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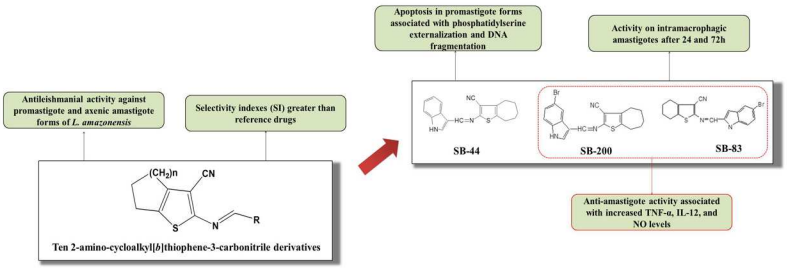

2-Amino-thiophene derivatives present antileishmanial activity mediated by apoptosis and immunomodulation *in vitro*

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Running title: Antileishmanial activity of new 2-amino-thiophene derivatives

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Abstract

This study evaluated the effects of 2-amino-thiophene derivatives on the promastigote and amastigote forms of *Leishmania (Leishmania) amazonensis* and their possible mechanisms of action. Initially, we evaluated the antileishmanial activity of ten 2-amino-thiophene derivatives on promastigote and axenic amastigote forms of *L. amazonensis* and their cytotoxicity against murine macrophages and human red blood cells. Three promising compounds were selected for studies of the cell death process using flow cytometry analysis and a DNA fragmentation assay. The effects of the compounds were assessed on intramacrophagic amastigotes, and the modulation of cytokine and NO production was investigated. All thiophene derivatives showed antileishmanial activity against promastigotes and axenic amastigotes with less toxicity for murine macrophages and human red blood cells. The best values were obtained for compounds containing a lateral indole ring. Docking studies suggested that these compounds played an important role in inhibiting trypanothione reductase (TryR) activity. The selected compounds SB-200, SB-44, and SB-83 induced apoptosis in promastigotes involving phosphatidylserine externalization and DNA fragmentation in a pattern similar to that observed for the positive control. Additionally, SB-200, SB-44, and SB-83 significantly reduced the infection index of macrophages by the parasites; for compounds SB-200 and SB-83 this reduction was associated with increased TNF-α, IL-12, and NO levels. This study demonstrated the effective and selective action of 2-amino-thiophene derivatives against *L. amazonensis*, resulting in apoptosis-like cell death and immunomodulation *in vitro*. The results suggest that they are promising compounds for the development of new leishmanicidal drugs.

Keywords: Leishmaniasis, drug development, mechanisms of action, synthetic chemistry

1. Introduction

Leishmaniasis is a complex of infectious diseases caused by more than 20 kinetoplastid protozoan parasites that belong to the Trypanosomatidae family and genus *Leishmania*. It affects approximately 12 million people in more than 98 countries distributed in five continents and is included in the neglected tropical diseases (NTD) group [1,2]. The clinical manifestations can vary from nodular and ulcerative skin lesions to the progressive mucocutaneous and visceral form, which is the most severe form and can potentially result in fatal disease [3]. *Leishmania (Leishmania) amazonensis* is found in the New World primarily in Latin America and is associated with different clinical forms of cutaneous leishmaniasis. It is the main agent of diffuse cutaneous leishmaniasis, which is commonly refractory to available treatments [4].

Currently, leishmaniasis chemotherapy has a reduced arsenal of drugs and is far from satisfactory. Most of the drugs exhibit severe toxic side effects, and increased chemoresistance of the parasite greatly reduces its sensitivity to these drugs [3]. The first-line and most widely used drugs for the treatment of leishmaniasis are pentavalent antimonials such as meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostan®). Pentavalent antimonials have been used since 1945; they require parenteral administration and are toxic and poorly tolerated drugs [5]. Second-line drug such amphotericin B, pentamidine, paromomycin and miltefosine are used in refractory and resistant cases, but these drugs have high costs and may be even more toxic than the antimonials [6]. Therefore, there is a dire need globally for the development of drugs that are more effective against the parasite and less toxic than conventional drugs.

Planning and synthesizing new drugs based on known structures is founded on the main objective of obtaining derivatives with greater therapeutic interest and higher activity,

bioavailability and metabolism that are more suited to therapeutic use. These drugs should exhibit less toxicity and may even acquire more appropriate pharmacotechnical features [7,8].

Among the compounds that have been tested as potential antileishmanial agents, thiophene derivatives are particularly interesting [9]. Thiophene derivatives have been identified as secondary metabolites in various organisms including fungi and plants, such as *Tagetes patula* and *Tagetes erecta* (Asteraceae) [10]. In medicinal chemistry, thiophenes are a versatile class of sulphur-containing heterocyclic compounds that meet the characteristics of a "privileged structure" in which a simple molecular framework is able to provide ligands for diverse targets [11]. Several reports confirm that small substitutions in the thiophene structure cause large differences in the biological and pharmacological profiles [12]. Properties described in the literature include: anti-inflammatory, antitumor, antinociceptive, anticonvulsant, and antiarrhythmic [13]. These structures also exhibit promising activity against microorganisms due to their antibacterial, antifungal, and antiprotozoal properties [12,14]. Some plant-extracted thiophene derivatives have been reported to be effective against the promastigote forms of *L. donovani*, *L. infantum*, *L. braziliensis*, and *L. amazonensis*, [9,15] thereby highlighting the potential of these molecules as agents for the treatment of leishmaniasis. However, studies of naturally occurring substances commonly present limitations in terms of the availability of the substance to be tested.

In this context, the present study evaluated the antileishmanial activity of 10 synthetic 2-amino-cycloalkyl[*b*]thiophene-3-carbonitrile derivatives against *L. amazonensis*, one of the etiological agents of American cutaneous leishmaniasis (ACL). These molecules present simple chemical structures and are easily synthesized using a low cost method via a two-step reaction. The reaction starts with the one pot reaction between malononitrile with a cyclic ketone and elemental sulphur in the presence of morpholine, followed by the Gewald reaction [16]. Among the molecules tested, three showed better selectivity indices to the parasite and

were evaluated on amastigotes internalized into macrophages. Finally, possible mechanisms of action responsible for the antileishmanial activity were evaluated.

2. Results

2.1 Synthetic Chemistry

Scheme 1 shows the reaction sequence used to obtain the thiophene derivatives and the chemical structures of the studied compounds. For the evaluation of the antileishmanial activity, all compounds were re-synthesized and their chemical structures were confirmed by comparison with their physico-chemical characteristics and signals in the NMR ^{1}H spectra. All compounds were obtained with a good yield (greater than 70%), demonstrating the good synthetic feasibility and choice of standardization of reaction conditions employed in the synthetic strategy.

2.2 X-Ray crystal structures of SB-83 and SB-200

The crystal structures of two of the thiophene derivatives (compounds SB-200 and SB-83) were solved by X-ray crystallography. The crystallographic parameters, crystal data, data collection details and structure refinement results for SB-200 and SB-83 were presented in Table 1.

Table 1 – Crystal data, data collection details and structure refinement results for SB-200 and SB-83.

Parameter	SB-200 - $C_{19}H_{16}BrN_3S$	SB-83 - $C_{18}H_{14}BrN_3S$
Molecular Weight (g/mol)	398.32	384.29
Space Group	$P2_1/n$	Pmnb
a (Å)	11.370(1)	6.769(1)
b (Å)	11.195(1)	10.160(2)
c (Å)	14.275(1)	24.968(4)
β (°)	106.026(1)	90.000 (0)
V (Å^3)	1746.5(3)	1717.3(1)
Z	4	4
D_c(g.cm^{-3})	1.515	1.486
Absorbance (μ.mm^{-1})	2.478	2.518
Independent reflections	4,143	2,206
Reflections $I>2\sigma(I)$	3,265	1,731
R-factor	0.0396	0.0429

The fractional atomic coordinates and isotropic temperature parameters (Å^2) for SB-200 and SB-83 are presented in the supplementary data. Figures 1 and 2 represent the stereodiagrams of the crystal structures of SB-200 and SB-83. The nonhydrogen atoms are drawn as ellipsoids that represent the probability (50%) that the atoms are located within the designated volumes. The interatomic bond distances (Å) and angles (°) for SB-200 and SB-83 are presented in the supplementary data. The view of the packing of SB-200 and SB-83 showing the intermolecular interactions that stabilize the crystal structures are presented in the supplementary data.

Figure 1. Projection of SB-200, showing atom labeling and 50% probability displacement ellipsoids.

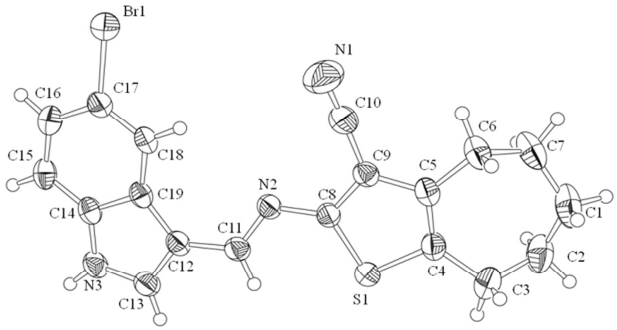
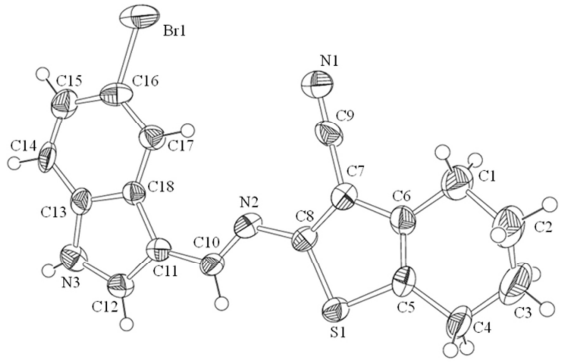


Figure 2. Projection of SB-83, showing atom labeling and 50% probability displacement ellipsoids.



2.3 *In vitro* antileishmanial efficacy of 2-amino-thiophenes and their cytotoxicity

In a preliminary experiment, ten 2-amino-thiophene derivatives were investigated. Their antileishmanial efficacy on the promastigote and axenic amastigote forms of *L. amazonensis* and their cytotoxicity against murine peritoneal macrophages and human red blood cells were evaluated to identify the most active and selective molecules against the parasite. Their IC_{50}, EC_{50}, CC_{50} and HC_{50} calculated values are shown in Table 2; meglumine antimoniate and amphotericin B were included as reference drugs.

All thiophene derivatives showed antileishmanial activity on promastigotes, with IC_{50} values ranging from 3.37 µM (SB-83) to 189.3 µM (SB-63) and EC_{50} values ranging from 15.82 µM (SB-44) to 212.73 µM (6CN10) when assayed against axenic amastigotes. Among the evaluated compounds, SB-200, SB-44, and SB-83 (which contain a lateral indole ring) showed higher antileishmanial activity. The antileishmanial activity was higher than meglumine antimoniate and lower than amphotericin B. With regards to their cytotoxic effects, all of the compounds showed lower toxicity for macrophages than for the different parasite forms (CC_{50} values ranging from 32.02 µM for SB-200 to 721.76 µM for SB-63). The haemolytic assays showed that the 2-amino-thiophene derivatives did not exhibit toxicity towards human red blood cells (HC_{50} values ranging from 624.8 µM for SB-81 to > 800 µM for the compounds 6CN10, 6CN09, SB-80, SB-63, SB-200, and SB-83). In contrast,

meglumine antimoniate was less toxic to the parasite than the macrophages (CC_{50} of 1035 mg/L) and human erythrocytes (HC_{50} of 429.5 mg/L), and amphotericin B presented high levels of cytotoxicity (CC_{50} of 0.18 µM and HC_{50} of 11.6 µM).

The best selectivity index values were again obtained for the compounds containing a lateral indole ring (SB-200, SB-44, and SB-83). The selective indices of these compounds compared to the reference drugs were particularly interesting because the selectivity of SB-200, SB-44, and SB-83 for the parasites was much more pronounced. For example, SB-83 was 840- and >14,836-fold more benign than meglumine antimoniate towards macrophages and erythrocytes, respectively. These values are highly representative of the *in vitro* potential efficacy of thiophenes compared to the reference drugs. In view of the promising results obtained with the thiophene derivatives containing an indole ring (the SB-200, SB-44, and SB-83 compounds) that showed the best selectivity indices for the parasite (Table 3), these molecules were selected for further study.

Table 2 – Antileishmanial activity, cytotoxic effects against mammalian cells, and selectivity index values calculated for thiophene derivatives, meglumine antimoniate and amphotericin B.

Compounds	Cytotoxicity		Antileishmanial activity					
	Macrophages	Red blood cell	Promastigotes			Axenic amastigotes		
	CC_{50} µM	HC_{50} µM	IC_{50} µM	SI_m	SI_{rb}	EC_{50} µM	SI_m	SI_{rb}
6CN10	228.86	>800	187.1	1.22	>4.27	212.73	1.07	>3.76
6CN09	211.43	>800	88	2.4	>9.1	167.2	1.26	>4.78
6CN01	141.1	682.1	61.5	2.29	11.09	124.4	1.13	5.4
SB-80	540.44	>800	139.44	3.87	>5.73	210.7	2.56	>3.79
SB-68	438.46	716.3	164.66	2.66	4.35	132.37	3.31	5.41
SB-63	721.76	>800	189.3	3.81	>4.22	192.15	3.75	>4.16
SB-81	196.01	624.8	49.12	3.99	12.71	102.8	1.9	6
SB-200	32.02	>800	3.65	8.77	>291.17	20.09	1.59	>39.8
SB-44	49.08	721.3	7.37	6.65	97.86	15.82	3.1	45.5
SB-83	113.4	>800	3.37	33.6	>237.38	18.5	6.12	>43.24
Meglumine antimoniate	1035[a]	429.5[a]	25740[a]	0.04	0.016	1017.4[a]	1.01	0.42
Amphotericin B	0.18	11.6	0.17	1.05	68.23	0.23	0.78	14.87

SI_m (selectivity index) = CC_{50}/IC_{50}
SI_{rb} (selectivity index) = HC_{50}/IC_{50}
[a] Values in mg/L

2.4 Docking

To determine the molecular mechanism responsible for the antileishmania activity of the 2-amino-thiophene derivatives, docking studies on trypanothione reductase (TryR) from *L. infantum* (PDB id: 2jk6) were performed. Table 3 shows the results of docking the thiophene derivatives into the catalytic site of TryR by presenting the binding energies (E_{dock}). The best ligands were compounds SB-200, SB-44 and SB-83, with E_{dock} values of –11.17, –

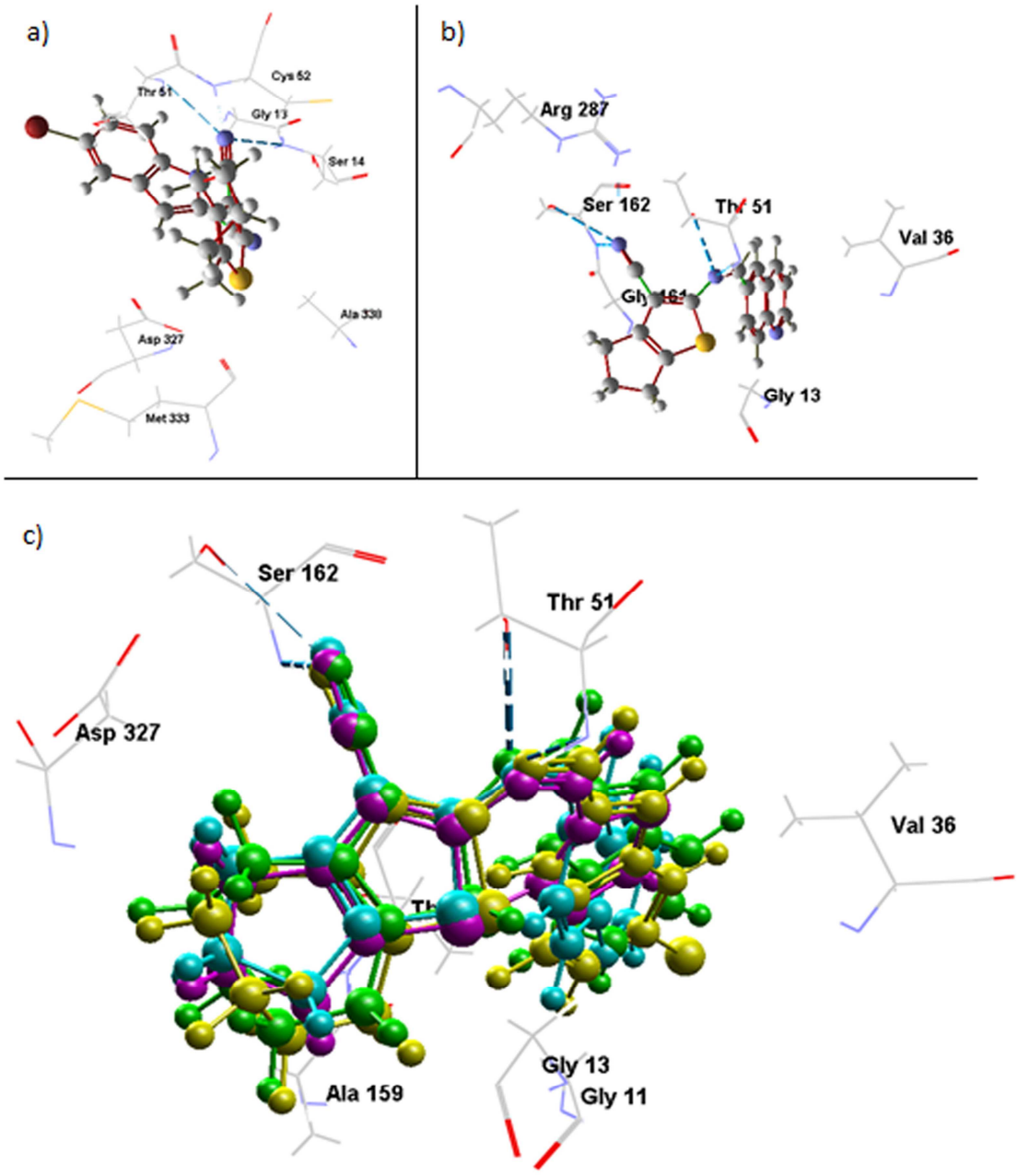
10.29 and –9.81 Kcal/mol, respectively. In contrast, the worse ligands were compounds SB-80 and SB-81, with E_{dock} values of –7.86 and –7.69 Kcal/mol, respectively.

Table 3 – Docking results of 2-amino-thiophene derivatives docked to Trypanothione reductase from *L. infantum* (PDB id: 2jk6).

Compound	Afinity energy (Kcal/mol)
6CN01	-8,79
6CN09	-8,60
6CN10	-9,36
SB63	-8,20
SB200	-11,17
SB44	-10,29
SB80	-7,86
SB81	-7,69
SB83	-9,81
SB68	-9,64

Docking analysis of the ligands into the TryR-binding site revealed that the 2-amino-thiophene derivatives fit well into the active site pocket made up of key residues (Figure 3). The main residues were Thr51 and Ser162, which interacted with all of the compounds through hydrogen bonds and acted as H-bond donors. Residue Thr51 made an H-bond with the sp nitrogen of the nitrile (–C≡N), and residue Ser162 stabilised the ligands though H-bonding with the sp^2 nitrogen of the imine group (–N=CH–). Other hydrophobic interactions with residues Gly11, Gly13, Val36, Ala159, Thr160 and Asp327 helped to stabilise the guest-host complexes and played an important role in the ligand binding affinity.

Figure 3 – a) Interaction of SB-200 with the TryR-binding site. Blue lines represent hydrogen bonds. b) Interaction of SB-81 with the TryR-binding site. Blue lines represent hydrogen bonds. c) TryR binding sites cluster with compounds SB-44 (green), SB-200 (yellow), SB-81 (blue) and SB-80 (purple).



2.5 Apoptotic-necrotic profiling in *Leishmania* promastigotes by selected thiophenes

To determine the cell death process in parasites exposed to compounds SB-200, SB-44, and SB-83, conventional flow cytometry was performed using Annexin V-FITC and PI. Viable cells remained unstained (Annexin V-FITC−/PI−). Early apoptotic cells showed Annexin V-FITC+/PI− staining patterns, whereas late apoptotic cells exhibited Annexin V-FITC+/PI+ staining patterns due to the loss of plasma membrane integrity [17]. Moreover, DNA fragmentation was analysed using the agarose gel electrophoresis assay. Representative dot-plots of Annexin V-FITC/PI staining are provided in Figure 4.

No death from initial apoptosis was observed (cells positive for annexin V and negative for PI) after treatment with the compounds SB-200, SB-44, SB-83 and amphotericin B (positive control). However, an increase in the number of promastigotes with late apoptosis (parasites staining positive for annexin V and PI) was observed following treatment with SB-200 (2x and 4x IC_{50}), SB-44 (4x IC_{50}), SB-83 (2x and 4x IC_{50}), and amphotericin B (1x, 2x, and 4x IC_{50}). Furthermore, necrotic cell death (cells negative for annexin V and positive for PI) was detected for compounds SB-200 (1x, 2x, and 4x IC_{50}), SB-44 (1x, 2x, and 4x IC_{50}), SB-83 (2x, and 4x IC_{50}), and amphotericin B (4x IC_{50}) (Table 4).

In a qualitative analysis using the DNA fragmentation assay by agarose gel electrophoresis, DNA fragmentation was detected in promastigote cells treated with SB-200, SB-44, and SB-83 at concentrations of 1x, 2x, and 4x the IC_{50} (Figure 5). The result was similar to that observed following treatment with amphotericin B at a concentration of 1x the IC_{50} using cellular death by apoptosis as a positive control. These results suggest that the cell death caused by the compounds SB-200, SB-44 and SB-83 in the *L. amazonensis* promastigote forms is associated with endonucleases activity, which is responsible for the DNA fragmentation that occurs as a result of apoptosis.

Figure 4 - Representative dot plots showing staining of *Leishmania amazonensis* promastigotes. *L. amazonensis* promastigotes were incubated at 26ºC for 4 h in the absence or presence of 2-amino-thiophene derivatives or amphotericin B at IC_{50}, 2x IC_{50} and 4x IC_{50} concentrations. Annexin/propidium iodide staining was performed and the samples were analysed by flow cytometry.

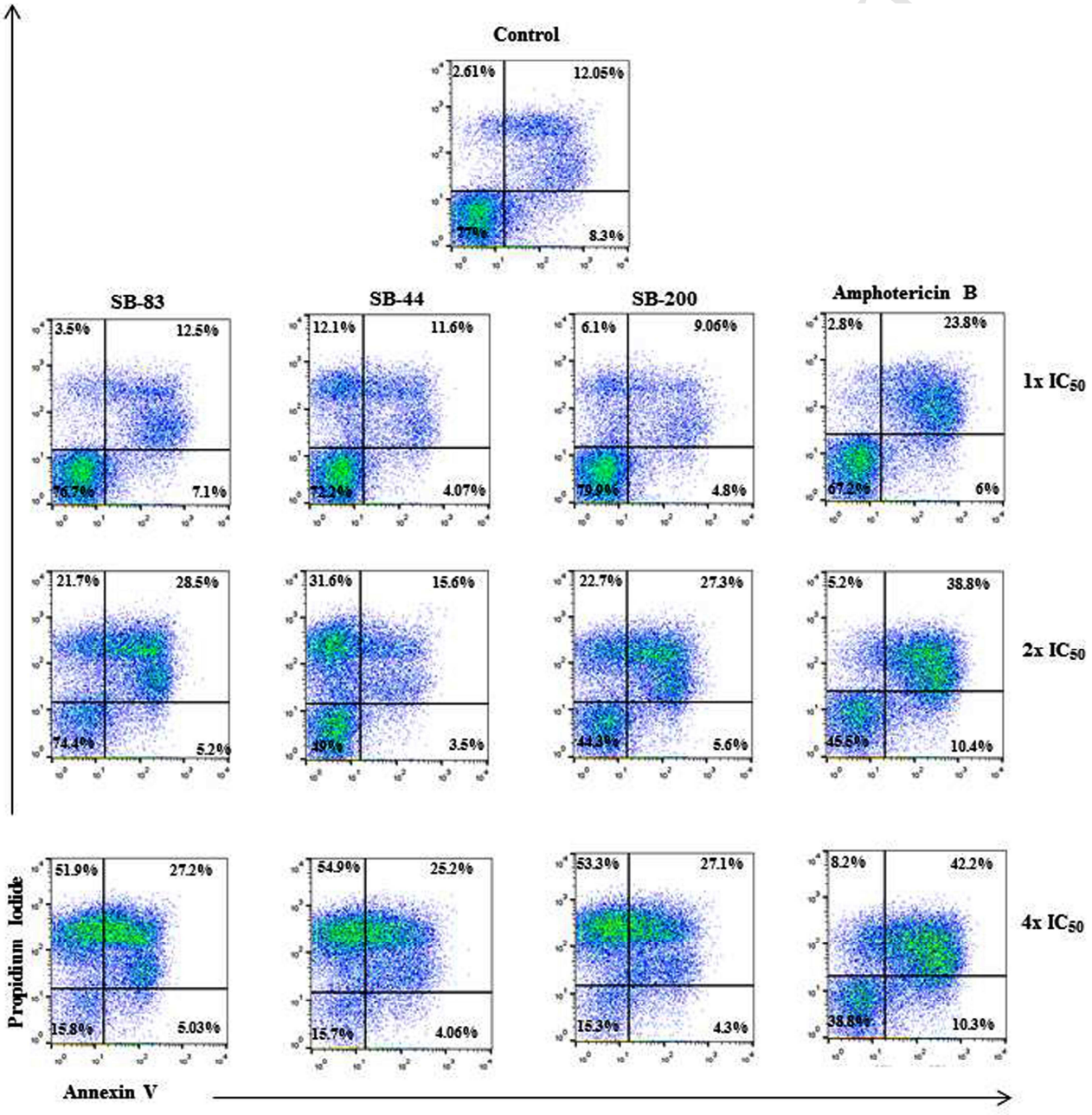
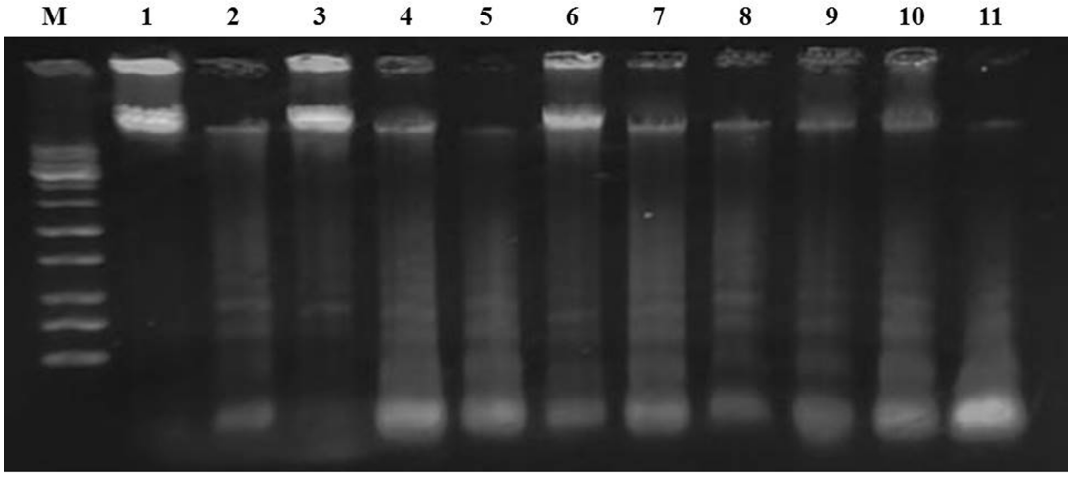


Table 4 - Flow cytometry analysis of *Leishmania amazonensis* promastigotes without treatment and treated with SB- 200, SB-44, SB-83 and the reference drug amphotericin B showing the percentage of annexin-V and propidium iodide (PI)-positive cells. The results represent the means ± S.E.M. of three experiments performed in triplicate. ([a]) $P < 0.05$ vs. control; ([b]) $P < 0.01$ vs. control; ([c]) $P < 0.001$ vs. control.

	Leishmania intracellular entities (% of cells)		
	Annexin	**Annexin/PI**	**PI**
Control	8.3	12.05	2.6
SB-200 1x IC_{50}	4.8	9.06	6.1[a]
SB-200 2x IC_{50}	5.6	27.3[b]	22.7[c]
SB-200 4x IC_{50}	4.3	27.1[b]	53.2[c]
SB-44 1x IC_{50}	4.07	11.6	12.1[a]
SB-44 2x IC_{50}	3.5	15.6	31.6[c]
SB-44 4x IC_{50}	4.06	25.2[b]	54.9[c]
SB-83 1x IC_{50}	7.1	12.5	3.5
SB-83 2x IC_{50}	5.2	28.5[b]	21.7[c]
SB-83 4x IC_{50}	5.03	27.2[b]	51.9[c]
Amph. B 1x IC_{50}	6	23.8[a]	2.8
Amph. B 2x IC_{50}	10.4	38.8[c]	5.2
Amph. B 4x IC_{50}	10.3	42.2[c]	8.6[a]

Figure 5 – The genomic DNA of *Leishmania amazonensis* promastigotes treated with different concentrations of SB-83, SB-44, and SB-200 or amphotericin B. The agarose gel (1%) was stained with gel red (1:500), and the analysis was performed under UV light. (M) – molecular marker 1 kb DNA (300 – 10,000 bp – Axygen); (1) – control; (2) – amphotericin B at a concentration of 1x IC_{50}; SB-200 at concentrations of 1x IC_{50} (3), 2x IC_{50} (4), and 4x IC_{50} (5); SB-44 at concentrations of 1x IC_{50} (6), 2x IC_{50} (7), and 4x IC_{50} (8); SB-83 at concentrations of 1x IC_{50} (9), 2x IC_{50} (10), and 4x IC_{50} (11).



2.6 *In vitro* efficacy of thiophenes SB-200, SB-44, and SB-200 against intramacrophagic amastigotes

To evaluate the action of selected thiophenes against intramacrophagic amastigotes, an *in vitro* model of infection of murine peritoneal macrophages was used. The results of the treatment with thiophenes SB-200, SB-44, and SB-83 on macrophages infected with *L. amazonensis* are presented in Figures 6 and 7. The obtained values revealed a significant and concentration-dependent reduction in the survival rate of amastigotes after 24h of treatment, resulting in EC_{50} values of 7.84, 10.5, and 8.17 for compounds SB-200, SB-44, and SB-83, respectively, while the reference drug meglumine antimoniate showed no effect. After 72 h of treatment, SB-200, SB-44, and SB-83 induced an even greater concentration-dependent reduction in the survival index, with EC_{50} values of 4.81, 9.52, and 6.4 μM, respectively. These thiophene derivatives had a much more significant effect than meglumine antimoniate after 72 h of exposure under the same experimental conditions; this reference drug was only able to induce a significant reduction in the survival index at a concentration of 300 mg/L and had an EC_{50} of 169.43 mg/L. Additionally, Figure 7 shows a qualitative representation of the effect of the thiophenes, which can be observed through the preserved integrity of macrophages after 72 h of treatment with different concentrations.

Figure 6 – Effects of SB-44, SB-200, SB-83, and the reference drug meglumine antimoniate on the survival index of amastigotes internalized in macrophages after 24 and 72 h of exposure. Murine macrophages were infected with promastigote forms of *Leishmania amazonensis* and treated at different concentrations of SB-44 (A), SB-200 (B), SB-83 (C) and meglumine antimoniate (D). The results represent the means ± S.E.M. of three experiments performed in triplicate. (*) $P < 0.05$ vs. control; (**) $P < 0.01$ vs. control; (***) $P < 0.001$ vs. control. C = control.

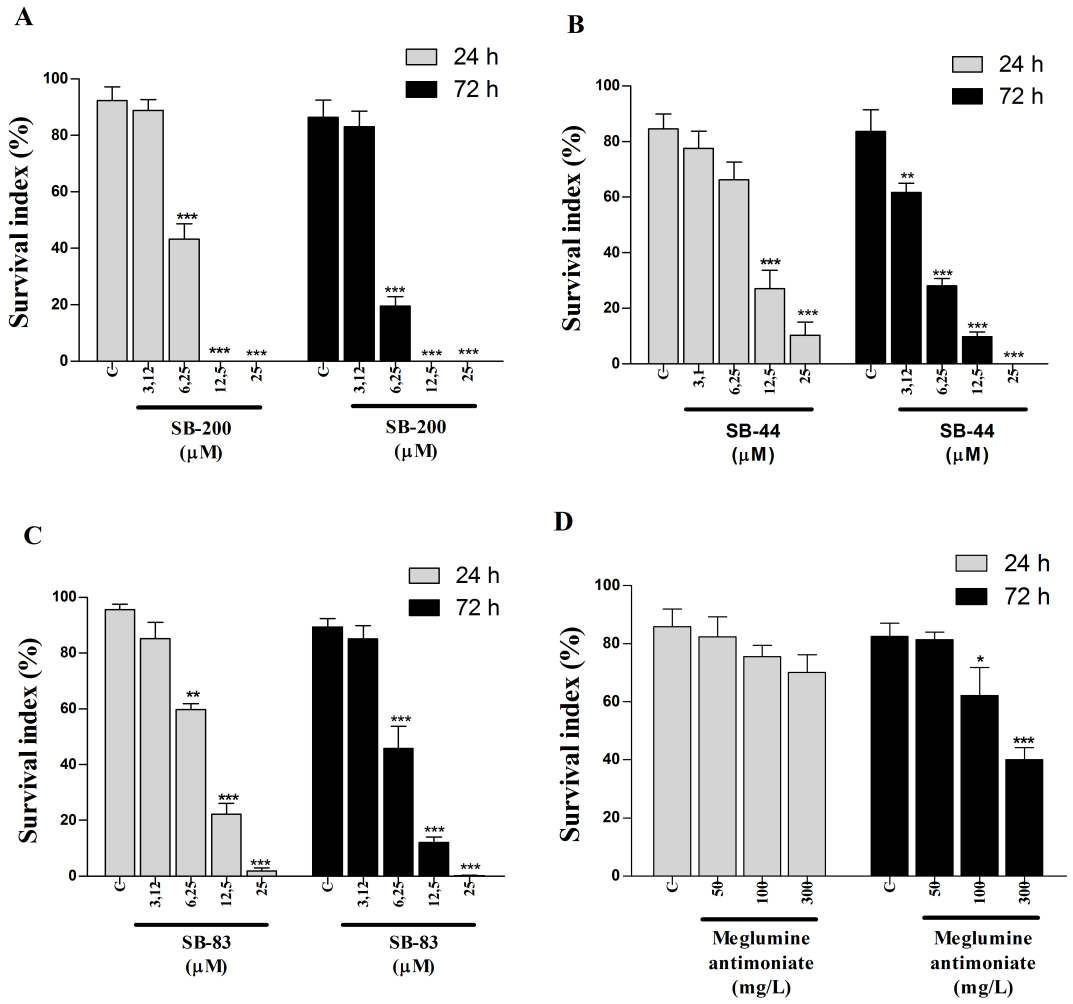
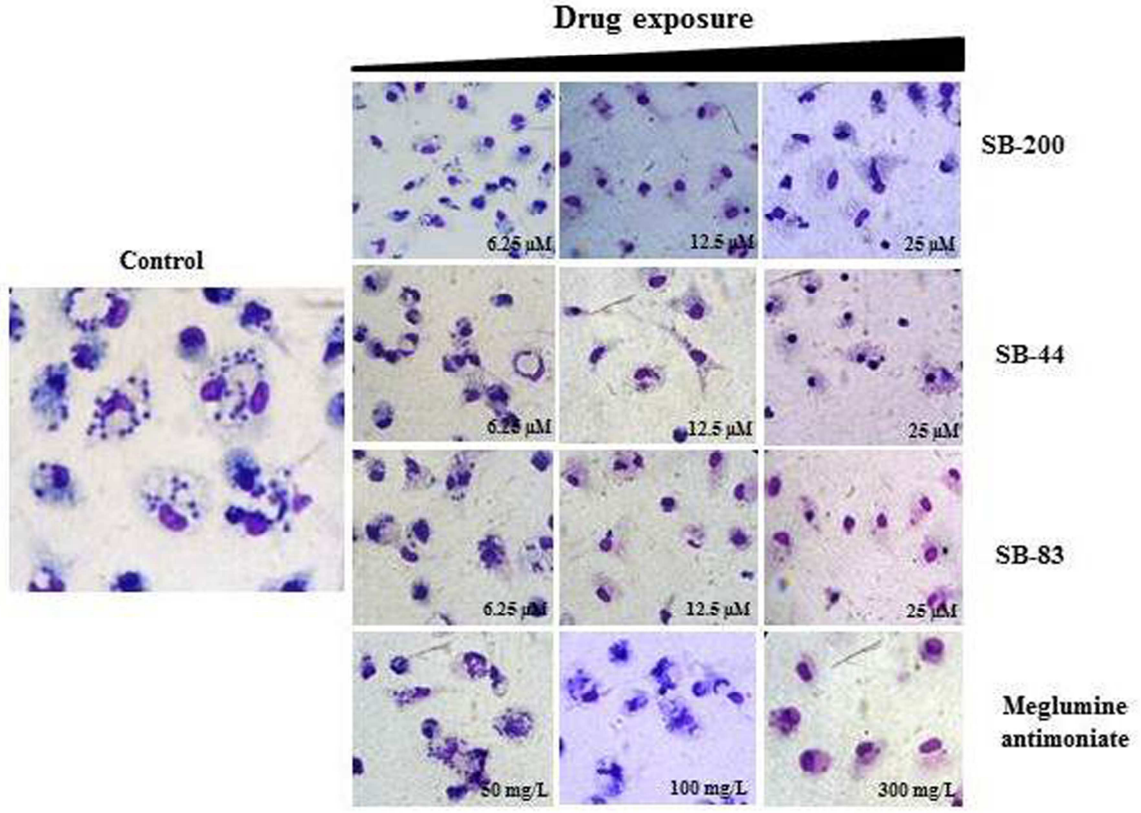


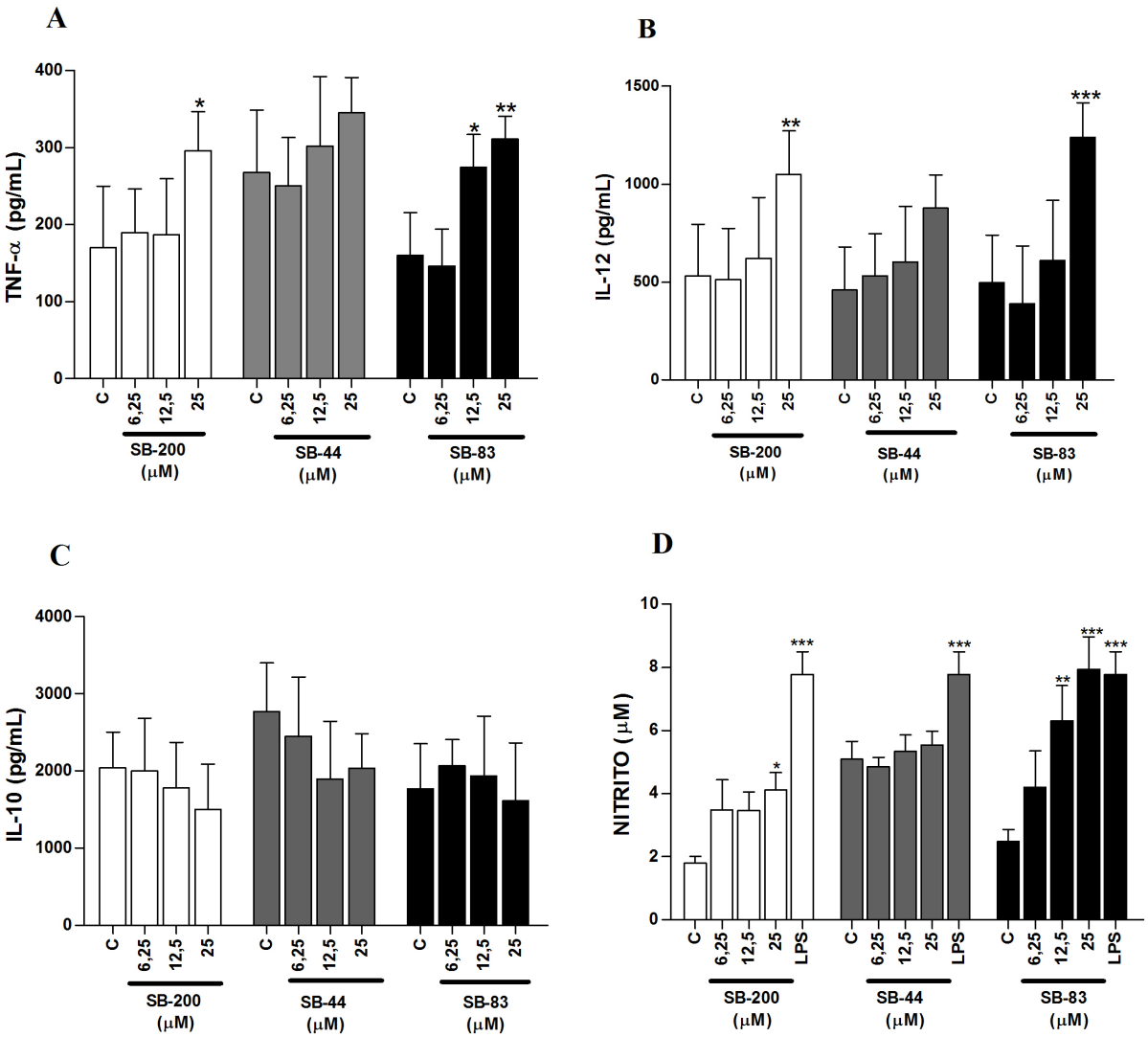
Figure 7 – Optical microscopy images of macrophages infected with promastigotes of *L. amazonensis* and treated with the 2-amino-thiophenes SB-200, SB-44, and SB-83 and the reference drug meglumine antimoniate for 72 h. The slides were observed at 1000x magnification.



2.7 Compounds SB-200, SB-44, and SB-200 induce a host-protective cytokine response and provide stimulus to increase NO levels

Due to the reduction in the infection rate of macrophages treated with the thiophene derivatives SB-44, SB-200 and SB-83, we investigated whether the antileishmanial activity was associated with an *in vitro* immunomodulatory action. SB-200 and SB-83 increased the production of TNF-α (Figure 8A), IL-12 (Figure 8B) and NO levels (Figure 8D) in murine macrophages infected with *L. amazonensis* without altering the level of IL-10 (Figure 8C) after 72 h of treatment. SB-44 did not affect the cytokine or NO levels under the evaluated conditions.

Figure 8 – Effects of SB-44, SB-200 and SB-83 on cytokine and nitric oxide (NO) production. The cytokines TNF-α (A), IL-12 (B), IL-10 (C) and NO (D) concentrations were measured in the supernatants from cultured macrophages infected with *Leishmania amazonensis* and treated with SB-44, SB-200, and SB-83 for 72 h at 37 °C and 5% CO_2. LPS - Bacterial lipopolysaccharide (10 mg/L). Data are presented as the mean ± S.E.M. of three experiments performed in triplicate. (*) $P < 0.05$ vs. control; (**) $P < 0.01$ vs. control; (***) $P < 0.001$ vs. control.



3. Discussion

Synthetic compounds or those derived from medicinal plants have been extensively evaluated for antileishmanial activity due to the urgent need for the discovery of new agents that are more effective and less toxic than the conventional drugs used for the treatment of

leishmaniasis [6,18]. Thus, we evaluated the antileishmanial efficacy of ten synthetic 2-amino-thiophene derivatives and their potential mechanisms of action. The thiophenes evaluated in this study were previously synthesized and shown to be promising antifungal [12] and antitumor agents [14]. These thiophenes have an additional advantage over natural compounds because they are synthesized with high purity and may be obtained on a large scale, including an industrial scale. The crystallographic data of compounds SB-200 and SB-83 confirms the chemical structures of the compounds in question and unequivocally provides the stereochemistry of the imine bond (CH = N), in both cases confirming the achievement of the *E* isomer. This information allowed us to obtain docking data with higher reliability and showed that the compounds could be obtained with very high purity (an indispensable condition to obtain single crystals of drugs).

As shown in Table 1, all ten evaluated thiophene derivatives inhibited the growth of *L. amazonensis* promastigotes, with SB-200, SB-44, and SB-83 presenting the highest activity. Studies with other thiophene derivatives also reported the antileishmanial potential of this class of chemical compounds against promastigote forms [19]. The necessity to minimize the use of laboratory animals in pharmacological and toxicological potential assessments of new products has led to the development of alternative *in vitro* methods. Thus, models involving axenic amastigotes are important for the screening of new drugs because the life stages of the parasite responsible for the different clinical manifestations of leishmaniasis must be considered [18]. Similar to the promastigote forms, all of the derivatives showed antileishmanial activity against the axenic amastigote forms. The compounds that showed enhanced antileishmanial activity against the promastigotes and axenic amastigotes (SB-200, SB-44, and SB-83) had in common the presence of an indole moiety ring that might be involved in the increase in antileishmanial activity. These compounds also showed increased antileishmanial activity compared to meglumine antimoniate, which was used as a reference

drug. Meglumine antimoniate showed higher antileishmanial activity against axenic amastigotes (IC_{50} of 1017.4 µM) than against promastigotes (IC_{50} of 25740 µM). These differences were also observed in previous studies and might be related to differences in the metabolic machinery and biochemistry of both stages [20,21].

To investigate the safety of 2-amino-thiophene derivatives on mammalian cells, we evaluated possible cytotoxic effects on murine peritoneal macrophages and human red blood cells. Experimental cytotoxic models involving macrophages are ideal for studies on antileishmanial activity because they are the major host cell type parasitized by *Leishmania* spp [22,23]. All compounds tested in this study were safer for macrophages than the reference drug, as evidenced by the CC_{50} values and selectivity indices. The presence of the indole ring appeared to greatly increase the selectivity indices of SB-200, SB-44, and SB-83, thereby contributing to the safety of these molecules. Other thiophenes that were evaluated for their murine macrophage cytotoxicity also presented higher selectivity for the parasite [9]. The selective action against *L. amazonensis* described for the thiophenes analysed in this work was remarkably higher than meglumine antimoniate, which was the first choice drug for leishmaniasis treatment; indeed, meglumine antimoniate presented higher cytotoxicity for the macrophages than for the parasite. The reference drug amphotericin B also exhibited high toxicity to macrophages despite having an antileishmanial activity superior to the evaluated compounds. This result can be compared to the *in vivo* effects of amphotericin B, which has high toxicity and induces many side effects (i.e., nephrotoxicity) despite being a drug of second choice. Therefore, amphotericin B is not an ideal drug [2].

Other cytotoxicity models have been investigated to determine the safety of new compounds with antileishmanial activity [21]. Cytotoxicity evaluations using red blood cells represent an *in vitro* model that is correlated with cell damage *in vivo* based on the injury or formation of pores in the plasma membrane [18]. Thus, the cytotoxicity of 2-amino-thiophene

derivatives against human red blood cells was also evaluated. A safety level was demonstrated in these cells because all thiophenes showed a lower percentage of haemolysis (624.8 – >800 µM) compared to meglumine antimoniate (429.5 mg/L) and amphotericin B (11.6 µM), which exhibited high toxicity. Other thiophene derivatives were found to be weakly toxic to erythrocytes, [24,25] demonstrating the safety of this compound class. Similar to the macrophage cytotoxicity results, the presence of the indolic ring increased the selectivity indices of SB-44, SB-200, and SB-83 against the promastigote and axenic amastigote forms with superior values compared to the reference drugs. The comparison with other studies confirmed the selectivity of thiophenes for parasites of the genus *Leishmania* compared to host cells [9,19]. Moreover, the safety of these molecules may be a strong indication of low toxicity *in vivo* because many studies have reported a correlation between *in vitro* cytotoxicity assays and acute toxicity in animals and humans [26].

Trypanothione reductase (TyrR) is an NADPH-dependent flavoprotein that is present only in protozoan parasites from the genera *Trypanosoma* and *Leishmania*. The absence of TyrR in humans makes it an attractive target for rational drug design targeting leishmaniasis and trypanossomiasis [27]. The trypanothione system is essential for parasite survival because the dithiol trypanothione system is required for the synthesis of DNA precursors, the detoxification of hydroperoxides, and the sequestration/export of thiol conjugates [27–29]. Computational tools are typically used to aid in the development and discovery of new drugs based on the chemical structures and properties of biological targets (enzymes) with the aim of decreasing illness [30].

The enzyme TryR from *L. infantum* (PDB id: 2jk6) is broadly used as a leishmaniasis target in docking studies. In our study, we found that several of our thiophene derivatives had lower E_{dock} values compared to other compounds described in the literature as promising inhibitors of TryR with high affinity. The E_{dock} values found for SB-200, SB-44 and SB-83 (–

11.17, –10.29 and –9.81 Kcal/mol, respectively) were smaller than: Taxifolin (–8.82 Kcal/Mol) [28]; a series of various tricyclic and quinone derivatives (between –6.70 and –9.50 Kcal/Mol) [31]; and chromene-2-thione and benzo[*f*]chromene-2-thione analogues (between –6.82 and –9.20 Kcal/Mol) [29]. These results indicate that these thiophene derivatives may be potent inhibitors of TryR. The study of Gundampati and Jagannadham [28], confirmed our docking results and also identified several amino acid residues including the Ser-162 residue as important for ligand binding to the TryR binding pocket. The association between the experimental data (*in vitro* antileishmanial activity of thiophenes; Table 2) with the docking study results (Table 3) shows a high correlation between the results, providing additional evidence that the possible mechanism of action of these molecules may occur through the inhibition of TryR. However, additional biological studies using the enzyme need to be performed to validate this hypothesis. In both studies (experimental and *in silico*), the compounds that showed more pronounced activity (lower IC_{50} and E_{dock} values) were the same: compounds SB-200, SB-44 and SB-83. Likewise, compounds 6CN09, SB63 and SB80 were the seventh, eighth and ninth best ligands in the docking study and in the inhibition of the axenic amastigote forms, respectively.

In the present study, compounds SB-200, SB-44, and SB-83 displayed a better antileishmanial capability and an increased safety level for mammalian cells. Based on these characteristics, we provide the first insights into the mechanism of cell death induced by 2-amino-thiophene derivatives in *L. amazonensis* using the compounds SB-200, SB-44, and SB-83. For this purpose, we initially investigated the apoptotic-necrotic profile using Annexin V-FITC/PI staining. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that is typically used to investigate the externalization of phosphatidyl serine due to a high affinity for this phospholipid. Double-staining with PI, which is a DNA intercalator that only travels through damaged membranes, allows apoptotic cells to be distinguished from necrotic cells

[17]. Our results demonstrated that our thiophene derivatives acted through a direct mechanism on promastigote forms that was associated with apoptosis and accompanied by secondary necrotic cell death. The reference drug amphotericin B, which was known to cause cell death by apoptosis in *Leishmania* spp. [32] and was used in this study as a positive control, showed the same profile. In a qualitative analysis, the DNA fragmentation assay using agarose gel electrophoresis showed that the effect of the compounds SB-200, SB-44, and SB-83 on promastigotes led to fragmentation of the oligonucleosomal DNA, which was similar to the results observed with amphotericin B and confirmed the involvement of apoptosis in the mechanism of action of these substances. Different studies have provided a large number of promising substances that promote antileishmanial activity by inducing apoptosis of the promastigote and amastigote forms of *Leishmania* spp. These mechanisms are mainly related to the externalization of phosphatidyl serine, DNA fragmentation, and depolarization of the mitochondrial membrane potential [17].

In addition to direct effects on the parasite, in this study we also investigated the effects of the compounds SB-200, SB-44, and SB-83 in a model of *in vitro* infection using murine peritoneal macrophages. We ascertained the potential of these molecules to modulate the immune response of host cells. Experimental models with intracellular amastigotes represent the most effective way to relate antileishmanial activity of a drug *in vitro* to its potential *in vivo* because macrophages are the primary cells parasitized by *Leishmania* spp. in mammalian hosts [33]. The 2-amino-thiophene derivatives SB-200, SB-44, and SB-83 were more effective in treating macrophages infected with *L. amazonensis* than meglumine antimoniate, and they were more sensitive against intramacrophagic amastigotes compared to axenic amastigotes. The difference in antileishmanial activity may be indicative of macrophage activation, which combines their microbicidal power with direct effects previously demonstrated for the three compounds [34].

Macrophages are the main phagocytic cells of the host. They perform their microbicidal functions through structural (phagocytosis, vacuolization, spreading, and increased lysosomal volume) and cellular changes (altered cytokine levels, changes in reactive oxygen species (ROS) and NO profiles, and matrix metalloproteinase secretion) [18]. To investigate the mechanisms by which SB-200, SB 44, and SB-83 diminished the macrophage infection rate, we determined the immunomodulator effect by analysing the production of TNF-α, IL-10, IL-12 and NO by infected macrophages treated with the thiophenes. Our results suggest that the anti-amastigote effect of compounds SB-200 and SB-83 is associated with increased TNF-α, IL-12, and NO; in contrast, SB-44 appears to exert its antileishmanial activity only through a direct parasite killing action. Successful elimination of parasites of the genus *Leishmania* have been reported to be dependent on the stimulation of the immune system by the test drug via activation of host-protective Th1-dominated immune responses with the production of protective cytokines (IFN-γ, TNF-α, and IL-12) [35]. TNF-α is a proinflammatory cytokine produced by many cell types, although it is primarily produced by macrophages. It induces the activation of different signalling pathways, including MAPK and NF-κB (a major transcription factor that modulates iNOS gene expression and results in the subsequent production of NO) [36]. IL-12 is a proinflammatory cytokine produced by various cells types such as NK cells, polymorphonuclear cells and macrophages that is involved in macrophage activation and the induction of the production of INF-γ, TNF-α and NO [37]. Experimental models of *L. amazonensis* infection of murine macrophages showed that INF-γ and IL-12 were essential for parasite control [38], which was in agreement with our findings. Gomes et al. [39] demonstrated that the involvement of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) activity of *L. amazonensis* in the increase in the parasite's survival was linked to the down-modulation of macrophage production of IL-12, TNF-a and NO but not IL-10. We observed in the present work that the decrease in the infection rate was

dependent on NO production. In response to the signal initiated by the activation of protective cytokines, infected cells produced microbicidal molecules such as ROS and NO, which played a crucial role in the elimination of the parasite *in vitro* and *in vivo* [40]. In terms of antileishmanial activity, NO is the principal effector molecule involved in the killing of established intracellular amastigotes [41]. Previous works demonstrated that the control of *L. amazonensis in vitro* and *in vivo* was associated with the combination of NO and the superoxide anion, resulting in production of peroxynitrite ($ONOO^-$), a powerful, highly reactive oxidant that acts as a leishmanicide. This finding is in contrast to other species such as *L. major*, in which NO acts alone with a high leishmanicidal effect [42].

4. Conclusions

In conclusion, our results reveal that 2-amino-thiophene derivatives exhibit selective antileishmanial activity against both stages of *L. amazonensis* with lower levels of cytotoxicity to host cells. The effects of the selected compounds SB-200, SB-44, and SB-83 against the promastigote forms is associated with apoptosis involving phosphatidylserine externalization and DNA fragmentation accompanied by secondary necrotic cell death. The three compounds were effective against macrophage infection, and the anti-amastigote activity of SB-200 and SB-83 was associated with the modulation of the host immune response. These results encourage the progression of studies on these compounds for the development of new leishmanicidal agents.

5. Experimental section

5.1 Chemicals

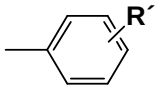
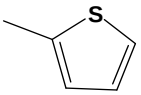
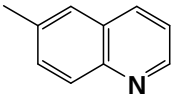
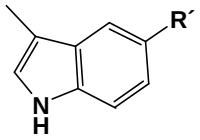
Schneider's medium, the antibiotics penicillin and streptomycin, tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) (MTT), dimethylsulfoxide

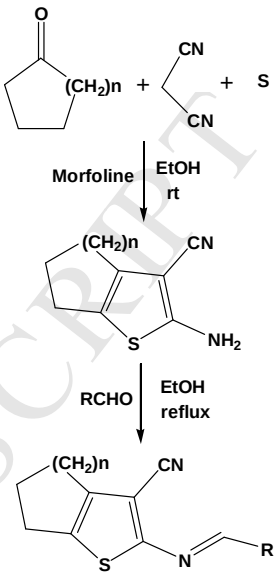
(DMSO, 99% purity) thioglycollate medium, sodium dodecyl sulphate (SDS), and Griess reagent (1% sulphanilamide in H_3PO_4 10% (v/v) in Milli-Q water) were purchased from Sigma Chemical (St. Louis, MO, USA). RPMI-1640 medium and foetal bovine serum (FBS) were obtained from Cultilab (São Paulo, SP, Brazil). The chemotherapy drug amphotericin B (Anforicin B®) was obtained from Cristália (São Paulo, SP, Brazil), and meglumine antimoniate (Glucantime®) was purchased from Aventis Pharma (São Paulo, SP, Brazil). The haematological stain kit Panótico Rápido was obtained from Laborclin (Curitiba, PR, Brazil). The sandwich ELISA assay kit and Annexin V-FITC/Propidium Iodide (PI) staining kit were obtained from eBioscience™ (San Diego, CA, USA).

5.2 Synthetic Chemistry

2-Amino-cycloalkyl[*b*]thiophene-3-carbonitrile derivatives (called simply thiophene derivatives in this work) were synthesized via a two-step reaction. The reaction started with the one pot reaction between malononitrile with a cyclic ketone and elemental sulphur in the presence of morpholine, followed by the Gewald procedure [16]. Treatment of the Gewald adducts with aromatic aldehydes in ethanolic medium and reflux afforded the target compounds 6CN01, 6CN09, 6CN10, SB-63, SB-80, SB-68, SB-81, SB-44, SB-83 and SB-200 according to previous procedures (Scheme 1). All compounds were previously synthesized and described by our group [12,14].

Scheme 1 - Synthesis and chemical structures of studied compounds.

Compound	n	R	R´
6CN01	2		H
6CN09	2		2-Br-5-OMe
6CN10	2		4-NO_2
SB-63	3		-
SB-80	1		-
SB-68	3		-
SB-81	1		-
SB-44	3		H
SB-83	2		5-Bromo
SB-200	3		5-Bromo



5.3 X-Ray crystal structures of SB-83 and SB-200

The crystals of the compounds SB-83 and SB-200 were obtained from ethanol solution using the slow evaporation method at 30°C under saturating conditions. The obtaining procedures of experimental data, cell refinements, data reduction and resolution of the crystal structure were performed according as described in Franscisco et al. [43] and are briefly described. The experimental data set was obtained using a Bruker Enraf - Nonius Kappa CCD diffractometer. The cell refinements were performed using the software Collect and Scalepack, and the final cell parameters were obtained on all reflections. Data were collected up to 28.2° in θ for SB-83 and up to 27.9° for SB-200, resulting in 15,624 and 15,822 Bragg reflections, respectively. Data reduction was performed using the software Denzo-SMN and Scalepack. Absorption was found to have a significant effect on both compounds (2.517 mm^{-1} for SB-83 and 2.478 mm^{-1} for SB-200); therefore, absorption correction was applied. The structure was solved using the software SHELXS-97 and refined using the software SHELXL-97. The C, N and O atoms were clearly solved and the full matrix least-squares refinement of these atoms with anisotropic thermal parameters was performed. The hydrogen

atoms were positioned stereochemically and refined with the riding model, except when they were found to be involved in hydrogen bonds on the electronic density map. The details concerning data collection and structure refinement were prepared using WinGX (version 1.70.01). The ORTEP-3 program was used to prepare the figures. The complete crystallographic data have been deposited at the Cambridge Crystallographic Data Center as supplementary publications no. CCDC 1029425 (SB-83) and no. CCDC 1027323 (SB-200). Copies of the data are available upon application to The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (e-mail: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk).

5.4 Docking Studies

The molecular modelling was performed as described in Scotti et al. [44]. Using the program Hyperchem v. 8.0.3, the chemical structures of the compounds of interest were drawn and their geometry was optimised using the MM+ force field. Afterwards, we performed a new geometry optimisation based on the semi-empirical method AM1 (Austin Model 1). The optimised structures were subjected to conformational analyses using the Spartan for Windows 10.0 software. We selected the Monte Carlo search method with 1,000 interactions, 100 cycles of optimisation, and 10 conformers with the lowest minimum energy. The dihedrals were evaluated by rotation in accordance with the standard (default) conditions of the program, in which the number of simultaneous variations was 1 to 8, acyclic chains were subjected to rotations from 60 to 180° and torsion rings were in the range of 30 to 120°. The conformers with the lowest minimum energy were selected and saved in .sdf format. The docking simulations were performed on the AutoDock 4.2 software. Receptor and ligand preparation was performed using VEGA ZZ 3.0.1 and MOLEGRO MOLECULAR VIEWER 2.5. Initially, the structures of the ligand and receptor were saved in pqbqt format to be used for docking calculations. PyRx 0.9 software was used to aid the steps of job submission and

the analysis of the results. The grid maps that represented the intact ligand in the actual docking trypanothione reductase (TryR) enzyme site were calculated with AutoGrid. The three dimensional grid box was created with a 60 Å grid size (x, y, z) and spacing of 0.300 Å. Each ligand was docked into this grid with the Lamarckian algorithm implemented in AutoDock. The genetic-based algorithm ran 12 simulations per substrate with 2,500,000 energy evaluations and a maximum number of 54,000 generations. The crossover rate was increased to 0.8, the rate of gene mutation was 0.02, and the number of individuals in each population was 200. All other parameters were left at the AutoDock default settings. The results for each calculation were analysed to obtain the affinity energy (Kcal/mol) values for each ligand conformation in its respective complex; probable structure inaccuracies were ignored in the calculations. To verify the number of hydrogen bonds and non-covalent interactions between each ligand conformation and the catalytic residues of the TryR enzyme, the programs PyMOL 1.4 and MOLEGRO MOLECULAR VIEWER 2.5 were employed [44].

5.5 *Leishmania* culture conditions

Promastigote forms of *Leishmania* (*Leishmania*) *amazonensis* (IFLA/BR/67/PH8) used in this work were maintained at 26ºC in supplemented Schneider's medium (20% heat-inactivated foetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/L streptomycin). The parasites were maintained as amastigotes *in vivo* in Swiss mice infected in the left hind paw [2]. Extracellular axenic amastigote forms were obtained following the methodology described previously [41]. Promastigote forms in stationary growth phase were differentiated into axenic amastigotes by the combination of a temperature increase (32ºC) and pH decrease (4.6).

5.6 Animals and peritoneal macrophages

Male and female Swiss mice (8-10 weeks old) were obtained from the biotherium section of the Professor Thomas George Animal House (Biotechnology Center, Federal University of Paraíba, João Pessoa, Brazil) and maintained under controlled temperature (21±1ºC; relative humidity 60%) and photoperiod (12-h light/dark cycle) conditions with free access to food pellets and water.

5.7 Activity assay against promastigotes and axenic amastigotes of *L. amazonensis*

Inhibition of promastigote growth was assayed as previously described [45]. Briefly, promastigotes (1 x 10^6 cells) were harvested in the exponential growth phase and incubated in supplemented Schneider´s medium in the presence and absence of different concentrations of the 2-amine-thiophene derivatives and the reference drugs meglumine antimoniate and amphotericin B. The cultures were kept for 72 h in a biological oxygen demand (B.O.D.) incubator at 25ºC; then, the effects of each compound were evaluated using a Neubauer haemocytometer. Axenic amastigotes were seeded into 96-well culture plates (1 x 10^6 per well) in the presence and absence of different concentrations of thiophenes and reference drugs at 32º for 24 h; the viability was evaluated by adding 10 µL of MTT (5 mg/mL). After a 4-h incubation, 10% SDS was added to dissolve the formazan crystals and the absorbance was evaluated using a spectrophotometer (Biosystems model ELx800, Curitiba, PR, Brazil) at 540 nm [18].

5.8 *In vitro* cytotoxicity on murine macrophages

Murine peritoneal macrophages were collected from the peritoneal cavity of Swiss mice 5 days after elicitation with 1 mL of thioglycollate medium (3% in distilled water) as described previously [46]. Animals were treated according to Resolution number 1000 (2012)

of the Federal Council of Veterinary Medicine, Brazil, and all experimental protocols were approved by the Ethics Committee for Animal Research (CEPA) of the Federal University of Paraíba (process number 0911/14).

Macrophage viability was determined using the MTT test [47]. Approximately 1 x 10^6 macrophages per well were seeded into 96-well plate plates with supplemented RPMI 1640 medium (10% heat-inactivated foetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/L streptomycin) in the presence or absence of thiophenes and reference drugs at 37ºC and 5% CO_2 for 24 h. After incubation, cytotoxicity was determined by adding 10 µL of MTT (5 mg/mL); after 4h, 10% sodium dodecyl sulphate (SDS) was added. Finally, readings were performed at 540 nm on a microplate reader.

5.9 Red blood cell lysis assay

The haemolytic activity of thiophenes and the reference drugs was determined using human red blood cells according to the method of Jain et al. [48]. Briefly, 80 µL of a 5% erythrocytes/PBS suspension was mixed with 20 µL of a series of concentrations of thiophenes, meglumine antimoniate, and amphotericin B and incubated at 37ºC for 1 h. From each suspension, 200 µL of phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, and 2.6 mM KCl, pH 7.2) was added to stop the haemolysis process and the sample were centrifuged for 10 min at 1000 x g. Then, the supernatant was collected and haemolysis was measured spectrophotometrically (540 nm). The haemolysis percentage was determined as $[(Abs_{sam} - Abs_{con})/(Abs_{tot} - Abs_{con}) \times 100$, where Abs_{sam} was the absorbance of the samples, Abs_{con} was the absorbance of the blank control (without drugs), and Abs_{tot} was the absorbance of total haemolysis (replacing the samples solution with an equal volume of Milli-Q water).

5.10 Apoptotic-necrotic profiling with Annexin V/PI staining

Promastigotes (1×10^6) in the logarithmic growth phase were incubated with or without 1x, 2x, and 4x IC_{50} values of thiophenes SB-200, SB-44, SB-83 and amphotericin B for 4 h, washed three times in cold PBS and re-suspended in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) according to the manufacturer's protocol. Then, the cells were stained using the FITC Annexin V/Dead Cell Apoptosis Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Stained cells were diluted in Annexin V-binding buffer (BD Biosciences, San Jose, CA, USA). Suspended cells were used to perform flow cytometry. Annexin V-FITC/PI-stained cells were analysed using a BD FACSCanto® II flow cytometer (BD Biosciences, San Jose, CA, USA). In total, 30,000 cells were analysed per measurement. The data were analysed using the FlowJo 10.0.7 software (TreeStar Inc., Ashland, OR, USA) [49].

5.11 DNA fragmentation assay by agarose gel electrophoresis

Qualitative analysis of DNA fragmentation from logarithmic growth-phase *L. amazonensis* promastigotes (untreated or treated with 1x, 2x, and 4x IC_{50} values of SB-200, SB-44, SB-83, and amphotericin B for 24 h) was performed by agarose gel electrophoresis. After exposure to the drugs, cells were subjected to DNA extraction using the chloroform/phenol method as previously described [2]. Genomic DNA was run on a 2% agarose gel containing the Gel Red Nucleic Acid Gel Stain at a 1:500 dilution (10,000x Biotium®) for 1 h at 100 mV and visualised using a UV transilluminator (ImageMaster®).

5.12 Treatment of infected macrophages

Peritoneal macrophages were harvested and plated on round glass coverslips in 24-well culture plates at a density of 1 x 10^6 cells/mL in supplemented RPMI 1640 medium for 2 h at

34ºC and 5% CO_2 to allow cell adhesion. Then, *L. amazonensis* stationary-phase promastigotes (in a ratio of 10 promastigotes to 1 macrophage) were added to each well. After 3 h of infection at 5% CO_2 and 34ºC, the wells were washed with PBS and new medium was added with different concentrations of SB-200, SB-44, SB-83 or meglumine antimoniate as the reference drug. Plates were incubated for 24 and 72 h; then, the coverslips were removed, fixed, and stained with the Panoptic staining kit. For each coverslip, 300 macrophages were analysed by light microscopy [36].

5.13 Estimation of Th1/Th2 cytokine and nitric oxide (NO) production

The supernatants of infected macrophages treated and untreated with thiophene derivatives were harvested after 24 and 72 h and stored at -20ºC for TNF-α, IL-10, IL-12, and NO production analysis [36]. Cytokine production was analysed by sandwich ELISA assay according to manufacturer's instructions. NO production was estimated from nitrite levels by the Griess reaction. LPS (10 mg/L) was used as a positive control. Cytokine (pg/mL) and NO (µM) concentrations were extrapolated from a standard curve with murine recombinant cytokines and sodium nitrite, respectively. At the end of the experiment, the analysis was performed using a microplate reader at 450nm (cytokines) and 540 nm (NO).

5.14 Statistical analysis

All assays were performed in triplicate and in 3 independent experiments. One-way ANOVA followed by the Bonferroni test was used to compare differences between groups using a p value <0.05 as the maximum level of statistical significance. GraphPad Prism® software version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis. The 50% inhibitory concentration (IC_{50}), 50% effective concentration (EC_{50}) 50% cytotoxic concentration (CC_{50}), and 50% haemolytic concentration (HC_{50}) values

were calculated using the probit regression model (SPSS program, version 13.0, Chicago, IL, USA).

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Conflict of interest

The authors declare they have no conflict of interests for this work.

Author contributions

Conceived, designed and performed the experiments: KAFR, CNSD, PLNN, JCR, MTS, LS, SRM, RCV, IAM, TSLK, TBO, MCAL, TLB, TMA, ROM, FJBMJ, MRO. Analyzed the data: KAFR, PLNN, FJBMJ, MRO. Wrote the paper: KAFR, FJBMJ, MRO. All the authors have read, approved and made substantial contributions for the manuscript.

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HIGHLIGHTS

- 10 thiophene derivatives showed antileishmanial activity *in vitro* to *L. amazonensis*;
- All thiophenes exhibited selectivity indexes (SI) greater than reference drugs;
- Selected compounds (**SB-200**, **SB-44**, and **SB-83**) induce apoptosis in promastigotes forms;
- **SB-200**, **SB-44**, and **SB-83** reduced the infection index of macrophages by *L. amazonensis*;
- **SB-200**, and **SB-83** stimulated TNF-α, IL-12, and NO production in macrophages infected.