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Original Article

Leishmanicidal activity of the venoms of the Scorpions *Brotheas amazonicus* and *Tityus metuendus*

Atividade leishmanicida dos venenos dos escorpiões *Brotheas amazonicus* e *Tityus metuendus*

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Abstract

Leishmaniasis is a vector-transmitted zoonosis caused by different species of the genus *Leishmania*, with a wide clinical spectrum. It is a public health problem aggravated by a series of limitations regarding treatment. In the search for new therapeutic alternatives, scorpion venoms are a source of multifunctional molecules that act against the natural resistance of pathogens. This work evaluated the antileishmanial potential of *Brotheas amazonicus* and *Tityus metuendus* venoms against the promastigote forms of *Leishmania amazonensis* e *Leishmania guyanensis*. The venoms of *B. amazonicus* and *T. metuendus* were evaluated for their constituents using Fourier Transform Infrared (FTIR). Growth inhibition and death of promastigotes were evaluated in the presence of diferente crude venom concentrations (100 µg/mL, 50 µg/mL, 10 µg/mL, 1 µg/mL) after one hour of incubation at 25 °C. The FTIR spectra of both venoms exhibited bands in approximate regions, revealing that both exhibit similar functional groups. Crude venom from both scorpion species showed similar or superior leishmanicidal effects to the standart drug N-methylglucamine antimoniate. At the highest concentration of 100 µg/mL, cultures of *L. guyanensis* treated with the venom of *B. amazonicus* showed the highest mortality percentages, above 28%, while *T. metuendus* venom showed the highest activity against *L. amazonensis*, with mortality above 7%. This preliminar study demonstrates that *B. amazonicus* and *T. metuendus* venoms can be important tools in the search for new drugs Against leishmaniasis. Next step involves evaluating the activity against the amastigote forms and purifying the venom proteins in order to identify the best anti-leishmania candidates.

Keywords: cutaneus leishmaniasis, *Leishmania amazonenses*, *Leishmania guyanensis*, Brazilian Amazon.

Resumo

A leishmaniose é uma zoonose de transmissão vetorial causada por diferentes espécies do gênero Leishmania, com amplo espectro clínico. É um problema de saúde pública agravado por uma série de limitações quanto ao tratamento. Na busca por novas alternativas terapêuticas, os venenos de escorpiões são uma fonte de moléculas multifuncionais que atuam contra a resistência natural dos patógenos. Este trabalho avaliou o potencial antileishmania dos venenos de *Brotheas amazonicus* e *Tityus metuendus* contra as formas promastigotas de *Leishmania amazonensis* e *Leishmania guyanensis*. Os venenos de *B. amazonicus* e *T. metuendus* foram avaliados quanto aos seus constituintes usando espectrometria no infravermelho por transformada de Fourier (FTIR). A inibição do crescimento e a morte de promastigotas foram avaliadas na presença de diferentes concentrações de veneno bruto (100 µg/mL, 50 µg/mL, 10 µg/mL, 1 µg/mL) após uma hora de incubação a 25 °C. Os espectros de FTIR de ambos os venenos exibiram bandas em regiões aproximadas, revelando que ambos exibem grupos funcionais semelhantes. O veneno bruto de ambas as espécies de escorpiões mostrou efeitos leishmanicidas semelhantes ou superiores ao antimoniato de N-metilglucamina padrão. Na maior concentração de 100 µg/mL, as culturas de *L. guyanensis* tratadas com o veneno de *B. amazonicus* apresentaram os maiores percentuais de mortalidade, acima de 28%, enquanto o veneno de *T. metuendus* apresentou a maior atividade contra *L. amazonensis*, com mortalidade acima de 7%. Este estudo preliminar demonstra que os venenos de *B. amazonicus* e *T. metuendus* podem ser ferramentas importantes na busca de novos fármacos contra a leishmaniose. O próximo passo envolve avaliar a atividade contra as formas amastigotas e purificar as proteínas do veneno para identificar os melhores candidatos anti-leishmania.

Palavras-chave: leishmaniose cutânea, *Leishmania amazonenses*, *Leishmania guyanensis*, Amazônia brasileira.

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1. Introduction

Leishmaniasis are vector-borne diseases with wide geographic distribution, with greatest occurrence in Asia, Africa and the Americas, where they are endemic in 18 countries (PAHO, 2019). They are caused by numerous digenetic species of the genus *Leishmania*, requiring at least two hosts to complete their development. Thus, a part of its cycle takes place in sandfly insects and the other in mammals (Sunter and Gull, 2017). They are classified as neglected tropical diseases (NTDs), occurring predominantly in poorer countries (Carvalho et al., 2019), transmitted by the bite of infected female sandflies during hematophagy (Alemayehu and Alemayehu, 2017). American cutaneous leishmaniasis (ACL) is a zoonosis that generates wounds on the skin, leaves scars and can progress to the nasal mucosa, mouth and throat (Carvalho et al., 2019). Brazil is among the countries with the highest number of cases in the world, caused by seven species of *Leishmania* recognized as agents of the disease, especially *L. amazonensis* and *L. guyanensis*, the latter being the main etiological agent in Amazonas, the Brazilian state with the highest incidence of ACL (Teles et al., 2019).

In general, pentavalent antimonials, especially antimoniate-N-methylglucamine (Glucantime), are the drugs of choice. But despite their effectiveness, they have a number of negative side effects (some common, others rare and associated with cumulative doses), such as muscle pain, gastrointestinal disorders, fever, skin reactions, cardiotoxicity, hepatotoxicity, nephrotoxicity and pancreatitis (Carvalho et al., 2019; Marques et al., 2019). Besides patients with heart, kidney and liver problems, the contraindication extends to pregnant women, since the drug can cross the transplacental barrier, affecting the fetal nervous tissue (Marques et al., 2019). The drugs of second choice are amphotericin B and pentamidine, which are equally toxic and of systemic administration (Carvalho et al., 2019). Since leishmaniasis is a multifactorial disease, it is common for its characteristics to vary according to the region, but there are numerous reports of therapeutic failure and relapse after clinical cure. Additionally, the metastatic forms do not respond well to traditional treatment (Carvalho et al., 2019). In recent years, treatment has also been limited by drug resistance, often by factors such as insufficient dosages, irregularity and premature discontinuation of treatment. Leishmaniasis remains a public health problem in several countries, with reports of the disease spreading to non-endemic areas (Marques et al., 2019).

In the search for potential agents with pharmacological functions, animal venoms have been studied. Tempone and Andrade Júnior (2001) observed the antileishmanial potential of the crude venom of the *Bothrops moojeni* snake against promastigote forms of *Leishmania* spp. Nunes et al. (2013) reported the effects of the BnSp-7 toxin from *Bothrops pauloensis* against promastigote and amastigote forms of *L. amazonensis*. Scorpion venoms also stand out as promising sources of biologically active peptides (Almaaytah and Albalas, 2014). The literature reports the activity of *Androctonus crassicauda* venom against *L. tropica* promastigotes (Yildiz Zeyrek et al., 2021). Borges et al. (2013) observed the leishmanicidal activity of the venoms of ten *Tityus* species against *L. mexicana*, in which *T. discrepans*, *T. gonzalespongai* and *T. perijanensis* caused more than 80% mortality.

In Brazil, the existing species are arranged in four families. The Buthidae family is the most abundant, with potentially dangerous species, such as those of the Tityus genus (Bertani et al., 2021), and has the highest number of species in the states of Bahia and Amazonas. Members of the Chactidae family are found almost exclusively in tropical forests, and in Brazil they restricted almost totally to the North region, such as species of the genus *Brotheas* (Brazil and Porto, 2010). Data in the literature demonstrate several biological activities of scorpion venoms or their derivatives, such as antibacterial (Dueñas-Cuellar et al., 2015; Marques-Neto et al., 2018), antifungal (Du et al., 2015), antiparasitic (Borges et al., 2006) and anticancer activities (Gómez Rave et al., 2019). Thus, the objective of this work was to evaluate the leishmanicidal activity of the venoms of the scorpions *B. amazonicus* and *T. metuendus* against the promastigote forms of *L. amazonensis* and *L guyanensis*.

2. Material and Methods

2.1. Venoms and strains of Leishmania spp.

The venoms of the scorpions *B. amazonicus* Lourenço, 1988 and *T. metuendus* Pocock, 1987 were extracted according to Batista et al. (2018), freeze-dried and stored at room temperature for later use (registration at SisGen: A5C939E). The strains *Leishmania (L.) amazonensis* IOC/L 575 (IFLA/BR/1967/PH8) and *Leishmania (V.) guyanensis* IOC/L 565 (MHDM/BR/1975/M4147) were provided by Dr. Maria das Graças Barbosa of Tropical Medicine Foundation Doctor Heitor Vieira Dourado – FMT/HDV, in Manaus, Amazonas, Brazil.

2.2. Scanning electron microscopy

Microscopy was performed at the Multiuser Center for the Analysis of Biomedical Phenomena (CMABIO). The promastigote forms were analyzed using a Jeol JSM-IT500HR scanning electron microscope, according to Souza (2007). The samples were washed with PBS 1x to remove excess culture medium and then fixed by submerging the samples in modified Karnovsky fixative (glutaraldehyde 2.5%, paraformaldehyde 2.5%, in potassium phosphate buffer 0.05 M) for two hours at room temperature. Subsequently, they were subjected to four successive washes in 0.05 M potassium phosphate buffer pH 7.2 for 10 minutes each.

Post-fixation was performed by submerging the samples for one hour in a 1% osmium tetroxide solution in 0.1 M potassium phosphate buffer pH 7.3, in the dark, followed by three washes with 0. 05 M. Then the samples were dehydrated with 30%, 50%, 70%, 80%, 90% and 100% ethanol solutions, keeping the samples submerged in each of the solutions for 10 minutes (this process was repeated three times in the 100% ethanol step). Afterwards, samples were spread on glass coverslips previously coated with poly-L-lysine, followed by drying in a Leica EM CPD300 critical-

point dryer for 90 minutes, ending with fixation on stubs with carbon tapes and submission to four minutes of metallization with gold in a JEOL Smart Coater.

2.3. Fourier Transform Infrared (FTIR)

The lyophilized venoms of *B. amazonicus* and *T. metuendus* were evaluated for their constituents using Fourier Transform Infrared – Attenuated Total Reflectance (FTIR-ATR). In this analysis, 2 mg of each sample were placed into an infrared equipment (Cary 360) and analyzed in the spectral range of 4000 to 400 cm^{-1}, with a resolution of 8 scans.

2.4. Leishmanicidal activity

The antileishmanial activity assay was performed at the Laboratory of Cellular Immunopharmacology of the Escola Normal Superior (LIFCEL – ENS/UEA). The growth of promastigote forms of *Leishmania* spp. was observed according to the protocol of Alves (2019) with modifications. The parasites were grown in cell culture flasks containing complete RPMI 1640 medium at a concentration of $1x10^6$ parasites/mL, and incubated at 25 °C, pH 7 for 10 days. Every 24 hours, 10 µL of the parasite culture was diluted in 740 µL of saline solution (NaCl 0.9%), followed by addition of 250 µL of 0.4% Trypan Blue dye for counting in a Neubauer chamber (RGB) with the aid of an optical microscope at 400x magnification. The arithmetic mean and standard deviation were used to generate the growth curves using the Excel® software (Microsoft).

The leishmanicidal activity of the venoms was determined according to the growth inhibition and mortality of the promastigote forms of *L. amazonensis* and *L. guyanensis*. Before each experiment, flagellar mobility and parasite morphology were observed with the aid of an inverted microscope. To carry out the bioassays, the promastigote forms were centrifuged at 1800 xg for 15 min at 4 °C, washed in sterile saline, diluted in 5 mL of RPMI medium and quantified in a Neubauer chamber, after adjustment to obtain a parasite concentration of $1.5x10^6$ parasites/mL. The ability to inhibit the growth or cause death of the promastigote forms was evaluated after the addition of different concentrations of the venoms (100 µg/mL, 50 µg/mL, 10 µg/mL and 1 µg/mL) in the presence of $1.5x10^6$ parasites/mL in 96-well microplates. The samples were resuspended in RPMI 1640 medium, while RPMI 1640 medium alone was used as negative control, and Glucantime ($3x10^4$ µg/mL) served as positive control, according to Alves (2019). The experiment was carried out in three replications, each in triplicate.

Growth inhibition and mortality of *L. amazonensis* and *L. guyanensis* promastigotes were monitored after one hour of incubation at 25 °C, according to a protocol adapted from Borges et al. (2006). To count the parasites, aliquots of 10 µL of the sample were solubilized in 740 µL of saline solution (1:10), stained with 250 µL of 0.4% Trypan blue, and from this solution 10 µL was removed and applied in the Neubauer chamber and observed under an optical microscope at 400x magnification. The percentage of live and dead parasites, mean and standard deviation were calculated.

3. Results

It was possible to extract venom from both scorpion species. Morphologically, *B amazonicus* had a yellowish-red telson and absence of thorn under the stinger, while *T. metuendus* was almost black, with small light spots on the trunk and legs (Figure 1) In nature, *B. amazonicus* is commonly found inside the trunks of trees fallen to the ground, while *T. metuendus* prefers to hide in the trunk or canopy of trees. The analysis of the promastigote forms of *L. amazonensis* and *L. guyanensis* by scanning electron microscopy revealed morphological characteristics typical of promastigote forms: elongated ovoid bodies and mobile

Figure 1. Characteristics of scorpions (A) *Tityus metuendus*, with small light spots on the trunk and legs, and (B) *Brotheas amazonicus*, with a yellowish-red telson.

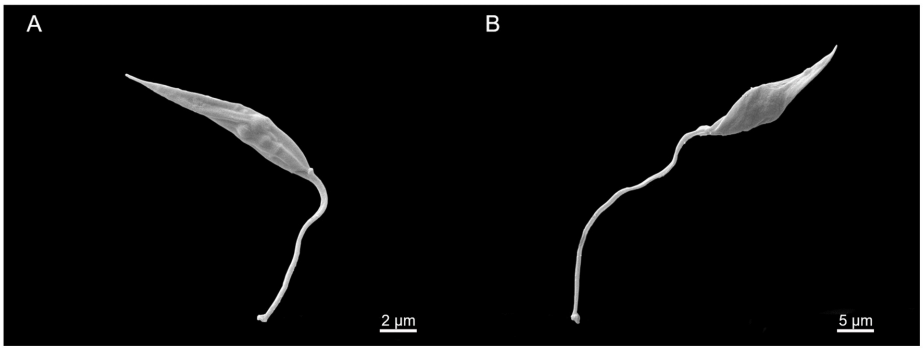

Figure 2. Promastigote forms of *L. amazonensis* (A) and *L. guyanensis* (B).

flagella (Figure 2). The parasitic growth curve was plotted to identify the different stages and development of *Leishmania* species and define the day for the experiments. Different growth patterns were observed. *L. amazonensis* had a logarithmic phase until the fifth day, and began the stationary phase on the sixth day, followed by decline until death phase from the eighth day of cultivation. Meanwhile, *L. guyanensis* had a logarithmic phase until the fourth day of cultivation, began the stationary phase on the fifth day and declined from the seventh day onward (Figure 3). Therefore, we chose the fourth day to carry out the tests, since it was in the transition from the log phase to the stationary phase of both species, during which the largest amounts of metacyclic promastigotes are produced, i.e., the most infective ones (Marques et al., 2019).

The FTIR spectra of both venoms exhibited bands in the regions 3278 and 3272 cm^{-1}, corresponding to hydrogen bonding vibrations (O-H); 2962, 2935 and 2932 cm^{-1}, representing C-H stretching vibrations (forming CH_2, CH_3, and CH bonds); 2367 and 2362 cm^{-1}, indicative of SH frequency; 1637 and 1628 cm^{-1}, characteristic of the amine group II (NH_2); 1542 cm^{-1}, amide peak; 1389 and 1386 cm^{-1}, related to the carbonyl group (C=O); 1081 and 1076 cm^{-1}, representing the ether group (C-O-C). The spectrum of *T. metuendus* venom showed a band in the 1235 region, corresponding to PO^{2-} vibrations (Figure 4).

Previous studies regarding the cytotoxicity of *B. amazonicus* and *T. metuendus* venoms, using the MRC5 human fibroblast line, showed cytotoxicity below 30% against the cells at the highest tested concentration, 100 µg/mL.

The venom of *B. amazonicus* caused a parasite mortality percentage similar to that observed in the control treatment (Glucantime) for *L. amazonensis* promastigotes at concentrations of 10 µg/mL, 50 µg/mL and 100 µg/mL (Table 1). At these concentrations, there was a statistically significant difference compared to the negative control, but there was no difference compared to the positive control (Figure 5A). This venom also caused a higher percentage of mortality of *L. guyanensis* promastigotes than Glucantime (6.65%) at concentrations of 10 µg/mL (8.07%), 50 µg/mL (15.04%) and 100 µg/ mL (28.86%), as can be seen in Table 1. These concentrations showed a statistically significant difference compared to the negative control, but only concentrations of 50 µg/mL and 100 µg/mL showed a

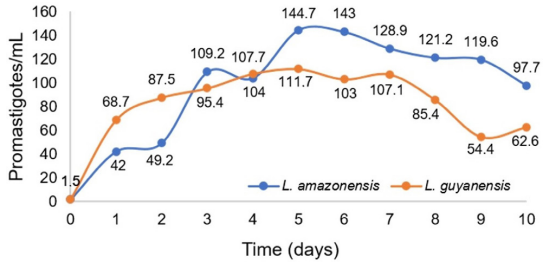

Figure 3. Growth curve of the promastigote forms of *L. amazonensis* and *L. guyanensis* after culture for 10 days.

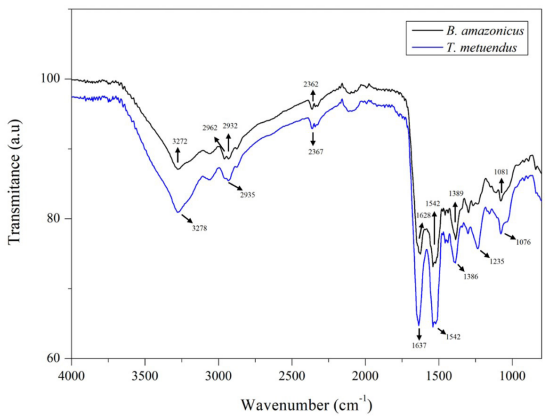

Figure 4. FTIR-ATR spectra of *B. amazonicus* and *T. metuendus* venoms.

statistically significant difference compared to the positive control at 1 hour, $P < 0.05$ (ANOVA), as seen in Figure 5B. The *T. metuendus* venom had activity against *L. amazonensis* promastigotes at all concentrations and was superior to the standard drug from a concentration of 10 µg/mL (Table 1), evidencing statistically significant difference to the negative control from this concentration. The concentrations of 50 µg/mL and 100 µg/mL produced a statistically significant difference in relation to the positive control regarding death of promastigotes (Figure 4A). The venom of *T. metuendus*

Table 1. Percentage of dead parasites in controls and at different concentrations of *B. amazonicus* and *T. metuendus* venoms.

Concentration	*B. amazonicus*		*T. metuendus*	
	L. amazonensis	*L. guyanensis*	*L. amazonensis*	*L. guyanensis*
Control (-)	1.56%	2.42%	1.16%	1.49%
1 µg/mL	2.74%	5.88%	2.68%	4.13%
10 µg/mL	3.45%	8.07%	4.23%	7.74%
50 µg/mL	4.52%	15.04%	6.06%	11.93%
100 µg/mL	4.96%	28.86%	7.45%	17.25%
Control (+)	**3.44%**	**6.65%**	**2.98%**	**4.85%**

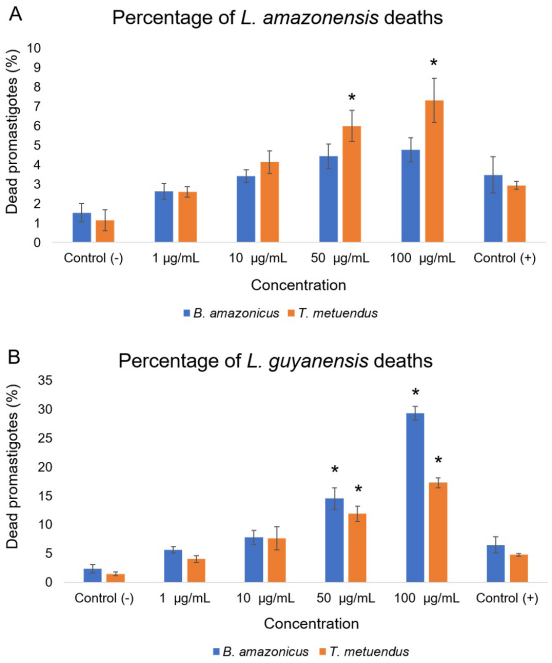

Figure 5. Number of dead parasites of (A) *L. amazonensis* and (B) *L. guyanensis* at different concentrations of *B. amazonicus* and *T. metuendus* venoms. *Indicates difference between concentration and positive control ($p<0.05$), Tukey test.

also caused higher mortality of *L. guyanensis* parasites than the drug, and statistically significant difference compared to the negative control from a concentration of 10 µg/mL. At the concentration of 50 µg/mL, a death percentage of 11.93% of the protozoa was observed, and at the concentration of 100 µg/mL the percentage was 17.25%, compared to 4.85% for Glucantime (Table 1), and both showed a statistically significant difference when compared to the positive control (Figure 4B).

4. Discussion

B. amazonicus is common in the region of Manaus, Amazonas, Brazil, while *T. metuendus* occurs in the Amazon region (Martins et al., 2021). One way to observe leishmanicidal biological action is through electron microscopy, whereby it is possible to observe structural changes in the length of the cell body and the flagellum (Sunter and Gull, 2017; Anversa et al., 2018), as well as elongated ovoid bodies, characteristic of the promastigote form.

The growth curve was plotted to determine the differences in the development of the species, and the best day to carry out the tests. The exponential growth of *L. guyanensis* and *L. amazonenses* occurred, respectively, until the fourth and fifth day. Similar results were reported, who observed exponential growth up to the sixth day of cultivation for both species (Campos, 2008). It was identified the logarithmic phase until the fourth day of cultivation for *L. amazonensis* and accelerated growth of *L. guyanensis* until the second day, with the start of the stationary phase on the third day of cultivation (Alves, 2019). We chose the fourth day of cultivation to carry out the tests with both species. The parasites collected that day were used in *in vitro* assays to evaluate the leishmanicidal action of the venoms.

The FTIR analysis was conductec to observe the occurrence of functional groups in the venoms of *B. amazonicus* and *T. metuendus*, revealing that both exhibit similar functional groups. According to Coates (2000), each molecule possesses a chatacteristic vibrational spectrum that acts as a "fingerprint" for identification, allowing the comparison of a "unknown" spectrum with previously recorded reference spectra.

Zhao et al. (2016), in their FTIR analysis of scorpions stings, observed that these are primarily composed of chitosan, detecting peaks attributed to O-H and C-H stretching vibrations, as well as amide I and amide II peaks. Ghorban Dadras et al. (2013), when comparing chitosan spectra with chitosan nanoparticle spectra containing *Androctonus crassicauda* venom, observed changes in the peak from 3261 to 3321 cm^{-1}, due to the formation of additional hydrogen bonds, along with N-H bending and carbonyl stretching in amide II.

Our results detected different leishmanicidal action between the tested venoms, with *T. metuendus* having greater action against *L. amazonensis* while the venom of *B. amazonicus* was the most effective against *L. guyanensis*. These results may be related to the bioactive potential of

each venom, or between the cultivated parasites, such as susceptibility and virulence (Mandal et al., 2015; Pinho et al., 2020). The venoms of both species are rich in compounds with therapeutic potential (Higa et al., 2014; Batista et al., 2018).

Cutaneous leishmaniasis is a disease lacking attention from healthcare services, considered a neglected illness, affecting populations in socioeconomic vulnerability (Brasil, 2021). Oliveira et al. (2021) reported a higher number of infections in individuals with low educational levels, often associated with the agricultural sector. Additionally, the population affected by the disease suffers from the social stigma resulting from disfiguring deformities and scars, significantly impacting their quality of life (Brasil, 2021). Ihsanullah et al. (2023) observed a higher percentage of lesions on the face, arms, and legs.

There was a difference in the percentage of dead *L amazonensis* and *L. guyanensis* parasites in relation to Glucantime. *L. amazonensis* was more resistant to the drug in this experiment, and therefore more difficult to treat. From the venom concentration of 10 µg/mL, there was a rising mortality percentage compared to the drug used as positive control (Glucantime $3x10^4$ µg/mL), in all treatments. Alves (2019) observed the mortality caused by this concentration of Glucantime after 72 hours of incubation, obtaining, approximately, 43% to 70% for *L. amazonensis* and 44% to 89% for *L. guyanensis*. Therefore, the low percentages of the standard drug in this experiment may be related to the short incubation time of one hour.

The antileishmanial screening performed in the present work showed the efficiency of the venoms of the scorpions tested in killing promastigotes. The inhibition results obtained through the leishmanicidal assay indicated that the venoms have a direct effect on *Leishmania in vitro*, showing that the venoms contain molecules that can be effective against the natural resistance of parasites. Antimicrobial peptides derived from scorpion venom have aroused great interest among researchers and in the pharmaceutical industry for the development of new drugs, due to their potent activity, low resistance rates and a unique mode of action (Solano-Godoy et al., 2021).

Different response patterns have been identified when testing the venom of *T. discrepans* against *Leishmania* species. The leishmanicidal activity was found to be dose-dependent in the sensitivity order *Leishmania (L.) mexicana* > *Leishmania (V.) braziliensis* > *Leishmania (L.) chagasi* (Borges et al., 2006). Species of the genus *Tityus* are found in different areas of Brazil, including urban areas, due to the ability of some of these animals to adapt to environments heavily modified by humans. Experimental studies have indicated that scorpions can produce peptides with antimicrobial, antiviral and antitumor potential (Batista et al., 2018; Rinaldi et al., 2021).

The highest percentage of promastigote mortality was caused by the venom of *B. amazonicus*. This scorpion in the Manaus region lives in leaf litter and inside fallen tree trunks, and has low toxicity to humans (Martins et al., 2021). The proteolytic activity of the *B. amazonicus* venom degraded the Aα and Bβ subunits of fibrinogen, and the *B. amazonicus* venom had low toxicity, making it a candidate for new drugs (Higa et al., 2014). On the other hand, mass spectrometry of the tryptic digestion of the soluble venom the venom of *T. metuendus* revealed an amino acid sequence of 111 different peptides. Search for similarities of the sequences indicated they were probably toxins of the sodium and potassium channels, metalloproteinases, hyaluronidases, endothelin and angiotensin converting enzymes, bradykinin potentiating peptide, hypothetical proteins, allergens, other enzymes, other proteins and peptides (Batista et al., 2018). To expand this investigation of the potential of animal venom against promastigote forms, *in vitro* toxicity testing on human cell lines is relevant, as new effective therapeutic strategies against *Leishmania* are currently lacking. Here, our tests to observe cell viability in the MRC5 human fibroblast line showed cell survival above 78% for *B. amazonicus* and 72% for *T. metuendus*, indicating low toxicity.

5. Conclusion

The venoms of the two scorpion species caused similar or higher percentages of death than the standard drug chosen to combat *Leishmania* spp. However, *B. amazonicus* venom showed better results against *L. guyanensis*, while *T. metuendus* venom was more effective against *L. amazonensis*, while both had leishmanicidal activity.

These results indicate that venoms of *B. amazonicus* and *T. metuendus* are promising in the search for new anti-leishmania agents. However, further studies are necessary to determine the potential of these venoms.

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