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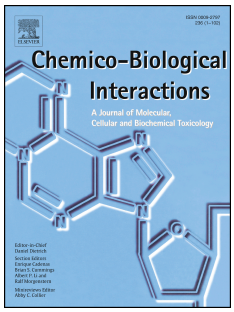

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Novel indol-3-yl-thiosemicarbazone derivatives: obtaining, evaluation of *in vitro* leishmanicidal activity and ultrastructural studies

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Abstract

Parasitic diseases still represent serious public health problems, since the high and steady emergence of resistant strains is evident. Because parasitic infections are distributed predominantly in developing countries, less toxic, more efficient, safer and more accessible drugs have become desirable in the treatment of the infected population. This is the case of leishmaniasis, an infectious disease caused by a protozoan of the genus Leishmania sp., responsible for triggering pathological processes from the simplest to the most severe forms leading to high rates of morbidity and mortality throughout the world. In the search for new leishmanicidal drugs, the thiosemicarbazones and the indole fragments have been identified as promising structures for leishmanicidal activity. The present study proposes the synthesis and structural characterization of new indole-thiosemicarbazone derivatives **(2a-j)**, in addition to performing *in vitro* evaluations through cytotoxicity assays using macrophages (J774) activity against forms of *Leishmania infantum* and *Leishmania amazonensis* promastigote as well as ultrastructural analyzes in promastigotes of *L. infantum*. Results show that the indole-thiosemicarbazone derivatives were obtained with yield values varying from 32.09 to 94.64%. In the evaluation of cytotoxicity, the indole-thiosemicarbazone compounds presented CC_{50} values between 53.23 and 357.97 μM. Concerning the evaluation against *L. amazonensis* promastigote forms, IC_{50} values ranged between 12.31 and> 481.52 μM, while the activity against *L. infantum* promastigotes obtained IC_{50} values between 4.36 to 23.35 μM. The compounds **2d** and **2i** tested against *L. infantum* were the most promising in the series, as they showed the lowest IC_{50} values: 5.60 and 4.36 respectively. The parasites treated with the compounds **2d** and **2i** showed several structural alterations, such as shrinkage of the cell body, shortening and loss of the flagellum, intense mitochondrial swelling and vacuolization of the cytoplasm leading the parasite to cellular unviability. Therefore, the indole-thiosemicarbazone

compounds are promising because they yield considerable synthesis, have low cytotoxicity to mammalian cells and act as leishmanicidal agents.

Keywords: *Leishmania infantum*; *Leishmania amazonensis;* thiosemicarbazone; indole derivatives; ultrastructural studies.

1. Introduction

World health remains affected by the consequences of infectious diseases, especially parasitic diseases, since they are one of the major causes of high morbidity and mortality. Therefore, it is necessary to search a form of controlling these diseases and, most of all, their outbreaks [1]. According to the World Health Organization (WHO), leishmaniasis, a parasitic disease caused by protozoan Leishmania spp. Is still classified as emerging and uncontrolled disease with an annual incidence of 1.5 to 2 million cases, posing risk of infection for more of 350 million people [2,3].

It is estimated that there are more than 22,000 cases of individuals infected with leishmaniasis in Brazil, predominantly in the Amazon region, where the insect vectors belonging to the genus Phlebotomus and Lutzomyia are found. These insects are responsible for the transmission of parasitosis [4]. Depending on the species at the time of infection, the parasite and the immune response of the infected host, the clinical manifestations may vary in cutaneous, mucocutaneous and visceral, the latter being the most aggressive, becoming fatal in 85- 90% of untreated patients [5-7].

The ideal treatment for leishmaniasis is still chemotherapy, through the use of pentavalent antimonial compounds (sodium stibogluconate and meglumine antimoniate), including second-choice compounds such as amphotericin B, pentamidine and miltefosine [8,9]. However, these therapies are quite toxic at the same time as they exhibit other

limitations such as the high cost and necessity of daily parenteral administration, which characterizes an invasive intervention in the long term [10-12]. In addition to the aforementioned drawbacks, there are also reports indicating the emergence of strains resistant to the treatment of leishmaniasis [13-15].

Hence, the search for new, more effective, less toxic, safe and more suitable leishmanicide chemotherapeutics is severely necessary. And it is above all in this context that several *in vitro* and *in vivo* studies have been developed using synthetic and leishmanicidal potential compounds already known.

A class of molecules that has been widely studied due to its chemical and pharmacological properties are the thiosemicarbazones, which stand out due to their leishmanicidal biological activity. Thiosemicarbazone derivatives have shown efficacy against promastigotes and amastigotes of *Leishmania* with possible mechanisms of action elucidated, acting in the depolarization of the mitochondria, interacting with the DNA, causing the death of the parasite by apoptosis, besides inhibiting proteins of the parasite [16-18].

Another fragment of interest in medicinal chemistry is the indole nucleus. As a privileged structure, it stands out in the antiparasitic activity because it constitutes several promising molecules of biological interest [19,20]. On the antimalarial activity (*Plasmodium falciparum*), the indole is indicated as a key point for the active site binding and histone deacetylase inhibition [21]. In addition, indole-containing compounds demonstrated activity against trypanosomatida trypanothione reductase (*Trypanosoma brucei*, *Trypanosoma cruzi* and Leishmania spp.) [22].

Recently, our research group has pointed to the leishmanicidal activity of new indole-thiophene hybrids to verify the action of these compounds, against promastigotes and amastigotes forms, in the immunomodulation and their role in the inhibition of trypanothione

reductase [23,24].This work investigated the chemical synthesis of new indole-thiosemicarbazone derivatives and evaluated their cytotoxic and biological profile through *in vitro* leishmanicidal investigation against the *Leishmania infantum* and *Leishmania amazonensis*.

2. Results

2.1 Chemistry

Synthesis of new indole-thiosemicarbazone derivatives was conducted in two steps (Scheme 1). First, intermediary compounds were obtained (thiosemicarbazide) through nucleophilic addition between substituted hydrazine and isothiocyanate. The second step consisted of a condensation reaction in which the synthesized thiosemicarbazide reacted to the substituted indole-carboxaldehyde in the presence of a catalyst quantity of hydrochloric acid forming interesting compounds with results that ranged from 32.09 to 94.64% of yield.

Analysis of the infrared spectrum showed that ligation (C-S) appears in the region (1197.67-1450.51) cm^{-1}, the band (C=N) was found in the region (1541-1628.36) cm^{-1}, which corroborated data from the literature in which the imino function appears in 1556-1630 cm^{-1} and the band (N-H) in the region (3105.8-3496). The possibility of tautomerism (thione-thiol) due to absence of bands in the region 2500-2600 cm^{-1} [25] was excluded.

NMR spectrum ^{1}H evidenced the presence of hydrogen in the ligation (HC=N) that is presented as singlet between. Signals referring to hydrogen in the methyl ($-CH_3$) group of indole between 2.41-2.50 ppm were also observed. In addition, signals between 11.22–11.64 and 11.18-12.35 ppm were attributed to hydrogen in the groups NH hydrazine and NH indole, respectively. The signal referring to NH phenyl appears between 8.01-9.69 ppm. This signal is more protected than the other NH signals due to the resonance effect likely to happen between Nitrogen and Caron in the thiocarbonyl (-NH-C=S; -N=C-SH) [26]. Therefore, after the

structural elucidation, the compounds that were planned and synthesized in this study were subjected to biological evaluation.

PLEASE, INSERT SCHEME 1 HERE

2.2 Biological assays *in vitro*

2.2.1 Cytotoxicity assay

Cytotoxicity analysis employed macrofage (J774) and allowed observation of cytotoxic variation between the compounds (53.23 to 357.97µM) (Table 1). Compounds **2a** and **2b** substituted in the *N-4* position of the thiosemicarbazone with hydrogen were the least cytotoxic in the series, showing values of respectively. These compunds displayed greater toxicity than drugs of reference that showed CC_{50} values of 37.6 and 219.05µM (AmB and Mtf).

Substitution of *N-4* position of the thiosemicarbazone with *p*-methyl-phenyl showed decrease in CC_{50} with values of 118,31 and 135,25µM for compounds **2g** and **2h** respectively. The other replacements (hydrogen, phenyl, *p*-ethyl-phenyl *p*-methoxy-phenyl) also showed decrease in toxicity, however they also displayed values of CC_{50} higher than 50µM.

2.2.2 Biological activity in *Leishmania infantum* promastigotes

All ten compounds assessed in the present investigation are structurally different in two aspects. The first differentiating aspect for compounds in the series are the substituents found bound to *N-4* position of the thiosemicarbazone (hydrogen, phenyl, *p*-methyl-phenyl, *p*-ethyl-phenyl *p*-methoxy-phenyl). The additional structural difference concerns the pattern of substitution of the indole ring (series 5-bromine-7-methyl/series 5-methyl-7-bromine). The biological repercussion promoted by these molecular modifications were investigated.

Leishmanicidal evaluation verified the efficacy of indole thiosemicarbazone on the promastigote forms of *Leishmania infantum.* All compounds in the series in the current investigation were able to inhibit the promastigote forms of with IC_{50} that ranged from de 4.36 to 23.25µM. Non-substitution (hydrogen) in the *N-4* position of the thiosemicarbazone displayed better results for compounds **2a** and **2b** with significant IC_{50} values of 16.70 and 13.51µM. However, substitution with *p*-methyl-phenyl group did not favor activity, significantly increasing as occurred for compound **2g** that obtained value of 23.35µM. The other substitutions with (phenyl, *p*-ethyl-phenyl, *p*-methoxy-phenyl) exhibited better activity in the molecule, particularly compound **2d** with phenyl substituent and **2i** with *p*-ethyl-phenyl substituent, both with bromine at C-5 and methyl at C-7 of the indole, displaying better leishmanicidal activity with IC_{50} values of 5.60 and 4.36µM respectively (Table 1).

Leishmanicidal evaluation concerning the promastigote forms of de *L. amazonensis* for all compounds in the series were also capable of inhibiting promastigote forms with IC_{50} values that ranged from 12.31 to > 481.52µM (Table 1). Substitution in the *N-4* position of the thiosemicarbazone with hydrogen, *p*-methyl-phenyl did not effectively increased Leishmanicidal activity displaying values of 87.76, 75.24 and 134.38µM for compounds **2a, 2f** and **2g.** Similar to *L. infantum* testing, phenyl substitutions and *p*-ethyl-phenyl displayed better results with IC_{50} values of 13.61 and 12.31µM for compounds **2c** and **2i**.

Evaluation of the selectivity index (SI) for compounds tested against *L. infantum* displayed better results for compounds **2a**; **2b**; **2d**; **2f** and **2i**, which displayed SI values of 14.24; 26.49; 17.97; 13.45; and 12,21 respectively. However, the compounds with the best results were the compounds **2d** and **2i** because in addition to displaying better selectivity to the parasite than a mammalian cell, they also displayed lower IC_{50}. Evaluation of compounds against *L. amazonensis* showed better results for derivatives **2b**, **2c**, **2h** and **2i** with values of

14.97; 4.67; 6,91 and 4, 32 respectively; all of the mentioned compounds displayed SI values higher than the standard drug (Miltefosine SI of 3,87).

The best compounds in the series were **2d** and **2i** as they presented good SI and the lowest IC_{50}. Thus, they were chosen for ultrastructural investigation of *L. infantum* and *L. amazonensis.*

PLEASE, INSERT TABLE 1

2.2.3 Structural analysis of *L. infantum* promastigotes

Ultrastructural evaluation used the compound with best leishmanicidal activity, compound **2d**, with substituent phenyl and compound **2i** with *p*-ethyl-phenyl both with bromine at C-5 and methyl at C-7 of the indole nucleus.

Ultrastructural analysis through scanning electron microscope (SEM) showed that untreated *L. infantum* promastigotes display fusiform morphology typical of the parasite in addition to a body surface topologically normal with long and free scourge (Figure 1 A-B). Promastigotes subjected to amphotericin B at 0.05 µM presented ultrastructural alterations such as shrinkage and deformation of the cell body and flagellum, alterations in the membrane with cell destruction (Figure 1 C-D).

PLEASE, INSERT FIGURE 1

L. infantum promastigotes treated with the compound **2d** at 5.6 µM and 11.2 µM concentrations displayed alterations on the parasite surface (Figure 2 A-D). Morphological alterations could be noted such as body cell shrinkage, which changed the fusiform shape to oval (Figure 2 A-B). In addition, the parasite membrane displayed undulation and

disintegration of the membrane (Figure 2 B-C) as well as shrinkage and flagellum loss (Figure 2 C-D). These alterations were similar to the ones observed in promastigotes subjected to amphotericin B at 0.05 µM.

PLEASE, INSERT FIGURE 2

Compound **2i** was also capable of causing alterations in *L. infantum* promastigotes for both concentrations 4.36 µM and 8.72 µM. Alterations were similar to the ones described after treatment with amphotericin B and compound **2d**. When subjected to 4.36 µM concentration, alterations in shape and size of promastigotes were noted with pronounced shrinkage of the body cell and flagellum retraction (Figure 3A), and the presence of several grooves in the outside surface of the protozoan was also noted (Figure 3B). Grooves were also noted at concentration 8.72 µM (Figure 3C) in addition to alterations in cell morphology and atypical cell division (Figure 3D).

PLEASE, INSERT FIGURE 3

Transmission electron microscopy (TEM) analysis showed that in the untreated group, *L. infantum* promastigotes displayed normal shape and morphology with well-preserved mitochondria, nucleus, flagellum pouch and flagellum (Figure 4 A-C). In cells treated with amphotericin B at 0.05 µM, it was noted the presence of mitochondrial swelling, pyknotic nucleus, intense vacuolization of the cytoplasm, electrodense particles and increase in chromatin condensation (Figure 4 D-F).

PLEASE, INSERT FIGURE 4

L. infantum promastigotes when subjected to treatment with compound **2d** at concentration (Figure 5 A-C) displayed alterations similar to the ones caused by amphotericin B at 0.05 µM. It was noted pyknotic nucleus, intense vacuolization of the cytoplasm, electrodense particles, intense mitochondrial swelling and disorganization of the kinetoplast. Subjected to treatment at 11.2 µM concentration, it was possible to observe the presence of electrodense particles, loss of cytoplasmatic content leading to cell destruction (Fig.5 D-F)

PLEASE, INSERT FIGURE 5

Compound **2i** for both 4.36 µM and 8.72 µM concentrations presented alterations in the cell cytoplasm, mitochondrial swelling with severe decrease in electrodensity of the matrix and appearance of concentric membrane structures within the organelles (Figure 6 A-F). Intense vacuolization was noted in the cytoplasm (Figure 6 A-B, D-E). The presence of autophagy vacuole (Figure 6 A-B) was noted at the smallest concentration, and at the highest it was noted the pyknotic nucleus with increase in condensation of chromatin, noting its marginalization.

PLEASE, INSERT FIGURE 6

3. Discussion

Cytotoxicity analysis of a compound is one of the criteria used to develop new drugs. A promising compound is classified when presenting low toxicity in mammalian cells [27]. All compounds in the indole-thiosemicarbazone tested in this investigation displayed low toxicity regarding mammal cells. Similar to Moreira et al. [28] who also tested cytotoxicity of

thiosemicarbazone derivatives and noted low toxicity values. Therefore, it can be inferred that thiosemicarbazone derivatives present potential in the production of new drugs.

All indole-thiosemicarbazone derivatives tested in this investigation were capable of inhibiting *L. infantum* and *L. amazonensis* promastigote forms. Such activity can be related to the presence of the indole group and substitutions at position *N-4* of thiosemicarbazones. The indole group has gained attention of the scientific community due to its therapeutic use [29]. Indole is commonly found in nature, in animal hormones as serotonin and melatonin and also present in plants in the constitution of some alkaloids [30]. Therefore, some natural and synthetical compounds containing indole have displayed antitumor [31], antiviral [32], antimicrobial [33], anti-inflammatory [34] and leishmanicidal [35].

Compounds **2c**, **2d** and **2i** presented the best values of IC_{50} when tested against *L. infantum*: 6.12; 5.60 and 4.36µM respectively. Compounds **2c** and **2i** also presented lower values when tested against *L. amazonensis*, with IC_{50} value of 13.61 and 12.31µM respectively. The compounds evaluated in this study have the disubstituted indole nucleus (5-CH_3/7-Br or 5-Br/7-CH_3). However, based on the results mentioned above we can conclude that the position of bromine or methyl group at the indole nucleus did not influence directly the leishmanicidal activity. Nonetheless, the importance of halogens as substituents has been described in the literature, such as the study by Hernandes et al. [36] with thiazolyl hydrazone against a *T. cruzi*, when the presence of halogens was noted to increase the interaction between the receptor and the drug, in addition to conserving permeability of the plasmatic membrane thus allowing the entrance of the drug into the cell. This justifies the greater activity of compounds with these groupings [37]. Furthermore, the presence of aromatic sessions and halogens in the molecules showed overall improvement in biological activity of the molecules against trypanosomatids [18, 38-41].

The compounds evaluated also presented structural difference with substituents in the *N-4* position of the thiosemicarbazone such as hydrogen and phenyl, *p*-methyl-phenyl, *p*-methoxy-phenyl, *p*-ethyl-phenyl and *p*-methoxy-phenyl groups. In the present study, substitutions with hydrogen, *p*-methoxy-phenyl and *p*-methyl-phenyl were not efficient to promote compound activity. Carvalho et al. [42] studied derivatives of thiosemicarbazone substituted in the *N-4* position with hydrogen and tested against *L. amazonensis* also not finding significant reduction in promastigotes. Substitution in the position *N-4* with *p*-methoxy-phenyl in works with thiosemicarbazone derivatives tested for *L. major* promastigotes such as ours, also did not present reduction in IC_{50} values [17]. In Melos et al. [43] thiosemicarbazone substituted in the position *N-4* with *p*-methyl-phenyl presented the best IC_{50} values but in the present study this substitution did not increase significantly the action of compounds **2g** and **2h** when tested against *L. infantum*. However, assessment against *L. amazonensis* showed compound **2h** displayed IC_{50} values of 19.58 and SI de 6.91, a higher selectivity index than the standard drug (Mtf 3.87).

Derivatives with substituents phenyl and *p*-ethyl-phenyl displayed the best activity for both strains (*L. infantum* compounds **2d** and **2i**) and (*L. amazonensis* compounds **2c** and **2i**), thus allowing the inference that the action is related to these substituents. The presence of substituents on the phenyl group at the *N-4* position of thiosemicarbazones was described in the literature and showed significant results against forms of *T. brucei* trypomastigotes [44]. Regarding the *p*-ethyl-phenyl, Saad et al. [45] noted moderate activity of compounds with this substituent. These results corroborate data found in the present study, in which compounds were also active. This can be related to substitution in the *N-4* position of the thiosemicarbazone, as also described by Pervez et al. [17].

Investigations on the ultrastructural alterations was conducted with two compounds chosen by their SI values as well as lower IC_{50}. Based on these criteria, two compounds stand

out when tested against, **2d** and **2i** with IC_{50} values of 5.60 and 4.36µM. The activity of these compounds can be seen through ultrastructural alterations that can lead to cell unviability thus reinforcing claims by Menna-Barreto et al. [46] who suggest that a great number of morphological alterations is always related to loss of cell viability and parasite death. Rounding and shrinkage of the cell body are described in the literature as characteristic events of death by apoptosis when despite alterations in the plasmatic membrane, it remains intact [47].

The alterations seen in the morphology of the parasites such as undulation in the membrane, changes in the form of promastigotes and shortening of the flagellum were also described in different studies. Dos Santos-Aliança et al. [48] assessed chemical compounds derivatives of phthalimido-thiazole against *L. infantum* promastigotes and observed the presence of undulations in the membrane. Brita et al. [16] tested thiosemicarbazone derivative of *S*-Limonene to find severe cell alterations in *L. amazonenses* at 13.9 µM concentrations. This was also found in the present study when *L. infantum* promastigotes were also subjected to compound **2i** at 8.72 µM concentration. Bilbão-Ramos et al. [49] investigated sulfonamide derivatives against *L. infantum* and observed changes in the morphology of the cell body and shortening of the flagellum.

Compounds **2d** and **2i** displayed leishmanicidal activity affecting the parasite mitochondria, an alteration that can be observed through TEM. Trypanosomatides are characterized for having a single branched mitochondrion along the body in which a region known as kinetoplast holds 30% of the DNA material of the protozoan known as k-DNA [50,51]. Therefore, the proper functioning of this organelle is important for the production of ATP and its alteration can lead to death which makes the mitochondria an important target for synthetic drugs [52,53]. Savoia et al. [54] report that the activity of a synthetic peptide caused severe mitochondrial swelling in strains of *L. infantum* and *L. amazonensis* as demonstrated

by the molecules in our study. Ferreira et al. [53] reported that piperine derivatives and phenyl-amide induced death of *L. amazonensis* promastigotes thus affecting directly the mitochondria physiology of the parasites. Based on both the excessive mitochondrial swelling that the indole-thiosemicarbazone induced and the literature, it is possible to claim that the parasite mitochondria is potential target for synthetic compounds.

Compounds **2d** and **2i** induced intense vacuolization and the presence of electrodense material. Such alterations were also present in the study by Ramírez-Macías et al. [55] who tested triazolopyrimidine against *L. braziliensis* promastigotes. De Mello et al. [56] used the same leishmania species to analyze chalcones and noted intense vacuolization as well as complete disorganization of the cytoplasm as occurred to compound **2d** at 11.2 µM concentration. Phenethylamine derivatives tested against *L. infantum* triggered the presence of electrodense particles and vacuolization which suggests that the presence of such alterations is related to cell death [57]. This finding corroborates investigations by Kaczanowski et al. [58] who studied eukaryotic cells and described alterations in the morphology of cells with vacuolization of cytoplasm as characteristic of cell death.

Therefore, it is possible to claim that compounds **2d** and **2i** are related to leishmanicidal activity and their mechanism of action implicates alterations in cell morphology and organization that leads the parasite to death. The death of parasites in this study presented characteristics of apoptosis such as mitochondrial swelling, condensation of chromatin and shrinkage of the cell body [59]. This is a significant type of cell death in parasites because it promots the removal of the parasite without inflammatory processes in the host organism [60, 61].

Finally, results allow the conclusion that indole-thiosemicarbazone compounds are likely prototypes for the development of leishmanicidal compounds. Further investigation is necessary to support the applicability of such compounds in the production of new drugs.

4. Experiment Section

4.1 Chemicals

The reagents used in this study are easily found and commercially available (Sigma-Aldrich, Acros Organics, VETEC). The fusion points were determined by Quimis 340 (Quimis, Brazil). The development of the reactions was analyzed through thin layer chromatography (TLC) (Merck, silica gel 60 F254 in aluminum foil) using ultraviolet light reading (254 or 366 nm). RMN spectrum were registered with Varian Unityplus (400 MHz for ^{1}H and 100 MHz for ^{13}C), the DMSO-d6 used as solvent was acquired from Sigma-Aldrich. IV spectrum were registered with spectrophotometer model Bruker IFS66 FT-IR (Bruker, Germany) using KBr disks. Chemical deviation is given in ppm and the multiplicities are given as s (singlet), d (duplet), t (triplet) and coupling (C) in Hertz. Mass spectrometry experiments were conducted in MALDI-TOF Autoflex III (Bruker Daltonics, Billerica, MA, USA).

4.1.1 General Synthesis to obtain thiosemicarbazide

Equimolar amounts of hydrazine hydrate 0.8361 mL (0.00061 mol) were added slowly in solution with equimolar amounts of substituted isothiocyanate 0.377 mL (0.00061 mol) and 20 of mL of dichloromethane. The reaction was kept in reflux for 3 hours at room temperature. The product was filtered, rinsed with dichloromethane and dried in vacuum desiccator [26].

4.1.2 General synthesis to obtain thiosemicarbazone

Equimolar amounts of substituted aromatic aldehydes 0,05 g (0.00021 mol), with 15mL of ethylic alcohol and hydrochloric acid as catalyst. After 20 minutes, the thiosemicarbazides were added in equimolar amounts. The reaction was kept in reflux for 24

hours, the product was filtered and purified through washings with ethylic alchol and dried in vacuum desiccator [26].

4.1.2.1 2-((5-bromo-7-methyl-1H-indol-3-yl)methylene)hydrazinecarbothioamide (2a)

Purple powder; MP: 239 °C; Yield: 37.54%; Rf: 0.46 (*n*-hexane/ethyl acetate 1:1). NMR ^{1}H (DMSO, $d_{6,}$ 400 MHz, ppm): δ 2.47 (s, 3H, CH_3), δ 7.16 (s, 1H, indole), δ 7.49 (s, 1H, NH_2), δ 7.885 (s, 1H, indole), δ 8.01 (s, 1H, NH_2), δ 8.12 (s, 1H, indole), δ 8.26 (s, 1H, indole), δ 11.18 (s, 1H, NH, indole), δ 11.82 (s, 1H, NH). NMR ^{13}C (DMSO, d_6, 100 MHz, ppm): δ 16.35, 111.09, 113.39, 121.27, 123.58, 125.13, 125.53, 131.78, 135.34, 140.39, 176.63. MS m/z (ES^+): 309.8850 $[M]^+$. IR (KBr, cm^{-1}): C=S (1367), C=N (1541), N-H (3428), N-H (3275).

4.1.2.2 2-((7-bromo-5-methyl-1H-indol-3-yl)methylene)hydrazinecarbothioamide (2b)

Orange powder; MP: 234 °C; Yield: 39.08%; Rf: 0.47 (*n*-hexane/ethyl acetate 6:4). NMR ^{1}H (DMSO, $d_{6,}$ 400 MHz, ppm): δ 2.43 (s, 3H, CH_3), δ 7.26 (s, 1H, indole), δ (s, 1H, NH_2), δ 7.825 (s, 1H, indole), δ 7.98 (s, 1H, indole), δ 8.03 (s, 1H, NH_2), δ 8.26 (s, 1H, CH), δ 11.19 (s, 1H, NH, indole), δ 11.71 (s, 1H, NH). NMR ^{13}C (DMSO, $d_{6,}$100 MHz, ppm): δ 20.71, 103.87, 111.79, 121.05, 125.75, 126.47, 131.79, 133.71, 140.39, 176.61. MS m/z (ES^+): 309.8950 $[M]^+$. IR (KBr, cm^{-1}): C=S (1380), C=N (1546), N-H (3496), N-H (3366).

4.1.2.3 2-((7-bromo-5-methyl-1H-indol-3-yl)methylene)-N-phenylhydrazinecarbothioamide (2c)

Dark yellow powder; MP: 174° C; Yield: 94.64%; Rf: 0.50 (*n*-hexane/ethyl acetate 4:6). NMR ^{1}H (DMSO, d_6, 400MHz) δ: 2.41 (s, 3H, CH_3), 7.18 (t, 1H, J= 7.2Hz, phenyl), 7.27 (s, 1H, indole), 7.37 (t, 2H, J=8Hz, phenyl), 7.69 (d, 2H, J=7.2Hz, phenyl), 7.96 (d, 1H, J=2.8Hz,

indole), 7.97 (s, 1H, indole), 8.38 (s, 1H, =CH-), 9.69 (s, 1H, NH), 11.64 (s, 1H, NH), 11.79 (s, 1H, NH). NMR ^{13}C (DMSO, d_6, 100MHz) δ: 20.85, 104.02, 111.60, 120.72, 124.55, 124.78, 125.98, 126.42, 128.13, 128.13, 131.40, 131.78, 133.69, 139.25, 140.43, 174.46. IR (KBr, cm^{-1}): C=S (1197.67), C=N (1533.79), N-H (3326.4). MS m/z (ES^+): 387,0210 $[M]^+$.

4.1.2.4 2-((5-bromo-7-methyl-1H-indol-3-yl)methylene)-N-phenylhydrazinecarbothioamide (2d)

Silver powder; MP: 199° C; Yield: 35.72%; Rf: 0.51 (ethyl acetate/*n*-hexane 7:3). NMR ^1H (DMSO, d_6, 400MHz) δ: 2.48 (s, 3H, CH_3), 7.17 (s, 1H, phenyl), 7.19 (s, 1H, indole), 7.37 (t, 2H, J=8Hz, phenyl), 7.71 (d, 2H, J=8Hz, phenyl), 7.98 (s, 1H, indole), 8.17 (s, 1H, indole), 8.38 (s, 1H, =CH-), 9.73 (s, 1H, NH), 11.62 (s, 1H, NH), 11.80 (s, 1H, NH). NMR ^{13}C (DMSO, d_6, 100MHz) δ: 16.35, 110.99, 113.38, 121.25, 123.76, 124.22, 124.72, 125.34, 125.48, 128.18, 131.88, 135.33, 139.28, 140.50, 174.37. IR (KBr, cm^{-1}): C=S (1267.33), C=N (1557.99), N-H (3410.7). MS m/z (ES^+): 387.0142 $[M]^+$.

4.1.2.5 2-((7-bromo-5-methyl-1H-indol-3-yl)methylene)-N-(4-methoxyphenyl)hydrazinecarbo-thioamide (2e)

White powder; MP: 206° C; Yield: 53.97%; Rf: 0.53 (*n*-hexane/ethyl acetate 5:5). NMR ^1H (DMSO, d_6, 400MHz) δ: 2.41 (s, 3H, CH_3), 3.76 (s, 3H, OCH_3), 6.93 (d, 2H, J= 8.8 Hz, phenyl), 7.26 (s, 1H, indole), 7.51 (d, 2H, J= 8.8 Hz, phenyl), 7.94 (d, 1H, J= 2.8 Hz, indole), 7.97 (s, 1H, indole), 8.37 (s, 1H, =CH-), 9.56 (s, 1H, NH), 11.53 (s, 1H, NH), 11.77 (s, 1H, NH). NMR ^{13}C (DMSO, d_6, 100MHz) δ: 20.87, 55.23, 104.01, 111.68, 113.32, 120.74, 126.00, 126.41, 126.77, 126.77, 131.38, 131.62, 132.22, 133.69, 140.23, 156.70, 174.99. IR (KBr, cm^{-1}): C=S (1241.91), C=N (1556.29), N-H (3149.1), N-H (3300.6), N-H (3439.2). MS m/z (ES^+): 418.9989 $[M]^+$.

4.1.2.6 2-((5-bromo-7-methyl-1H-indole-3-yl)methylene)-N-(4-methoxyphenyl)hydrazinecarbothioamide (2f)

White powder; MP: 192° C; Yield: 71.31%; Rf: 0.48 (ethyl acetate/*n*-hexane 7:3). NMR ^{1}H (DMSO, d_6, 400MHz) δ: 2.47 (s, 3H, CH_3), 3.76 (s, 3H, OCH_3), 6.94 (d, 2H, J= 8.4 Hz, phenyl), 7.16 (s, 1H, indole), 7.52 (d, 2H, J= 8.8 Hz, phenyl), 7.96 (s, 1H, indole), 8.16 (s, 1H, indole), 8.37 (s, 1H, =CH-), 9.60 (s, 1H, NH), 11.51 (s, 1H, NH), 11.86 (s, 1H, NH). NMR ^{13}C (DMSO, d_6, 100MHz) δ: 16.40, 55.26, 111.08, 113.38, 121.28, 123.74, 125.37, 125.51, 126.55, 131.76, 132.26, 135.35, 140.37, 156.68, 174.91. IR (KBr, cm^{-1}): C=-S (1239.92), C=N (1555), N-H (3311.5). MS m/z (ES^+): 419.0001 $[M]^+$.

4.1.2.7 2-((5-bromo-7-methyl-1H-indole-3-yl)methylene)-N-(p-tolyl)hydrazinecarbothioamide (2g)

Light green powder; MP: 195° C; Yield: 40.34%; Rf: 0.45 (*n*-hexane/ethyl acetate 6:4). NMR ^{1}H (DMSO, d_6, 400MHz) δ: 2.30 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 7.17 (d, 2H, J= 8.4 Hz, phenyl), 7.23 (s, 1H, indole), 7.57 (d, 2H, J= 8.4 Hz, phenyl), 8.05 (s, 1H, indole), 8.06 (s, 1H, indole), 8.34 (s, 1H, =CH-), 9.93 (s, 1H, NH), 9.93 (s, 1H, NH), 12.35 (s, 1H, NH). NMR ^{13}C (DMSO, d_6, 100MHz) δ: 16.33, 20.51, 113.35, 117.79, 120.40, 121.23, 124.31, 124.47, 125.35, 126.30, 128.64, 135.33, 136.71, 138.77, 140.35, 174.45, 185.11. IR (KBr, cm^{-1}): C=S (1450.51), C=N (1628.36), N-H (3107.3), N-H (3171.7). MS m/z (ES^+): 401.0279 $[M]^+$.

4.1.2.8 2-((7-bromo-5-methyl-1H-indole-3-yl)methylene)-N-(p-tolyl)hydrazinecarbothioamide (2h)

Light yellow powder; MP: 195° C; Yield: 32.09%; Rf: 0.60 (*n*-hexane/ethyl acetate 5:5). NMR ^{1}H (DMSO, d_6, 400MHz) δ: 2.40 (s, 3H, CH_3), 3.36 (s, 3H, CH_3), 7.16 (d, 2H, J= 8 Hz,

phenyl), 7.32 (s, 1H, indole), 7.54 (d, 2H, J= 8 Hz, phenyl), 7.90 (s, 1H, indole), 8.28 (s, 1H, indole), 8.29 (s, 1H, =CH-), 9.93 (s, 1H, NH), 11.59 (s, 1H, NH), 12.26 (s, 1H, NH). NMR ^{13}C (DMSO, d_6, 100MHz) δ: 20.85, 40.12, 104.32, 111.65, 118.51, 120.05, 124.62, 125.89, 125.99, 126.40, 127.24, 128.60, 131.38, 133.20, 133.79, 136.69, 139.11, 185.28. IR (KBr, cm^{-1}): C=S (1199.82), C=N (1618.41), N-H (3105.8). MS m/z (ES^+): 401.0220 $[M]^+$.

4.1.2.9 2-((5-bromo-7-methyl-1H-indole-3-yl)methylene)-N-(4-ethylphenyl)hydrazinecarbothioamide (2i)

Light yellow powder; MP: 195° C; Yield: 87.79%; Rf: 0.60 (*n*-hexane/ethyl acetate 7:3). NMR ^{1}H (DMSO, d_6, 400MHz) δ: 1.20 (t, 3H, J= 7.2 Hz, CH_3), 2.50 (s, 3H, CH_3), 2.61 (q, 2H, J= 7.2 Hz, CH_2), 7.17 (s, 1H, indole), 7.21 (d, 2H, J= 8 Hz, phenyl), 7.59 (d, 2H, J= 8 Hz, phenyl), 7.98 (s, 1H, indole), 8.17 (s, 1H, indole), 8.38 (s, 1H, =CH-), 9.65 (s, 1H, NH), 11.60 (s, 1H, NH), 11.86 (s, 1H, NH). NMR ^{13}C (DMSO, d_6, 100MHz) δ: 15.69, 16.37, 27.68, 111.02, 113.37, 121.25, 123.76, 124.37, 125.34, 125.47, 125.47, 127.46, 127.46, 131.83, 135.34, 136.90, 140.33, 140.38, 174.42. IR (KBr, cm^{-1}): C=S (1266.85), C=N (1551.13), N-H (3258.7), N-H (3623). MS m/z (ES^+): 415,0590 $[M]^+$.

4.1.2.10 2-((7-bromo-5-methyl-1H-indole-3-yl)methylene)-N-(4-ethylphenyl)hydrazinecarbothioamide (2j)

Yellow powder; MP: 210° C; Yield: 59.17%; Rf: 0.52 (*n*-hexane/ethyl acetate 5:5). NMR ^{1}H (DMSO, d_6, 400MHz) δ: 1.19 (t, 3H, J= 7.6 Hz, CH_3), 2.41 (s, 3H, CH_3), 2.61 (q, 2H, J= 7.6 Hz, CH_2), 7.20 (d, 2H, J= 8.4 Hz, phenyl), 7.27 (s, 1H, indole), 7.57 (d, 2H, J= 8 Hz, phenyl), 7.95 (s, 1H, indole), 7.97 (s, 1H, indole), 8.38 (s, 1H, =CH-), 9.62 (s, 1H, NH), 11.60 (s, 1H, NH), 11.78 (s, 1H, NH). NMR ^{13}C (DMSO, d_6, 100MHz) δ: 15.69, 20.85, 27.68, 104.03, 111.64, 120.72, 124.67, 125.97, 126.40, 127.41, 131.38, 131.73, 133.70, 136.88, 140.29,

140.37, 174.53. IR (KBr, cm^{-1}): C=S (1264.92), C=N (1549.13), N-H (3170), N-H (3294.2). MS m/z (ES^+): 415.0548 $[M]^+$.

4.2 Biologic Assay

4.2.1 Macrophage Culture

Macrophage (J774) were cultivated in RPMI (Sigma Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS) and kept in incubator stove at 5% CO_2, at 37 ºC.

4.2.2 Parasite Culture

Promastigote forms of *Leishmania infantum* (strain MHOM/BR/70/BH46) and *Leishmania amazonensis* (strain WHOM/00LTB0016) and were kept in culture medium supplemented with 20% FBS and 1% penicillin-streptomycin (Sigma Aldrich, St. Louis, USA) cultivated at 26ºC. Promastigote forms were used in the exponential growth phase in all steps of the experiment [48]

4.2.3 Cytotoxicity Analysis of compounds

Cytotoxicity in mammal cells was evaluated through tests with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium (MTT) bromide. Macrophage were cultivated in 96-well cell culture plate at 6 x 10^5 cell/plate concentrations and incubated in atmosphere of 5% CO_2 at 37 °C. After 24h, the supernatant was removed and the cells were incubated in the presence of several concentrations of the compounds (200 to 6µg/mL) for 72h. Absorbance reading of the solubilized formazan crystals was conducted with ELISA Benchmark Plus (Bio-Rad, California, USA) with wavelength of 490nm. The concentration was capable of causing 50% of loss for cellular viability determined by linear regression analysis. Each

experiment was conducted in biological duplicate. Macrophage incubated in culture medium and in standard drugs (Miltefosine and Amphotericin B) were used as negative and positive control, respectively [62].

4.2.4 Biological activity in *Leishmania infantum* and *Leishmania amazonensis*

Promastigote forms *L. infantum* and *L. amazonensis* were collected, counted and diluted in Schneider medium supplemented with 20% of FBS at 2 x 10^6 cell/mL concentration. Next, the cells were incubated in different concentrations of the compounds (25 to 1.56 µg/mL) for *L. infantum* promastigote forms and 200 a 6µg/mL for *L. amazonensis* forms of both for 72h. Parasites incubated in medium culture and standard drug (Miltefosine and Amphotericin B) were used as negative and positive control, respectively. Culture growth was monitored through counting using Neubauer chamber (iNCYTO C-Chip DHC-N01, Cheonan-Si, South Korea). The concentration that inhibited 50% of parasite growth (IC_{50}) was determined after 72h through linear regression. Biological duplicate was conducted for each experiment. Selectivity Index (SI) was determined as the ratio between CC_{50} and IC_{50} values and obtained for each compound [48].

4.2.5 Ultrastructural Analysis

The compounds that displayed the best SI results associated to the best IC_{50} value were selected for ultrastructural analysis. Therefore, given the established pattern, compounds **2d** and **2i** tested against *L. infantum* were selected. Promastigote forms of untreated *L. infantum* (negative control) treated with amphotericin B at 0.05 µM (positive control) and treated with concentrations 5.6 and 11.2 µM to compound **2d** and 4.36 and 8.72 µM to compound *2i*, were washed and fixed in solution 2.5% glutaraldehyde and 4% of formaldehyde in sodium

cacodylate buffer 0.1 M at pH 7.2. These were processed for transmission and scanning electronic microscopy.

For SEM, promastigotes were placed to adhere in dishes previously covered with poly-L-lysine (Sigma Aldrich, St. Louis, USA). After 20 minutes, the dishes were washed with cacodylate buffer 0.1M to remove non-adherent cells and post-fixed for 1 hour in solution 1% osmium tetroxide (OsO_4) in cacodylate buffer. Next, cells were dehydrated in crescent series of ethanol and critical-point-dried with HCP-2 (Hitachi, Tokyo, Japan) assembled in metallic support, sputter-coated with 20nm of gold in JFC-1100 and examined in scanning electron microscope JEOL T-200.

In TEM, parasites were washed and post-fixed for 1h 1% osmium tetroxide (OsO_4) in cacodylate buffer. Samples were then dehydrated in crescent series of acetone, infiltrated and incorporated in EPON (Sigma Aldrich, St. Louis, USA). The material was sectioned in layers of 70nm with ultramicrotome Leica EMUC6 (Leica, Wetzlar, Germany), and contrasted against and analyzed in transmission electron microscope TecNai G2 Spirit TEM (FEI, Hillsboro, USA) uranyl acetate and lead.

4.2.6 Statistical Analysis

Linear regression was conducted using software SPSS 8.0 (IBM Co., New York, USA) for Windows.

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Conflict of Interest

The authors declare that there were no competing interests.

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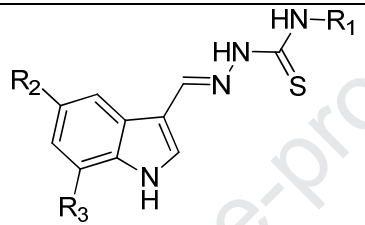
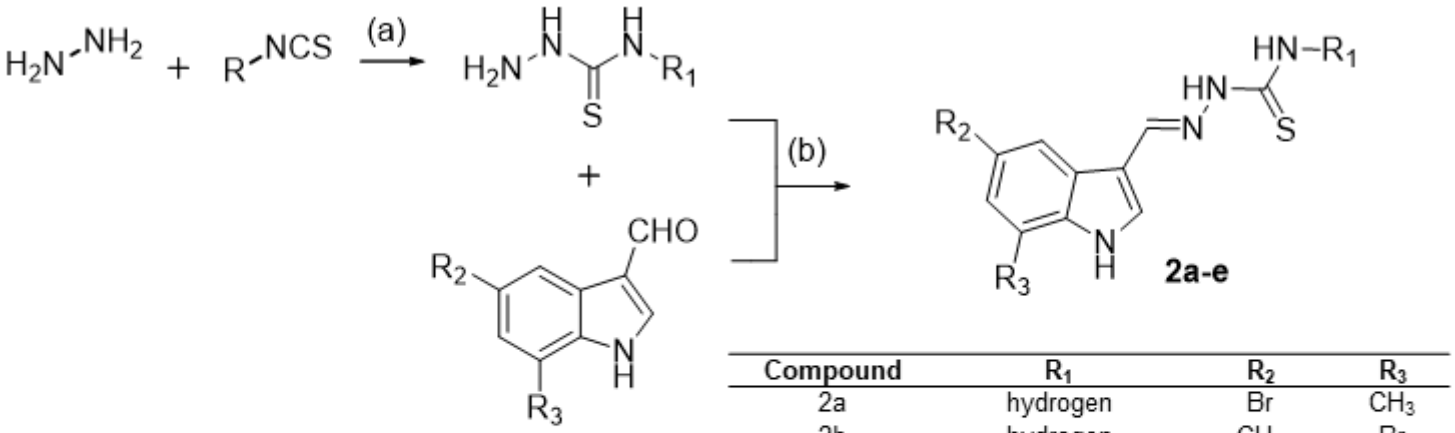
Table 1. Analysis of cytotoxic potential, *in vitro* leishmanicidal activity on *L. infantum* and *L. amazonensis* promastigotes and selectivity index of indole-thiosemicarbazone derivatives.



Compound	μM					R_1	R_2	R_3
	IC_{50} (a)	IC_{50} (b)	CC_{50}	IS (a)	IS (b)			
2a	16.70	87.76	237.76	14.24	2.71	hydrogen	Br	CH_3
2b	13.51	23.92	357.97	26.50	14.97		CH_3	Br
2c	6.12	13.61	63.54	10.38	4.67	phenyl	CH_3	Br
2d	5.60	46.63	100.65	17.97	2.16		Br	CH_3
2e	8.43	33.16	88.18	10.46	2.66	*p*-methoxy-phenyl	CH_3	Br
2f	8.93	75.24	120.08	13.45	1.60		Br	CH_3
2g	23.35	134.38	118.31	5.07	0.88	*p*-methyl-phenyl	Br	CH_3
2h	11.46	19.58	135.25	11.80	6.91		CH_3	Br
2i	4.36	12.31	53.23	12.21	4.32	*p*-ethyl-phenyl	Br	CH_3
2j	11.97	>481.52	93.17	7.78	0.19		CH_3	Br

AmB	0.05	------	37.6	752	------	-------	------	------
Mtf	------	56.67	219.05	------	3,87	------	------	------

IC_{50} (a) - concentration inhibiting 50% of growth of promastigote forms of *L. infantum*; IC_{50} (b) - concentration inhibiting 50% of growth of promastigote forms of *L. amazonensis*. CC_{50} - concentration capable of causing loss of viability in 50% of macrophages (J774); SI (a) - Selectivity Index - CC_{50} Macrophage/IC_{50} (a); SI (b) - Selectivity Index - CC_{50} Macrophage/IC_{50} (b); AmB - Amphotericin B; Mtf - Miltefosine.



Compound	R_1	R_2	R_3
2a	hydrogen	Br	CH_3
2b	hydrogen	CH_3	Br
2c	phenyl	CH_3	Br
2d	phenyl	Br	CH_3
2e	*p*-methoxy-phenyl	CH_3	Br
2f	*p*-methoxy-phenyl	Br	CH_3
2g	*p*-methyl-phenyl	Br	CH_3
2h	*p*-methyl-phenyl	CH_3	Br
2i	*p*-ethyl-phenyl	Br	CH_3
2j	*p*-ethyl-phenyl	CH_3	Br

Scheme 1. Synthesis of indol-thiosemicarbazone derivatives (**2a-j**). Reagents and conditions: (a) $CHCl_2$, room temperature; (b) hydrochloric acid (few drops), EtOH, room temperature. Scheme according to De Oliveira et al. (2015) with modifications.

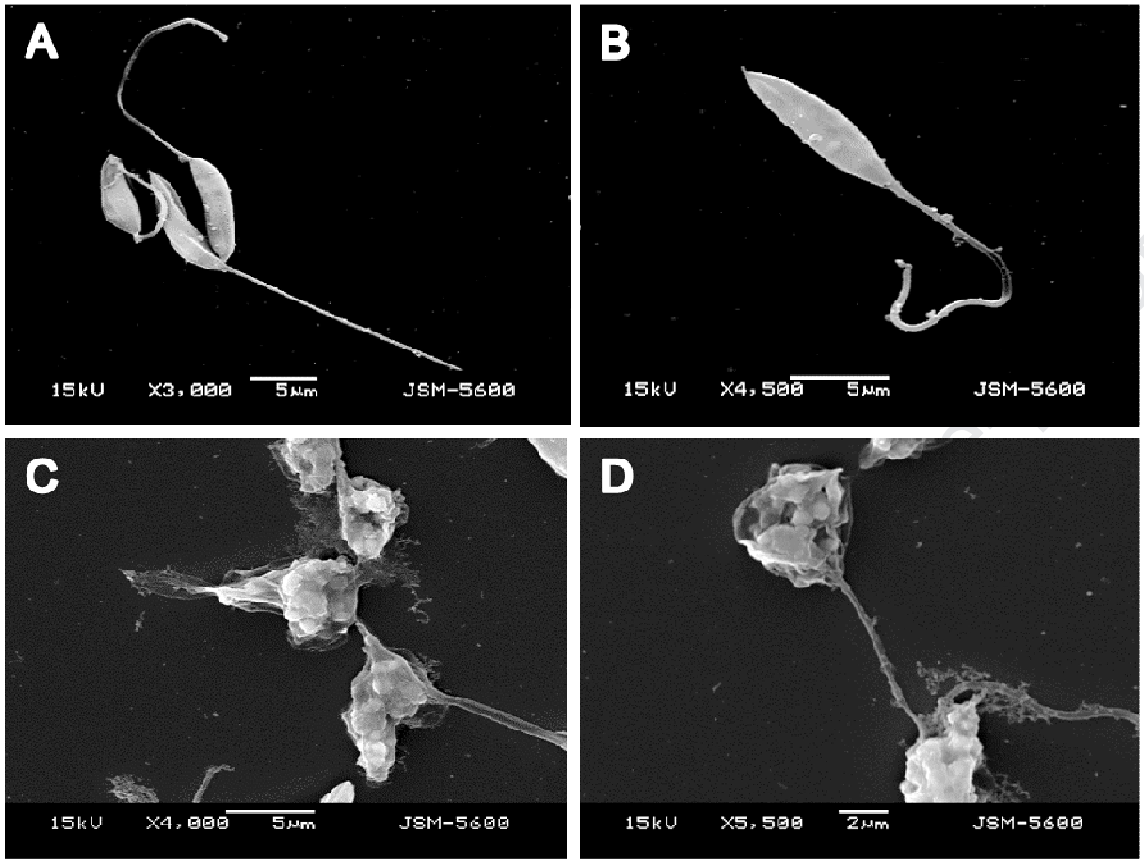


Figure 1. Electromicrographs of promastigote forms of *Leishmania infantum*. (A-B) Untreated promastigotes showing preserved morphology with fusiform body and elongated free flagellum. (C-D) Promastigotes submitted to treatment with amphotericin B presenting morphometric changes in the body and flagellum of the parasite.

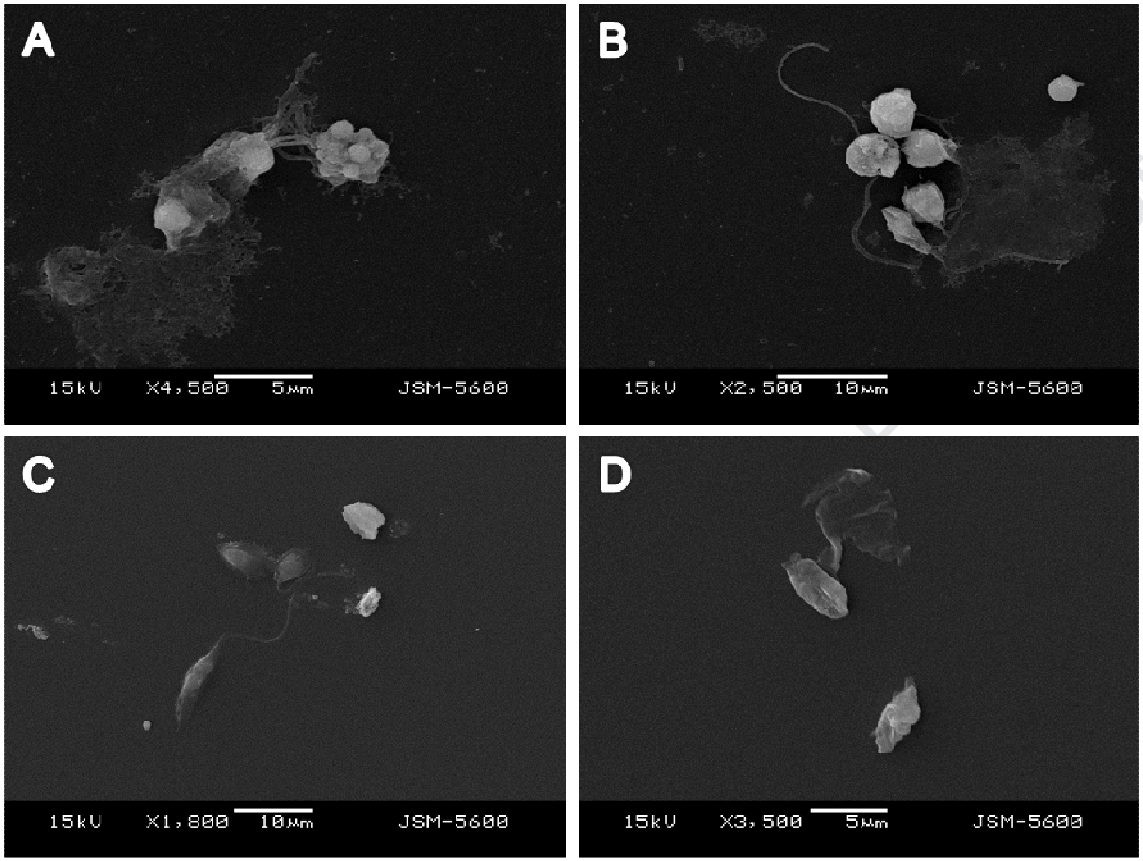


Figure 2. Electromicrographs of promastigote forms of *L. infantum* treated with compound **2d** 5.6 μM (A-B) and 11.2 μM (C-D). Observe changes in the body and flagella of the parasite.

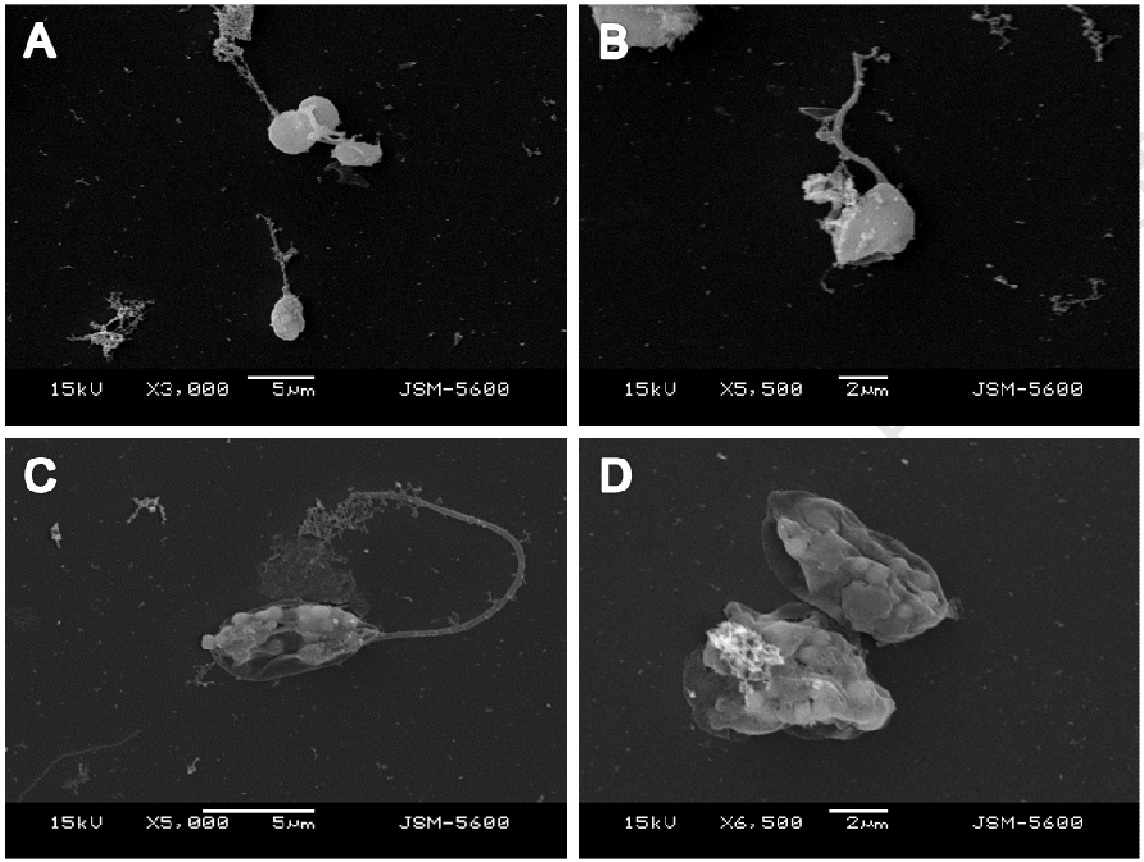


Figure 3. Electromicrographs of promastigote forms of *L. infantum* treated with compound **2i** 4.36 µM (A-B) and 8.72 µM (C-D). Observe changes in the body and flagella of the parasite.

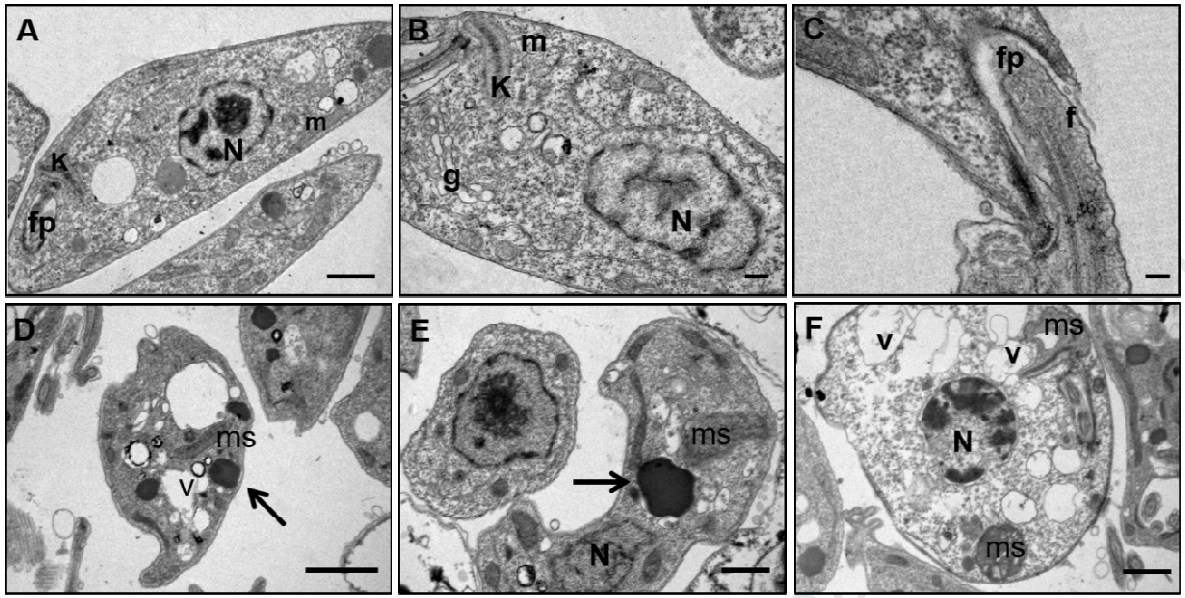


Figure 4. Electromicrographs of promastigote forms of *Leishmania infantum*. (A-C) Untreated promastigotes showing preserved organelles and morphology. (D-F) Promastigotes treated with amphotericin B (0.05µM), showing changes in morphology and cell organelles such as mitochondria swelling (ms) and presence of vacuoles (v) and electron-dense particles (arrow). (fp) flagellar pocket, (m) mitochondria, (N) nucleus e (f) flagellum. Bars = 1µm

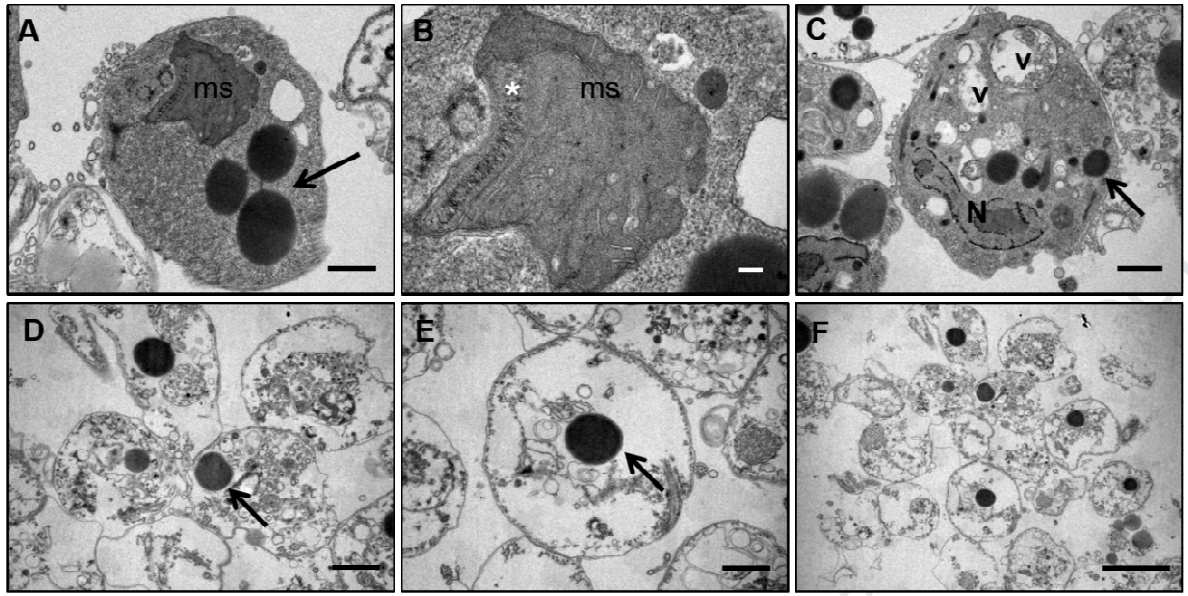


Figure 5. Electromicrographs of promastigote forms of *L. infantum* treated with compound **2d**. (A-C) Promastigotes treated with the compound at 5.6 μM showing changes in morphology and cell organelles such as mitochondria swelling (ms), kinetoplast disorganization (*), electron-dense particles (arrow), picnotic nucleus (N) and presence of vacuoles (v). (D-F) Promastigotes treated with the compound at 11.2 μM showing completely destroyed cells completely destroyed cells with only electrondense particles. Bars = 1μm

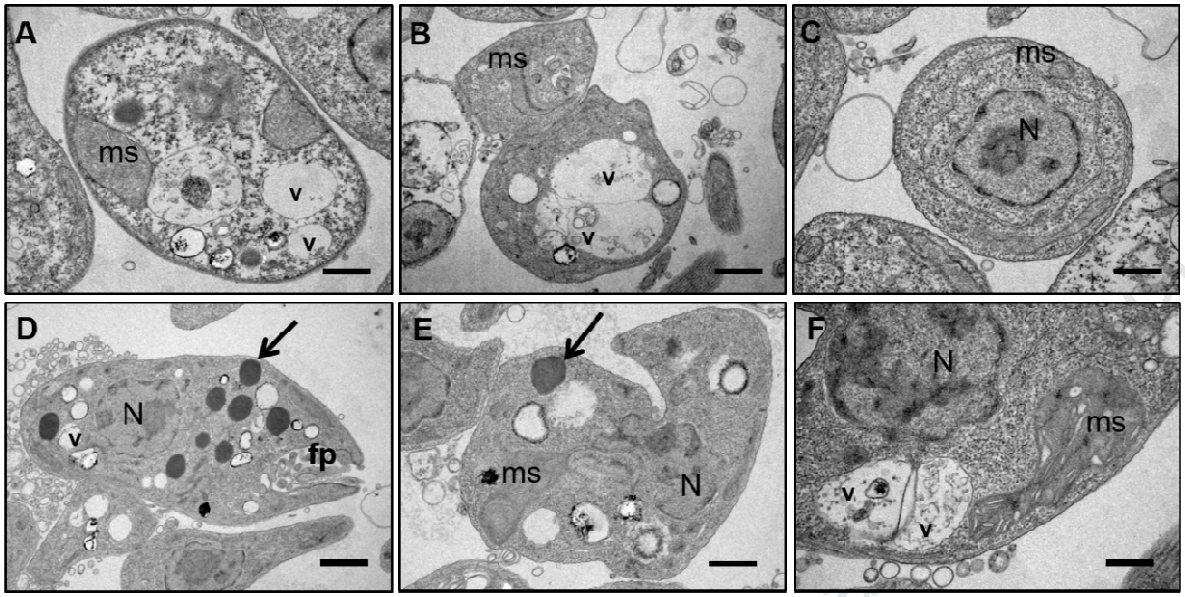


Figure 6. Electromicrographs of promastigote forms of *Leishmania infantum* treated with compound **2i**. (A-C) Treated with the compound at 4.36 µM and (D-F) treated with the compound at 8.72 µM. Observe mitochondria swelling (ms), electron-dense particles (arrow), picnotic nucleus (N) and presence of vacuoles (v). Bars = 1µm

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: