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TREATMENT AND PROPHYLAXIS - ORIGINAL PAPER

In vitro evaluation and in vivo efficacy of nitroimidazole-sulfanyl ethyl derivatives against *Leishmania* (*V.*) *braziliensis* and *Leishmania* (*L.*) *mexicana*

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Abstract

The aim of this study was to synthesize several small molecules of the type 5-nitroimidazole-sulfanyl and evaluate biological properties against the main *Leishmania* species that cause cutaneous leishmaniasis in Venezuela. Final compounds **4**–**7** were generated through simple nucleophilic substitution of 1-(2-chloroethyl)-2-methyl-5-nitroimidazole **3** with 2-mercaptoethanol, 1-methyl-2-mercaptoethanol, and 2-thyolacetic acid derivative. Compound **8** was synthesized via a coupling reaction between **7** and (*S*)-Methyl 2-amino-4-methylpentanoate hydrochloride. The inhibitory concentrations of (**3, 4, 7, 8**) against *Leishmania* (*L.*) *mexicana* and (*V.*) *braziliensis* in promastigotes and experimentally infected macrophages were determined by in vitro activity assays. Compounds **7** and **8** shown high activity against both species of *Leishmania* and were selected for the in vivo evaluation. Animals were infected with promastigotes of the two species and divided into four groups of ten (10) animals and a control group. Intralesional injection way was used for the treatment. The parasitological diagnostic after treatment was obtained by PCR using species specific oligonucleotides. The two *Leishmania* species were susceptible to compounds **7** and **8** in vivo assays. The results indicated that both compounds reduce significantly (96%) the size of the lesion and cure 63% of the mice infected with *L (L) mexicana* or *L (V) braziliensis* as was determined by PCR. The results are indicating that both compounds may represent an alternative treatment for these two *Leishmania* species.

Keyword *Leishmania* · Metronidazole · Nitroimidazole · Sulfanyl · PCR

Introduction

Leishmaniasis is a neglected tropical disease (NTD) caused by protozoa of the genus *Leishmania*; the disease is endemic in 98 countries with an overall prevalence of 12 million cases and an annual mortality rate of more 59.000 deaths (WHO 2019). Over 20 *Leishmania* species are known to be infective to humans and are transmitted by the bite of infected female phlebotomine sandflies, thus giving three main clinical manifestations of leishmaniasis: visceral (VL), cutaneous (CL), and mucocutaneous (MCL). It is estimated that approximately 0.2 to 0.4 million of new VL cases and 0.7 to 1.2 million of new CL cases occur each year (WHO 2019). Since there are currently no effective vaccines that protect against the disease, leishmaniasis control relies on chemotherapy. The recommended first-line therapies include pentavalent antimony compounds, such as sodium stibogluconate and meglumine antimoniate, although these drugs present several disadvantages, such as toxicity, high costs,

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prolonged treatment, and parenteral or intralesional routes of administration. The second-line treatments include pentamidine and amphotericin B, but their use is limited because of toxicity and cost, even though lipid and liposomal formulations of amphotericin B have been developed to reduce this toxicity. Recently, the oral administration of miltefosine has been used for the treatment of VL in some countries, but despite its great efficacy, miltefosine is not free either from toxicity as it shows teratogenic potential (Singh et al. 2016; Sangshetti et al. 2015; Dietze et al. 2001; Agrawal and Singh 2006; Soto et al. 2001; Monge and López 2015). Several compounds that show leishmanicidal activity are currently in different stages of development. Among them, a few classes of compounds, such as the 8-aminoquinolinic sitamaquine (Loiseau et al. 2011), the 7-aminoimidazoquinolinic imiquimod (Seeberger et al. 2003), the triazolic posaconazole (Al-Abdely et al. 1999), as well as some natural product derivatives, such as licochalcone A (Zhai et al. 1999), have been revealed as potential new drugs for antileishmania therapy. The synthesis of several molecules showing leishmanicidal activity and that of new lead compounds, such as β-carboline alkaloids (Stefanello et al. 2014), piperoylaminoacid conjugates (Singh et al. 2010), heteroretinoid-bis(benzylidene)ketones (Tiwari et al. 2015), and bispyridinium cyclophanes, has also been described (Gómez-Pérez et al. 2015). The Drugs for Neglected Diseases *initiative* (DND*i*) and Takeda will work on the "lead optimization" of the aminopyrazole series (DND*i*. Org. 2021). Considering toxicity issues, increasing parasite resistant, increasing failure rates of current treatments, and the lack of effective clinic agents against leishmaniasis, there is an urgent need in the development of effective and safe new drugs for treating leishmaniasis in their different clinical forms. In this sense, also, attention has been directed to those drugs that contain a nitro group, for their use for the treatment of a wide variety of diseases, including antineoplastic, antibacterial, antiparasitic, tranquilizer, fungicide, and insecticide as well as herbicide agents (Ang et al. 2017; Nepali et al. 2019). In particular, nitro containing imidazol like 5-nitroimidazole, metronidazole clinically, or fexinidazole and its sulfonic metabolite now in clinical trial were shown to have effective antileishmanial efficacy (Somaratne et al. 2019; Wyllie et al. 2012; de Morais-Texaira et al. 2019; Burrows et al. 2014). Another example is related to delamanid, a nitroimidazooxazole demonstrated effective antileishmanial activity resulting in a total cure in a mouse model of VL (Patterson et al. 2016; Wyllie et al. 2016; Andrews et al. 2014).

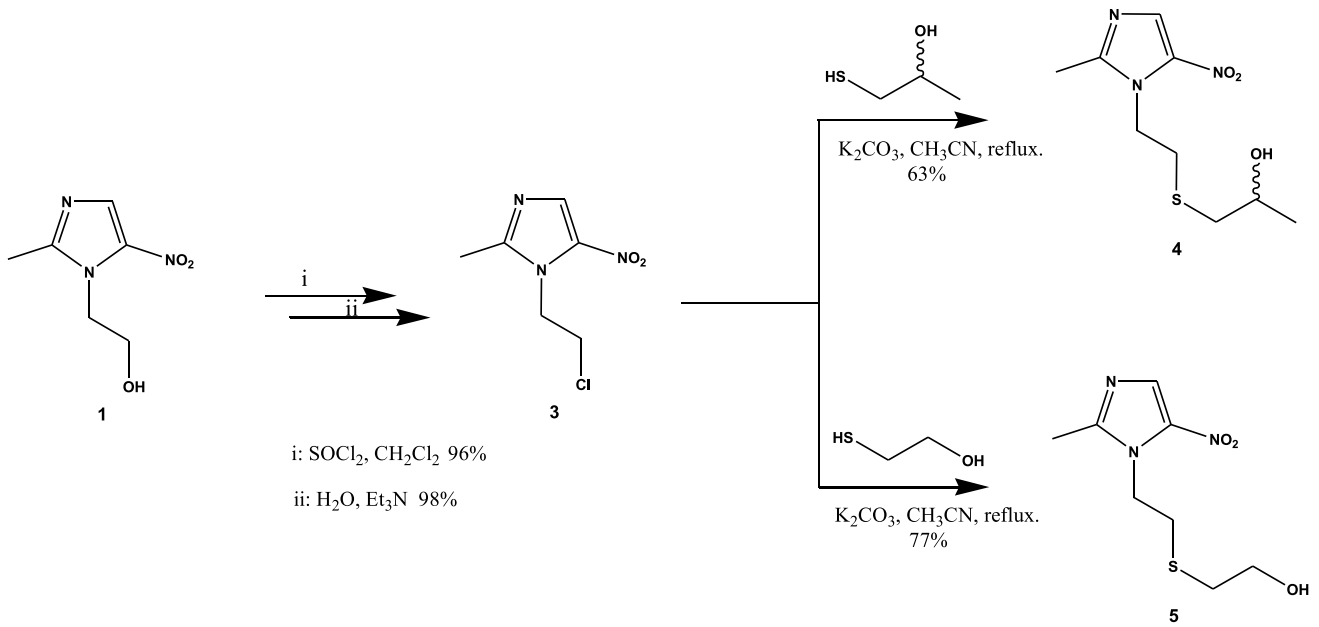
In biological systems, nitro groups require enzymatic reduction to induce the therapeutic and cytotoxic effects, either by oxidative stress or through the formation of adducts between a protein or non-protein thiol and some intermediate metabolites. This reduction is normally mediated by nitroreductases using flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) as prosthetic groups and nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) as reducing agents (Macherey and Dansette 2015).

In the search for more effective alternatives to the currently used antileishmanial drugs, some research groups have been introducing the hybridization strategy to obtain new structures with potential activity. The results have led to some leading compounds (Otero et al. 2017; Baquedano et al. 2014; Upadhyay et al. 2019). To throw light on this scenario, our research group has been synthesized a set of novel metronidazole analogues featuring an heterocyclic and basic 5-nitroimidazole head linked to a substituted benzoic acid through a dialkyl sulfur chain (Rodríguez et al. 2020). The choice of this sulfur-containing spacer was related to our previous successful experience with 7-chloroquinolin-4-ylthio derivatives (Rodrigues et al. 2012). In view of the above considerations, here, we are reporting the synthesis and the biological results related to a series of metronidazole analogues against *Leishmania* (*V.*) *braziliensis* and *Leishmania* (*L.*) *mexicana* in vitro and their efficacy in vivo.

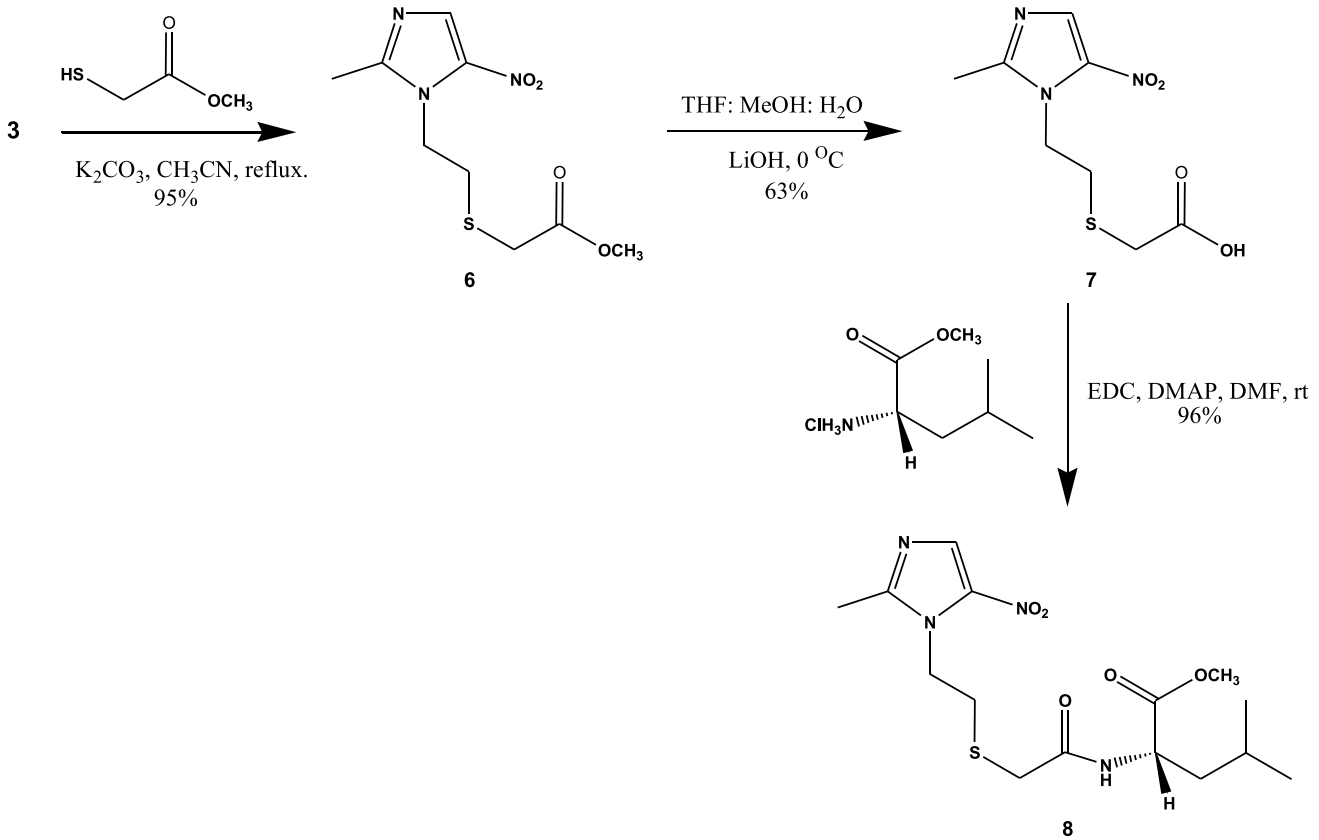
Materials and methods

Chemicals

All solvents and reagents used for the synthesis were of analytical grade and were purchased from different chemical suppliers. Thin-layer chromatography (TLC) was carried out on Merck silica F254 0.255-mm plates, and spots were visualized by UV fluorescence at 254 nm. At final compounds, the impurities were removed by either recrystallization or silica gel column chromatography. A Thomas micro hot stage apparatus was used to obtain the melting points and is uncorrected. The IR spectra were recorded on a Shimadzu™ model 470 spectrophotometer using NaCl pills or KBr pellets. Optical rotations were measured with an ATAGO™ Polarimeter model POLAX-2L. NMR specters were obtained using a JEOL Eclipse™ 270 MHz for ^{1}H-NMR and at 67.9 MHz for ^{13}C-NMR using $CDCl_3$ or DMSO-d6 and are reported in ppm downfield from the residual $CHCl_3$ or DMSO (δ 7.25 or 2.50 for ^{1}H NMR and 77.0 or 39.8 for ^{13}C NMR, respectively). A Perkin Elmer™ 2400 CHN elemental analyzer was used to obtain the elemental analyses, and the results were within ± 0.4% of the predicted values. The general synthesis of compounds **2**–**8** is presented in Schemes 1 and 2, and the synthetic procedures are presented below. Compounds **2**, **3,** and **5** were prepared according to known procedures (Salahuddin et al. 2012; Rodríguez et al. 2020).



Scheme 1 Synthesis of (*S*,*R*) 1-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethylthio]propan-2-ol (**4**) and 2-[2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethylthio]ethanol (**5**)



Scheme 2 Synthesis of methyl 2-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethylthio]acetate (**6**), 2-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethylthio]acetic acid (**7**) and (*S*) methyl 2-{2-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethylthio]acetamido}-4-methyl-pentanoate (**8**)

Synthesis and characterization of 4, 6–8

(S,R) 1-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethylthio] propan-2-ol (4)

A stirred solution of **3** (0.5 g, 2.65 mmol), 1-mercapto-2-propanol (0.26 mL, 2.7 mmol), and potassium carbonate (0.8 g, 5.6 mmol) in 20 mL of acetonitrile was refluxed for 12 h and concentrated under vacuum. The residue was partitioned between ethyl acetate and water, and the organic layer was separated and washed with brine, dried over anhydrous $MgSO_4$, and concentrated to yield crude product which was purified by column chromatography AcOEt/hexane 1:1 as eluent to give pure product as oil, yield 72%, IR NaCl υ (cm^{-1}): 3344, 2976, 1529, 1468, 1427; ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 1.22 (d, 3H, CH_3 $J=6.3$ Hz), 2.48 (dd, 1H, H_8 $J=13.5$, 8.3 Hz), 2.57 (s, 3H, CH_3), 2.66 (dd, 1H, H_8 $J=13.5$, 6.3 Hz), 2.93 (t, 2H, CH_7 $J=7.3$ Hz), 3.88 (m, 1H, H_9), 4.49 (t, 2H, H_6 $J=7.3$ Hz), 7.97 (s, 1H, H_4); ^{13}C-NMR ($CDCl_3$, 67.9 MHz) δ: 14.2 (CH_3), 22.4 (CH_3), 32.2 (C_7), 41.6 (C_6), 46.5 (C_8), 66.8 (C_9), 131.6 (C_4), 138.4 (C_5), 150.2 (C_2). Anal. calcd. for $C_9H_{15}N_3O_3S$: C, 44.07; H, 6.16; N, 17.13. Found: C, 43.98; H, 6.18; N, 17.29.

Methyl 2-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethylthio] acetate (6)

A solution of methyl thyoglicolate (0.56 g, 5.3 mmol) and potassium carbonate (0.73 g, 5.3 mmol) in 100 mL of acetonitrile was stirred for 15 min at room temperature; following the addition of **3** (1.0 g, 5.3 mmol), the mixture was refluxed for 12 h and concentrated under vacuum. The residue was partitioned between ethyl acetate and water; the organic layer was separated and washed with brine, dried over anhydrous $MgSO_4$, and concentrated to yield a crude product which was purified by column chromatography AcOEt/hexane 1:1 as eluent to give pure product as a solid pale yellow, yield 95%, m.p.: 46 °C, IR KBr υ (cm^{-1}): 3120, 3008, 1747, 1523, 1468; ^{1}H- RMN ($CDCl_3$, 270 MHz) δ: 2.57 (s, 3H, CH_3), 3.02 (t, 2H, H_7, $J=7.2$ Hz), 3.21 (s, 2H, H_8), 3.72 (s, 3H, CH_3O), 4.53 (t, 2H, H_6, $J=7.2$ Hz), 7.96 (s, 1H, H_4); ^{13}C-RMN ($CDCl_3$, 67.9 MHz) δ: 14.2 (CH_3), 32.2 (C_7), 33.7 (C_9), 46.0 (C_6), 56.2 (OCH_3), 132.1 (C_4), 138.5 (C_5), 150.3 (C_2), 170.4 (C=O); Anal. calcd. for $C_9H_{13}N_3O_4S$: C, 41.69; H, 5.05; N, 16.21. Found: C, 41.73; H, 5.02; N, 16.37.

2-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethylthio]acetic acid (7)

To a mixture of **6** (1.3 g, 5 mmol) in THF:H_2O (3:1) was added LiOH (0.18 g, 7.5 mmol), the mixture was stirred at 0 °C for 1 h. After the reaction time, the solvent was eliminated at reduced pressure, 20 ml of water was then added, and a saturated solution of $KHSO_4$ was added drop by drop until a pH between 2 and 3 was reached. The resulting mixture was extracted with 2×50 mL portions of ethyl acetate, and the organic phases were joined, washed with distilled water 2×50 ml, with brine, dried over anhydrous $MgSO_4$, filtered and concentrated to yield pure product as a powdered yellow, yield 84%, m.p.: 164–166 °C, IR KBr υ (cm^{-1}): 3160–2144, 1705, 1542, 1478, 1424; ^{1}H-RMN (DMSO-d6, 270 MHz) δ: 2.48 (s, 3H, CH_3), 2.97 (t, 2H, H_7, $J=7.0$ Hz), 3.31 (s, 2H, H_8), 4.49 (t, 2H, H_6, $J=7.0$ Hz), 8.03 (s, 1H, H_4), 12.66 (brs, 1H, OH); ^{13}C-RMN (DMSO-d6, 67.9 MHz) δ: 14.5 (CH_3), 31.6 (C_7), 33.8 (C_9), 45.7 (C_6), 133.5 (C_4), 138.8 (C_5), 151.8 (C_2), 171.7 (C=O). Anal. calcd. for $C_8H_{11}N_3O_4S$: C, 39.18; H, 4.52; N, 17.13. Found: C, 39.20; H, 4.53; N, 17.35.

(S) Methyl 2-{2-[2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethylthio]acetamido}-4-methyl-pentanoate (8)

A solution of **7** (0.150 g, 0.6 mmol) in dry DMF (2 mL) at – 10 °C was treated with *N*-(3-dimethylaminopropyl)-*N´*-ethylcarbodiimide hydrochloride (EDCI) (0.153 g, 0.8 mmol) and 4-(dimethylamino)-pyridine (DMAP) (0.05 g, 0.4 mmol) dissolved in dry DMF (1 mL). The mixture was left shaking at – 10 °C for 30 m. The (*S*)-Methyl 2-amino-4-methylpentanoate hydrochloride (Colmenares et al. 2020) (0.118 g, 0.65 mmol) dissolved in dry DMF (1 mL) were slowly added to the mixture over 30 m. The resulting mixture was left stirring for 12 h at room temperature. Next, water was added, and the aqueous fraction was extracted with CH_2Cl_2 (2×10 mL). The organic layer was washed with 10% sodium bicarbonate (2×8 mL), dried over anhydrous sodium sulfate and filtered. The solvent was evaporated under reduced pressure. The compound **8** was purified using column chromatography using as eluent hexane:ethyl acetate (9:1). Yield 96%, m.p.: 59–60 °C, IR KBr υ (cm^{-1}): 3.221, 3124, 1763, 1625, 1598, 1543; $[\alpha]_D^{rt}$ + 16.0 (*c* 1.0 $CHCl_3$); ^{1}H-RMN ($CDCl_3$, 270 MHz) δ: 0.89 (d, 6H, $2CH_3$ $J=4.8$ Hz), 1.55–1.63 (m, 3H, $H_{10,11}$), 2.49 (s, 3H, CH_3), 2.95 (t, 2H, H_7 $J=7.4$ Hz), 3.21 (s, 2H, H_8), 3.67 (s, 3H, OCH_3), 4.46–4.55 (m, 3H, $H_{6,9}$), 6.83 (d, 1H, NH $J=7.9$ Hz), 7.90 (s, 1H, H_4); ^{13}C-RMN ($CDCl_3$, 67.9 MHz) δ: 14.2 (CH_3), 21.7 (CH_3), 22.7 (CH_3), 24.9 (C_{11}), 31.8 (C_7), 35.3 (C_8), 40.9 (C_7), 45.5 (C_6), 51.1 (C_{10}), 52.2 (OCH_3), 132,9 (C_4), 138.4 (C_5), 150.7 (C_2), 168.8 (NC=O), 173.1 (CH_3OC=O). Anal. calcd. for $C_{15}H_{24}N_4O_5S$: C, 48.37; H, 6.50; N, 15.04. Found: C, 48.32; H, 6.55; N, 15.27.

Culture and maintenance of parasite

International reference strains of *L. (V.) braziliensis* (MHOM/BR/75/M2903) and *L. (L.) mexicana* (MHOM/

BZ/82/Bel21) were thawed and cultured in RPMI 1640 medium (Gibco_BRL) at room temperature with 10% fetal bovine serum (FBS) inactivated by heating at 56 °C for 30 min. Finally, antibiotics (penicillin/streptomycin), at concentrations of 100 and 1000 units, respectively, were added. For the experiments, parasites were collected in the logarithmic phase of growth (fifth day of culture) by centrifugation at 3,000 rpm, washed three times with saline phosphate buffer, pH 8.0. The pellet was re-suspended in fresh medium, and the parasites were adjusted to a concentration of 1×10^6 cells/mL (Brito et al. 2006).

Antileishmanial activity on promastigote proliferation

Each compound was diluted to a concentration of 50 mg/mL in appropriate solvent (DMSO), and, subsequently, dilutions between 10 and 500 µg/mL were prepared for the experiments. Different concentrations of each compound were used for the different species of *Leishmania*, i.e., *L. (V.) braziliensis* or *L. (L.) mexicana* to investigate the response of the parasite to each compound. A daily sample of 5 µL was taken for cell counting. The count was performed in triplicate for 7 days, until the culture reaches the stationary phase of growth. The effect of each compound over the different *Leishmania* species was evaluated (Brito et al. 2006).

Calculation of cell viability and LC_{50}

To evaluate the effect of the compounds on cell viability and in order to calculate the LC_{50}, two methods were used:

Indirect method

Parasites were incubated with various concentrations of the respective compound for 18–24 h; thereafter, 10 µL (10 mg/mL) of methyl-thiazole tetrazolium (MTT, Sigma) were added and incubated for 4 h. After incubation, the reaction was stopped with lysis buffer (50% isopropyl alcohol, 10% SDS), and then the optical density (OD) was measured at 570 nm in a spectrophotometer (Biorad). Cell viability is directly proportional to OD, since the higher number of living cells has greater color intensity, because they have high capacity to metabolize the MTT. For each experiment, different controls were used, including cells treated with solvent only, controls without and with meglumine antimoniate (i.e., drug of choice in the treatment of leishmaniasis). The effect of each compound on the growth of the parasites in relation to controls was used to estimate the concentration that causes the death of 50% of the cells in a given time (LC_{50}) (Rodríguez et al. 2020).

Direct method

This method is based on the comparison between two doses that we call X_1 and X_2, such that the density of parasites Y_1 to the X_1 dose is greater than half of the density found in the control (I); and the density of parasites Y_2 found to the dose X_2 is less than half of the control. Then we can calculate the lethal concentration 50 (LC_{50}) using the algorithm previously described by (Huber and Koella 1993). For promastigote, 1×10^6 parasites were added in 2 mL of medium SDM 79 at pH 7.2 supplemented with 10% fetal serum and 100 µL of penicillin–streptomycin in sterile 6-well plates and treated with different concentrations of the compound, then incubated at 26 °C; 5 µL of each sample was taken daily. Viability was determined by counting the cells stained with trypan blue.

In macrophage sensitivity assays

To obtain infected macrophages, we used J774-G8 mouse macrophage cell line, which were grow in monolayers before used. Macrophages were infected with *Leishmania* promastigotes in a 10:1 ratio and incubated at 35 °C. After 24 h, unbound promastigotes are discarded. At 72 h the promastigotes are counted inside the cell with Giemsa stain (Brito et al. 2006). Once the infection is established, infected cells are used for trials with the different compounds. The compounds to be evaluated were placed at the previously established concentrations, and their effect was evaluated at 24 and 72 h, using MTT (3–4,5-dimethylthiazol-2-bromide of diphenyl tetrazolium), for which macrophages infected and treated with the different concentrations of the drug were incubated for 18–24 h; then, 10 µL (10 mg/ml) of methylthiazole tetrazolium (MTT, Sigma) is added and incubated for 4 h. After incubation, the reaction is stopped with lysis buffer solution (50% isopropanol, 10% SDS), and then the OD is measured at 570 nm, in a spectrophotometer. The cellular viability is directly proportional to the OD since to the greater number of living cells, greater color intensity, because they have greater capacity to metabolize the MTT, transforming it into formazan crystals that generate the color (Huber and Koella 1993).

In vivo assays

Balb/c mice between 6 and 8 weeks of age were inoculated on the foot pad with 1×10^6 *Leishmania* promastigotes of the different species to be evaluated *L. (V.) braziliensis* and *L. (L.) mexicana*. The progress of the infection was evaluated weekly, taking the measurement of the lesion (nodule) with a digital caliper (Rodriguez et al. 2002); groups of 10 mice were used for each compound to be evaluated. Once the disease was found in the mice, treatment was carried out

with the selected compounds **7** and **8**. A group of mice were treated with Glucantime™, which is the drug indicated for the treatment of human leishmaniasis.

Compounds **7** and **8** were dispersed in water with 5% (w/v) Tween 80. The obtained dispersion was stirred to create a fine suspension. The formulation was kept at 4 °C and in the dark when not being used. The diluent was prepared with 5% (w/v) Tween 80 in water.

Intramuscular treatment was administering for a period of 5 weeks using 50 µg of the compounds each week, on the back flanks of the infected mice. After treatment, measurements were made on the lesion site. A control group do not received any treatment.

Parasitological and clinical efficacy of compounds 7 and 8

After the last measurement, 8 mice treated with compound **8** were taken at random, and biopsies were obtained from the foot pad for parasitological diagnosis by the polymerase chain reaction (PCR), using the protocol described by (Rodríguez et al. 2002). Briefly, this procedure involves isolating DNA from a biopsy taken at the site of the lesion and using it to amplify the DNA of the parasite that may be present, using species specific oligonucleotides in a reaction that also contain nucleotides and the enzyme Taq polymerase. The reaction is carried out in a thermal cycler with the appropriate program required for amplification of the DNA fragment unique to the species of *Leishmania* to be diagnosed. After treatment, no side effect was observed.

Statistical analysis

The statistical analysis was performed using a GraphPad Prism, version 5.3 program. The difference was considered significant when the P value was ≤ 0.05.

Animal research ethical aspects

All animals were handled according to local and national regulations, and the research protocols were approved by the Institute of Biomedicine Committee on Animal Research.

Results and discussion

Synthesis and characterization of 5-nitroimidazole derivatives

Our synthesis work began from metronidazole™ **1**, whose primary alcohol was substituted by a chlorine atom. Following known procedures, upon reaction with thionyl chloride, **1** was converted into the corresponding hydrochloride salt **2**, which was then treated with water and Et_3N until pH 11, to obtain the 1-(2-chloroethyl)-2-methyl-5-nitroimidazole **3** with a yield of 96% (Scheme 1) (Salahuddin et al. 2012). Subsequent nucleophilic substitution of **3** with 1-mercapto-2-propanol, 2-mercaptoethanol, or methyl thyoglicolate furnished the thioether-linked metronidazole analogue **4**, **5,** or **6**, respectively. The compounds were obtained in good yield (Schemes 1 and 2). Compound **7** was obtained from **6** through alkaline hydrolysis. The compound **8** was synthesized via a coupling reaction between **7** and (*S*)-Methyl 2-amino-4-methylpentanoate hydrochloride, in the presence of EDCI and DMAP in CH_2Cl_2. The title compound was isolated in excellent yield after purification by column chromatography (Scheme 2). The chemical structures of all synthesized compounds were confirmed on the basis of their IR and NMR spectral data, and their purity was ascertained by microanalysis. In ^{1}H NMR spectra, the signal of the respective protons of the compounds were checked on the basis of their chemical shifts, multiplicities, and coupling constants. All compounds showed a single signal ranging from δ H 7.9 to 8.5 ppm, which was assigned to the H-4 of the imidazole ring. The aliphatic signals expected at upfield shifts were found from δ H 2.9 to 4.5 ppm. The ^{13}C NMR spectra showed characteristic signals of the 5-nitroimidazole core, with one signal resonating at δ C 140–165 ppm, which was attributed to C-5, as well as two signals observed in the δ C 138–152 and 124–140 ppm regions, which were assigned to C-2 and C-4, respectively. For the carboxyl group, another characteristic signal was observed further downfield around δ C 165–166 ppm.

In vitro antileishmanial activity

To identify the potential of 5-nitroimidazole derivatives against American cutaneous leishmaniasis (CL), the 8 compounds were tested against *L. mexicana* and *L. braziliensis* parasites. Initially, we measured the antiproliferative activities of these compounds against promastigote of *L. mexicana* and *L. braziliensis* parasites results are shown in Figs. 1 and 2 (Tables 1a, 1b, 2a, 2b supplementary material). In general, both species were sensitive to the action of the tested 5-nitroimidazole derivatives. In Fig. 1A and B (*L. mexicana*), we observed that compounds **3**, **4**, **7**, and **8** are inhibiting the growth of the parasite when we compared with the control (no treated parasites); similar effect was observed with *L. braziliensis* (Fig. 2A and B). However for both species, the compound **8** has a high activity at 500 µg/ml for both species; we observed that all parasites are dead after 24 h in the presence of the compound.

To evaluate the activity of the tested 5-nitroimidazole derivatives, in the host cell of the *Leishmania* parasite, an in vitro cytotoxicity evaluation on J774-G8 cell line macrophage was performed for the compounds **3**, **4**, **7**, and **8**.

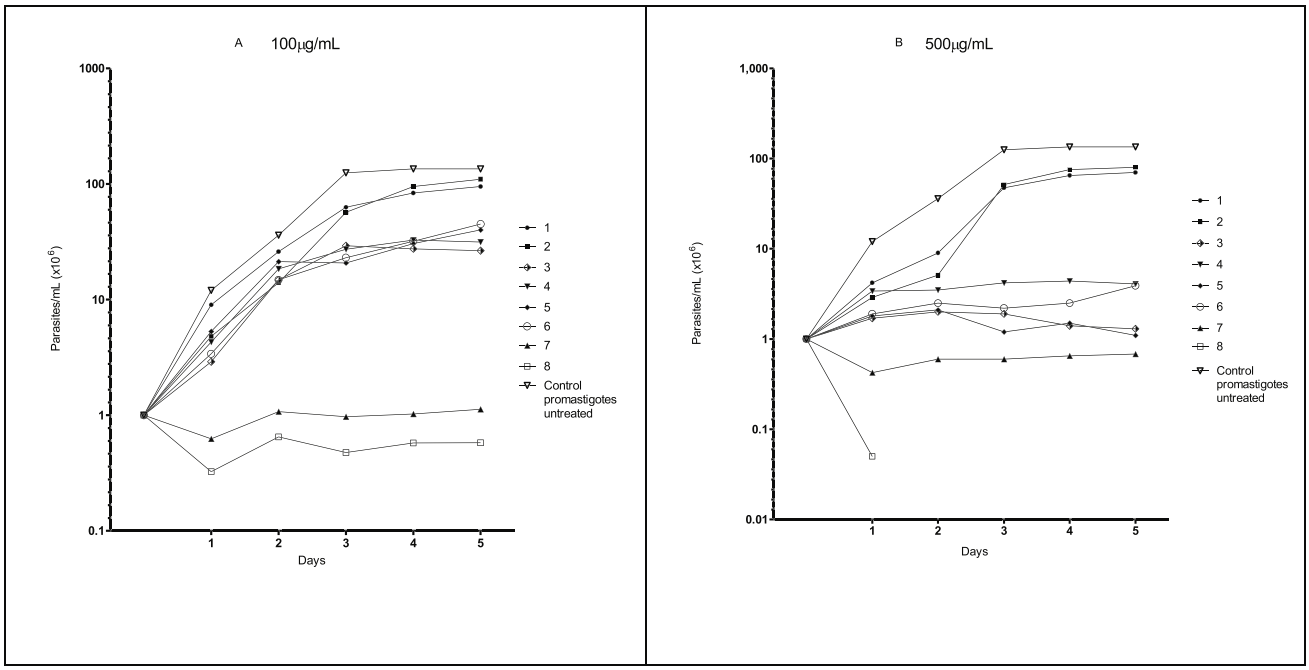


Fig. 1 Promastigotes of *L. (L.) mexicana* treated whit compounds **1**–**8**, (**1** = MNZ™). **A** 100 µg/mL. **B** 500 µg/mL; in this concentration compound **8** killed all parasites after 24 h in the presence of the compound. Parasites were counted every day during 5 days

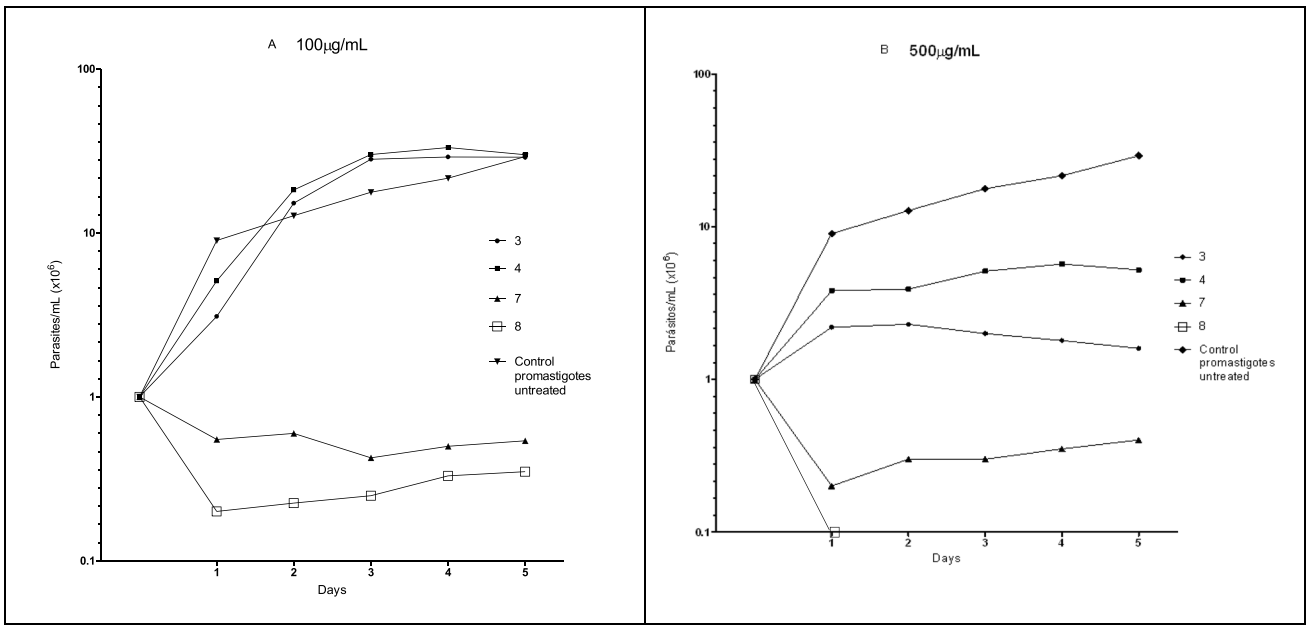


Fig. 2 Promastigotes of *L. (V.) braziliensis* treated whit compounds **3**, **4**, **7**, and **8**. **A** 100 µg/mL. **B** 500 µg/mL; in this concentration compound **8** killed all parasites after 24 h. Parasites were counted every day during 5 days

The results are presented in Table 1; this suggested that compounds **3**, **4**, **7**, and **8** represented the most attractive antileishmanial candidates for further assays due to their lower toxicity in the macrophages.

To continue with the in vitro evaluation of the tested 5-nitroimidazole derivatives, experiments against intracellular amastigote of *L. mexicana* and *L. braziliensis* was carried out for the derivatives **3**, **4**, **7**, and **8**. The intracellular

Table 1 Effect of compounds 3, 4, 7, and 8 on J774-G8 macrophages, IC_{50} values on amastigote of *L. (L.) mexicana* and *L. (V.) braziliensis*, and selectivity indexes

No	Macrophages J774-G8 (LC_{50} µg/mL)[a]	Amastigote *L. (L.) mexicana* (IC_{50} µg/mL)[b]	Amastigote *L. (V.) braziliensis* (IC_{50} (µg/mL)[b]	S. I *Lm*[c]	S. I *Lb*[c]
3	> 60	66	75	ND	ND
4	36.43 ± 4.09	40	47	0.9	0.8
7	21.70 ± 2.17	22	26	1	0.8
8	33.44 ± 5.77	18	21	1.9	1.6

[a]LC_{50}: Letal concentration, the results are the means of three independent experiments

[b]IC_{50}: Inhibitory concentration, the results are the means of five independent experiments

[c]S.I. *L m*: selectivity index on *L. mexicana*; S.I. *L b*: selectivity index on *L. braziliensis*

ND not determined

amastigote model is one of the most important assays for the in vitro leishmanicidal evaluation of a candidate due that this intracellular form is the main responsible of the clinical manifestations of the disease (Chang and McGwire 2002). We evaluated amastigote of *L. mexicana* and *L. braziliensis* due to that these two species are the main responsible of the CL in America (90% of all cases) (WHO 2019). Traditionally, the intracellular amastigotes are produced in situ from macrophage in presence of promastigote parasite under specific conditions (pH 5.5 at 37 °C).

The intracellular amastigotes were exposed to selected compounds **3**, **4**, **7**, and **8**; its corresponding effect was evaluated at 48 h post treatment. In general, it should be noted that the intracellular amastigote form was more resistant than promastigote form for the tested compounds **3** and **4**; however it was sensitive to compounds **7** and **8**; results are shown in Table 1.

In vivo antileishmanial activity of compounds 7 and 8

The results indicate that of the 8 compounds evaluated, 4 of them had the greatest activity on the parasites; however only 2 showed parasiticidal action at low concentrations; the compound **7** 2-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethylthio] acetic acid, with a IC_{50} of 22 and 26 µg/mL for intracellular removal of *L. mexicana* and *L. braziliensis*, respectively, and compound **8** (*S*) methyl 2-{2-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethylthio]acetamido}-4-methyl-pentanoate, with a IC_{50} of 18 µg/mL for *L. mexicana* and 21 µg/mL for *L. braziliensis*. The results obtained in the lesions of experimentally infected Balb/c mice indicate that both compounds reduce significantly (96%) (Figs. 3, 4, 5, and 6) (Tables 4–7 supplementary material) and cured 63% of the mice infected with *L (L) mexicana* or *L (V) braziliensis*; this was evidenced

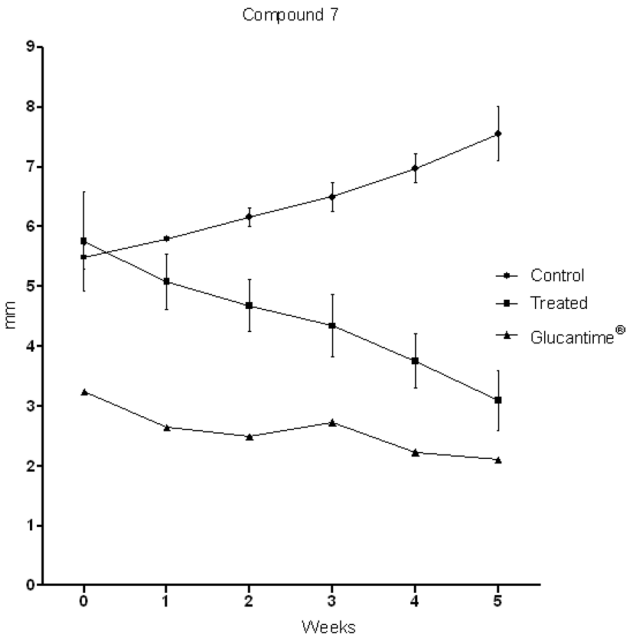


Fig. 3 Balb-c mice were inoculated in the foot pad with promastigotes of *L. (L.) mexicana* and treated with compound **7**. Control: untreated Balb/c mice. Glucantime™: Balb/c mice treated with this drug. Size of the lesion was measured every week after treatment during 5 weeks

both by the disappearance of the lesions and by the absence of parasites in the parasitological diagnosis both in culture and by PCR (Fig. 7).

Conclusion

A series of novel metronidazole™ (MNZ) derivatives has been synthesized and tested as antileishmanial agents against promastigotes and amastigotes of *L. (L.) mexicana*

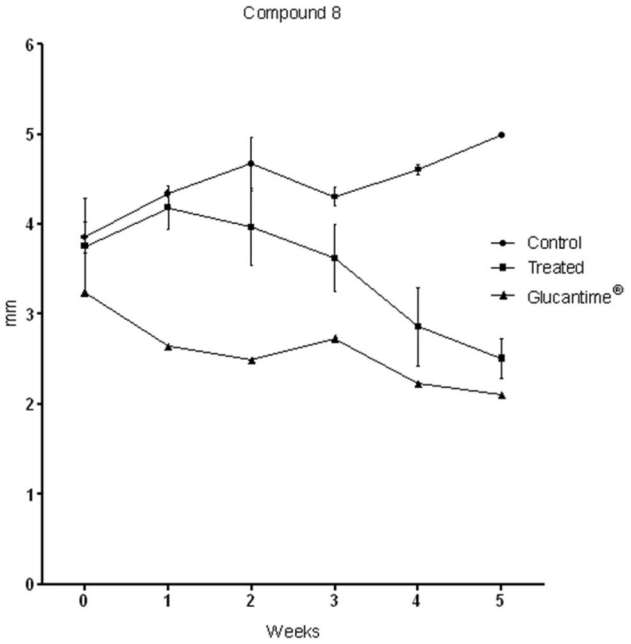


Fig. 4 Balb/c mice inoculated in the foot pad with promastigotes of *L. (L.) mexicana* and treated with compound **8**. Control: Untreated Balb/c mice. Glucantime™: Balb/c mice treated with this drug. Size of the lesion was measured every week after treatment, during 5 weeks

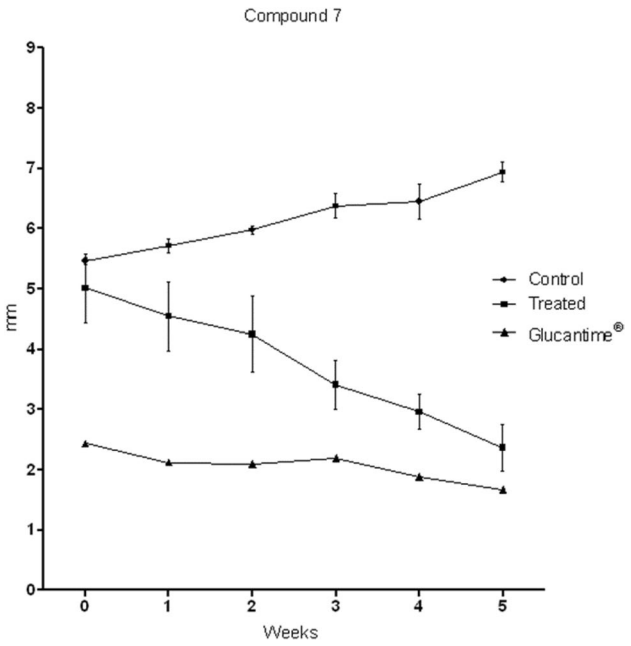


Fig. 5 Balb/c mice inoculated in the foot pad with promastigotes of *L. (V.) braziliensis* and treated with compound **7**. Control: Untreated Balb/c mice. Glucantime™: Balb/c mice treated with this drug. Size of the lesion was measured every week during 5 weeks

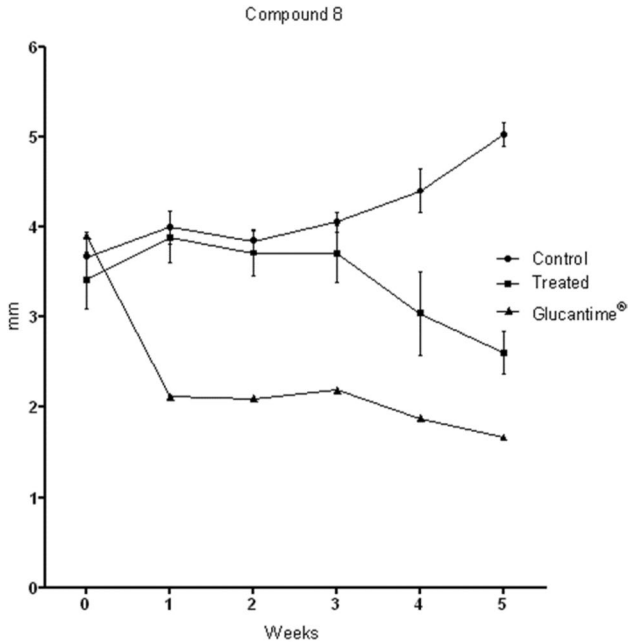


Fig. 6 Balb-C mice inoculated in the foot pad with promastigotes of *L. (V.) braziliensis* and treated with compound **8**. Control: Untreated balb-C mice. Glucantime™: Balb-C mice treated with this drug. Size of the lesion was measured every week during 5 weeks

and *L. (V.) braziliensis*. Among the tested compounds, the derivatives **7** and **8** have shown a significant in vitro and in vivo activity superior to that of the parent drug MNZ®, which may result from several independent or combined causes. The natures either of the A group or of the B substituent were crucial in the identification of the most appropriate pharmacophores. As can be seen in Fig. 8, the A group is represented by an S-alkyl substitution, while the B group consisted of a hydroxy attached at 2-position of the alkyl core, carboxy free or accoupled with an amino. In general, from the most active derivatives **7** and **8**, two important molecular pharmacophores were identified: (a) the inclusion of a S atom attached at 2-position of N-alkyl group of the 5-nitroimidazole ring as A substituent and (b) the incorporation of a carboxy group free or forming an amide with N-alkyl terminal as B substituent, which could lead to an improvement of the amphiphilic character of the molecules, leading to an increase in concentration of the compound inside the parasite form, thus increasing the interactions with leishmanial functional proteins. Regarding the influence of the rest of S-alkyl terminal B group, we can mention that (a) the incorporation of a primary hydroxyl or ester groups as B substituent caused a significant loss of the effect compounds **5** and **6**, (b) the effect of

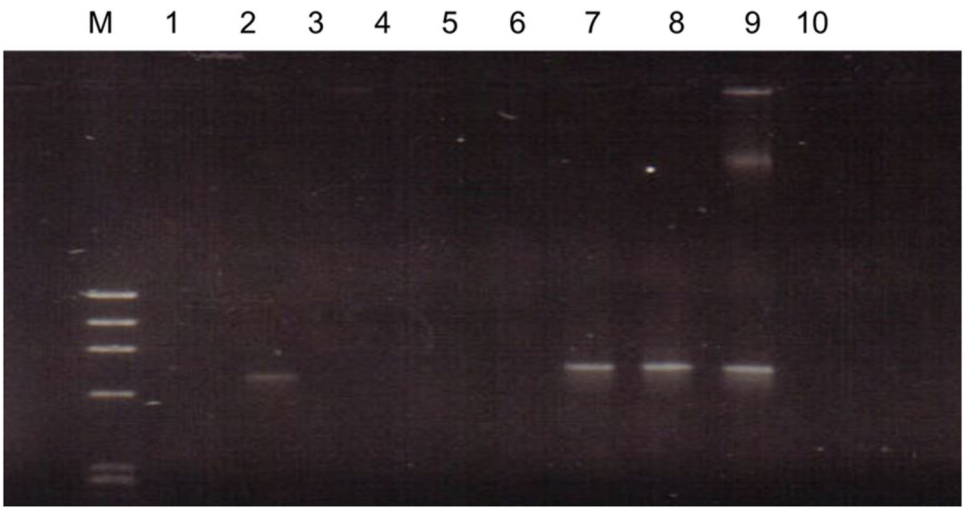


Fig. 7 Parasitological diagnosis using PCR and samples obtained from the food pad of the treated mice with compound **8**. Lanes 2, 7, and 8 resulted positives for *L. (V.) braziliensis* and 1, 3, 4, 5, and 6 were negatives with no parasites. Lane 9: positive control (DNA extracted from promastigotes); lane 10: negative control (Reaction mix with no DNA) M: Molecular weight marker

a secondary hydroxyl resulted in marginal antileishmanial activity compound **4**, and (c) the effect of hydroxy metronidazole MNZ™ **1** or chlorine atoms **2** and **3** attached at 2-position of N-alkyl group of the 5-nitroimidazole ring as A substituent a loss or marginal of antileishmanial activity was observed.

Even if additional assays related to toxicity on other human cell lines, genotoxicity experiments, and mechanism of action will be required to estimate their real potential, these biological results revealed that these two compounds constitute promising candidates in the search for improved therapies against *L. (L.) mexicana* and *L (V.) braziliensis*.

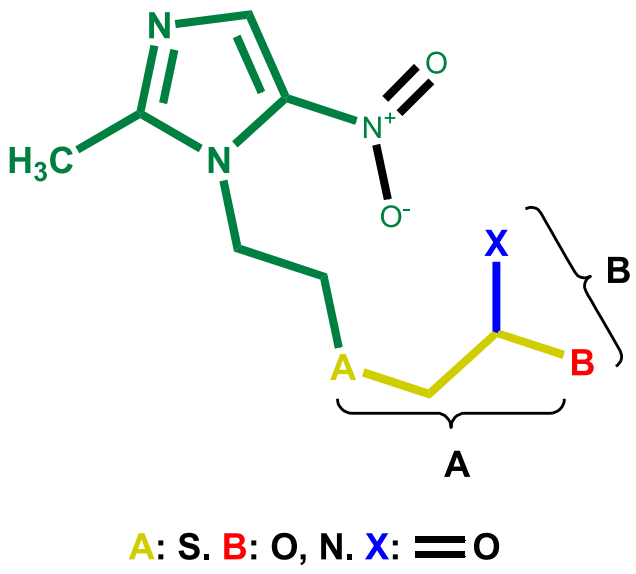


Fig. 8 Proposal for the most appropriate pharmacophorus for potential leishmanicides with these structural features

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Declarations

Conflict of interest The authors declare no competing interests.

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