

4.2.4. 6-Methyl-3-(3',4'-dihydroxybenzoyl)coumarin (**4**): Pale Yellow; Yield: 77%; ^{1}H NMR (300 MHz, DMSO) δ 10.11 (s, 1H, OH), 9.46 (s, 1H, OH), 8.19 (s, 1H, H-4), 7.66 – 7.44 (m, 2H, H-2', H-6'), 7.44 – 7.14 (m, 3H, H-5, H-7, H-8), 6.81 (d, *J* = 8.2 Hz, 1H, H-5'), 3.37 (s, 3H);

4.3. ORAC-FL

The ORAC-FL assays 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 molecule fluorescein (FL) associated to its incubation in presence of AAPH, was estimated from fluorescence (F) and absorbance (A) measurements, respectively. 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 200 µL final volume. FL (40 nM, final concentration) and coumarin-chalcone hybrids solutions in methanol with a range of concentration 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 for FL final concentration). 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 and AAPH using methanol instead of the antioxidant solution and five calibration solutions using Trolox (0.5 μM to 2.5 μM for Fl) as antioxidant were also used in each assay. The inhibition capacity was expressed as ORAC-FL values, and is quantified by integration of the area under the curve (AUCNET). 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.

4.4. Scavenging assays

A mixture of 100 μL water with 50 μL NaOH (final concentration 4 mM) follow by addition of 50 μL DMPO spin trap (30 mM final concentration) and 50 μL H_2O_2 30% and finally 50 μL of antioxidant compound dissolved in N,N-dimethylformamide. The mixture was put in EPR Cell and we recorded spectrum after five minutes of reaction. All derivatives were studied to 3 mM final concentration.

4.5. Trypanosomal 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]-2,5diphenyltetrazolium bromide) assay, using 0.22 mg mL^{-1} phenazine metosulfate (as electron carrier). In this colorimetric assay for testing the antitrypanosomal activity, the coumarin-chalcone hybrids, dissolved in DMSO were added to 3 x 10^6 parasites mL^{-1} at 10 and 100 μmol L^{-1} final concentrations in RPMI 1640 culture medium (5% bovine fetal serum) for 24 h at 28°C. DMSO final concentration was less than 0.1% v/v. Likewise, nifurtimox was added as positive control. 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 10% sodium dodecyl sulfate/0.1 mmol L^{-1} HCl and incubated overnight. After incubation time, we determined the number of viable parasites by absorbance measures 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.

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