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Effect of substituents in the A and B rings of chalcones on antiparasite activity

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Abstract

Chalcones are a group of natural products with many recognized biological activities, including antiparasitic activity. Although a lot of chalcones have been synthetized and assayed against parasites, the number of structural features known to be involved in this biological property is small. Thus, in the present study, 21 chalcones were synthesized to determine the effect of substituents in the A and B rings on the activity against *Leishmania braziliensis*, *Trypanosoma cruzi*, and *Plasmodium falciparum*. The compounds were active against *L. braziliensis* in a structure-dependent manner. Only one compound was very active against *T. cruzi*, but none of them had a significant antiplasmodial activity. The electron-donating substituents in ring B and the hydrogen bonds at C-2′ with carbonyl affect the antiparasitic activity.

KEYWORDS

antiplasmodial, B ring electron-donating substituents, C-2′ hydrogen bond, leishmanicidal, trypanocidal

1 | INTRODUCTION

Leishmaniasis, trypanosomiasis, and malaria are vector-borne parasitic diseases with the highest global impact. Besides being fatal, they seriously affect the quality of life of millions of people by causing deterioration of health and social stigma. These diseases have become one of the challenges of the World Health Organization (WHO) and their treatment is essential to achieve the goals of sustainable development in 2030.[1]

In the absence of a vaccine and the difficulties of implementing a vector control program, current antiparasitic control tools have focused on chemotherapeutic treatments. The need for new pharmacological alternatives for the control of parasitic diseases caused *by Leishmania* spp., *Trypanosoma cruzi*, and *Plasmodium falciparum* is associated with different factors, including the few drugs available, the reduced effectiveness due to microbial resistance, extensive treatments, and high toxicity. In the case of leishmaniasis treatment, pentavalent antimonial (sodium stibogluconate and meglumine antimoniate), miltefosine, amphotericin B, and pentamidine isethionate are approved drugs (Figure 1). All these drugs have associated toxicity problems with considerable adverse side effects and high costs, mainly due to hospital care[2]; all these drawbacks cause low adherence to treatment. For American trypanosomiasis (Chagas disease), only two drugs are available, nifurtimox and benznidazole, both with bioavailability and efficacy problems during the chronic phase of the disease, reduced tolerance, and moderate or severe adverse reactions.[3] In the case of malaria, quinolinic derivatives and artemisinin are recommended for treatment, and their activity is being affected by the emergence of resistant *Plasmodium* strains.[4] For these reasons, the continuous work for the discovery of biologically active molecules against these parasites is still essential.

In search of antiparasitic drugs, several natural products have been previously reported. Particularly, chalcone-type compounds

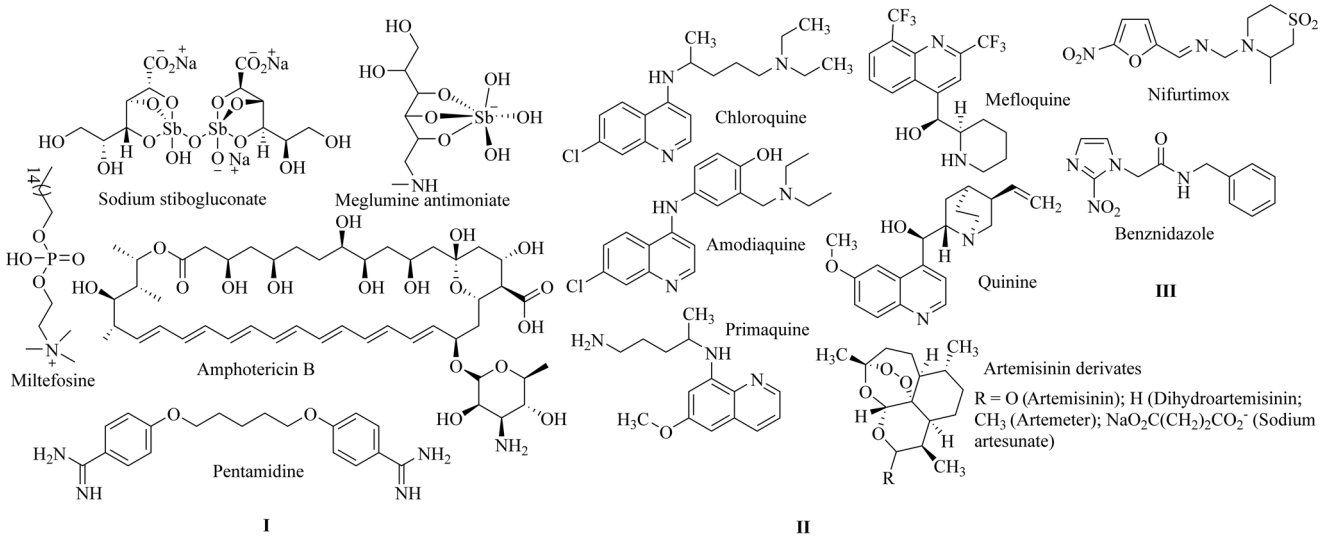


FIGURE 1 Chemical structures of the main drugs used as alternatives in the treatment of leishmaniasis (I), malaria (II), and Chagas disease (III)

(1,3-diaryl-2-propen-1-one) exhibited activity against *Leishmania* species,[5–9] *T. cruzi*,[9,10] and *P. falciparum*,[11,12] in addition to antioxidant, cytotoxic,[10,13] antiproliferative,[14,15] antitumoral,[16,17] antimicrobial,[18] antiulcer, and anti-inflammatory activities,[19] among others. Thus, a wide range of synthetic procedures had been carried out to establish structure–activity relationships in these compounds, but the structure requirements involved in these activities are still practically unknown. In this study, we report the in vitro activity of 21 chalcone derivatives, with H-bond formation and electron-donating substituents in the A and B rings, respectively, against *Leishmania braziliensis*, *T. cruzi*, and *P. falciparum*.

TABLE 1 The synthesis of chalcone derivatives (according to Scheme 1)

Compound type			Substituent (C atom)				
I, Y = H	II, Y = OH	III, Y = NH_2	2	3	4	5	6
1	8	15	H	H	H	H	H
2	9	16	H	OCH_3	OCH_3	OCH_3	H
3	10	17	H	OCH_3	H	OCH_3	H
4	11	18	H	H	NO_2	H	H
5	12	19	H	H	F	H	H
6	13	20	H	H	Cl	H	H
7	14	21	H	H	OCH_3	H	H

2 | RESULTS

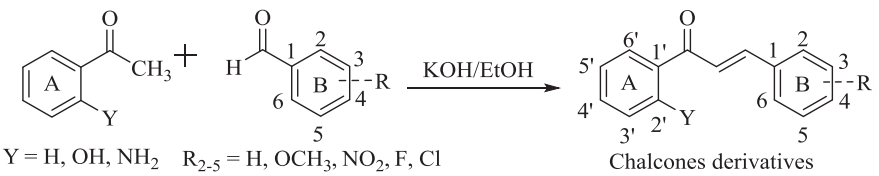
2.1 | Chemistry

Through a Claisen–Schmidt condensation reaction (Scheme 1), three types of chalcone derivatives were synthesized according to different substituents in C-2′ of ring A: H, OH, and NH_2. Also, substitutions with electroattractant groups (NO_2) and electron donors (OCH_3, F, and Cl) were placed in the B ring (Table 1).

All compounds were isolated by acidification of the reaction mixture, followed by dichloromethane extraction and chromatographic separation. The compounds were identified by infrared (IR), ^{1}H NMR (nuclear magnetic resonance), ^{13}C NMR, and high-resolution mass spectroscopy (HRMS), with relatively good yields between 50% and 96%. These compounds have already been described in the literature; however, there are no reports of biological activity against *L. braziliensis*, *T. cruzi*, and *P. falciparum*.

2.2 | Antiparasite activities and cytotoxicity

On the basis of a promising activity, with an EC_{50} value <20 μM (equivalent to 4 μg/ml), it was observed that the synthesized compounds were more active against *L. braziliensis* as compared with



SCHEME 1 The synthetic strategy for the preparation of chalcones derivatives

T. cruzi and *P. falciparum* (Table 1). In general, compounds of type I, Y–H, were more active against intracellular amastigotes of *L. braziliensis*, as 12 of the 21 synthesized compounds showed an EC_{50} value <20 μM. This concentration was lower with compounds **2**, **3**, **7**, **10**, **15**, **16**, and **20**, which exhibited an EC_{50} value <10 μM. Compounds **11**, **13**, and **21** showed a moderate activity against *L. braziliensis*, and the remaining compounds, **8**, **14**, and **19**, exhibited weak activity against the *Leishmania* parasite (Table 1). All compounds were more active than AMB.

Only compounds **8**, **10**, **13**, **16**, and **17** showed high activity against *T. cruzi*, and the most powerful was **17**, with $EC_{50} = 8.1 \pm 1.1$ μM, which was seven times more active than BNZ. A moderate activity against *T. cruzi* was observed in compounds **4**, **5**, **6**, **9**, **12**, **15**, **18**, **19**, **20**, and **21**, whose activity was higher than 20 μM, but lower than 50 μM. For compounds **3** and **7**, it was not possible to determine the exact EC_{50} values against *T. cruzi*, given its toxicity to U-937 host cells, whereas the remaining compounds, **1**, **2**, **11**, and **14**, exhibited a low activity against *T. cruzi* amastigotes (Table 2). However, in *P. falciparum*, all compounds displayed a wide range of activity, from marginal to a lack of activity, with EC_{50} values ranging from 59.2 ± 0.2 μM to 908.9 ± 53.0 μM.

Although all compounds were cytotoxic for U-937 cells, with LC_{50} values lower than 83 μM, when the activity and cytotoxicity were correlated, compound **17** was identified as the most promising compound, exhibiting an SI of 10.13 for *T. cruzi* and 3.6 for *L. braziliensis*, followed by compounds **3**, **10**, **15**, and **16**, with SI values of 3.0, 2.9, 2.8 and 2.6, respectively (Table 2).

3 | DISCUSSION

The search for drugs against neglected diseases is a persistent need for millions of people, due to the reduced number of drugs available, toxicological effects, and parasite resistance. Although natural products have been a good alternative as medicine sources, the drug development process is affected by the low availability of natural substances, as bioactive substances are usually found in low concentrations. For this reason, natural products are taken as templates, whose structures are modified and optimized by organic synthesis. Natural chalcones are relatively simple structures, with a very varied spectrum of activities, including antiparasitic, and can be easily synthesized with excellent yields.

In this study, we describe the synthesis of three types of chalcone derivatives, which have the substituents H, OH, or NH_2 in C-2′, whereas, in ring B, they have groups with different electron-donating and electron-withdrawing properties, such as OCH_3, NO_2, and halogens (Cl, F). In vitro effects of these substances on *L. braziliensis*, *T. cruzi*, and *P. falciparum* were evaluated.

The results found showed that the synthesized molecules have a high selectivity; thus, *P. falciparum* was insensitive to them, unlike what was detected in *T. cruzi*, which exhibited a good activity with some compounds. Greater sensitivity was observed on the amastigotes of *L. braziliensis*. Hence, the discussion of the effects of the molecules will focus on the results exhibited on *L. braziliensis* and *T. cruzi*.

The effects of the compounds were analyzed according to the structural parameters that guided their synthesis: the C-2′ substitutions of ring A and ring B. Usually the biological effect of the chalcones has been attributed to their ability to act as Michael acceptors of biological macromolecules, due to the α,β-unsaturated carbonyl system.[20,21] The results of this study indicate that this mechanism may be responsible for cytotoxicity, which was very high for all compounds, and besides, for the activity and selectivity against *L. braziliensis*. Thus, most of the compounds of the series I (Y = H, **1–7**) showed high leishmanicidal activity, with $EC_{50} < 20.0$ μM, and compounds of the series III of the amine (Y = NH_2, **8–14**) exhibited this activity to a lesser extent. Type II compounds, which have a hydroxyl group in C-2′ (Y = OH, **15–21**), exhibited a poor activity; this fact could be explained on the basis of the reactivity of the conjugated carbonyl system, as the formation of hydrogen bonds with the carbonyl decreases the reactivity and, therefore, the ability to form Michael adducts with biological molecules (Figure 2). The failure to establish an H-bonding in the compounds of the series I maintains the reactivity of carbonyl. As for the hydroxyl group, it forms a stronger hydrogen bond, negatively affecting the reactivity of the system and, therefore, its activity.

In this regard, Bello et al.[22] synthesized several polymethoxylated chalcones substituted in the A ring, whereas ring B possessed groups like ours in different positions. It was found that substitution in ring A, especially trimethoxylation in *ortho* and *meta* positions with respect to carbonyl positions of the unsaturated system, significantly improved activity against *L. braziliensis*, whereas ring B did not influence that activity. Although the authors make an analysis of molecular orbitals to rationalize this activity, it is likely that the inductive donor effect on carbonyl and, therefore, its reactivity as part of the Michael adduct is the actual cause.

Similarly, licochalcone A (3-dimethylallyl-4,4′-dihydroxy-6-methoxychalcone) that has hydroxyl groups in C-4 and C-4′ was active in animal models of *L. major* and *L. donovani*, and also exhibited antimalarial activity against *P. yoelii* in mice,[23] but in our case, all the evaluated compounds showed a low activity against *P. falciparum*, with EC_{50} values >50 μM. In that case, the hydrogen bridge is not involved, but there is possibly an electron-donating effect of the OH group on the conjugate system. However, the contribution of the B ring is another factor that also seems to be involved in the activity. Compounds **2**, **9**, and **16**, which have a C-4′–OCH_3 group, are more active, as this group acts as an electron donor system to the carbonyl system. Nevertheless, polymethoxylation at adjacent positions in that carbon atom, as in **7**, **14**, and **21**, does not improve activity, except in the first compound, in which the lack of ability to form hydrogen bonds seems to be essential, as it was mentioned already. The latter is in agreement with the results observed for the other compounds substituted in position C-4′–OH. Thus, for example, compounds **5**, **12**, and **19**, which have a powerful electron-attracting atom such as fluorine, do not show significant activity against amastigotes of *L. braziliensis*, as the Michel acceptor is destabilized. In the case of the

TABLE 2 In vitro cytotoxicity and antiparasite activity of chalcones

Compound	Cytotoxicity (LC_{50}: μM ± SEM)	Antiparasitic activity (EC_{50}: μM ± SEM)			SI[a]		
		L. braziliensis[b]	*P. falciparum*[c]	*T. cruzi*[d]	*L. braziliensis*	*T. cruzi*	*P. falciparum*
1	27.9 ± 0.5	**15.0 ± 1.4**[e]	768.9 ± 72.0	99.9 ± 39.0	1.9	0.3	0.03
2	18.3 ± 0.3	**9.6 ± 1.0**	127.1 ± 15.0	77.8 ± 6.4	1.9	0.2	0.14
3	19.4 ± 0.1	**7.4 ± 0.1**	485.6 ± 125.0	>11.2[f]	2.6	<1.7	0.04
4	22.5 ± 0.4	**18.6 ± 2.0**	724.3 ± 68.0	35.2 ± 3.5	1.2	0.6	0.03
5	23.0 ± 0.1	22.6 ± 3.1	477.3 ± 101.0	31.4 ± 3.9	1.0	0.7	0.05
6	21.5 ± 0.1	**17.4 ± 2.9**	908.9 ± 53.0	29.3 ± 4.1	1.2	0.7	0.02
7	22.3 ± 0.0	**9.7 ± 0.8**	310.4 ± 34.0	>12.6	2.3	<1.8	0.07
8	37.9 ± 2.7	56.2 ± 4.9	134.3 ± 23.2	24.5 ± 0.4	0.7	1.5	0.30
9	20.4 ± 1.0	**15.0 ± 0.1**	59.2 ± 0.2	45.5 ± 5.7	1.4	0.4	0.34
10	19.4 ± 0.4	7.0 ± 1.4	93.6 ± 3.5	19.7 ± 3.2	2.8	1.0	0.20
11	26.3 ± 1.9	33.8 ± 13.4	105.2 ± 4.0	85.7 ± 10.4	0.8	0.3	0.25
12	24.0 ± 0.8	**22.3 ± 9.0**	112.4 ± 4.5	47.1 ± 11.9	1.1	0.5	0.21
13	25.2 ± 1.2	29.5 ± 9.7	76.3 ± 5.4	24.0 ± 0.4	0.9	1.1	0.33
14	30.7 ± 1.6	>30.7	286.9 ± 38.2	71.2 ± 70.4	<1.0	0.4	0.11
15	27.3 ± 1.3	**9.4 ± 3.1**	135.8 ± 3.1	41.2 ± 5.8	2.9	0.8	0.07
16	17.6 ± 0.3	**5.7 ± 0.0**	256.4 ± 48.0	22.0 ± 2.6	3.1	0.5	0.14
17	82.3 ± 1.4	22.6 ± 0.2	590.6 ± 38.0	**8.1 ± 1.1**	3.6	10.2	0.14
18	23.1 ± 0.4	**15.7 ± 2.6**	69.4 ± 6.7	44.0 ± 3.4	1.5	0.6	0.33
19	28.6 ± 2.0	65.1 ± 31.9	119.5 ± 12.9	45.6 ± 7.5	0.4	0.6	0.24
20	24.1 ± 0.4	**8.2 ± 1.9**	73.1 ± 3.1	43.6 ± 8.2	2.9	0.6	0.33
21	25.7 ± 1.2	41.1 ± 16.7	220.5 ± 21.0	26.9 ± 4.7	0.6	1.0	0.12
AMB	41.6 ± 6.8	0.3 ± 0.1	NA	NA	138.7	NA	NA
CQ	300.8 ± 10.1	NA	0.6 ± 0.0	NA	NA	NA	501.3
BNZ	>768.5	NA	NA	56.5 ± 1.5	NA	>16.8	NA
DOX	0.6 ± 0.1	NA	NA	NA	NA	NA	NA

Note: The data show the average values ± standard deviation (SEM), in μM, of the cytotoxicity in U-937 cells and the antiparasite activity for each compound.

Abbreviations: AMB, amphotericin B for *Leishmania braziliensis*; BNZ, benznidazole; CQ, chloroquine; DOX, doxorubicin; EC_{50}, Half maximal effective concentration; LC_{50}, cytotoxicity expressed as LC_{50}, NA, not applicable; SI, selectivity index.

[a]SI = LC_{50} U-937/EC_{50}.

[b]*Leishmania braziliensis* amastigotes.

[c]*Plasmodium falciparum*.

[d]*Trypanosoma cruzi* amastigotes.

[e]In bold: Active compounds (EC_{50} < 20 μM; equivalent to 4 μg/ml).

[f]Exact concentration value was not determined, because the toxic concentration was higher than the active concentration.

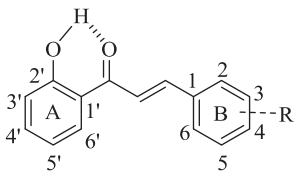


FIGURE 2 The effect of H-bonding on the reactivity of chalcones like Michael acceptors

NO_2 group, which has effects on *meta* positions, as in compounds **4**, **11**, and **18**, the effect of the hydrogen bridge remains predominant.

Several substances exhibited moderate activity against *T. cruzi*, and contrary to what was observed for *L. braziliensis*, the compounds that can form hydrogen bonds with carbonyl, such as **10** and **17** (C-2′–OH and C-2′–NH_2, respectively), had the best trypanocidal activity. The latter was especially interesting, as it showed an intense trypanocidal activity, with an EC_{50} value of 8.12 μM. Additionally, this

compound has relatively good activity against amastigotes of *L. braziliensis*, with an EC_{50} value of 22.6 μM. Similarly, 2′-hydroxy chalcones containing methoxy groups were very active against *T. brucei* and *T. cruzi*. Against this parasite, there are very few drugs available in the market, and it also exhibits high resistance, as evidenced in the reduced effect of BNZ, which was used as a control.

Therefore, it seems, that the mechanism of action of the chalcones is diverse because the formation of Michael adducts does not explain the effects observed in this study, as the most active compounds against *L. braziliensis* were **2–4**, **7**, **10**, **15**, **16**, and **20**. Moreover, this leishmanicidal effect is different from that observed in *T. cruzi*, as, in the latter, the active compounds were **6**, **8**, **10**, **13**, **16**, and **21**, and especially compound **17**. This leishmanicidal effect was depending on the type of substituent on C-2′, as well as those on the B ring. *P. falciparum* was virtually insensible to all synthesized substances.

Fifteen of the 21 compounds synthesized in this study presented EC_{50} values lower than that shown by benznidazole, with compound **17** standing out and being seven times more active against *T. cruzi*. On the contrary, none of the compounds assessed for *L. braziliensis* had an activity higher or similar to amphotericin B. Nevertheless, it is important to highlight that the purpose of this study is to identify compounds with a promising activity that allows their selection as hit compounds and then to continue the studies in animal models to validate these selected compounds as active pharmaceutical ingredients.

4 | CONCLUSION

Some chalcones with selective activity against *Leishmania* and *Trypanosoma* parasites were synthesized in this study. The role of several substituents in the A and B ring in this biological activity was demonstrated; these facts must be considered in the design of antiparasitic chalcones. Moreover, these compounds were obtained by a simple route and with high yields, which would eventually allow them in the amounts for confirmatory assays in animal models of leishmaniasis and trypanosomiasis.

5 | EXPERIMENTAL

5.1 | Chemistry

5.1.1 | General

All commercially available reagents and solvents were obtained from commercial suppliers and used without further purification. Benzaldehydes and acetophenones (98–99%) were purchased from Sigma Chemical Co. (St. Louis, MO). Thin-layer chromatography (TLC), with silica gel 60 F254-impregnated aluminum sheets (0.25 mm; Merck, Darmstadt, Germany), was used to check the progress or reactions, and compounds were detected under UV light (254, 360 nm) after spraying with vanillin (3% in H_2SO_4) and heating at 110°C. The chromatographic separations were performed using preparative column chromatography with silica gel 60 (200–300 mesh; Merck, Darmstadt, Germany). The melting points were determined using a Mel-Temp apparatus (Electrothermal, Staffordshire, UK). Fourier transform infrared (FTIR) spectra were obtained on a Bruker Alpha FTIR spectrometer (Bruker Optic GmbH, Ettlingen, Germany). ^1H, ^{13}C, and two-dimensional NMR spectra of the synthetic compounds were recorded on a Bruker Fourier 300 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany), operating at 300 MHz for ^1H and 75 MHz for ^{13}C NMR, using $CDCl_3$ (Sigma) as the solvent and tetramethylsilane as an internal standard. Chemical shifts (δ) are reported in ppm, and the coupling constants (J) are reported in Hz. High-resolution mass spectra were obtained using a UHR-QqTOF (Ultra-High-Resolution Qq-Time-Of-Flight) mass spectrometer (Impact II-Bruker), with an electrospray ionization source in the positive ion mode.

The InChI codes of the investigated compounds, together with some biological activity data, are provided as Supporting Information.

5.1.2 | General procedure for the preparation of chalcone derivatives

The synthesis was conducted according to experimental procedures described in the literature.[24–27] Briefly, a mixture of acetophenone derivative (1 mmol) and benzaldehyde derivative (1.05 mmol) was dissolved in ethanol (20 ml) and kept at room temperature, with magnetic stirring for 5 min. Then, a KOH/EtOH solution (1.1 mmol on 20 ml) was added dropwise, and stirring was continued at 40°C for 12 hr. Further, the reaction mixture was poured into ice water and acidified with 1 M HCl. Then, compounds were extracted with dichloromethane, and finally, compounds were purified using column chromatography with silica gel 60, eluting with hexane/ethyl acetate 2:1.

*1,3-Diphenyl-2-propen-1-one (**1**)*

Yield 70%, m.p.: 55–56°C; IR (KBr) υ/cm: 3,059.61, 1,966.81, 1,905.40, 1,822.23, 1,661.41, 1,604.31, 1,574.43, 1,495.76, 1,446.77, 1,339.86, 1,311.40, 1,286.70, 1,217.96, 1,178.51, 1,153.83, 1,073.00, and 977.14; ^1H NMR (300 MHz, $CDCl_3$) δ = 8.03 (d, J = 7.2 Hz, 2H), 7.82 (d, J = 15.7 Hz, 1H), 7.69–7.61 (m, 2H), 7.61–7.47 (m, 4H), and 7.46–7.40 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 190.89, 145.18, 138.50, 135.18, 133.12, 130.88, 129.28, 128.95, 128.82, 128.78, and 122.37. HRMS (ESI) m/z, calculated for $C_{15}H_{12}O$ $[M+H]^+$ 209.0960; found 209.0957.

*1-Phenyl-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (**2**)*

Yield 88%, m.p.: 141–142°C; IR (KBr) υ/cm: 3,065.75, 2,996.02, 1,720.91, 1,660.78, 1,577.87, 1,503.92, 1,461.00, 1,420.16, 1,320.10, 1,280.13, 1,249.37, 1,215.71, 1,185.83, 1,128.96, 1,018.96, 1,081.91, 984.49, and 832.40; ^1H NMR (300 MHz, $CDCl_3$) δ = 8.01 (d, J = 7.0 Hz, 2H), 7.72 (d, J = 15.6 Hz, 1H), 7.63–7.46 (m, 3H), 7.40 (d, J = 15.6 Hz, 1H), 6.86 (s, 2H), 3.92 (s, 6H), and 3.90 (s, 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ = 191.42, 153.76, 145.34, 140.68, 138.54, 131.99, 130.65, 128.91, 128.78, 121.73, 105.91, 61.29, and 56.49. HRMS (ESI) *m/z*, calculated for $C_{18}H_{18}O_4Na$ $[M+Na]^+$ 321.1097; found 321.1035.

*3-(3,5-Dimethoxyphenyl)-1-phenylprop-2-en-1-one (**3**)*

Yield 80%, m.p.: 80–82°C; IR (KBr) υ/cm: 2,947.80, 2,835.42, 1,665.84, 1,604.70, 1,460.84, 1,444.19, 1,428.42, 1,346.73, 1,323.47, 1,287.68, 1,249.68, 1,202.87, 1,153.53, 1,062.67, 1,017.55, 973.03, and 937.93; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.01 (d, *J* = 7.5 Hz, 2H), 7.72 (d, J 15.7 Hz, 1H), 7.63–7.42 (m, 4H), 6.78 (s, 2H), 6.53 (s, 1H), and 3.84 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 190.99, 161.41, 145.24, 138.50, 137.11, 133.19, 129.00, 122.97, 106.68, 103.10, and 55.84; HRMS (ESI) *m/z*, calculated for $C_{17}H_{16}O_3$ $[M+H]^+$ 269.1172; found 269.1170.

*3-(4-Nitrophenyl)-1-phenylprop-2-en-1-one (**4**)*

Yield 88%, m.p.: 138–140°C; IR (KBr) υ/cm: 2,930.33, 1,658.88, 1,608.64, 1,516.58, 1,446.66, 1,337.15, 1,219.91, 1,106.27, 1,016.02, and 983.59; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.29 (d, *J* = 8.8 Hz, 2H), 8.04 (d, *J* = 7.5 Hz, 2H), 7.80 (d, *J* = 8.5 Hz, 3H), 7.69–7.59 (m, 2H), and 7.54 (t, *J* = 7.3 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 190.02, 148.90, 141.88, 141.39, 137.86, 133.74, 129.29, 129.18, 128.95, 126.04, and 124.58. HRMS (ESI) *m/z*, calculated for $C_{15}H_{11}NO_3Na$ $[M+Na]^+$ 276.0631; found 276.0634.

*3-(4-Fluorophenyl)-1-phenylprop-2-en-1-one (**5**)*

Yield 75%, m.p.: 79–81°C; IR (KBr) υ/cm: 3,067.62, 3,037.36, 1,970.60, 1,907.19, 1,602.84, 1,574.84, 1,507.89, 1,446.07, 1,414.27, 1,340.71, 1,317.28, 1,214.03, 1,159.36, 1,100.53, 1,031.12, 1,013.42, 940.75, 836.71, 820.28, 777.05, 754.72, 716.28, and 688.41; ^{1}H NMR (300 MHz, CDCl3) δ = 8.02 (d, *J* = 7.2 Hz, 2H), 7.78 (d, *J* = 15.7 Hz, 1H), 7.70–7.56 (m, 3H), 7.55–7.41 (m, 3H), and 7.12 (t, *J* = 8.6 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 190.66, 164.37 (d, *J* = 251.8 Hz), 143.84, 138.42, 133.19, 131.44 (d, *J* = 3.2 Hz), 130.68 (d, *J* = 8.4 Hz), 128.80 (d, *J* = 13.4 Hz), 122.05 (d, *J* = 2.2 Hz), 116.45, and 116.09. HRMS (ESI) *m/z*, calculated for $C_{15}H_{11}FO$ $[M+H]^+$ 227.0866; found 227.0800.

*3-(4-Chlorophenyl)-1-phenylprop-2-en-1-one (**6**)*

Yield 80%, m.p.: 113–114°C; IR (KBr) υ/cm: 1,657.86, 1,602.92, 1,488.23, 1,446.13, 1,402.67, 1,333.54, 1,217.23, and 1,092.03; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.01 (d, *J* = 7.1 Hz, 2H), 7.74 (d, *J* = 15.7 Hz, 1H), 7.62–7.42 (m, 6H), and 7.37 (d, *J* = 8.5 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 190.43, 143.54, 138.24, 136.66, 133.60, 133.22, 129.87, 129.49, 128.94, 128.77, and 122.64; HRMS (ESI) *m/z*, calculated for $C_{15}H_{11}ClO$ $[M+H]^+$ 243.0571; found 243.0570.

*3-(4-Methoxyphenyl)-1-phenylprop-2-en-1-one (**7**)*

Yield 87%, m.p.: 80–82°C; IR (KBr) υ/cm: 3,013.96, 2,954.87, 2,942.51, 2,902.20, 2,841.91, 1,657.84, 1,600.28, 1,511.88, 1,445.81, 1,419.03, 1,339.03, 1,304.35, 1,263.99, 1,213.97, 1,170.42, 1,017.57, 984.70, and 824.98; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.01 (d, *J* = 7.1 Hz, 2H), 7.79 (d, *J* = 15.6 Hz, 1H), 7.67–7.46 (m, 5H), 7.42 (d, *J* = 15.6 Hz, 1H), and 6.94 (d, *J* = 8.7 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 191.01, 162.04, 145.10, 138.76, 132.93, 130.60, 128.92, 128.78, 127.96, 120.13, 114.77, and 55.77; HRMS (ESI) *m/z*, calculated for $C_{16}H_{14}O_2$ $[M+H]^+$ 239.1065; found 239.1065.

*1-(2-Hydroxyphenyl)-3-phenylprop-2-en-1-one (**8**)*

Yield 88%, m.p.: 89°C; IR (KBr) υ/cm 3,038.37, 1,689.91, 1,605.69, 1,577.23, 1,460.33, 1,405.64, 1,373.27, 1,342.48, 1,303.56, 1,227.87, 1,180.59, 985.49, and 957.38; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 11.83 (s, 1H), 7.00–6.86 (m, 2H), 6.73–6.62 (m, 3H), 6.54–6.38 (m, 4H), and 6.08–5.88 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 193.79, 163.63, 145.54, 136.48, 134.62, 130.99, 129.71, 129.10, 128.72, 120.13, 120.04, 118.92, and 118.69. HRMS (ESI) *m/z*, calculated for $C_{15}H_{12}O_2$ $[M+H]^+$ 225.0910; found 225.0908.

*1-(2-Hydroxyphenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (**9**)*

Yield 90%, m.p.: 158°C; IR (KBr) υ/cm: 2,942.71, 2,843.01, 1,685.83, 1,586.53, 1,461.40, 1,423.37, 1,391.45, 1,329.92, 1,206.04, 1,127.90, and 992.47; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.85 (s, 1H), 7.93 (d, *J* = 8.1 Hz, 1H), 7.85 (d, *J* = 15.4 Hz, 1H), 7.52 (dd, *J* = 15.7, 10.1 Hz, 2H), 7.03 (d, *J* = 8.3 Hz, 1H), 6.95 (t, *J* = 8.0 Hz, 1H), 6.88 (s, 2H), 3.92 (s, 6H), and 3.71 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 193.85, 163.90, 153.84, 145.99, 141.09, 136.72, 130.38, 129.94, 120.32, 119.56, 119.15, 118.98, 106.21, 61.37, and 56.58; HRMS (ESI) *m/z*, calculated for $C_{18}H_{18}O_5$ $[M+H]^+$ 315.1227, found 315.1228.

*3-(3,5-Dimethoxyphenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**10**)*

Yield 55%, m.p.: 102–103°C; IR (KBr) υ/cm: 3,010, 1,702.31, 1,604.87, 1,578.03, 1,476.68, 1,272.76, 1,201.18, 1,159.09, 1,072.45, and 828.94; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.81 (s, 1H), 7.89 (d, *J* = 7.9 Hz, 1H), 7.80 (d, *J* = 15.4 Hz, 1H), 7.57 (d, *J* = 15.4 Hz, 1H), 7.48 (t, *J* = 7.8 Hz, 1H), 7.01 (d, *J* = 8.9 Hz, 1H), 6.92 (t, *J* = 7.6 Hz, 1H), 6.77 (s, 2H), 6.52 (s, 1H), and 3.82 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 193.91, 163.84, 161.34, 145.71, 136.72, 129.97, 120.76, 120.22, 119.14, 118.86, 107.36, 103.28, and 55.73; HRMS (ESI) *m/z*, calculated for $C_{17}H_{17}O_4$ $[M+H]^+$ 285.1121; found 285.1120.

*1-(2-Hydroxyphenyl)-3-(4-nitrophenyl)prop-2-en-1-one (**11**)*

Yield 72%, m.p.: 141.8–142.1°C; IR (KBr) υ/cm: 3,363.81, 2,923.21, 1,798.98, 1,640.58, 1,575.96, 1,272.99, and 1,179,85; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.59 (s, 1H), 8.30 (d, *J* = 8.8 Hz, 2H), 7.99–7.88 (m, 2H), 7.88–7.76 (m, 3H), 7.60–7.51 (m, 1H), 7.06 (d, *J* = 8.4 Hz, 1H), and 6.98 (t, *J* = 7.6 Hz, 1H); ^{13}C NMR (75 MHz, CDCl3) δ = 193.33, 164.09, 149.08, 142.46, 141.02, 137.40, 130.02, 129.49, 124.65, 124.44, 120.10, 119.47, and 119.20; HRMS (ESI) *m/z*, calculated for $C_{15}H_{11}NO_4$ $[M+Na]^+$ 292.0580; found 292.0594.

*3-(4-Fluorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**12**)*

Yield 56%, m.p.: 79–80°C; IR (KBr) υ/cm: 1,639.00, 1,579.40, 1,508.20, 1,341.87, 1,270.33, 1,208.23, 1,159.59, 1,024.16, and 829.07; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.80 (s, 1H), 7.97–7.81 (m, 2H), 7.73–7.59 (m, 3H), 7.52 (dd, *J* = 15.5, 7.0 Hz, 2H), 7.13 (t, *J* = 8.6 Hz, 2H), 7.03

(d, J = 8.4 Hz, 1H), and 6.95 (t, J = 7.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 193.86, 163.95, 164.61 (d, J = 252.6 Hz), 144.47, 136.82, 131.18 (d, J = 11.8, 3.4 Hz), 130.96 (d, J 8.7, 0.0 Hz), 129.93, 120.25, 120.12, 119.22, 119.00, and 116.59 (d, J = 22.0 Hz); HRMS (ESI) *m/z*, calculated for $C_{15}H_{11}FO_2$ $[M+H]^+$ 243.0815; found 243.0815.

*3-(4-Chlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**13**)*

Yield 93%, m.p.: 149°C; IR (KBr) υ/cm: 2,958.90, 1,640.86, 1,563.97, 1,487.07, 1,440.54, 1,406.14, 1,368.17, 1,341.36, 1,303.75, 1,263.06, 1,205.60, 1,159.31, 1,090.61, 1,022.97, 983.97, and 819.6; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.76 (s, 1H), 7.94–7.75 (m, 2H), 7.69–7.56 (m, 3H), 7.51 (t, J = 7.8 Hz, 1H), 7.41 (d, J = 8.5 Hz, 2H), 7.04 (d, J = 8.3 Hz, 1H), and 6.95 (t, J = 7.6 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 193.80, 163.95, 144.28, 137.20, 136.91, 133.39, 130.13, 129.95, 129.68, 120.87, 120.24, 119.26, and 119.03. HRMS (ESI) *m/z*, calculated for $C_{15}H_{11}ClO_2$ $[M+H]^+$ 259.0520; found 259.0549.

*1-(2-Hydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**14**)*

Yield 85%, m.p.: 91°C; IR (KBr) υ/cm: 2,969.64, 2,934.24, 1,639.09, 1,564.79, 1,512.46, 1,303.75, 1,260.68, 1,211.64, 1,162.63, 1,030.42, 985.69, and 828.91; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.95 (s, 1H), 7.98–7.85 (m, 2H), 7.64 (d, J = 8.6 Hz, 2H), 7.59–7.45 (m, 2H), 7.05–6.92 (m, 4H), and 3.87 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 194.05, 163.89, 162.38, 145.73, 136.52, 130.92, 129.89, 127.69, 120.46, 119.12, 118.94, 117.92, 114.86, and 55.81. HRMS (ESI) *m/z*, calculated for $C_{16}H_{14}O_3Na$ $[M+Na]^+$ 227.0835; found 227.0868.

*1-(2-Aminophenyl)-3-phenylprop-2-en-1-one (**15**)*

Yield 88%, m.p.: 50–54°C; IR (KBr) υ/cm: 3,442.86, 3,383.19, 3,078.44, 1,643.38, 1,617.30, 1,576.07, 1,340.49, 1,209.27, 1,156.99, and 739.12; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.87 (d, J = 8.2 Hz, 1H), 7.75 (d, J = 15.6 Hz, 1H), 7.68–7.58 (m, 3H), 7.41 (d, J = 5.2 Hz, 3H), 7.30 (t, J = 7.7 Hz, 1H), and 6.71 (dd, J = 7.7, 4.0 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 191.94, 151.25, 143.17, 135.50, 134.57, 131.27, 130.35, 129.14, 128.51, 123.36, 119.28, 117.56, and 116.11. HRMS (ESI) *m/z*, calculated for $C_{15}H_{13}NO$ $[M+H]^+$ 224.1069, found 224.1068.

*1-(2-Aminophenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (**16**)*

Yield 50%, m.p.: 128°C; IR (KBr) υ/cm: 3,468.67, 3,320.11, 2,991.19, 2,959.13, 2,938.87, 2,836.37, 1,644.19, 1,576.27, 1,542.03, 1,461.90, 1,357.04, 1,320.46, 1,286.48, 1,125.59, 1,012.88, 868.25, and 830.22; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.89 (d, J = 8.3 Hz, 1H), 7.68 (d, J = 15.5 Hz, 1H), 7.52 (d, J = 15.5 Hz, 1H), 7.30 (t, J = 7.7 Hz, 1H), 6.87 (d, J = 2.0 Hz, 2H), 6.80–6.67 (m, 2H), and 3.93 (m, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 191.72, 153.63, 151.24, 143.29, 140.19, 134.50, 131.17, 131.01, 122.60, 119.22, 117.56, 115.99, 105.63, 61.18, and 56.39; HRMS (ESI) *m/z*, calculated for $C_{18}H_{19}NO_4$ $[M+H]^+$ 314.1386; found 314.1386.

*1-(2-Aminophenyl)-3-(3,5-dimethoxyphenyl)prop-2-en-1-one (**17**)*

Yield 63%, m.p.: 119–122°C; IR (KBr) υ/cm: 3,422.12, 3,301.63, 1,650.14, 1,584.38, 1,451.88, 1,291.08, 1,248.01, 1,202.06, 1,164.68, and 1,070.73; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.85 (d, J = 7.1 Hz, 1H), 7.70–7.52 (m, 2H), 7.30 (t, J = 7.7 Hz, 1H), 6.77 (d, J = 2.2 Hz, 2H), 6.74–6.67 (m, 2H), 6.54–6.49 (m, 1H), and 3.84 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 191.97, 161.33, 151.16, 143.28, 137.48, 134.71, 131.36, 123.92, 119.38, 117.72, 116.31, 106.49, 102.62, and 55.79; HRMS (ESI) *m/z*, calculated for $C_{17}H_{18}NO_3$ $[M+H]^+$ 284.1281; found 284.1282.

*1-(2-Aminophenyl)-3-(4-nitrophenyl)prop-2-en-1-one (**18**)*

Yield 75%, m.p.: 145°C; IR (KBr) υ/cm: 3,460.29, 3,337.72, 1,646.71, 1,615.70, 1,578.40, 1,544.81, 1,411.48, 1,340.16, 1,207.79, 1,159.54, 1,109.31, 1,005.95, 986.22, 773.52, and 743.76; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.25 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.3 Hz, 1H), 7.78–7.70 (m, 4H), 7.31 (t, J = 7.7 Hz, 1H), and 6.76–6.67 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 190.91, 151.66, 148.59, 141.84, 139.98, 135.20, 131.29, 129.02, 127.34, 124.49, 118.75, 117.75, and 116.28. HRMS (ESI) *m/z*, calculated for $C_{15}H_{12}N_2O_3$ $[M+H]^+$ 269.0920; found 269.0920.

*1-(2-Aminophenyl)-3-(4-fluorophenyl)prop-2-en-1-one (**19**)*

Yield 59%, m.p.: 90°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.92–7.87 (m, 1H), 7.75 (d, J = 15.7 Hz, 1H), 7.70–7.64 (m, 2H), 7.59 (d, J = 15.6 Hz, 1H), 7.38–7.32 (m, 1H), 7.20–7.11 (m, 2H), 6.81–6.71 (m, 2H), and 6.37 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 191.0, 164.0, 161.0, 151.0, 141.69, 136.0, 134.44, 131.01, 130.20, 126.0, 117.39, 116.23, 116.16. and 115.0. HRMS (ESI) *m/z*, calculated for $C_{15}H_{12}FNO$ $[M+H]^+$ 242.0975; found 242.0974.

*1-(2-Aminophenyl)-3-(4-chlorophenyl)prop-2-en-1-one (**20**)*

Yield 85%, m.p.: 94°C; IR (KBr) υ/cm: 3,472.61, 3,325.33, 1,643.98, 1,612.83, 1,570.41, 1,492.35, 1,444.31, 1,404.72, 1,336.60, 1,292.96, 1,264.03, 1,210.78, 1,156.94, 1,088.42, 1,026.48, 1,008.26, 981.60, and 842.88; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.902 (d, J = 1.2 Hz, 1H), 7.874–7.588 (m, 4H), 7.438–7.304 (m, 3H), 6.766–6.719 (m, 2H), and 6.40 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 191.36, 151.15, 141.46, 135.96, 134.52, 133.79, 131.02, 129.45, 129.19, 123.57, 118.80, 117.40, and 115.96. HRMS (ESI) *m/z*, calculated for $C_{15}H_{12}ClNO$ $[M+H]^+$ 258.0678; found 258.0678.

*1-(2-Aminophenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**21**)*

Yield 56%, m.p.: 67–68°C; IR (KBr) υ/cm: 3,455.33, 3,329.60, 3,076.14, 1,684.40, 1,644.34, 1,613.86, 1,572.25, 1,541.77, 1,510.32, 1,420.68, 1,353.03, 1,338.23, 1,305.89, 1,287.80, 1,247.72, 1,211.78, 1,158.14, 1,112.04, and 978.75; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.86 (d, J = 8.3 Hz, 1H), 7.72 (d, J = 15.5 Hz, 1H), 7.59 (d, J = 8.7 Hz, 2H), 7.50 (d, J = 15.5 Hz, 1H), 7.32–7.23 (m, 1H), 6.93 (d, J = 8.7 Hz, 2H), 6.73–6.63 (m, 2H), 6.30 (s, 2H), and 3.85 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 192.84, 161.36, 150.90, 142.86, 134.13, 130.95, 130.01, 128.03, 120.82, 119.35, 117.32, 115.89, 114.38, and 55.44. HRMS (ESI) *m/z*, calculated for $C_{16}H_{16}NO_2$ $[M+H]^+$ 254.1175; found 254.1174.

5.2 | Biological assays

The compounds were assayed in vitro for cytotoxicity on mammalian cells and for leishmanicidal activity against intracellular amastigotes

of *L. braziliensis*. The trypanocidal activity was carried out in intracellular amastigotes of *T. cruzi*. Their antiplasmodial activity was determined on nonsynchronized *P. falciparum* cultures. The antiprotozoal activities were classified grouped according to EC_{50} values, based on the hit criteria proposed by Katsuno et al.[26] into high (EC_{50} < 10 μM), moderate (10 μM < EC_{50} < 50 μM), and low activity (EC_{50} > 50 μM).

5.2.1 | Cytotoxic activity

Cytotoxicity of the compounds was evaluated in the human monocyte cell line (U-937 ATCC CRL-1593.2) at the exponential growth phase, adjusted at 1×10^5 cells/ml in RPMI-1640 medium (GIBCO Invitrogen, Basel, Switzerland) enriched with L-glutamine (200 mM), 10% inactivated fetal bovine serum (FBS), 1% penicillin (10,000 UI/ml) and streptomycin (10,000 mg/ml). The compounds were added as a 100-μl solution in the same medium. For each compound, six serial 1:2 dilution concentrations, starting at 368 μM, were assayed. Doxorubicin was assayed as a control drug under the same dilution pattern, starting at 18 μM, respectively. Afterward, cells were incubated for 72 hr at 37°C and 5% CO_2, and cell viability was assayed by the MTT reduction assay according to the optical density (OD) at 570 nm of the resulting reduction of formazan in a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA).[17] Nonspecific absorbance was corrected by subtracting the OD of the blank. Cells exposed to doxorubicin were used as positive controls, whereas unexposed cells were used as negative controls.[28]

5.2.2 | Antileishmanial activity

The antileishmanial activity was evaluated in intracellular amastigotes of *L. braziliensis* as follows. U-937 human cells at a density of 3×10^5 cells/ml in Rosewell Park Memorial Institute (RPMI)-1640 medium and 0.16 μM phorbol-12-myristate-13-acetate were dispensed in a 24-well microplate and then infected with stationary *L. braziliensis* promastigotes transfected with the green fluorescent protein gene (MHOM/CO/88/UA301-EGFP), at a 15:1 parasite/cell ratio. Plates were incubated at 34°C and 5% CO_2 for 3 hr, and cells were then washed twice with phosphate-buffered saline (PBS) to eliminate noninternalized parasites. Fresh RPMI-1640 medium was added into each well (1 ml) and the plates were incubated again for 24 hr. Afterward, the RPMI-1640 medium was replaced with compound at four serial dilutions (250, 125, 62.5, and 31.25 μM) dissolved in a fresh culture medium, and plates were incubated at 34°C and 5% CO_2 for 72 hr. Then, cells were detached from the plate with 100 μl of ethylenediaminetetraacetic acid/trypsin (0.25%) solution, centrifuged (500*g*, 5 min, 4°C), and washed with 1 ml of cold PBS. Cells were then suspended in 500 μl of 0.4% paraformaldehyde in PBS and analyzed by flow cytometry (Cytomics™ FC 500; Coulter), expressed as median fluorescence intensity. Nonspecific fluorescence was corrected by subtracting the FL1 channel in noninfected cells. Infected cells exposed to amphotericin B were used as controls as a positive control (antileishmanial drug) in the assay, whereas infected cells incubated in the absence of any compound or drug were used as controls for infection (negative control).[29]

5.2.3 | Antiplasmodial activity

The antiplasmodial activity was evaluated in vitro on asynchronous cultures of *P. falciparum* (3D7 strain), maintained under standard culture conditions.[30] The activity of each compound was assessed by measurement of the lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant, as previously described.[31] *P. falciparum* cultures were adjusted to 0.5% parasitemia and 1% hematocrit in RPMI-1640 medium enriched with 1% Albumax II. Then, in each well of a 96-well plate, 100 μl of parasite suspension was dispensed and subsequently exposed against 100 μl of four serial dilutions of compounds (100, 25, 3.125, and 0.78 μM), chloroquine (CQ) was applied as a positive antiplasmodial drug control. Parasites unexposed to any compound were used as controls for both growth and viability (negative control). Plates were incubated for 72 hr at 37°C in an N_2 (90%), CO_2 (5%), and O_2 (5%) atmosphere. After incubation, the plates were harvested and parasites were subjected to three 20-min freeze–thaw cycles. Meanwhile, 100 μl of Malstat reagent (400 μl Triton X-100 in 80 ml deionized water, 4 g L-lactate, 1.32 g Tris-buffer reagent, and 0.022 g acetylpyridine adenine dinucleotide in 200 ml deionized water; pH 9.0) and 25 μl of NBT/PES solution (16 g/l nitroblue tetrazolium salt and 0.8 g/l phenazine methosulfate) were added to each well of a second flat-bottom 96-well microtiter plate. Cultures in each well were lysed by five freeze–thaw cycles, the lysate was homogenized by repetitive pipetting, and 15 μl from each well was transferred into a replica plate containing Malstat and NBT/PES solution. After 1 hr of incubation in the dark, color development of the LDH reaction was monitored at 650 nm in a Varioskan, Thermo spectrofluorometer. Nonspecific absorbance was corrected by subtracting the value of the blank.

5.2.4 | Antitrypanosomal activity

T. cruzi (Tulahuen strain transfected with the β-galactosidase gene) metacyclic trypomastigotes were cultured at 26°C for 10 days in modified NNN (Novy–McNeal–Nicolle) medium. U-937 cells were seeded in 96-well dishes at a density of 2.5×10^4 cells in 100 μl of medium/well and exposed to PMA, as described above. After transformation to macrophages, cells were infected using a ratio of five parasites per cell and incubated in RPMI-1640 medium with 10% FBS for 24 hr at 37°C and 5% CO_2. Two washes with PBS removed noninternalized parasites, and then fresh medium was added, containing each of the six serial diluted concentrations of each testing compound or benznidazole. After 72 hr of incubation under identical

conditions, the viability of intracellular amastigotes was determined by measuring the β-galactosidase activity. For this, 100 μM of chlorophenol red-β-D-galactopyranoside and 0.1% Nonidet P-40 were added to each well and incubated for 4 hr at 37°C and at 24°C protected from light. Then, measurement β-galactosidase activity was measured at 570 nm by a Varioskan, Thermo spectrophotometer. Nonspecific absorbance (blank) was subtracted from the measurement. Infected cells exposed to benznidazole (50, 12.5, and 3.125 μM) were used as controls for antitrypanosomal activity (positive control) and nontreated cells as controls for infection (negative control). Determinations were done in triplicate with at least two independent experiments.[32]

5.2.5 | Data analysis

All experiments were performed in triplicate. The cytotoxicity was expressed as the Lethal Concentration 50 (LC_{50}), whereas the antiprotozoal activity was calculated as the median Effective Concentration (EC_{50}). Both LC_{50} and EC_{50} values were calculated by the Probit analysis. The LC_{50} values in U-937 cells and the EC_{50} values in *P. falciparum* were determined according to the percentage of inhibition of cell or parasite viability, whereas the EC_{50} values in *L. braziliensis*, *T. cruzi* amastigotes, and blood-stage *P. falciparum* parasites were determined according to the percentage of inhibition, as described previously.[28]

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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