Bioorganic & Medicinal Chemistry Letters 22 (2012) 5569–5573

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Antitrypanosomal and antioxidant properties of 4-hydroxycoumarins derivatives

Fernanda Pérez-Cruz [a], Silvia Serra [b,c,*], Giovanna Delogu [c], Michel Lapier [a], Juan Diego Maya [d], Claudio Olea-Azar [a], Lourdes Santana [b], Eugenio Uriarte [b]

[a] Departamento de Química Inorgánica y Analitica, Laboratorio de radicales libres y antioxidantes, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Casilla 233, Santiago 1, Chile
[b] Departamento de Química Orgánica, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain
[c] Dipartimento di Scienze della Vita e dell'Ambiente, Università degli Studi di Cagliari, Via Ospedale 72, 09124 Cagliari, Italy
[d] Departamento de Farmacología Molecular y Clínica, Facultad de Medicina, Universidad de Chile, Santiago, Chile

ARTICLE INFO

Article history:
Received 26 May 2012
Revised 30 June 2012
Accepted 4 July 2012
Available online 13 July 2012

Keywords:
4-Hydroxycoumarins
Trypanosoma cruzi
Chagas disease
Antioxidant properties

ABSTRACT

In the present communication we prepared a series of six 4-hydroxycoumarin derivatives, isosters of quercetin, recognized as an antioxidant natural compound, with the aim of evaluating the antitrypanosomal activity against *Trypanosoma cruzi*, the parasite responsible for Chagas disease, and the antioxidant properties. We have used the 4-hydroxycoumarin moiety (compound **1**) as the molecular template for the synthesis of compounds **2–7**. These derivates have shown moderate trypanocidal activity. However they have been proved to be good antioxidants. In particular compound **7** is the most active antioxidant and it is, therefore, a potential candidate for a successful employment in conditions characterized by free radicals overproduction.

Phenolic compounds are one of the major families of secondary metabolites in plants and they include a broad group of molecules. They have shown important properties such as antioxidant and can act protecting from degenerative diseases in which reactive oxygen species (ROS) are involved.[1–4] These compounds contain one or more aromatic benzene rings with one or more hydroxyl groups and their properties are related to their chemical structure. Coumarins are one of the most abundant molecules of naturally occurring poliphenolic compounds. A lot of them have been identified from natural sources, especially from green plants. Due to their structural features they are important building blocks in the natural product and synthetic chemistry areas. Coumarins are recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, enzymatic inhibition and anticarcinogenic properties.[5–13] The hydroxycoumarins are typical phenolic compounds and can act as potent metal chelators and free radical scavengers.[14,15] Flavonoids are another important group of natural phenolic compounds that are known for their biological activities, including the important antioxidant properties.[16] The quercetin is one of the most powerful and effective antioxidant flavonoid present in nature.[17]

Antioxidants are a class of substances of many diverse chemical structures that are capable of decrease or prevent oxidation of other sensitive molecules through different mechanisms like chelation of active metal ions, free radicals scavenging or inhibition of pro-oxidant enzymes.[18] Free radicals are very unstable molecules responsible of aging, cellular membrane and DNA damage, and possibly serious diseases like cancer and heart condition. It has been found that many hydroxycoumarins affect the formation and scavenging of ROS, exhibiting antioxidant tissue-protective properties.[19,20]

Furthermore ROS produce oxidative stress that contributes to Chagas disease progression.[21] This illness, known also as American Trypanosomiasis, is caused by the protozoan parasite, *Trypanosoma cruzi (T. cruzi)*.[22] It is a serious threat to health in Central and South America, and it is one of the most important emerging parasitic disease in developed countries.[23,24] The parasite has a complex life cycle, which involves obligatory passage through four stages.[25,26] The infection is transmitted to humans predominantly by insect vector, blood transfusion or by transmission from mother to fetus. Current treatment is based on old and quite unspecific drugs: nifurtimox (Nfx) and benznidazole (Bzn). In fact this therapy is highly toxic and usually ineffective in the chronic stages.[27–30] 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 parasite, immune-mediated cytotoxic reactions, and

* Corresponding author.
E-mail address: silvserra@tiscali.it (S. Serra).

0960-894X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2012.07.013

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.[31]

Given the known antioxidant properties attributed to the coumarin skeleton and to the flavonoid compounds like quercetin (Fig. 1), which presents a hydroxyl group in the pyron ring, in the present work a series of six 4-hydroxycoumarin derivatives were synthesized (compounds **2**–**7**).

In fact these compounds can be considered isosters of the mentioned flavonoid. We have used the 4-hydroxycoumarin (compound **1**) as the molecular template in the design and the synthesis of these derivatives that present also an aryl group at C3 (Fig. 1).

Hydroxy, methoxy, methyl and/or chloro substituents were introduced in the 3-phenyl ring, whereas the six position in the coumarin moiety was not substituted/or substituted with a chloro atom.

Then the antitrypanosomal activity against *Trypanosoma cruzi* and the antioxidant properties of compounds **1**–**7** were assessed.

The key step for the synthesis of the 3-arylcoumarin skeleton was achieved by a palladium-catalyzed Suzuki coupling reaction between phenyliodonium zwitterion salts and the conveniently substituted phenyl boronic acids.[32–36] Initially we have synthesized the different phenyliodonium coumarinate species (**I**–**II**) that are electrophilic molecules with a positive charge at iodine atom delocalized to the neighboring oxygen.[35] Then we have carried out the palladium-catalyzed coupling reaction using $Pd(OAc)_2$ as catalyst and P(*t*-Bu)$_3$ as ligand to afford the compounds **2**–**7** in good yields.[32,33,36]

The reaction conditions are delineated in the Scheme 1.

The antitrypanosomal activity for all synthesized compounds was evaluated using MTT assays.[37,38]

The results reported in Table 1 have shown that derivatives **2**, **4**, and **5** respectively with any substituent, a chlorine atom or a methyl group at *p*-position in the 3-phenyl ring present antitrypanosomal activity. However, these compounds have less activity compared with positive control nifurtimox. On the other hand the simple 4-hydroxycoumarin (compound **1**) and compounds **3**, **6** and **7** with methoxy or hydroxyl substituents in the 3-phenyl ring do not show any activity.

In the present series is clearly observed how the absence of the 3-phenyl ring or the introduction of methoxy or hydroxyl groups in this causes a considerably decrease of antitrypanosomal activity. On the other hand the activity improves enormously in presence of a *p*-chloro atom in the 3-phenyl ring of the 4-hydroxycoumarin. In fact the compound **4** that presents this substituent is the best of

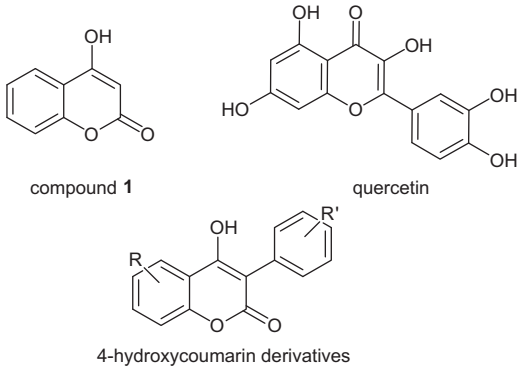
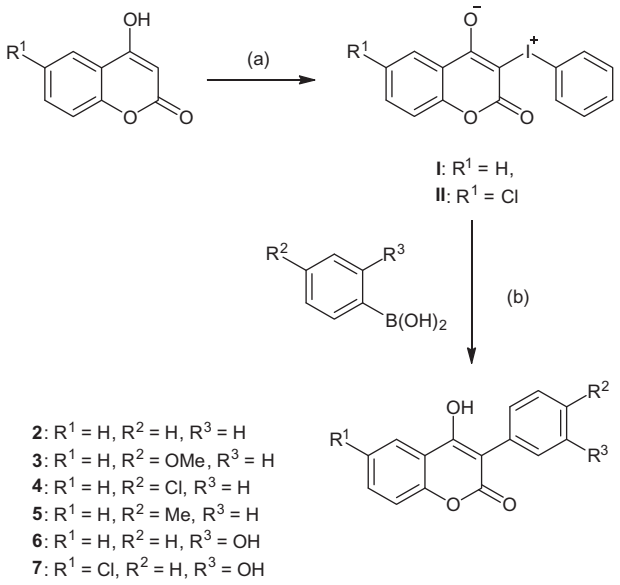


Figure 1. Chemical structure of compound **1**, quercetin and 4-hydroxycoumarin derivatives.



Scheme 1. Reagents and conditions: (a) $PhI(OAc)_2$, Na_2CO_3, H_2O, rt, 14 h; (b) $Pd(OAc)_2$, P(*t*-Bu)$_3$, LiOH, DME/$H_2$0, rt, 24–48 h.

Table 1
% Trypanocidal activity results for the compounds **1**–**7** and nifurtimox (Nfx)

Compounds	% Trypanocidal activity at (10 μmol L^{-1})
1	[a]
2	21.1 ± 0.4
3	1.6 ± 0.2
4	30.7 ± 2.2
5	16.7 ± 6.9
6	3.9 ± 3.3
7	[a]
Nfx	52.5 ± 2.2

[a] Inactive at concentration tested.

the evaluated series with around 30% of trypanocidal activity at 10 μmol L^{-1}.

These preliminary findings and especially the antitrypanosomal capacity found for compound **4**, encourage us to the future structural optimization of these compounds.

The capacity of scavenging peroxyl radicals was studied through the oxygen radical absorbance capacity method using fluorescence-based technology of detection measurements (ORAC-FL).[39,40] This assay gives an relative index refering to the hydrosoluble standard molecule (trolox), a vitamine E derivative. Results are expressed as ORAC values and are tabulated in Table 2.[41] They take account the induction time, initial rate and the range of total inhibition obtained for each value.[42]

The oxidation occurs due to exposition of the fluorophore, in this case fluorescein (FL), to the peroxyl radical leading to decay of fluorescence emission through time.[40] In ORAC assays, the loss

Table 2
ORAC values and (%) scavenging of hydroxyl radical calculated for compounds **1**–**7**

Compounds	ORAC values	% scavenging
1	4.2 ± 0.26	61.9 ± 3.4
2	4.4 ± 0.16	45.1 ± 2.6
3	5.7 ± 0.30	46.3 ± 2.5
4	3.9 ± 0.15	31.3 ± 4.7
5	6.5 ± 0.23	34.6 ± 4.5
6	4.9 ± 0.35	40.2 ± 3.4
7	7.7 ± 0.54	100 ± 4.5

of fluorescence of fluorescein generally shows an induction time and is reliant on the oxidation potential of antioxidant molecules. It refers to the time in which the FL is protected against peroxyl radicals in presence of antioxidant molecules. This behavior is associated to competitive reaction between radical both probe and antioxidant.[43] We obtained profile of fluorescence measure at 528 nm versus the incubation time at different concentration for all 4-hydroxycoumarin derivatives. Then the area under the curve (AUC) was calculated for all studied compounds.

The highest ORAC values are found for compounds **5** and **7** (6.5 and 7.7, respectively). The results are comparable with quercetin (7.28) and catechin (6.76), used as reference compounds.[39]

Figure 2a shows the kinetic profiles obtained in presence of increasing concentration of compound **7**. For all coumarin derivatives the area under the curve (AUC_{NET}) of the kinetic profiles depend on the concentration of the additive as it is presented in Figure 2b.

The different ORAC values are related to the substituents present in the coumarin skeleton. We have used the commercially 4-hydroxycoumarin, compound **1**, as patron molecule. When it was added a phenyl group at 3 position (compound **2**), the delocalization of semiquinone radical improved, influencing ORAC value. In the compound **3**, in which the phenyl ring presents a *p*-methoxy group, and in the compound **5** with a *p*-methyl group, an increment in the antioxidant capacity was observed. This fact can be explained taking into account the strong electron donating effect that increases the electronic density around the hydroxyl group, favoring the hydrogen atom transfer mechanism (HAT). On the contrary in the compound **4**, that presents as substituent on its phenyl ring an electron withdrawing group like a chloro atom, the antioxidant capacity decreases. The positive effect, although smaller, is observed for the compound **6** with a *m*-hydroxyl group on the 3-phenyl ring. This substituent, together with one chlorine atom on the 6 position of the coumarin skeleton leads to the compound **7**, which resulted to be the best of the evaluated series.

So it has been found that the presence, the number and the position of electron donating groups (hydroxyl, methoxy or methyl) on the 3-phenyl ring and the presence of an electron withdrawing group (chlorine atom) at 6 position on the coumarin skeleton are important structural factors that contribute to the increment of the antioxidant capacity of these 4-hydroxycoumarins.

In order to study the antioxidant reactivity of all 3-phenyl-4-hydroxycoumarin derivatives synthesized against hydroxyl radicals, we have adapted a non-catalytic and competitive Fenton system in which the spin trap DMPO and the antioxidant molecules compete for hydroxyl radicals.[44,45]

The ESR spectrum shows four hyperfine lines, due to the DMPO-OH adduct formation. The intensity of the spectrum decreases

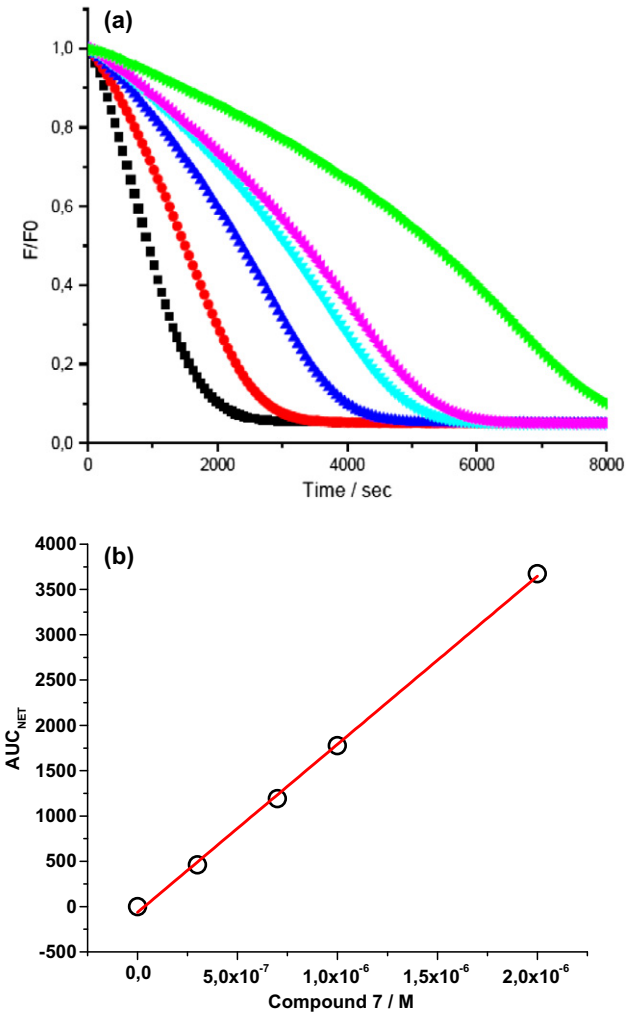


Figure 2. (a) ORAC profile for compound **7**; (b) graphic AUC_{NET} versus concentration compound **7**.

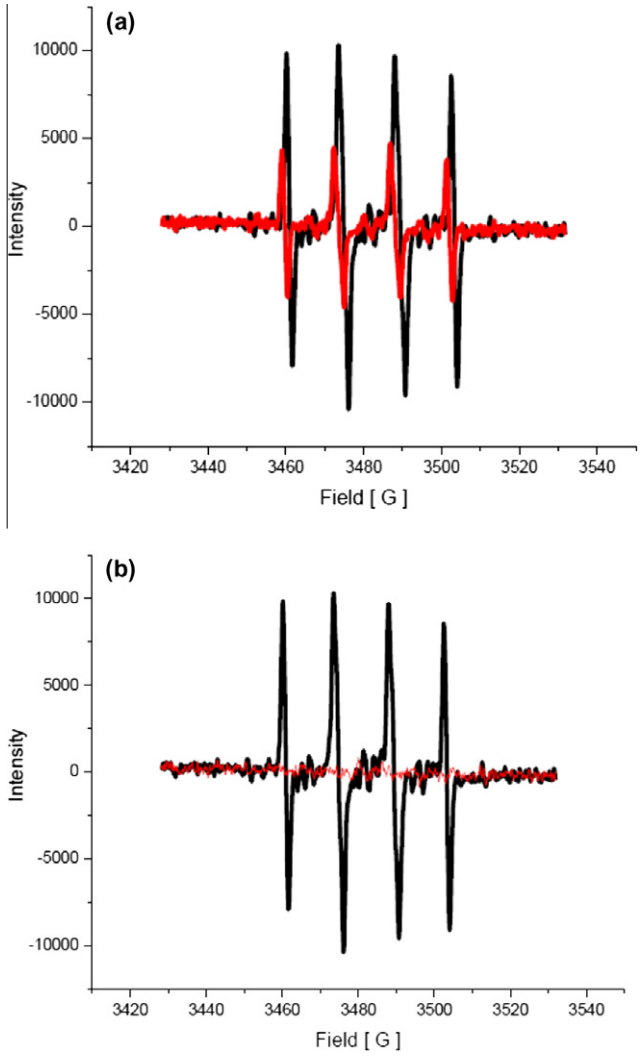


Figure 3. (a) ESR spectrum for adduct DMPO-OH without antioxidant molecule (black) and in presence of compound **1** (red); (b) ESR spectrum for adduct DMPO-OH without antioxidant molecule (black) and in presence of compound **7** (red).

when a 3-aryl-4-hydroxycoumarin derivative is added into the system. Figure 3a shows the experiment carried out for the compound **1** in which a 61.9% of scavenging hydroxyl radicals was obtained. In the spectrum of Figure 3b we can observe for the compound **7** a total scavenging. This response was observed for all derivatives and the percentage of the hydroxyl radical scavenging activity is illustrated in Table 2.[41] The order of best reactivity against hydroxyl radical was **7** > **1** > **3** > **2** > **6** > **5** > **4**.

In conclusion, the values obtained in the study for the antitrypanosomal activity have not shown experacted results. Only compounds **2**, **4**, and **5** have an appreciable activity but still lower than nifurtimox, used in the treatment of Chagas disease. We can use these results for a structural optimization of these series of compounds. On the other hand, we have confirmed the good antioxidant activity of 4-hydroxycoumarin derivatives. Their antioxidant activity is significantly affected by the introduction of a phenyl moiety at the C3 position. Also when a substituent with electron donating effect is present in the phenyl group, the antioxidant capacity increases. The results of the antioxidant assay using ESR showed higher reactivity against hydroxyl radical for compound **1** and **7**. A very interesting finding is that derivative **7** is very reactive and present good antioxidant capacity against hydroxyl and peroxyl radicals. Based of these results, we can conclude that the compound **7** is a potential candidate for a successful employment in conditions characterized by an overproduction of free radicals.

Acknowledgements

Thanks to Italian Ministry (PRIN 2008, F21J10000010001), Spanish Ministry (PS09/00501) and Xunta de Galicia (09CSA030203PR). S. Serra thanks Regione Sardegna for the grant (PR-MAB-A2009-613), Fernanda Pérez-Cruz gratefully acknowledges CONICYT-Chile for Doctoral fellowship and Scholarship support for doctoral theses CONICYT-Chile N° 24110059. Claudio Olea-Azar acknowledges to FONDECYT (Chile) for project 1110029. Juan Diego Maya acknowledges to FONDECYT 1090078 and Anillo ACT 112.

References and notes

1. Tyagi, Y. K.; Kumar, A.; Raj, H. G.; Vohra, P.; Gupta, G.; Kumari, R.; Kumar, P.; Gupta, R. K. *Eur. J. Med. Chem.* **2005**, *40*, 413.
2. Hamdi, N.; Puerta, C.; Valerga, P. *Eur. J. Med. Chem.* **2008**, *43*, 2541.
3. Panteleon, V.; Kostakis, I. K.; Marakos, P.; Pouli, N.; Andreado, I. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5781.
4. Guardado-Yordi, E.; Pérez-Molina, E.; Matos, M. J.; Uriarte-Villares, E. In Nutrition, Well-Being and Health; Bouayed, J. and Bohn, T. Eds.; InTech, 2012, Chapter 2.
5. Borges, F.; Roleira, F.; Milhazes, N.; Santana, L.; Uriarte, E. *Curr. Med. Chem.* **2005**, *12*, 887.
6. Borges, F.; Roleira, F.; Milhazes, N.; Uriarte, E.; Santana, L. *Front Med. Chem.* **2009**, *4*, 23.
7. Matos, M. J.; Terán, C.; Pérez-Castillo, Y.; Uriarte, E.; Santana, L.; Viña, D. *J. Med. Chem.* **2011**, *54*, 7127.
8. Matos, M. J.; Santana, L.; Uriarte, E.; Delogu, G.; Corda, M.; Fadda, M. B.; Era, B.; Fais, A. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3342.
9. Viña, D.; Matos, M. J.; Yáñez, M.; Santana, L.; Uriarte, E. *Med. Chem. Commun.* **2012**, *3*, 213.
10. Secci, D.; Carradori, S.; Bolasco, A.; Chimenti, P.; Yáñez, M.; Ortuso, F.; Alcaro, S. *Eur. J. Med. Chem.* **2011**, *46*, 4846.
11. Ostrov, D. A.; Hernández Prada, J. A.; Corsino, P. E.; Finton, K. A.; Le, N.; Rowe, T. C. *Antimicrob. Agents Chemother.* **2007**, *51*, 3688.
12. Melagraki, G.; Afantitis, A.; Igglessi-Markopoulou, O.; Detsi, A.; Koufaki, M.; Kontogiorgis, C.; Hadjipavlou-Litina, D. J. *Eur. J. Med. Chem.* **2009**, *44*, 3020.
13. Viña, D.; Matos, M. J.; Ferino, G.; Cadoni, E.; Laguna, R.; Borges, F.; Uriarte, E.; Santana, L. *ChemMedChem* **2012**, *7*, 464.
14. Fylaktakidou, K. C.; Hadjipavlou-Litina, D. J.; Litinas, K.; Nicolaides, D. N. *Curr. Pharm. Des.* **2004**, *10*, 3813.
15. Kostova, I. *Mini-Rev. Med. Chem.* **2006**, *6*, 365.
16. Procházková, D.; Boušová, I.; Wilhelmová, N. *Fitoterapia* **2011**, *82*, 513.
17. Coşkun, Ö.; Kanter, M.; Armutçu, F.; Çetin, K.; Kaybolmaz, B.; Yazgan, Ö. *Eur. J. Gen. Med.* **2004**, *1*, 37.
18. Nordberg, J.; Arnér, E. S. *Free Radic. Biol. Med.* **2001**, *31*, 1287.
19. Symeonidis, T.; Chamilos, M.; Hadjipavlou-Litina, D. J.; Kallitsakis, M.; Litinas, K. E. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1139.
20. Nishiyama, T.; Ohnishi, J.; Hashiguchi, Y. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 1127.
21. De Oliveira, T. B.; Pedrosa, R. C.; Filho, D. W. *Int. J. Cardiol.* **2007**, *116*, 357.
22. El Sayed, N. M.; Myler, P. J.; Bartholomeu, D. C., et al *Science* **2005**, *309*, 409.
23. Schofield, C. J.; Jannin, J.; Salvatella, R. *Trends Parasitol.* **2006**, *22*, 583.
24. Lepesheva, G. I.; Zaitseva, N. G.; Zhou, W.; Liu, J.; Hill, G. C.; Warterman, M. R. *J. Biol. Chem.* **2006**, *281*, 3577.
25. Bern, C.; Montgomery, S. P.; Herwaldt, B. L.; Rassi, A., Jr.; Marin-Neto, J. A.; Dantas, R. O.; Maguire, J. H.; Acquatella, H.; Morillo, C.; Kirchhoff, L. V.; Gilman, R. H.; Reyes, P. A.; Salvatella, R.; Moore, A. C. *J. Am. Med. Assoc.* **2007**, *298*, 2171.
26. Castro, J. A.; de Mecca, M. M.; Bartel, L. C. *Hum. Exp. Toxicol.* **2006**, *25*, 471.
27. Jakson, Y.; Alirol, E.; Jetaz, L.; Wolff, H.; Combescure, C.; Chappuis, F. *Clin. Infect. Dis.* **2010**, *51*, e69.
28. Perez-Molina, J. A.; Perez-Ayala, A.; Moreno, S.; Fernandez-Gonzalez, M. C.; Zamora, J.; Lopez-Velez, R. *J. Antimicrob. Chemother.* **2009**, *64*, 1139.
29. Bern, C. *N. Eng. J. Med.* **2011**, *364*, 2527.
30. Cerecetto, E.; Gonzalez, M. *Curr. Top. Med. Chem.* **2006**, *22*, 583.
31. Gupta, S.; Wen, J. J.; Garg, N. J. Oxidative Stress in Chagas Disease. *Interdiscip. Perspect. Infect. Dis.* 2009, 190354.
32. Zhu, Q.; Wu, J.; Fathi, R.; Yang, Z. *Org. Lett.* **2002**, *4*, 3333.
33. Goldoni, L.; Cravotto, G.; Penoni, A.; Tollari, S.; Palmisano, G. *Synlett* **2005**, *6*, 927.
34. Serra, S.; Ferino, G.; Matos, M. J.; Vázquez-Rodríguez, S.; Delogu, G.; Viña, D.; Cadoni, E.; Santana, L.; Uriarte, E. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 258.
35. *General procedure for the preparation of 3-phenyliodonium coumarinates (**I**–**II**)*: iodobenzene diacetate (10 mmol) was suspended in a solution of Na_2CO_3 (10 mmol) in water (100 mL) and was stirred for 30 min at room temperature. To this solution was added a mixture of the corresponding 4-hydroxycoumarin (10 mmol) and Na_2CO_3 (10 mmol) in water (100 mL). After the mixture was stirred at room temperature for 14 h, the precipitate was collected by filtration, washed with water (5 × 20 mL) and dried under vacuum. The resulting white solid was used without further purification.
36. *General procedure for the preparation of 3-aryl-4-hydroxycoumarins (**2**-**7**)*: a degassed solution of appropriated phenyl boronic acid (1.21 mmol) and P(*t*-But)$_3$ (0.109 mmol) in DME and H_2O (4:1, 12.5 mL) was added to a mixture of iodonium ylide (0.55 mmol), LiOH/H_2O (1.65 mmol) and $Pd(OAc)_2$ (0.027 mmol) under argon at room temperature. After being stirred at the same temperature for 24–48 h. The resulting mixture was purified by FC (hexane/ethyl acetate, 7:3) to give the desired compound.
*4-Hydroxy-3-(3′-hydroxyphenyl)coumarin (**6**)*. It was obtained with yield 58%. Mp: 265–267 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 6.76 (m, 2H, H4′, H6′), 7.41 (m, 5H, H6, H7, H8, H2′, H5′), 7.94 (s, 1H, H5). ^{13}C NMR (DMSO-d_6) δ (ppm): 80.3, 93.0, 101.2, 106.7, 115.1, 116.7, 118.3, 122.1, 124.2, 124.5, 129.5, 132.8, 152.7, 157.5, 160.1. MS *m*/*z* (%): 254 (M^+, 48), 134 (26), 121 (100), 65 (28). Anal. Calcd for $C_{15}H_{10}O_4$: C, 70.86; H, 3.96. Found: C, 70.88; H, 3.98.
*6-Chloro-4-hydroxy-3-(3′-hydroxyphenyl)coumarin (**7**)*. It was obtained with yield 69%. Mp: 283–285 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 6.76 (t, *J* = 7.1 Hz, 2H, H4′, H6′), 7.14–7.25 (m, 1H, H2′), 7.37–7.48 (m, 1H, H8), 7.64–7.80 (m, 2H, H7, H5′), 7.96 (s, 1H, H5). ^{13}C NMR (DMSO-d_6) δ (ppm): 92.1, 107.3, 115.2, 118.7, 121.9, 123.3, 128.4, 129.4, 132.3, 151.3, 152.5, 157.4, 159.4, 161.8, 164.9. MS *m*/*z* (%): 288 (M^+, 65), 155 (63), 154 (26), 134 (100). Anal. Calcd for $C_{15}H_9ClO_4$: C, 62.41; H, 3.14. Found: C, 62.39; H, 3.12.
37. Muelas, S.; Suárez, M.; Pérez, R.; Rodríguez, H.; Ochoa, C.; Escario, J. A.; Gómez-Barrio, A. *Mem. Inst. Oswaldo Cruz* **2002**, *97*, 269.
38. *Trypanocidal activity was evaluated against the T. cruzi trypomastigote stage (clone Dm28c)*. It was measured through the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay, using 0.22 mg mL^{-1} phenazine metosulfate (as electron carrier). In this colorimetric assay for testing the antitrypanosomal activity, the 3-aryl-4-hydroxycoumarin derivatives, dissolved in DMSO were added to 10^7 parasites mL^{-1} at 10 μmol L^{-1} final concentration for 24 h at 37 °C. DMSO final concentration was less than 0.1% v/v. Likewise, nifurtimox was added as positive control. After incubation time, we determined the number of viable parasites by absorbance measures at 570 nm in a microplate spectrophotometer.
39. Ou, B.; Hampsch-Woodill, M.; Prior, R. L. *J. Agric. Food Chem.* **2001**, *49*, 4619.
40. 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). 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 reaction was carried out in 75 mM sodium phosphate buffer (pH 7.4), and 200 μL final volume. FL (40 nM, final concentration) and 3-aryl-4-hydroxycoumarin 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, 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 antioxidant were also used in each assay. The inhibition capacity was expressed as Equivalents Trolox (T_{equiv}), 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. Data processing was performed using Origin Pro 8 SR2 (Origin Lab Corporation, USA).

41. Statistical analyses were conducted using GraphPad Prism 5 software (GraphPad Software, Inc. San Diego, CA). The data are expressed as means ± SD. The experimental data were analyzed by one-way analysis of variance (ANOVA), and differences between groups were assessed using Tukey's post-test. The level of significance was set at p <0.05, and all experiments were replicated three times.
42. Niki, E. *Free Radical Biol. Med.* **2010**, *49*, 503.
43. Bisby, R. H.; Brooke, R.; Navaratnam, S. *Food Chem.* **2008**, *108*, 1002.
44. Yoshimura, Y.; Inomata, T.; Nakazawa, H.; Kubo, H.; Yamaguchi, F.; Ariga, T. *J. Agric. Food Chem.* **1999**, *47*, 4653.
45. We mixed 150 μL of *N*,*N*-dimethylformamide with 50 μL NaOH (final concentration 3 mM) follow by addition of 50 μL DMPO spin trap (30 mM final concentration) and finally 50 μL Hydrogen peroxide 30%. The mixture was put in EPR Cell and we recorded spectrum after five minutes of reaction. All derivatives were studied to 1 mM final concentration.