Med Chem Res
DOI 10.1007/s00044-016-1729-1

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and in vitro evaluation of leishmanicidal activity of 7-hydroxy-4-phenylcoumarin derivatives

Isael A. Rosa[1] · Letícia de Almeida[2] · Karina F. Alves[2] · Marcos J. Marques[2] · Antônio M. Fregnan[3] · Claudinei A. Silva[1,4] · Juliana O. S. Giacoppo[5] · Teodorico C. Ramalho[5] · Diogo T. Carvalho[3] · Marcelo H. dos Santos[6]

Received: 29 March 2016 / Accepted: 30 September 2016

Abstract Eight coumarin derivatives (**2–8**) were synthesized from 7-hydroxy-4-phenylcoumarin **1** and were evaluated for their in vitro leishmanicidal activity against promastigote and amastigote forms of *Leishmania amazonensis*, as well their toxicity in murine macrophages. Compounds **4** and **7** showed the most significant results against promastigote forms of *L. amazonensis*. They were at least three-fold more active than **1** and Compound **4** was as effective as Amphotericin B. Compound **4,** a 7-*O*-prenylated derivative, and **7**, a tetra-*O*-acetyl-β-D-glucopyranosyl derivative, presented IC_{50} values of 21.35 and 10.03 μM against promastigote and IC_{50} values of 58.10 and 34.93 μM, respectively against amastigote forms. Furthermore, they do not cause toxicity in mammalian or *Leishmania* cells in vitro. This study shows that these coumarin derivatives are potential prototypes for the development of novel drugs with leishmanicidal activity.

Keywords Coumarin derivatives · Leishmaniasis · *Leishmania amazonensis*

Introduction

Leishmaniasis is a neglected tropical disease (NTD) that represents the most common illnesses of the world's poorest people (Hotez et al. 2012). This disease has been reported in 88 countries on five continents, Africa, Asia, Europe, North America, and South America (van Assche et al. 2011), among which the majority are represented by developing countries (Bhargava and Singh 2012). The outcome of the disease is divided into several clinical presentations: cutaneous leishmaniasis (CL), muco-cutaneous atleishmaniasis (MCL), visceral leishmaniasis (VL) also known as Kala-azar and post-Kala-azar dermal leishmaniasis (Beattie and Kaye 2011). It is a parasitic and vector-borne disease caused by several different species of protozoan parasites of the genus *Leishmania*, which maintain their life cycle through transmission between an insect (phlebotomine sandfly) and a mammalian host (Kaye and Scott 2011). The parasite exists in two distinct forms: a promastigote form found in the vector and an amastigote form, which develops intracellularly in the susceptible mammalian host (van Griensven and Diro 2012).

Electronic supplementary material The online version of this article (doi:10.1007/s00044-016-1729-1) contains supplementary material, which is available to authorized users.

✉ Claudinei A. Silva
clalvess@ufg.br

✉ Marcelo H. dos Santos
marcelo_hs@yahoo.com.br

[1] Laboratório de Fitoquímica e Química Medicinal – LFQM, Universidade Federal de Alfenas, Alfenas-MG 37130-000, Brazil

[2] Laboratório de Parasitologia/Biologia Molecular de Microrganismos, Universidade Federal de Alfenas, Alfenas-MG 37130-000, Brazil

[3] Laboratório de Pesquisa em Química Farmacêutica – LQFar, Faculdade de Ciências Farmacêuticas, Universidade Federal de Alfenas, Alfenas-MG 37130-000, Brazil

[4] CIEXA-JAT, Universidade Federal de Goiás, Regional Jataí, Jataí-GO 75801-615, Brazil

[5] Departamento de Química, Universidade Federal de Lavras, Lavras-MG 37200-000, Brazil

[6] Departamento de Química, Universidade Federal de Viçosa, Viçosa-MG 36570-000, Brazil

Published online: 27 October 2016

Current therapeutics for leishmaniasis include pentavalent antimony (sodium stibogluconate or meglumine antimoniate), the polyene amphotericin B (as the deoxycholate salt or a liposomal formulation), the alkyl phosphocholine miltefosine and the aminoglycoside paromomycin (Croft and Olliaro 2011; Seifert et al. 2011). However, those drugs are far from satisfying the current demands in individuals, who live in a region where *Leishmania* infections are endemic, due to their cost (Fidalgo and Gille 2011) variation in the sensitivity of *Leishmania* species to drugs, drug-induced toxicity, and variation in host immune response-drug interaction (Croft et al. 2006)

The search for new bioactive molecules in natural products has proven to be a powerful approach for discovery and development of new drugs for the treatment of neglected human diseases (Cragg 1997; Ndjonka et al. 2013). Thus, the use of natural compounds for the treatment of various diseases caused by parasites, such as the causative agents of trypanosomiasis, leishmaniasis, schistosomiasis, lymphatic filariasis and onchocerciasis, have stimulated the research for new agents. Among the bioactive molecules some alkaloids, chalcones, lactones, tetralones, saponins and coumarins have been explored. (Akendengue et al. 1999; Cragg 1997; Ndjonka et al. 2013; Vieira et al. 2001). Since coumarins have been prospected for their in vitro and in vivo leishmanicidal properties, this compound class can be exploited to discover new effective, low toxic drugs for the treatment of leishmaniasis (Brenzan et al. 2007; Napolitano et al. 2004; Tiuman et al. 2012).

A compound belonging to the class of coumarins, 7-geranyloxycoumarin, showed significant growth inhibition against the tropical parasite *Leishmania major* (Napolitano et al. 2004). Also, prenylated coumarins auraptene, umbelliprenin, and galbanic acid, isolated from *Ferula szowitsiana* (Apiaceae), showed in vitro antileishmanial activity against promastigotes of *L. major* (Singh et al. 2014). Isopropenyl coumarins isolated from the extracts of the leaves of *G. panamensis* T. S. Elias (Rutaceae) demonstrated significant inhibition against axenic amastigote forms of *Leishmania panamensis* (Arango et al. 2010). Another coumarin-type, (-) mammea A/BB, purified from the crude extract of *Calophyllum brasiliense* leaves has shown significant activity against both forms of *Leishmania amazonensis*, and it was recently used to assess the treatment of lesions in animal models (Brenzan et al. 2007, 2008). These studies encouraged us to investigate the leishmanicidal activity of new synthetic coumarin derivatives to warrant higher potency against these human pathogens.

The aim of this work was to obtain and evaluate the leishmanicidal activity against both forms, promastigotes and amastigotes,of *L. amazonensis* of eight coumarin derivatives. We also evaluate the cytoxicity of this compound in mammalian cells (murine peritoneal macrophages).

Materials and methods

Chemistry

The reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 (Merck®). Melting points were determined by using a Mettler melting point apparatus (Mettler-Toledo, Leicester, UK) and were not corrected. NMR spectra were recorded on BRUKER Avance DPX 200 or BRUKER Avance DRX 400 spectrometers. Chemical shifts (δ) were reported in parts per million (ppm) with reference to tetramethylsilane (TMS). The coupling constants (J) were reported in hertz (Hz) and signal multiplicities were reported as singlet (s), doublet (d), doublet of doublet (dd), multiplet (m) and sextet (sext).

Synthesis of 7-Hydroxy-4-phenylcoumarin (1)

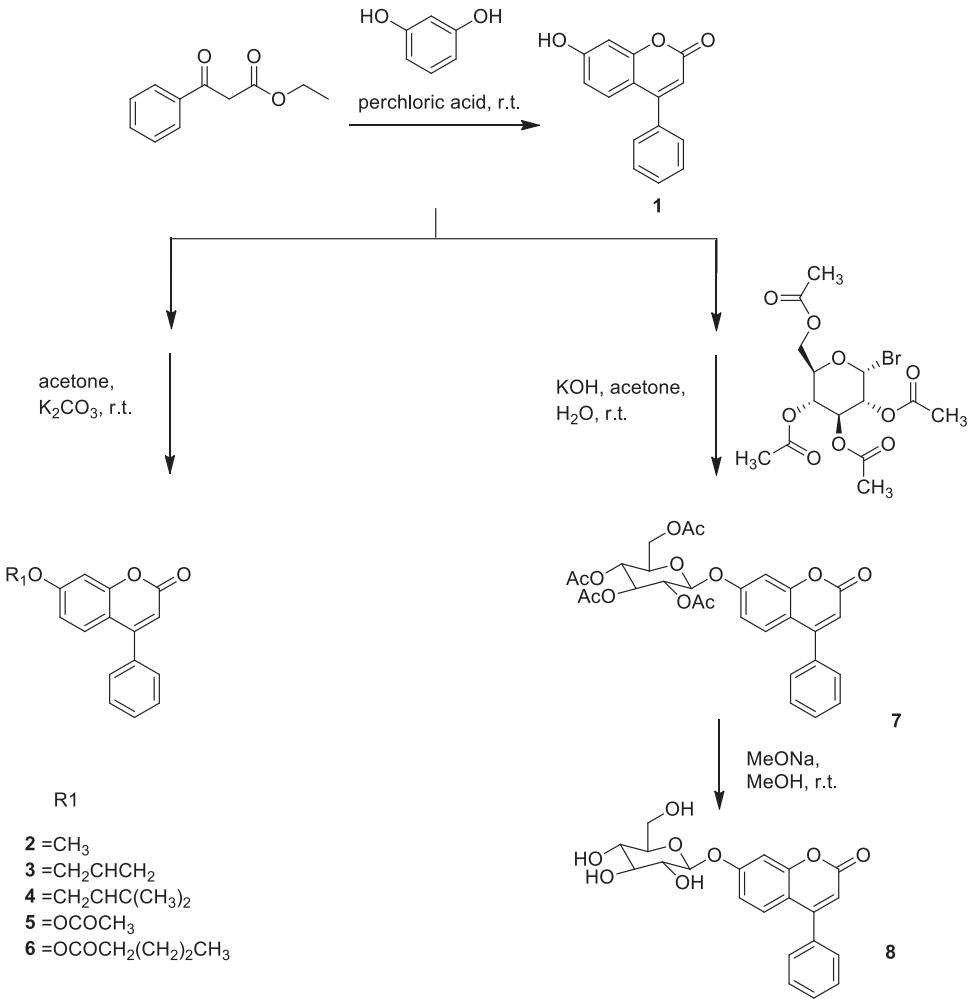
Equimolar amounts of resorcinol and ethyl benzoyl acetate were stirred in perchloric acid at room temperature for 1.5 h. The reaction mixture was then poured in crushed ice under constant stirring; the solid product **1** thus formed was isolated by suction filtration and was purified by recrystallization from hot ethanol.

*Synthesis of O-alkyl (**2**, **3** and **4**) and O-acyl (**5** and **6**) coumarin derivatives*

Potassium carbonate (2 eq.) and the alkyl or acyl halide (1 eq.) were added to a stirred solution of **1** (1 eq.) in acetone (Scheme 1). The reaction mixture was kept under stirring at room temperature and was monitored by TLC (hexane/ethyl acetate, 8:2 v/v). The reaction mixture was then extracted with ethyl acetate (3 × 20 mL). The organic layers were combined, dried over anhydrous sodium sulfate, filtered and the solvent evaporated under reduced pressure. Products were obtained in good yields and were used further without needing purification.

*Synthesis of coumarin glucosides (**7** and **8**)*

Equimolar amounts of potassium hydroxide and **1** were stirred in an acetone-water mixture (1:1, v/v) under room temperature until total solubilization. Then 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl bromide (0.3 eq.) was added in acetone solution. This mixture was left under the same reaction conditions and was monitored by TLC (dichloromethane/hexane, 9:1, v/v). Acetone was then eliminated under reduced pressure and the raw product was extracted



Scheme 1 Rote for the synthesis of 7-Hydroxy-4-phenylcoumarin (**1**), *O*-alkyl (**2**, **3** and **4**), *O*-acyl (**5** and **6**) coumarin derivatives and coumarin glucosides (**7** and **8**)

with dichloromethane (3 × 20 mL). The organic phase was washed with NaOH (10 % w/v/ 3 × 20 mL) and then dried with anhydrous sodium sulfate. The solvent was eliminated under reduced pressure and the pure product **5** was obtained after recrystallization from water/ethanol (6.5:3.5, v/v). Following, Compound **5** was dissolved in a 1 mol L^{-1} solution of MeONa in dry MeOH (2 mL/100 mg of compound). The solution was stirred at room temperature and the reaction was followed by TLC (ethyl acetate/ hexane, 8:2 v/v). The solution was then made neutral by using Amberlite® IRA-120 ion exchange resin, and the filtrate cooled to 0 °C when the pure and solid product **6** was obtained after filtration and cold methanol washings (Conchie et al. 1957; Zweckmair et al. 2014).

7-Hydroxy-4-phenylcoumarin (**1**) (Kuarm et al. 2012) White crystalline solid (yield 89 %); m.p. 254 °C; IR (KBr) υ_{max} (cm^{-1}) 3097 (υ O–H); 1711.22 (υ C=O); 1238.17 (υ C–O–C). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 6.19 (*s*,1H, H3), 6.81–6.82 (*d*, 1H, *J* = 4.0 Hz H8), 6.84–6.85 (*dd*, 1H, *J* = 2.0, 4.0, 2.0 Hz, H6) 7.31–7.33 (*d*, 1H, *J* = 8.0 Hz, H6), 7.56–7.61 (*m*, 5H, H Ph); ^{13}C NMR (50 MHz, DMSO-d_6): δ 162.47 (C-2), 161.27 (C-7), 156.62 (C-4), 141.82(C-9), 136.22 (C-1′), 130.66 (C-5), 129.90 (C-3′ and C5′), 129.45 (C-2′ and C-6′), 129.17(C-4′), 114.29(C-3), 111.72 (C-10), 111.39 (C-6), 103.76 C-8). HRMS *m/z* 261.0611 $C_{15}H_{10}O_3Na^+$ (calcd. 261.0528).

7-*O*-Methyl-4-phenylcoumarin (**2**) (Rizzi et al. 2006) White solid (yield 56 %); m.p. 111–112 °C; IR (KBr) υ_{max} (cm^{-1}) 3062 (υ C–H Ph); 1743.36 (υ C=O); 1207.44 (υ C–O–C). ^{1}H NMR ($CDCl_3$, 400 MHz):δ 7.43–7.52 (m, 5H, H Ph), 7.38–7.40 (*d*, 1H, *J* = 8.0 Hz H5) 6.89–6.90 (*d*, 1H, *J* = 2.4 Hz H6) 6.78–6.81 (*dd*, 1H, *J* = 2.8, 4.0, 2.8 Hz, H8), 6.22 (*s*, 1H, H3), 3.89 (*s*, 3H, H7′ OCH_3) ^{13}C NMR ($CDCl_3$, 100 MHz):δ 163.17 (C-2), 161.56 (C-7), 156.39 (C-4), 156.17 (C-9), 135.95 (C-1′), 129.92 (C-4′), 129.17 (C-3′ and C5′), 128.72(C-2′ and C-6′), 128.33, (C-5) 112.90 (C-10), 112.68 (C-3), 112.24 (C-6), 101.46 (C-8), 56.14(C-7′). HRMS *m/z* 275.0049 $C_{16}H_{12}O_3Na^+$ (calcd. 275.0684).

7-*O*-allyl-4-phenylcoumarin (**3**) (El-Ansary et al. 2012) White solid (yield 75 %); m.p. 183–184 °C; IR (KBr) υ_{max}

(cm^{-1}) 3079.41 (υ C–H_2); 3020.58 (υ C–H Ph) 1729.21 (υ C=O); 1124.52 (υ C–O–C) 748.39 and 623.02 (δ C–H Ph). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 7.41–7.51(*m*, 6H), 6.89–6.90 (*d*, 1H, $J = 4.0$ Hz H8) 6.79–6.87 (*dd*, 1H, $J = 2.0$; 10.0; 2.0 Hz H6), 6.22 (*s*, 1H), 5.95–6.14 (*ddd*, 1H $J = 6.0$, 12.0, 18.0 Hz H8′) 5.32–5.49, (*m*, 2H) 4.60–4.62 (*d*, 2H, $J = 4.0$ Hz, H7). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 162.04 (C-2), 161.60 (C-7), 156.24 (C-4), 156.16 (C-9), 135.87 (C-1′), 132.44 (C-8′), 129.93 (C-4′), 129.16 (C-3′ and C5′), 128.71 (C-2′ and C-6′), 128.32 (C-5), 118.94 (C-9′), 113.18 (C-3), 112.95 (C-10), 112.25 (C-6), 102.29 (C-8) , 69.60 (C-7′). HRMS *m/z* 301.0515 $C_{18}H_{14}O_3Na^+$ (calcd. 301.0841).

7-*O*-Prenyl-4-phenylcoumarin (**4**) (Subramanyam Raju and Subba Rao 1974) White crystalline solid (yield 57 %); m.p. 202–205 °C; IR (KBr) υ_{max} (cm^{-1}) 3032.10 (υ C–H_2); 2862.36 and 2966.52 (υ C–H_3); 1708.93 (υ C=O); 1149.57 (υ C–O–C). ^{1}H NMR ($CDCl_3$, 400 MHz):δ 7.43–7.52 (*m*, 5H, H Ph) 7.38 (*d*, 1H, $J = 8.8$ Hz H5) 6.89–6.90 (*d*, 1H, $J =$ 2.4 Hz H6), 6.78–6.81 (*dd*, 1H, $J = 4.0$, 6.0, 4.0 Hz, H8), 6.21 (*s*, 1H, H3), 5.47–5.49 (*t*, 1H $J = 4.0$, 4.0 Hz H8′, $OCH_2\underline{C}H\underline{C}(CH_3)_2$) 4.58–4.60 (*d*, 2H $J = 8.0$ Hz, H7′, $OC\underline{H}_2CHC(CH_3)_2$), 1.81 (*s*, 3H, H11′, $OCH_2CHC(C\underline{H}_3)_2$) 1.77 (*s*, 3H, H10′,$OCH_2CHC(C\underline{H}_3)_2$), ^{13}C NMR ($CDCl_3$, 100 MHz): δ 162.54 (C-2), 162.41(C-7), 156.28 (C-4), 156.24 (C-9), 139.71 (C-1), 135.93(C-9′), 129.90(C-4′), 129.15(C-3′C5′), 128.72(C-2′C6′), 128.26 (C-5), 118.93 (C-8′), 113.32 (C-3), 112.72 (C-10), 112.05 (C-6), 102.10 (C-8), 65.75 (C-7′), 26.17 (C-10′), 18.64 (C-11′). HRMS *m/z* 329.0928 $C_{20}H_{18}O_3$ Na^+ (calcd. 329.1154).

7-*O*-Acetyl-4-phenylcoumarin (**5**) (Subramanyam Raju and Subba Rao 1974) White solid (yield 68 %); m.p. 211–213 °C; IR (KBr) υ_{max} (cm^{-1}) 3077.48 (υ C–H_3); 1762.15 and 1733.07 (υ C=O); 1142.84 (υ C–O–C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.44–7.54 (*m*, 5H, H Ph), 7.50–7.48 (*d*, 1H, $J =$ 2.4 Hz H8), 7.19–7.20 (*d*, 1H, $J =$ 2.4 Hz H5), 6.99–7.02 (*dd*, 1H, $J = 2.4$, 6.4, 2.4 Hz, H-6), 6.35 (*s*, 1H, H3), 2.34 (*s*, 1H, H-7′).^{13}C NMR ($CDCl_3$, 100 MHz): δ 168.47 (C-2), 160.21 (C-7′), 155.03 (C-4), 154.60 (C-7), 152.97 (C-9), 130.85 (C-1′), 129.58 (C-3′ and C-5′), 128.73 (C-2′ and C-6′), 128.14 (C-4′), 127.65 (C-5), 117.84 (C-6), 114.31 (C-8), 110.47 (C-3), 20.87 (C-8′). HRMS *m/z* 303.0515 $C_{17}H_{12}O_4$ Na^+ (calcd. 303.0634).

7-*O*-butanoyl-4-phenylcoumarin (**6**) White solid (yield 68 %); m.p. 198–199 °C; IR (KBr) υ_{max} (cm^{-1}) 3030.10 (υ C–H_2); 2951.09 (υ C–H_3); 1757.15 and 1739.79 (υ C=O); 1165.00 (υ C–O–C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.43–7.53 (*m*, 5H, H Ph), 7.18–7.19 (*d*, 1H, $J = 2.0$ Hz H5), 6.98–7.00 (*dd*,1H, $J =$ 2.4, 8.8, 2.4 Hz, H6), 6.34 (*s*, 1H, H3), 2.60–2.56 (*t*, 2H, $J =$ 4.0, 4.0 Hz H7′), 1.60–1.85, (sext, 2H, $J = 8.0$, 8.0, 8.0, 8.0, 8.0 Hz, H8′) 1.04–1.07 (*t*, 3H, $J = 8.0$, 8.0 Hz H9′) ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.77 (C-6′), 160.81 (C-2), 155.62 (C-4), 155.19 (C-7), 153.69 (C-9), 135.46 (C-1′), 130.14 (C-4′), 129.29 (C3′ and C5′), 128.72 (C2′ and C6′), 128.17(C-5), 118.45 (C-6), 117.15(C-10), 114.82 (C-8), 111.03 (C-3), 36.50 (C-8′), 18.66 (C-9′), 13.92 (C-10′). HRMS *m/z* 331.1186 $C_{19}H_{16}O_4$ Na^+ (calcd. 331.0947).

7-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-4-phenylcoumarin (**7**) (Garazd et al. 2005) Light yellow solid (yield 61 %); m.p. 180 °C; IR (KBr) υ_{max} (cm^{-1}) 3471 (υ C–H_2); 3078.33, 2970.38 and 2881.85 (υ C–H_3); 1752.22 and 1739.79 (υ C=O); 1157.29 (υ C–O–C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.52–7.39 (*m*, 5H, H Ph), 7.02 (*d*,1H, $J =$ 6.4Hz, H-5, 6.87), 6.87–6.84 (*dd*,1H, $J = 2.4$, 6.4, 2.4 Hz, H-6), 6.27 (*s*, 1H, H3), 5.35–5.28 (*m*, 1H, H6″), 5.17–5.19 (*m*, 1H, H-4″), 4.27–4.30 (*m*, 1H, H-2″), 4.17–4.20 (*m*, 1H, H-1″), 3.90–3.94 (*m*, 1H, H-5″), 2.12 (*s*, 3H, H-8″), 2.06 (*s*, 6H, H-14′; H10′), 2.04 (*s*, 3H, H-11″) ^{13}C NMR ($CDCl_3$, 100 MHz): δ170.93 (C-13″), 170.49 (C-7″), 169.73 (C-9″), 169.56 (C-12″), 161.02(C-2), 159.66 (C-9), 155.92 (C-7), 155.81 (C-4), 135.58 (C-10), 130.10 (C-3), 129.27(C-6′ and C-2′), 128.67 (C-5′ C-3′), 128.56(C-4′), 114.87 (C-1′), 114.30 (C-8), 113.50 (C-6), 104.54 (C-5), 98.71 (C-1″), 72.93 (C-2″), 72.83 (C-3″), 71.31 (C-4″), 68.45(C-5″), 62.17 (C-6″), 21.03 (C-14″), 20.98 (C-8″), 20.91 C-10″C-11″). HRMS *m/z* 591.1538 $C_{29}H_{28}O_{12}$ Na^+ (calcd. 591.1479).

7-*O*-(β-D-glucopyranosyl)-4-phenylcoumarin (**8**) (Garazd et al. 2005) White solid (yield 69 %); m.p. 233–234 °C; IR (KBr) υ_{max} (cm^{-1}), 3456.44 (υ O–H); 2885.51 (υ C–H_2); 1701.22 (υ C=O); 1161.15 (υ C–O–C). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.50–7.56 (*m*, 5H, H Ph), 7.33–7.35 (*d*, 1H, $J = 8.8$ Hz H5) 7.13, (*s*, 1H, H5″) 6.97–6.99 (*d*, 1H $J =$ 8.8 Hz) 6.25 (*s*, 1H, H3), 5.46–5.59 (*m*, 4H, H7″H8″H9″ H10″), 5.02–5.03 (*d*, 1H, $J = 4$,0 Hz H5″) 3.70–3.14 (*m*, 4H, H6″H4″H3″ H2″), ^{13}C NMR (DMSO-d_6, 100 MHz) δ 161.25 (C-2), 160.90 (C-3), 155.98 C-9), 135.85 (C-1″), 130.64 (C-8), 129.81 (C-6′, and C-2′), 129.39 (C-5′ and C-3′), 128.71 (C-4′), 114.70 (C-1′), 113.68 (C-7), 112.83 (C-6), 104.60 (C-5), 100.96 (C-10), 78.13 (C-5″), 77.43 (C-2″), 74.06 (C-4), 70.56 (C-3″), 61.58 (C-6″). HRMS *m/z* 423.1375 $C_{21}H_{20}O_8$ Na^+ (calcd. 423.1056).

Biological evaluation

Leishmanicidal in vitro activity against promastigotes

Promastigote forms of *L. amazonensis* (MHOM/BR/71973/M2269) were cultivated in Schneider's Drosophila medium (Sigma, USA) supplemented with 10.0 % (v/v) heat-inactivated fetal bovine serum and 1.0 % penicillin (10,000 UI/ mL)/streptomycin (10.0 mg/mL) (Sigma, USA). Cells were counted in Neubauer's chamber and adjusted to a concentration of 1×10^6 cells/mL. *Leishmania* promastigotes were re-suspended in fresh medium and harvested on a

24-well plate. Coumarin derivatives, solubilized in dimethylsulfoxide (DMSO), were added to promastigote culture plates and incubated for 72 h at 25 °C. After that, the surviving parasites were counted in a Neubauer's chamber and compared with controls for the determination of 50.0 % inhibitory growth concentration (IC_{50}). All tests were performed in triplicate and Amphotericin B (Eurofarma) was used as the reference drug (Maciel-Rezende et al. 2013).

Leishmanicidal in vitro activity against intracellular amastigotes

Murine peritoneal macrophages were maintained in RPMI-1640 medium, supplemented with 10.0 % heat-inactivated fetal bovine serum and 1.0 % penicillin (10,000 UI/mL)/streptomycin (10 mg/mL). Cells, at 8×10^5 cells/mL concentration, were cultivated in 24-well plates on 13 mm glass slides (Nunc, USA). After 30 min for adhesion, the cells were infected with *L. amazonensis* promastigotes at a multiplicity of infection of 10:1 (parasite/macrophage). The plates were incubated in a 5.0 % CO_2 air mixture at 37 °C for 24 h. The nonphagocytosed promastigotes were then removed while washing, and the compounds were added. After 72 h, chamber slides were fixed in absolute methanol, stained with Giemsa and examined under an oil immersion objective lens of a light microscope. At least 200 macrophages were counted per well for calculating the percent inhibition for the determination of IC_{50} value. All tests were performed in triplicate on three different occasions and Amphotericin B (Eurofarma) was used as the reference drug (Pereira et al. 2010).

Evaluation of cytotoxicity

For the cytotoxicity assay a suspension of 8×10^5 cells/mL of murine peritoneal macrophages, in RPMI 1640 medium, supplemented with 10.0 % heat-inactivated fetal bovine serum and 1.0 % penicillin (10,000 UI/mL)/streptomycin (10 mg/mL) were added to each well of 24-well plates, on 13 mm glass slides. The plates were incubated in a 5.0 % CO_2 air mixture at 37 °C for cell adhesion. After 24 h, the non-adherent cells were removed by washing with the RPMI 1640 medium. Thus, several concentrations of compounds (in the range of 0.05–160.0 µg/mL) were added to the wells containing the cells. All target compounds were solubilized in DMSO at a final concentration of 0.6 % v/v and the plates were incubated for another 72 h. The non-adherent cells were then removed by washing with the RPMI 1640 medium and 50.0 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well at a concentration of 5.0 mg/mL, followed by incubation for another 4 h, as described by Mossman (Mosmann 1983). After this, the medium was removed and 1 mL of DMSO was added to each well and homogenized for 15 min. Next, the absorbance of each individual well, minus the control value, was calculated at 570 nm according to the following formula.

$$\% \text{ inhibition} = \left(\frac{OD_{control} - OD_{drugs}}{OD_{control}} \right) \times 100$$

Each experiment was performed in triplicate, and the percentage of viable cells was calculated in relation to control cultures in the medium with only DMSO at the concentration of 0.6 % v/v.

Statistical analysis

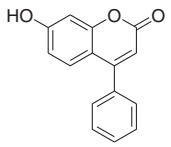
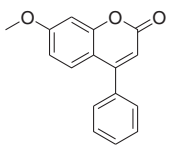
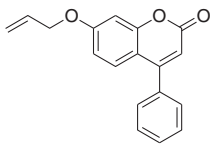
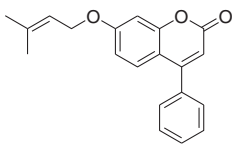
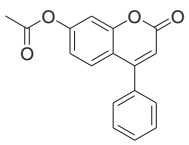
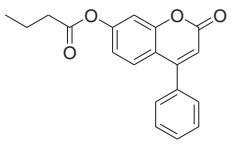
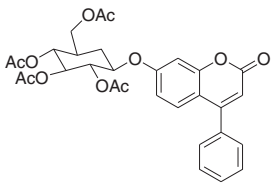
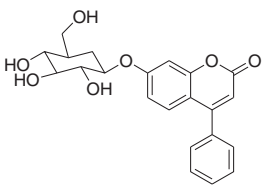
The leishmanicidal activity of the compounds was expressed as the concentration that inhibits the growth of 50.0 % of protozoan forms. Statistical analysis was performed using nonlinear regression to obtain the IC_{50} and CC_{50} (cytotoxic concentration for 50.0 % of macrophages) values, followed by variance analyses and Tukey's test. Differences were significant when the p value was lower than 0.05.

Evaluation of lipophilicity by LogP (octanol/water) and ab initio calculations

Lipophilicity values were estimated through theoretical determination of LogP (o/w) by using the XLOGP3 program (Urzúa et al. 2008). The QikProp program calculates the LogP (o/w) values from regression equations using experimental data and molecule physical descriptors (hydrogen bond counts, atom types and charges, rotor counts, etc.) through Monte Carlo statistical mechanics simulations (Jorgensen and Duffy 2000) calculated lipophilicity expressed by LogP (o/w) of the studied compounds are shown in Table 1. Actually, partition coefficient data are quite useful to estimate the solubility of a solute in a specific solvent (Yalkowsky 1999) (Fig. 1).

The previous predictions of LogP (o/w) values were confirmed by ab initio theoretical calculations of Lipophilicity values by using Gibbs free energies of solvation of the chemical species. In this methodology, we initially carried out a conformational analysis using Monte Carlo (MC)-simulated annealing (from $T = 5000$ K to $T = 300$ K) (da Cunha et al. 2009; Hohenberg and Kohn 1964). The selected conformations were subsequently refined at the DFT level. Thus, each selected conformer was then fully optimized by DFT with the B3LYP functional and basis set 6-311+g (d, p). The solvent effect was taken into account using PCM calculations (Miertus et al. 1981).

Table 1 Chemical structure and biological activity of coumarin derivatives against both forms of *L. amazonensis*, compared to amphotericin

Compound	Chemical structure	Promastigote IC_{50} (μM)	Amastigote IC_{50} (μM)	Amastigote cytotoxicity	LogP^{a}//P^{b}
1		70.10 ± 5.56	93.60 ± 3.32	>671.59	3.51//3.2
2		58.19 ± 1.71	83.05 ± 2.56	>634.27	3.63//3.75
3		37.37 ± 0.45*	91.81 ± 1.09	>574.92	4.40//4.60
4		21.35 ± 0.83*	58.10 ± 1.58	>522.26	5.33//6.20
5		45.33 ± 0.88*	112.11 ± 2.01	>570.88	3.06//3.12
6		38.63 ± 0.89*	89.38 ± 3.1	>518.92	4.12//5.70
7		10.03 ± 1.21*	34.93 ± 1.86	>281.43	3.69//3.71
8		91.78 ± 1.69	n.d.	>399.62	1.55//1.58
Amphotericin B		5.09	6.15	27.05	–

n.d. not determined

* Values marked with one asterisk means that the derivative differs statistically from their starting compound when $p < 0.05$ by Tukey's test

[a] Calculated lipophilicity expressed by Log *P* (oct/wat) using XLOGP3 program version 3.0.1

[b] Calculated lipophilicity expressed by Log *P* (oct/wat) using Gibbs free energy of solvation from Gaussian program

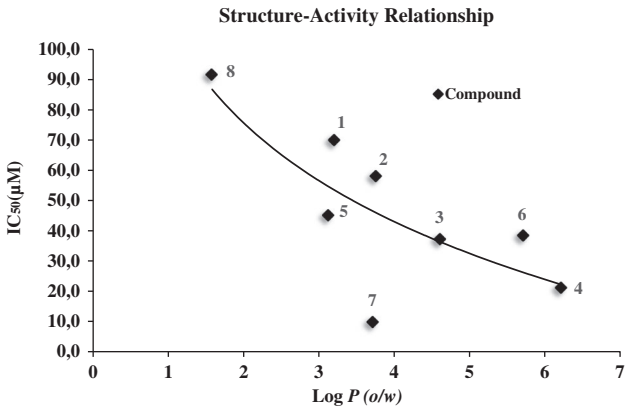


Fig. 1 Structure–activity relationship by IC_{50} values to promastigote forms and lipophilicity expressed by Log *P* (o/w) by using Gibbs free energy of solvation from Gaussian program

Results and discussion

In order to prepare a variety of coumarin derivatives, Coumarin **1** was prepared as the starting compound. As depicted in Scheme 1, Compound **1** was synthesized via Pechmann reaction (Pechmann 1884), which is the most widely used method to obtain coumarins. This reaction involves the use of simple starting materials, such as phenols and β-ketoesters in acidic medium and generally works well at room temperature. The advantages of this procedure are short reaction times, operational simplicity and high product yields. Many coumarins are readily purified by crystallization, such as those synthesized in this work, which were recrystallized from a mixture of ethanol/water (7:3 v/v) furnishing high purity products without using column chromatography purification. All derivatives were obtained in good yields. The structural assignments of all synthesized compounds were based on ^{1}H and ^{13}C NMR, IR and mass spectra analyses.

A series of synthetic coumarins is shown here that were evaluated in vitro against both forms of *L. amazonensis* and the results support the leishmanicidal potential of this class of compounds. The studies reported by Kappagoda and co-workers with antiparasitic coumarin derivatives were focused on coumarins bearing a 5,7-dihydroxy skeleton, a phenyl group attached to C-4 and prenyl or acyl substituents attached to C-6 or C-8 (Kappagoda et al. 2011). In this study, the phenyl group attached to the C-4 position was maintained and the nature of substituents at the C-7 position was varied. The most promising derivatives were **4** and **7**, which exhibited good activity against both promastigote and amastigote forms of *Leishmania*. Concentrations up to 281.43 µM showed no cytotoxicity and the same profile was observed in tests beyond these concentrations. Derivative **8** showed high IC_{50} value against promastigote forms but was not selected to evaluate its activity against intracellular form of the parasite. All results are shown in Table 1.

Compound **4,** which presented an *O*-prenyl group at position C-7, as well as **7,** with a penta-*O*-acetyl-glucopyranosyl group in this same position, were the most active coumarins against promastigote forms of *L. amazonensis* (IC_{50} values = 21.35 and 10.03 µM, respectively). Both compounds were also the most effective against amastigote forms (IC_{50} values = 58.10 and 34.93 µM, respectively). Moreover they presented lower cytotoxicity than the reference drug, Amphotericin B (cytotoxicity value = 27.05 µM). Compounds **1**, **2**, **3**, **5** and **6**, which present free phenol, *O*-methyl, *O*-allyl, *O*-acetyl and *O*-butanoyl groups, respectively, were the least active against both promastigote and amastigote forms. The presence of free-hydroxyls β-D-glucopyranosyl group at position C-7 does not seem to be a promising alternative considering the leishmanicidal activity, since Compound **8** was not active against promastigotes of *L. amazonensis*. On the other hand, it was observed that per-*O*-acetyl glucopyranosyl moiety, present in Compound **7**, had an important effect on the desired biological action. Comparing the chemical structures of Compounds **7** and **8**, one may suggest that lipophilicity is an important issue for the leishmanicidal activity of the Compound **7** (Fig. 1).

Pereira and co-workers showed that benzophenones with higher lipophilicity present higher activities against *Leishmania* species, which could in fact be related to the ease of entering the macrophage and reaching the parasite (Pereira et al. 2010). Maciel-Rezende and co-workers recently evidenced that an increase in the size of side chain substituents led to a proportional increase in lipophilicity of benzophenone derivatives and leishmanicidal potency (Maciel-Rezende et al. 2013). Indeed, these results indicate that changing the substituents in the coumarin nucleus to influence on the polarity of the molecule also changes its biological activity. The results of this study suggest that some chemical features, as lipophilicity, in the synthesized coumarins are relevant for the leishmanicidal activity, but further investigations must be performed to confirm this hypothesis.

In order to shed some light on the experimental results, a computational study was developed to evaluate the physicochemical properties of the synthesized compounds to check the influence of the nature of the environment on the stability of the molecules and to obtain data that could allow proposing the biological action mechanism of these drugs. In this regard, we carried out partition coefficient calculations. Currently, the CCl_4/water and *n*-octanol/water biphasic systems are the mimic models most widely used for the water-membrane system (Liu et al. 2011). It should be kept in mind that the partition coefficient is a very important issue of the Lipinski's rule-of-five. In 1997, Lipinski and colleagues postulated some structural and

electronic features that are associated with orally active drugs in humans, which is denominated as the "rule-of-five" (Lipinski et al. 2012).

In fact, from the theoretical side, a consolidate methodology for the LogP calculation is to use information of the free energy of solvation in water and in octanol or CCl_4 to estimate the partition coefficient. From Gibbs free energies of solvation in two different phases at temperature T, one can calculate the corresponding partition coefficient, according to the following expression: where $\Delta_{hyd}G$ is the hydration free energy and $\Delta_{solv}G$ is the Gibbs free energy of solvation in 1-octanol.

$$\mathrm{Log}P = \frac{\Delta_{\mathrm{hyd}}G - \Delta_{\mathrm{solv}}G}{2.303\,\mathrm{RT}}$$

In accordance with Table 1, Compounds (**4**) and (**7**) reveal the high hydrophobicity for both theoretical methodologies used. Interestingly, those compounds were the most active coumarins against promastigote and amastigote forms of *L. amazonensis*. Therefore, this pattern clearly points out and reinforces the proposition regarding the biological action of phenylcoumarins. In fact, the interaction with the membrane likely plays an important role in the biological action mechanism of these drugs.

The evaluation of the coumarin derivatives in relation to toxicity in mammalian cells showed no significant toxicity. These results indicate that Compounds **4** and **7** showed the most significant results against promastigote and amastigotes forms of *L. amazonensis* in vitro and no toxicity. Coumarin derivatives may be potential new drugs for the treatment of leishmaniasis that could become available for low-income populations. This study is part of a continued search for new drugs with high activity and few side effects that can be used to treat diseases associated with protozoan parasites, such as leishmaniasis.

Conclusions

Eight coumarin derivatives (Compounds **1–8**) were prepared in good chemical yields and their biological activity against *L. amazonensis* was tested. The most distinguished results seem to be related to Compounds **4** and **7** against both promastigote and amastigote forms of *L. amazonensis.* These results indicated that probability that the variation of the substituents on the coumarin scaffold seems to influence on the polarity of the molecule and its biological activity. The potency could in fact be related to the facility to penetrate the macrophage and reach the parasite. Our theoretical findings from LogP (o/w) calculations support this proposal. Thus, these series of derivatives could be explored as candidates for development of new drug prototypes against leishmaniasis. Further studies should be conducted to better understand the action mechanism of these molecules and their effectiveness against amastigote forms of *Leishmania*.

Acknowledgments The authors are grateful to Rede Mineira de Química (RQ-MG) (CEX-RED-00010-14), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Financiadora de Estudos e Projetos (FINEP), Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the financial support.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

Akendengue B, Ngou-Milama E, Laurens A, Hocquemiller R (1999) Recent advances in the fight against Leishmaniasis with natural products. Parasite 6:3–8

Arango V, Robledo S, Séon-Méniel B, Figadère B, Cardona W, Sáez J, Otálvaro F (2010) Coumarins from Galipea panamensis and their activity against Leishmania panamensis. J Nat Prod 73:1012–1014

Beattie L, Kaye PM (2011) Leishmania–host interactions: What has imaging taught us?. Cell Microbiol 13:1659–1667

Bhargava P, Singh R (2012) Developments in diagnosis and antileishmanial drugs. Interdiscip Perspect Infect Dis 2012:626838

Brenzan MA, Nakamura CV, Dias Filho BP, Ueda-Nakamura T, Young MCM, Cortez DAGC (2007) Antileishmanial activity of crude extract and coumarin from calophyllum brasiliense leaves against Leishmania amazonensis. Parasitol Res 101:715–722

Brenzan MA, Nakamura CV, Dias Filho BP, Ueda-Nakamura T, Young MCM, Corrêa AG, Alvim Júnior J, dos Santos AO, Cortez DAGC (2008) Structure–activity relationship of (-) Mammea A/BB derivatives against leishmania amazonensis. Biomed Pharmacother 62:651–658

Conchie J, Levvy GA, Marsh CA (1957) Methyl and phenyl glycosides of the common sugars. Adv Carbohydr Chem 12:157–187

Cragg GM (1997) Natural products in drug discovery and development. J Nat Prod 60:52–60

Croft SL, Olliaro P (2011) Leishmaniasis chemotherapy-challenges and opportunities. Clin Microbiol Infect 17:1478–1483

Croft SL, Sundar S, Fairlamb AH (2006) Drug resistance in Leishmaniasis. Clin Microbiol Rev 19:111–126

da Cunha EFF, Sippl W, Ramalho TC, Antunes OAC, de Alencastro RB, Albuquerque MG (2009) 3D-QSAR CoMFA/CoMSIA models based on theoretical active conformers of HOE/BAY-793 analogs derived from HIV-1 protease inhibitor complexes. Eur J Med Chem 44:4344–4352

El-Ansary SL, Hussein MM, Rahman DEA, Hamed MIAL (2012) Synthesis and docking studies of furobenzopyrones of potential antimicrobial and photochemotherapeutic activities. Life Sci J 9:1114–1125

Fidalgo LM, Gille L (2011) Mitochondria and trypanosomatids: Targets and drugs. Pharmaceut Res 28:2758–2770

Garazd YL, Garazd MM, Khilya VP (2005) Modified coumarins. 19. Synthesis of Neoflavone D-Glycopyranosides. Chem Nat Compd 41:663–668

Hohenberg P, Kohn W (1964) Inhomogeneous electron gas. Phys Rev 136:B864–871

Hotez PJ, Savioli L, Fenwick A (2012) Neglected tropical diseases of the middle East and North Africa: review of their prevalence,

distribution, and opportunities for control ed. Serap Aksoy. PLoS Negl Trop Dis 6:e1475

Jorgensen WL, Duffy EM (2000) Prediction of drug solubility from Monte Carlo simulations. Bioorg Med Chem Lett 10:1155–1158

Kappagoda S, Singh U, Blackburn BG (2011) Antiparasitic therapy. Mayo Clin Proc 86:561–583

Kaye P, Scott P (2011) Leishmaniasis: complexity at the host-pathogen interface. Nat Rev Microbiol 9:604–615

Kuarm BS, Madhav JV, Rajitha B (2012) Xanthan sulfuric acid: an efficient and recyclable solid acid catalyst for Pechmann condensation. Synth Commun 42:1770–1777

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (2012) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 64(SUPPL):4–17

Liu X, Testa B, Fahr A (2011) Lipophilicity and Its relationship with passive drug permeation. Pharmaceut Res 28:962–977

Maciel-Rezende CM, de Almeida L, Costa ED'M, Pires FR, Alves KF, Viegas Junior C, Dias DF, Doriguetto AC, Marques MJ, dos Santos MH (2013) Synthesis and biological evaluation against Leishmania amazonensis of a series of Alkyl-substituted benzophenones. Bioorg Med Chem 21:3114–3119

Miertus SE, Scrocco E, Tomasi J (1981) Electrostatic interaction of a solute with a continuum. A direct utilization of AB initio molecular potentials for the prevision of solvent effects. Chem Phys 55:117–129

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Napolitano HB, Silva M, Ellena J, Rodrigues BDG, Almeida ALC, Vieira PC, Oliva G, Thiemann OH (2004) Aurapten, a Coumarin with growth inhibition against Leishmania major promastigotes. Braz J Med Biol Res 37:1847–1852

Ndjonka D, Rapado LN, Silber AM, Liebau E, Wrenger C (2013) Natural products as a source for treating neglected parasitic diseases. Int J Mol Sci 14:3395–3439

Pereira IO, Marques MJ, Pavan AL, Codonho BS, Barbiéri CL, Beijo LA, Doriguetto AC, D'Martin EC, dos Santos MH (2010) Leishmanicidal activity of benzophenones and extracts from Garcinia Brasiliensis Mart. fruits. Phytomedicine 17:339–345

Rizzi E, Dallavalle S, Merlini L, Pratesi G, Zunino F (2006) Short synthesis of cytotoxic 4-arylcoumarins. Synth Commun 36:1117–1122

Seifert K, Munday J, Syeda T, Croft SL (2011) In vitro interactions between sitamaquine and Amphotericin B, sodium stibogluconate, miltefosine, paromomycin and pentamidine against Leishmania Donovani. J Antimicrob Chemother 66:850–854

Singh N, Mishra BB, Bajpai S, Singh RK, Tiwari VK (2014) Natural product based leads to fight against Leishmaniasis. Bioorg Med Chem 22:18–45

Subramanyam Raju M, Subba Rao NV (1974) Search for physiologically active compounds. Part XXIV. synthesis of 7, 8-Furano, Pyrono and 3-Methyl-4-Phenylcoumarins. Proc Indian Acad Sci Sect A 79:223–229

Tiuman TS, Brenzan MA, Ueda-Nakamura T, Dias Filho BP, Cortez DGC, Nakamura CV (2012) Intramuscular and topical treatment of cutaneous Leishmaniasis lesions in mice infected with Leishmania amazonensis using Coumarin (-) Mammea A/BB. Phytomedicine 19:1196–1199

Urzúa A, Echeverría J, Rezende MC, Wilkens M (2008) Antibacterial properties of 3 H-spiro[1-benzofuran-2,1′-cyclohexane] derivatives from Heliotropium Filifolium. Molecules 13:2385–2393

v. Pechmann H (1884) Neue Bildungsweise Der Cumarine. Synthese Des Daphnetins I. Ber Deut Chem Ges 17:929–936

van Assche T, Deschacht M, da Luz RAI, Maes L, Cos P (2011) Leishmania-macrophage interactions: Insights into the redox biology. Free Radic Bio Med 51:337–351

van Griensven J, Diro E (2012) Visceral leishmaniasis. Infect Dis Clin N Am 26:309–322

Vieira PC, Mafezoli J, Pupo MT, Fernandes JB, da Silva MFGF, de Albuquerque S, Oliva G, Pavão F (2001) Strategies for the isolation and identification of trypanocidal compounds from the rutales. Pure Appl Chem 73:617–622

Yalkowsky SH (1999) Solubility and solubilization in aqueous media, 1st ed. American Chemical Society, Washington, DC

Zweckmair T, Becker M, Ahn K, Hettegger H, Kosma P, Rosenaua T, Potthast A (2014) A novel method to analyze the degree of acetylation in biopolymers. J Chromatogr A 1372:212–220