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Synthesis of chalcone analogues with increased antileishmanial activity

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Abstract—Eighteen analogues of an active natural chalcone were synthesized using xanthoxyline and some derivatives, and these analogues were tested for selective activity against both promastigotes and intracellular amastigotes of *Leishmania amazonensis* in vitro. Three analogues (**10**, **12**, and **19**) containing nitro, fluorine or bromine groups, respectively, displayed increased selective activity against the parasites as compared with the natural chalcone. The nitrosylated chalcone **10** was also tested intralesionally in infected mice and was found to be as effective as Pentostan reference drug at a dose 100 times higher than that of the chalcone in controlling both the lesion growth and the parasite burden.

1. Introduction

Leishmaniases are a group of diseases caused by different species of the protozoan parasite *Leishmania*, which affect more than 12 million people worldwide.[1] The clinical manifestations may range from single cutaneous lesions to fatal visceral leishmaniasis. Conventional chemotherapy relies on multiple parenteral injections with pentavalent antimonials that are considerably toxic and prone to induce resistance. Second-line drugs, such as Amphotericin B and its lipid formulations, are either too toxic or expensive for routine use in developing countries. At the same time, the efficacy of Miltefosine against cutaneous leishmaniasis remains to be ascertained.[2,3] These facts call for safer, cheaper, and more effective new antileishmanial drugs.

In the past decade, chalcones emerged as a new class of antileishmanial agents.[4–7] Chalcones are structurally simple compounds of the flavonoid family and are present in a variety of plant species with a vast spectrum of pharmacological activities including antibacterial, antifungal, immunosuppressive, and antinociceptive properties.[8–10] One of the most studied antileishmanial chalcones is licochalcone A isolated from the *Glycyrrhiza* spp. Chinese plant, which inhibits the parasite enzyme fumarate reductase.[11] However, licochalcone A and some synthetic derivatives have also been shown to affect human cell functioning by inhibiting lymphocyte proliferation and cytokine production.[12]

We have previously reported the selective activity of the 2′,6′-dihydroxy-4′-methoxychalcone (DMC) isolated from the inflorescences of *Piper aduncum* against intracellular amastigotes of *Leishmania amazonensis*[13] and the improvement of its therapeutic activity in mice by encapsulation in biodegradable nanospheres.[14] Aiming at developing compounds with improved antileishmanial activity, we synthesized a similar active chalcone (2′-hydroxy-4′,6′-dimethoxychalcone, compound **2**) by using xanthoxyline, a natural compound present in the leaves and stems of *Sebastiana chottiana*[15] with reported antibacterial,[16] antifungal,[17] and antioedematogenic[18] properties. In this work, other 17 chalcone analogues were then synthesized by base-catalyzed condensation

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of appropriate aldehyde and xanthoxyline or modified xanthoxyline.

The chalcones were tested in vitro against *L. amazonensis*, a causative agent of cutaneous leishmaniasis, using both the promastigote forms found in the insect vector and the intracellular amastigote forms found in mammalian hosts. Cytotoxicity against murine macrophages was evaluated for drug selectivity. One of the most active chalcones was also tested for evaluation of efficacy in mice with cutaneous leishmaniasis.

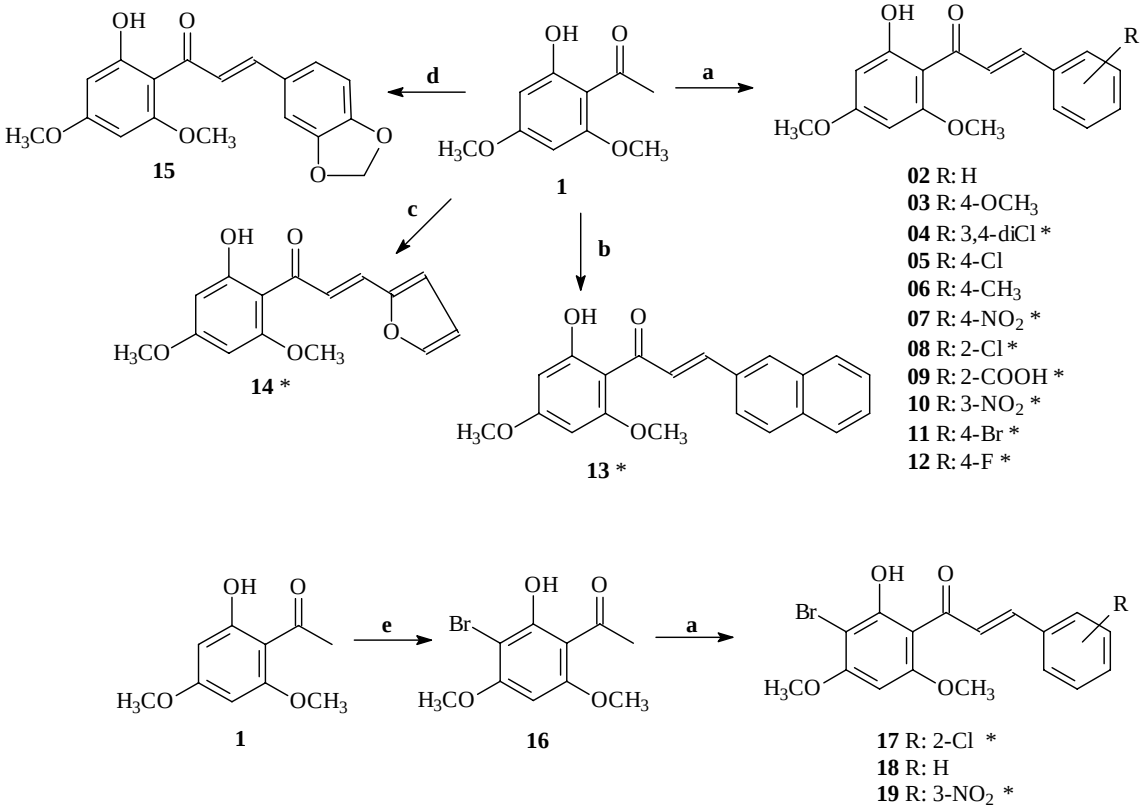
2. Results and discussion

2.1. Chalcones

The natural chalcone DMC was purified from Piper aduncum as previously described.[13] The 18 synthetic chalcones were prepared as shown in Scheme 1. All the starting materials were commercially obtained (Merck), except for xanthoxyline (**1**) and 2-hydroxy-3-bromo-4,6-dimethoxyacetophenone (**16**), which were prepared as previously described.[18] This method allowed the preparation of a variety of, especially, B-ring-substituted compounds.

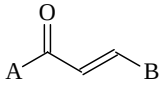
2.2. In vitro antileishmanial activity

Table 1 displays IC_{50} values of natural and synthetic chalcones against promastigotes and intracellular amastigotes of *L. amazonensis*. Pentostan® and Pentamidine® were used as reference drugs for antipromastigote and antiamastigote activities, respectively. The toxicity against murine macrophages (low IC_{50} values), as measured by the release of the cytoplasmic enzyme lactate dehydrogenase, was also considered to be indicative of unselective drug toxicity. The results showed that despite its high activity against intracellular amastigotes (IC_{50} = 4 μM), the natural chalcone DMC displayed a slight toxicity against the host mammalian cell (IC_{50} = 67.4 μM). These effects were similar to those produced by Pentostan®, in agreement with what was previously found.[13] The first compound synthesized in this work was **2**, and it presented similar activity against intracellular parasites compared to DMC (**1**). This result indicated that methoxy moiety at ring A in **2** is bioequivalent to hydroxy group in DMC and for easiness of synthesis, compound **2** served as a template for further modifications. Xanthoxyline 1 and its derivative 2-bromoxanthoxyline **16** (Scheme 1) were both devoid of antileishmanial activity (not shown), indicating that their reported antibacterial[16] and antifungal[17] properties were not extensive to the protozoan leishmania and that the phenyl vinyl structure was important for the antileishmanial activity. Almost all substitutions at the *para* position of B ring reduced both the antileishmanial and the antimacrophage toxicity (compounds **3**, **5**, **6**, **7**, and **11**). It can be observed that not only electron-acceptor but also electron-donor substituents at position 4 reduced the antiamastigote activity relating to DMC, while electron-acceptor substituents, such as the nitro group at position 3 (**10**) and chlorine atom at position 2 (**8**), exhibited, practically, the same activity. This is



Scheme 1. Reagents: (a) CHO-Ph-X, NaOH/EtOH; (b) 2-naphthaldehyde, NaOH/EtOH; (c) 2-furaldehyde, NaOH/EtOH; (d) 3,4-(methylenedioxy)benzaldehyde; (e) Br_2/ AcOH. *New compounds.

Table 1. In vitro antileishmanial activity



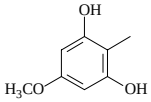
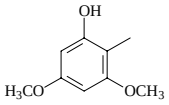
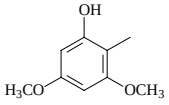
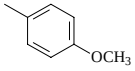
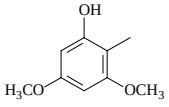
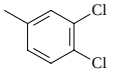
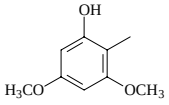
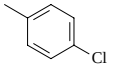
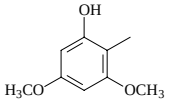
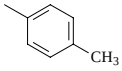
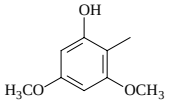
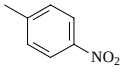
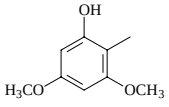
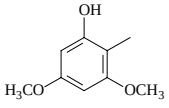
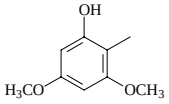
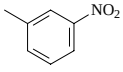
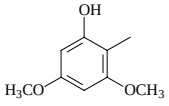
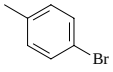
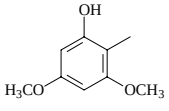
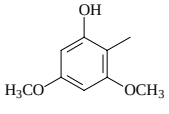
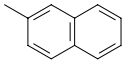
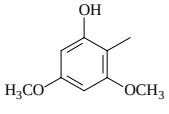
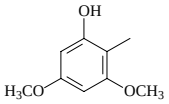
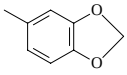
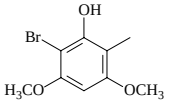
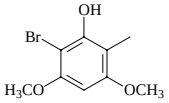
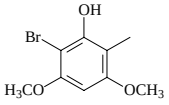
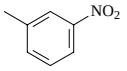
Chalcone	A	B	IC_{50} (μM)		
			Promastigotes	Amastigotes	Macrophages
DMC			25.3 ± 0.5	4.0 ± 0.4	67.3 ± 0.8
2			21.4 ± 0.2	8.4 ± 0.3	65.6 ± 1.0
3			72.6 ± 0.6	27.9 ± 0.1	>100
4			11.9 ± 0.8	3.8 ± 0.2	8.0 ± 0.0
5			>100	>100	>100
6			>100	>100	>100
7			>100	>100	>100
8			5.1 ± 0.3	3.6 ± 0.3	54.2 ± 1.5
9			0.8 ± 0.5	28.5 ± 0.3	7.6 ± 0.8
10			0.7 ± 0.1	15.8 ± 0.4	>100
11			59.8 ± 0.9	>100	>100
12			0.8 ± 0.0	4.3 ± 0.8	>100
13			38.0 ± 0.3	3.4 ± 0.5	16.5 ± 2.3
14			5.0 ± 0.9	3.4 ± 0.4	64.0 ± 0.0

Table 1 (*continued*)

Chalcone	A	B	IC_{50} (μM)		
			Promastigotes	Amastigotes	Macrophages
15			0.4 ± 0.1	3.6 ± 0.2	18.3 ± 0.0
17			0.9 ± 0.1	14.2 ± 0.1	9.5 ± 0.1
18			46.7 ± 0.1	4.1 ± 0.7	58.0 ± 1.0
19			0.5 ± 0.4	6.3 ± 0.5	>100
Pentostan			nd	4.4 ± 0.2	72.0 ± 1.0
Pentamidine			6.0 ± 0.5	nd	nd

Arithmetic means ± SD ($n = 3$).
nd = not determined.

consistent with the presence of a steric effect at position 4. The fact that the fluorine atom at position 4 did not reduce the activity may be attributed to the small size of this atom. The lack of reduction in the activity exhibited by compounds **13** and **15** that possess a carbon and an oxygen atom, respectively, at position 4, can be attributed to two factors: (i) both atoms are smaller than the chloride atom, which is the smallest substituent that promotes a reduction in activity at position 4, (ii) the carbon and oxygen atoms are vertices of an aromatic and pentacyclic ring, respectively, and thus their bonding electrons are far from the place where the steric hindrance at position 4 is stronger.

It is interesting to note that while the NO_2 group at position 4 renders the molecule inactive, the same group at position 3 conserves the activity and (**10**, **19**) confers increased selectivity irrespective of the presence of a bromine atom in the A ring. This finding indicates that chalcones with a NO_2 substituent at the *meta* position of the B ring should be investigated for the development of highly selective antileishmanial compounds.

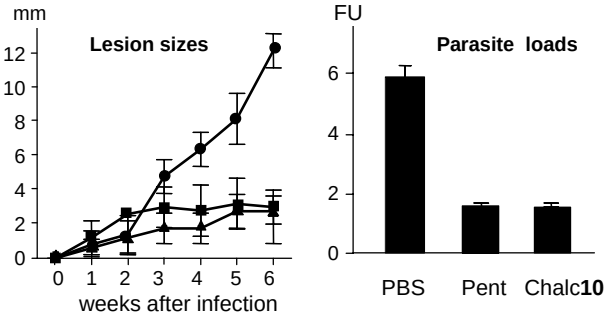


Figure 1. Effectiveness of chalcone 10 in the treatment of murine cutaneous leishmaniasis. BALB/c mice were infected with *Leishmania amazonensis*-GFP in the ear. From day 7 of infection they were treated twice a week with chalcone **10** (▲, 2 μg/dose), Pentostan (■, 200 μg/dose) or 10 μL of vehicle alone (●, PBS + 1% DMSO) for 4 weeks. The lesion sizes were measured at the indicated times and expressed in millimeters (mm). The parasite loads in the lesion lysates were measured on day 45 of infection and expressed as specific fluorescence units (FU). Mean ± SD ($n = 5$).

2.3. In vivo antileishmanial activity

One of the most potent analogues, chalcone **10**, was tested in mice infected with *L. amazonensis*. We found that intralesional treatment with nitrosylated chalcone **10** effectively controlled the growth of the lesions (Fig. 1). The potency of **10** was much superior to that of Pentostan® reference drug, considering that the former in a dose 100 times smaller than that of the latter produced the same protective effect. The reduction in lesion size was not due to an antiinflammatory effect, since the number of parasites was also significantly reduced (right panel). Insertion of bromine in ring A (**19**) enhances the antileishmanial activity of compound **10** (Table 1), probably by promoting increased permeation of the molecule through the macrophage membrane. Experiments are underway to evaluate whether that chalcone is more effective than **10** in treating cutaneous leishmaniasis.

3. Conclusions

Altogether, those findings point to a significant increase in the antileishmanial activity of the natural chalcone DMC and **2** due to substitutions in their B ring with fluorine or a nitro group at *para* and *meta* positions,

respectively. The resulting compounds **10**, **12**, and **19** are non-toxic to the host cells and retain high antiparasite activity, suggesting that they act on specific and critical parasite targets. We have supporting evidence that these three chalcones do not interfere with parasite fumarate reductase activity even at supraoptimal concentrations,[19] which indicates that their mechanism of action for *Leishmania* is different from that for other chalcones such as licochalcone A.[12] The potent selective activity and simple synthesis of these chalcones suggest that they are potential candidates for the development of new antileishmanial drugs. This is supported by the finding that local injections with small doses of compound **10** were more effective than Pentostan® reference drug in treating murine cutaneous leishmaniasis.

4. Experimental

4.1. Synthesis

The chalcones were synthesized as shown in Scheme 1. All the starting materials were commercially obtained (Merck), except for xanthoxyline (**1**) and 2-hydroxy-3-bromo-4,6-dimethoxyacetophenone (**16**), which were prepared as previously described.[18] The substituted chalcone derivatives **2**–**15** were prepared by stirring a solution of xanthoxyline (1) (0.18 g; 0.92 mMol), EtOH (15 mL), NaOH (0.1 g; 2.5 mMol, with a minimum of H_2O), and an appropriate aldehyde (0.95 mMol) at room temperature for 1–23 h. The substituted chalcone derivatives **17**–**19** were prepared by stirring a solution of compound **16** (0.2 g; 0.73 mMol), EtOH (15 mL), NaOH (0.1 g; 2.5 mMol, with a minimum of H_2O), and an appropriate aldehyde (0.95 mMol) at room temperature for 2–3 h. All the crude products were isolated by acidification of the cool diluted solution and recrystallized from ethyl ether and hexane (chalcones **4**, **7**, **8**, **9**, **10**, **11**, **12**, **13**, **17**, and **19** and **14** are new). Chalcone **2** was previously described by Kimura;[20] chalcones **5**, **15**, **16**, and **18** have been described by Cechinel and co-workers,[18] whereas chalcones **03** and **06** were described by Morio and co-workers.[21]

4.2. Physico-chemical data of synthesized compounds

The purified chalcones were obtained in yields of 16–89%. Their structures were identified using infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), and elementary analyses. Melting points were determined with a Microquimica MG APF-301 apparatus and are uncorrected. IR spectra were recorded with a FT Perkin Elmer 16 PC spectrometer on KBr disks. NMR (^{1}H and ^{13}C NMR) spectra were recorded on a Brucker Ac-200 F (200 MHz) with tetramethylsilane as an internal standard. Elementary analyses were obtained on a Perkin Elmer 2400. Percentages of C and H were in agreement with the product formula (within ±0.4% of theoretical values). The purity of the synthesized substances was analyzed by thin-layer chromatography (TLC) using Merck silica pre-coated aluminum plates 200 μm in thickness with several solvent systems of different polarities. Compounds were visualized with ultraviolet light (254 nm) and using ferric chloride solution followed by heat as developing agent and purified by recrystallization from ethyl ether and hexane. ^{1}H NMR spectra revealed that all the chalcones, except for compounds **3**, **7**, and **15**, were geometrically pure and configured E ($J_{H\alpha-H\beta}$ = 15–16 Hz).[18]

4.2.1. Compound 2: 2′-hydroxy-4′,6′-dimethoxychalcone. IR (KBr) 1628 (C=O), 1586 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 14.34 (s, 1H, OH), 7.87 (d, 1H J = 15.34 Hz, Hβ), 7.76 (d, 1H, J = 15.34 Hz, Hα), 7.50 (d, 2H, J = 8.06 Hz, H2, H6), 7.20 (d, 2H, J = 8.06 Hz, H3, H5), 6.10 (d, 1H, J = 2.36 Hz, H3′), 5.96 (d, 1H, J = 2.36 Hz, H5′), 3.91 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193.40 (C=O), 169.03 (C2′), 166.82 (C4′), 163.18 (C6′), 143.23 (Cβ), 141.22 (C4), 133.50 (C1′), 130.30 (C2,C6), 129.08 (C3,C5), 127.17 (C1), 107.04 (Cα), 94.48 (C3′), 91.94 (C5′), 56.53 (OCH_3), 56.27 (OCH_3). Anal. Calcd for $C_{17}H_{16}O_4$: C, 71.82; H, 5.67; found: C, 71.57; H, 5.68. Yield = 52%; mp = 85–86 °C.

4.2.2. Compound 3: 4-methoxy-2′-hydroxy-4′,6′-dimethoxychalcone. IR (KBr) 1622 (C=O), 1580 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 14.41 (s, 1H, OH), 7.80 (s, 2H, H2, H3), 7.57 (d, 1H J = 8.65 Hz, Hβ), 6.93 (d, 1H, J = 8.65 Hz, Hα), 6.11 (d, 1H, J = 2.3 Hz, H3′), 5.96 (d, 1H, J = 2.3 Hz, H5′), 3.92 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 192.55 (C=O), 168.33 (C2′), 165.98 (C4′), 162.41 (C6′), 161.32 (C4), 142.44 (Cβ), 130.07 (C1′,C2,C6), 128.27 (C1), 125.07 (Cα), 114.31 (C3,C5), 93.76 (C3′), 91.18 (C5′), 55.79 (OCH_3), 55.53 (OCH_3), 55.35 (OCH_3). Anal. Calcd for $C_{18}H_{18}O_5$: C, 68.78; H, 5.77; found: C, 68.53; H, 5.77. Yield = 32%; mp = 109–110 °C.

4.2.3. Compound 4: 3,4-dichloro-2′-hydroxy-4′,6′-dimethoxychalcone. Yellow solid; mp = 120–123 °C; UV λ_{max} 343 (3.80); IR (KBr) 1622 (C=O), 1586 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 14.14 (s, 1H, OH), 7.85 (d, 1H, J = 15.60 Hz, Hβ), 7.62 (d, 1H, J = 15.60 Hz, Hα), 7.43–7.51 (m, 3H, H2, H3, H6), 6.14 (d, 1H, J = 2.3 Hz, H3′), 5.97 (d, 1H, J = 2.3 Hz, H5′), 3.92 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 191.96 (C=O), 168.47 (C4′), 166.53 (C6′), 162.45 (C2′), 139.23 (Cβ), 135.19 (C1), 133.75 (C3), 133.12 (C4), 132.04 (C5), 130.83(C2), 129.67 (Cα), 106.22 (C1′), 93.81 (C3′), 91.37 (C5′), 55.94 (OCH_3), 55.64 (OCH_3). Anal. Calcd for $C_{17}H_{14}Cl_2O_4$: C, 57.81; H, 4.00; found: C, 57.62; H, 4.09. Yield = 47%.

4.2.4. Compound 5: 4-chloro-2′-hydroxy-4′,6′-dimethoxychalcone. IR (KBr) 1630 (C=O), 1566 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 14.23 (s, 1H, OH), 7.87 (d, 1H J = 15.52 Hz, Hβ), 7.71 (d, 1H, J = 15.52 Hz, Hα), 7.53 (d, 2H, J = 8.49 Hz, H3, H5), 7.37 (d, 2H, J = 8.49 Hz, H2, H6), 6.11 (d, 1H, J = 2.26 Hz, H3′), 5.96 (d, 1H, J = 2.26 Hz, H5′), 3.91 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 192.28 (C=O), 168.40 (C2′), 166.31 (C4′), 162.42 (C6′),

140.41 (Cβ), 129.41 (C3, C5), 129.08 (C2, C6), 106 (Cα), 93.76 (C3′), 91.26 (C5′), 55.83 (OCH_3), 55.57 (OCH_3). Anal. Calcd for $C_{17}H_{15}ClO_4$: C, 64.06; H, 4.74; found: C, 64.26; H, 4.69. Yield = 69%; mp = 173–175 °C.

4.2.5. Compound 6: 4-methyl-2′-hydroxy-4′,6′-dimethoxychalcone. IR (KBr) 1622 (C=O), 1586 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.34 (s, 1H, OH), 7.87 (d, 1H J = 15.34 Hz, Hβ), 7.76 (d, 1H, J = 15.34 Hz, Hα), 7.5 (d, 2H, J = 8.06 Hz, H6), 7.2 (d, 2H, J = 8.06 Hz, H3, H5), 6.1 (d, 1H, J = 2.36 Hz, H3′), 5.96 (d, 1H, J = 2.36 Hz, H5′), 3.91 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193.4 (C=O), 169.03 (C2′), 166.82 (C4′), 163.18 (C6′), 143.23 (Cβ), 141.22 (C4), 133.50 (C1′), 130.30 (C2, C6), 129.08 (C3,C5), 127.17 (C1), 107.04 (Cα), 94.48 (C3′), 91.94 (C5′), 56.53 (OCH_3), 56.27 (OCH_3). Anal. Calcd for $C_{18}H_{18}O_4$: C, 72,47; H, 6.08; found: C, 72.31; H, 6.14. Yield = 70%; mp = 125–126 °C.

4.2.6. Compound 7: 4-nitro-2′-hydroxy-4′,6′-dimethoxychalcone. Orange solid; mp = 295–296 °C; UV λ_{max} 379 (3.80) R (KBr) 1664 (C=O), 1584 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.26 (d, 1H, J = 8.85 Hz, Hβ), 7.98 (d, 1H, J = 8.85 Hz, Hα), 7.25 (s, 2H, H3, H5), 6.75 (s, 2H, H2, H6), 6.42 (s, 1H, H3′), 6.17 (s, 1H, H5′), 3.97 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 192.21 (C=O), 172 (COOH), 169.51 (C4′), 163.08 (C6′), 160.05 (C2′), 149.55 (C4), 147.22 (C1), 139.05 (Cβ), 131.24 (C2, C6), 123.82 (Cα), 122.44 (C3, C5), 107.21 (C1′), 94.41 (C3′), 89,52 (C5′), 56.62 (OCH_3), 56.54 (OCH_3). Anal. Calcd for $C_{17}H_{15}NO_6$: C, 62.00; H, 4.59; N, 4.25; found: C, 61.98; H, 4.56; N, 4.23. Yield = 16%.

4.2.7. Compound 8: 2-chloro-2′-hydroxy-4′,6′-dimethoxychalcone. Yellow solid; mp = 136-137 °C; UV λ_{max} 336 (3.68); IR (KBr) 1630 (C=O), 1556 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.25 (s, 1H, OH), 8.19 (d, 1H, J = 15.67 Hz, Hβ), 7.91 (d, 1H, J = 15.67 Hz, Hα), 7.30–7.75 (m, 4H, H3, H4, H5, H6), 6.15 (d, 1H, J = 2.36 Hz, H3′), 6.0 (d, 1H, J = 2.36 Hz, H5′), 3.88 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193.01 (C=O), 169.14 (C4′), 167.08 (C6′), 163.17 (C2′), 138.55 (Cβ), 136.05 (C1), 134.53 (C2), 131.35 (C4), 130.94 (C3), 130.73 (C6), 128.49 (C5), 127.65(Cα), 106 (C 1′), 94.49 (C3′), 91.99 (C5′), 56.56 (OCH_3), 56.30 (OCH_3). Anal. Calcd for $C_{17}H_{15}ClO_4$: C, 64.06; H, 4.74; found: C, 63.87; H, 4.80. Yield = 89%.

4.2.8. Compound 9: 2-[-3-(2′-Hydroxy-4′,6′-dimethoxyphenyl)-3-oxoprop- 1-enyl]benzoic acid. Yellow solid; mp = 160–161 °C; UV λ_{max} 291 (3.82) IR (KBr) 1640 (C=O), 1560 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 13.99 (s, 1H, OH), 8.44 (d, 1H, J = 15.49 Hz, Hβ), 7.81 (d, 1H, J = 15.49 Hz, Hα), 7.96 (d, 1H, J = 7.87 Hz, H3), 7.47-7.81 (m, 3H, H4, H5, H6), 6.12 (d, 1H, J = 2.39 Hz, H3′), 6.09 (d, 1H, J = 2.38 Hz, H5′), 3.93 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 199.66 (C=O), 167.15 (C6′, C4′), 162.43 (C2′), 149.67 (Cβ), 133.94 (C1), 128.93 (C3, C5), 125.50 (C2), 125.04 (C4, C6), 122.43 (Cα), 106 (C1′), 93.57 (C3′), 90.60 (C5′), 55.44 (OCH_3), 55.30 (OCH_3). Anal. Calcd for $C_{18}H_{16}O_6$: C, 65.85; H, 4.91; found: C, 65.64; H, 4.84. Yield = 44%.

4.2.9. Compound 10: 3-nitro-2′-hydroxy-4′,6′-dimethoxychalcone. Orange solid; mp = 171–172 °C; UV λ_{max} 335 (3.74); IR (KBr) 1640 (C=O), 1580 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.09 (s, 1H, OH), 8.46 (s, 1H, H6), 8.22 (d, 1H, J = 7.75 Hz, H4), 7.98 (d, 1H, J = 15.68 Hz, Hβ), 7.84-7.88 (m, 2H, H3, H4), 7.74 (d, 1H, J = 15.68 Hz, Hα), 6.12 (s, 1H, H3′), 5.98 (s, 1H, H5′), 3.94 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 191.78 (C=O), 168.46 (C4′), 166.45 (C6′), 162.45 (C2′), 148.71 (C3), 138.68 (Cβ), 137.43 (C1), 134.01 (C6), 130.51 (C5), 129.78 (Cα), 123.99 (C4), 122.08 (C2), 106.20 (C1′), 93.84 (C3′), 91.37 (C5′), 55.82 (OCH_3), 55.56 (OCH_3). Anal. Calcd for $C_{17}H_{15}NO_6$: C, 62.00; H, 4.59; N, 4.25; found: C, 62.08; H, 4.55; N, 4.26. Yield = 57%.

4.2.10. Compound 11: 4-bromo-2′-hydroxy-4′,6′-dimethoxychalcone. Orange solid; mp = 150–151 °C; UV λ_{max} 338 (4.08); IR (KBr) 1632 (C=O), 1588 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.21 (s, 1H, OH), 7.87 (d, 1H, J = 15.51 Hz, Hβ), 7.68 (d, 1H, J = 15.51 Hz, Hα), 7.47–7.51 (m, 4H, H2, H3, H5, H6), 6.11 (s, 1H, H3′), 5.96 (s, 1H, H5′), 3.91 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193 (C=O), 169.13 (C4′), 167.07 (C6′), 163.15 (C2′), 141.47 (Cβ), 135.21 (C1), 132.79 (C3, C5), 130.36 (C2, C6), 128.34 (Cα), 124.88 (C4), 106.99 (C1′), 94.1 (C3′), 92.03 (C5′), 56.57 (OCH_3), 56.30 (OCH_3). Anal. Calcd for $C_{17}H_{15}BrO_4$: C, 56.22; H, 4.16; found: C, 56.04; H, 4.21. Yield = 38%.

4.2.11. Compound 12: 4-fluoro-2′-hydroxy-4′,6′-dimethoxychalcone. Yellow solid; mp = 140–141 °C; UV λ_{max} 339 (4.00); IR (KBr) 1632 (C=O), 1572 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 13.72 (s, 1H, OH), 7.01–7.42 (m, 6H, Hβ, Hα, H2, H3, H5, H6), 6.08 (d, 1H, J = 2.22 Hz, H3′), 6.91 (d, 1H, J = 2.22 Hz, H5′), 3.83 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193.05 (C=O), 169.08 (C4′), 166.93 (C6′), 163.12 (C2′), 141.69 (Cβ), 132.48 (C1), 130.73(C2, C6), 128.12 (Cα), 116.43 (C3, C5), 106.91 (C1′), 94.45 (C3′), 91.94 (C5′), 56.52 (OCH_3), 56.26 (OCH_3). Anal. Calcd for $C_{17}H_{15}FO_4$: C, 67.54; H, 5.00; found: C, 67.50; H, 5.04. Yield = 87%.

4.2.12. Compound 13: 1-(2′-Hydroxy-4′, 6′-dimethoxyphenyl)-3-(2-naphthyl)prop-2-en-1-one. Yellow solid, mp = 110–112 °C; UV λ_{max} 336 (4.05); IR (KBr) 1638 (C=O), 1586 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.35 (s, 1H, OH), 7.47–7.99 (m, 9H, Hβ, Hα, H2, H4, H5, H6, H7, H9, H10), 6.13 (d, 1H, J = 2.26 Hz, H3′), 5.99 (d, 1H, J = 2.26 Hz, H5′), 3.95 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 193.25 (C=O), 169.14 (C4′), 166.93 (C6′), 163.20 (C2′), 143.17 (Cα), 134.86 (C2), 134.11 (C9), 133.77 (C10), 129.29 (C4, C8), 128.44 (C1, C5), 127.83 (C7), 127.35 (C6), 124.41 (C3, Cα), 107.07 (C1′), 94.50 (C3′), 91.99 (C5′), 56.30, 56.60 (OCH_3). Anal. Calcd for $C_{21}H_{18}O_4$: C, 75.43; H, 5.43; found: C, 75.52; H, 5.47. Yield = 30%.

4.2.13. Compound 14: 3-(2-furyl)-1-(2′-hydroxy-4′,6′-dimethoxyphenyl)prop-2-en-1-one. Yellow solid; mp = 92–94 °C; UV λ_{max} 363 (3.42); IR (KBr) 1626 (C=O), 1586 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 7.79 (d, 1H, J = 15.37 Hz, Hβ), 7.57 (d, 1H, J = 15.37 Hz, Hα), 7.51 (s, 1H, H2), 6.67 (d, 1H, J = 3.31 Hz, H4), 6.50 (t, 1H, J = 1.68 Hz, H3), 3.91 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 192.74 (C=O), 169.09 (C4′), 166.85 (C6′), 163.21 (C2′), 152.93 (C2), 145.32 (Cβ, C5), 129.63 (Cα), 116.11 (C4), 113.20 (C3), 106.99 (C1′), 94.91 (C3′), 91.88 (C5′), 56.45 (OCH_3), 56.24 (OCH_3). Anal. Calcd for $C_{15}H_{14}O_5$: C, 65.69; H, 5.1; found: C, 65.76; H, 5.18. Yield = 52%.

4.2.14. Compound 15: 3,4-methylenedioxy-2′-hydroxy-4′,6′-dimethoxychalcone. IR (KBr) 1625 (C=O), 1592 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.36 (s, 1H, OH), 7.73 (s, 3H, H2, H5, H6), 7.09 (d, 1H J = 8.1 Hz, Hβ), 6.83 (d, 1H, J = 8.1 Hz, Hα), 6.10 (d, 1H, J = 2.26 Hz, H3′), 6.07 (s, 2H, O-CH_2-O), 5.96 (d, 1H, J = 2.26 Hz, H5′), 3.91 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3) ^{13}C NMR ($CDCl_3$) δ 193 (C=O), 169.07 (C2′), 166.78 (C4′), 163.12 (C6′), 143.11 (Cβ), 107.28 (Cα), 102.21 (CH_2), 94.47 (C3′), 91.92 (C5′), 56.55 (OCH_3), 56.26 (OCH_3). Anal. Calcd for $C_{18}H_{16}O_6$: C, 65.85; H, 4.91; found: C, 65.82; H, 4.89. Yield = 32%; mp = 153–154 °C.

4.2.15. Compound 17: 2-chloro-3′-bromo-2′-hydroxy-4′,6′-dimethoxychalcone. IR (KBr) 1624 (C=O), 1556 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.67 (s, 1H, OH), 8.17 (d, 1H J = 15.58 Hz, Hβ), 7.81 (d, 1H, J = 15.58 Hz, Hα), 7.28–7.70 (m, 4H, H3, H4, H5, H6), 6.05 (s, 1H, H5′), 3.98 (s, 6H, OCH_3) ^{13}C NMR ($CDCl_3$) δ 193.25 (C=O), 163.92 (C2′), 163 (C4′), 162.9 (C6′), 139.49 (Cβ), 106 (Cα), 92.80 (C3′), 87.93 (C5′), 57.08 (OCH_3), 56.85 (OCH_3). Anal. Calcd for $C_{17}H_{14}$ $BrClO_4$: C, 51.35; H, 3.55; found: C, 51.45; H, 3.58. Yield = 47%; mp = 210–212 °C.

4.2.16. Compound 18: 3′-bromo-2′-hydroxy-4′,6′-dimethoxychalcone. IR (KBr) 1628 (C=O), 1558 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.83 (s, 1H, OH), 7.85 (d, 1H J = 15.74 Hz, Hβ), 7.76 (d, 1H, J = 15.74 Hz, Hα), 7.38–7.60 (m, 5H, H2, H3, H4, H5, H6), 6.02 (s, 1H, H5′), 3.98 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3) ^{13}C NMR ($CDCl_3$) δ 193.43 (C=O), 163.84 (C2′), 162.94 (C4′), 162.63 (C6′), 143.97 (Cβ), 135.94 (C1′), 131.0 (C1), 107.53 (Cα), 92.58 (C3′), 87.81 (C5′), 57 (OCH_3), 56.76 (OCH_3). Anal. Calcd for $C_{17}H_{15}$ BrO_4: C, 56.22; H, 4.16; found: C, 56.05; H, 4.14. Yield = 40%; mp = 178–180 °C.

4.2.17. Compound 19: 3-nitro-3′-bromo-2′-hydroxy-4′,6′-dimethoxychalcone. Orange solid; mp = 264 °C; UV λ_{max} 288 (3.25)IR (KBr) 1632 (C=O), 1558 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$) δ 14.55 (s, 1H, OH), 8.48-7.60 (m, 6H, Hβ, Hα, H2, H3, H4, H6), 6.10 (s, 1H, H5′), 4.03 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ 199.99 (C=O), 163.14 (C4′), 163.09 (C6′), 162.39 (C2′), 148.30 (C3), 140.20 (Cβ), 134.85 (C1), 131.00 (C6), 130.40 (C5), 124.81 (Cα), 107.02 (C1′), 92.12 (C3′), 87.16 (C5′), 56.76 (OCH_3), 56.18 (OCH_3). Anal. Calcd for $C_{17}H_{14}BrNO$: C, 50.02; H, 3.46; N, 3.43; found: C, 50.10; H, 3.44; N, 3.40. Yield = 71%.

4.3. Biological assays

4.3.1. In vitro antileishmanial activity. The antileishmanial activities of the test compounds were determined in vitro against both the insect promastigote forms and the intramacrophage amastigote forms of *L. amazonensis* (Josefa strain) transfected with the green fluorescence protein (GFP).[22] For antipromastigote activity, fluorescent promastigotes were plated in triplicate at 10^5 parasites/ well with varying concentrations of test compounds (0, 1, 10, and 100 μM) in a final volume of 200 μL of medium containing 5% serum and 1% hybri-max dimethyl sulfoxide (DMSO, Sigma). After 72 h at 27 °C, the fluorescence intensity of the cultures was measured using a plate-reader fluorometer (Fluoroskan) set at 435 nm excitation/538 nm emission. For antiamastigote activity, 10^6 mouse peritoneal macrophages were infected with 5×10^6 fluorescent promastigotes for 4 h at 37 °C, washed, and cultured for a further 72 h in 500 μL with the test compounds in 1% DMSO. Controls were 1% DMSO alone. The fluorescence intensity of the cell monolayers was measured for each drug concentration as for promastigotes. Maximum and minimum inhibitory activities were fluorescence units of uninfected macrophages and infected cells without drugs, respectively. The IC_{50} (the concentration required to induce half of the maximum activity) values were calculated by linear regression of the mean fluorescence intensities. Two reference drugs were used: Pentostan® (sodium stibogluconate, GlaxoSmithKline: antiamastigote activity) and Pentamidine® (pentamidine isothionate, Aventis: antipromastigote activity), since Pentostan® is not active against promastigotes.

4.3.2. Cytotoxicity against macrophages. For cytotoxicity against mammalian cells, adherent mouse peritoneal macrophages were cultured for 48 h at 37 °C with varying concentrations of the test compounds. The release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium was measured using an assay kit (Doles Reagentes, Brazil). Maximum and minimum release values were cells cultured with 2% Triton X-100 or 1% DMSO, respectively. The IC_{50} values were calculated by linear regression analysis.

4.3.3. In vivo antileishmanial activity. BALB/c mice were infected with *L. amazonensis*-GFP in the ear. From day 7 of infection they were locally injected twice a week during 4 weeks with compound **10** (2 μg/dose), Pentostan (200 μg/dose) or 10 μL vehicle alone (phosphate-buffered saline—PBS—plus 0.5% DMSO). The lesion sizes were measured at the indicated times with a dial caliper and expressed in millimeters. The parasite loads in the individual lesions were measured on day 45 of infection as described previously[23] and expressed as arbitrary fluorescence units after deduction of the background fluorescence intensity of the contralateral uninfected ears (1.6 ± 0.2 units).

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