Evaluation of the *in vivo* and *in vitro* effect of *Libidibia ferrea* on *Leishmania (Leishmania) amazonensis*

Avaliação do efeito in vivo e in vitro de *Libidibia ferrea* sobre *Leishmania (Leishmania) amazonensis*

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RESUMO
Atualmente, o arsenal terapêutico disponível para o tratamento da leishmaniose é limitado. Assim, o objetivo deste estudo foi avaliar a eficácia antileishmania in vitro e in vivo do extrato metanólico e da fração diclorometano de Libidibia ferrea (DCM). Para atingir esse objetivo, foi determinada a citotoxicidade desses extratos em ensaios in vitro. Posteriormente, seus efeitos em uma infecção por Leishmania (Leishmania) amazonensis de macrófagos foram avaliados. Estudos in vivo foram realizados com hamsters dourados infectados com L. (L.) amazonensis, os quais foram tratados com uma microemulsão contendo a DCM. Além disso, essa fração também foi avaliada em combinação com Glucantime®. Os extratos reduziram as infecções por macrófagos e induziram a reversão da supressão de IL-6 em células infectadas. Observou-se redução significativa no volume da lesão dos animais tratados intralesionalmente com a combinação de DCM+20 mg/SbV/kg/dia. Tomados em conjunto, os resultados obtidos mostram que os extratos de L. ferrea não foram citotóxicos para macrófagos e limitaram a infecção dos macrófagos. A microemulsão contendo a fração diclorometano de L. ferrea aumentou os efeitos curativos do Glucantime®.

Palavras-chave: Produtos naturais; Microemulsão; Tratamento da leishmaniose;

ABSTRACT
Currently, the therapeutic arsenal available for the treatment of leishmaniasis is limited. Thus, the aim of this study was to evaluate the in vitro and in vivo antileishmanial efficacy of the methanolic extract and dichloromethane fraction of Libidibia ferrea (DCM). To achieve this goal, the cytotoxicity of these extracts was determined in in vitro assays. Subsequently, its effects on a Leishmania (Leishmania) amazonensis infection of macrophages were evaluated. In vivo studies were performed with golden hamsters infected with L. (L.) amazonensis, which were treated with a microemulsion containing DCM. Furthermore, this fraction was also evaluated in combination with Glucantime®. The extracts reduced macrophage infections and induced reversal of IL-6 suppression in infected cells. There was a significant reduction in the volume of the lesion in the animals treated intralesionally with the combination of DCM+20 mg/SbV/kg/day. Taken together, the results obtained show that L. ferrea extracts (DCM) were not cytotoxic to macrophages and limited macrophage infection. The microemulsion containing the dichloromethane fraction of L. ferrea enhanced the curative effects of Glucantime®.

Keywords: Natural products; Microemulsion; Leishmaniasis treatment;
INTRODUÇÃO

Cutaneous leishmaniasis (CL) is a disease of worldwide distribution, and affects at least 92 countries (WHO, 2021). A recent report stated that most cases occur in Afghanistan, Algeria, Colombia, Iran, Syria and Brazil (DNDi, 2018). This disease is caused by the protozoa of the genus Leishmania, which are transmitted to mammalian hosts through the bites of sandflies (Blanco, 2017). The therapeutic arsenal and treatment regimen for CL in Brazil follows the recommendations of the Brazilian Ministry of Health, and consists of the use of meglumine antimoniate (Glucantime®), pentamidine isethionate (Pentacarinat®) or amphotericin B (Ambiosome®) (Brasil, 2017). However, the efficacy of these drugs is compromised by the occurrence of parasitic resistance, the lengthy treatment period, and drug toxicity in patients (Bastos et al., 2016).

According to Brazil (2017), CL is characterized by a single or multiple skin ulcer, which may have metastasis through the hematogenous spread as its main complication, and causes destruction of mucous tissue, especially in the nasopharynx, which is known as mucosal leishmaniasis. Some studies have shown that the increase in the cytokine IL-6 may be associated with the risk of development of the mucosal form, and it is therefore essential to investigate the modulation of this cytokine in the different treatments used for cutaneous leishmaniasis.

To address the need for new therapeutic compounds, several studies have concentrated efforts on the search for active ingredients from natural products (Garzon et al., 2021). Among these natural products, plant-derived products have attracted attention because they include unique secondary metabolites that have a diversity of chemical structures (Jensen et al., 2017).

In this context, the plant species Libidibia ferrea has attracted the interest of the scientific community due to reports of its antidiarrheal, anticatarrhal, anti-inflammatory (Falcão et al., 2019), and antileishmanial (Comandolli-Wyrepkowski et al., 2017) properties, among others. Previous studies have demonstrated the potential of using L. ferrea in the treatment of CL via topical formulations (Comandolli-Wyrepkowski et al., 2017).

In the study conducted by Jensen (2020), the characterization of dichloromethane was carried out, and methyl gallate and a phenylpropanoic acid derivative were isolated. According to the study, the substances indicated promising antileishmanial activity and no cytotoxic profile. These promising observations indicate that additional studies need
to be carried out to identify and evaluate the antileishmanial bioactive substances found in L. ferrea and optimize skin permeation of these compounds (Jensen, 2020).

Studies on the use of microemulsion systems have shown promise, and some of the advantages of these systems include the possibility of optimizing the permeation and the controlled release of bioactive molecules (Ghorbanzadeh et al., 2019). In addition, Jensen (2020) suggests that further studies should be developed that combine the standard treatment for CL (Glucantime®) with microemulsion-based formulations containing L. ferrea in order to investigate a possible augmentation of the effectiveness of Glucantime®.

Therefore, the aim of this study was to evaluate the in vitro antileishmanial efficacy of the methanolic extract and the dichloromethane fraction of L. ferrea in infections of macrophages. A microemulsion was developed containing the dichloromethane fraction of L. ferrea, which was evaluated in in vivo studies for its effectiveness alone and in association with Glucantime® in the experimental treatment of cutaneous leishmaniasis.

MATERIAL AND METHODS

Origin and maintenance of parasites

The strains used in this study were Leishmania (Leishmania) amazonensis (MHO/BR/2009/IM5584), cryopreserved in the bank of strains at the Laboratory of Leishmaniasis and Chagas Disease/COSAS/INPA and Leishmania (Leishmania) amazonensis (MHOM/BR/77/LTB0016), cryopreserved in the Laboratory of Microbiology and Cell Science, University of Florida, USA. For the biological assays, the parasites were grown in Novy-MacNeal-Nicolle medium (NNN) and culture was expanded in complete RPMI medium 1640 (Himedia), which was supplemented with 10% inactivated fetal calf serum (iFCS) and gentamycin (40 μg.mL-1).

Plant material and fractionation

The collection of Libidibia ferrea was carried out on a private property in the Parque Dez de Novembro neighborhood, Manaus, Amazonas, (3º 4’ 34” S 60º 0’ 11” W),
during the fruiting period (June and July). The specimens were sent to the Botany Department of the Instituto Nacional de Pesquisas da Amazônia (INPA), identified as Libidibia ferrea (Mart. ExTul.) LP Queiroz – Fabaceae: Caesalpinoideae, and deposited in the INPA herbarium under voucher specimen number 2461509.

The fruits were sorted, the seeds were removed and the other parts of the fruit were dried in a forced circulation oven at a temperature below 45 °C. After drying, the material was pulverized, macerated and extracted with the solvents hexane and methanol (VETEC®), with three extractions being carried out with each of the solvents (Comandolli-Wyrepkowski et al., 2017). The dichloromethane fraction (DCM) of the L. ferrea extract was obtained by liquid-liquid partition, using 20 g of the methanolic extract solubilized in 200 mL of H2O:MeOH, then the fraction was concentrated using a rotary evaporator (Yamamoto®) (Jensen, 2020).

Preparation of topical formulations

Two microemulsions were prepared. The first was a microemulsion containing the DCM fraction of L. ferrea (DCM) at 2% and the second was a placebo microemulsion without the fraction. Both microemulsions were developed using isopropyl alcohol, Tween® 20, distilled water, oleic acid, with or without the DCM fraction of L. ferrea, and kept under agitation for 2 minutes until a homogeneous solution was obtained (Jensen, 2020). The patent for the formulation is registered at the National Institute of Industrial Property (INPI) Brazil, under process number BR 10 2019000809 1.

In vitro biological cytotoxicity assay

Murine RAW 264.7 macrophages, cryopreserved in the Laboratory of Microbiology and Cell Science, University of Florida, USA, were cultured (105 cells/mL) in 96-well plates in RPMI 1640 (Sigma Chemical Co. St. Louis, USA), which was supplemented with 10% heat-inactivated fetal bovine serum - FBS (LGC Biotecnologia, São Paulo, Brazil) and 50 µg mL−1 gentamicin (Novafarma, Brazil) for 10 hours. After this period, the cells were exposed to different drug preparations: methanolic extract of L. ferrea (EXT); dichloromethane fraction of L. ferrea (FRA); Glucantime® (GL) and Pentacarinat® (PTM). All compounds were tested at 0, 50 and 1.6 µg.mL−1, and were
incubated at 37 oC for 24 hours. After this incubation period, the MTT reagent (3-[4,5-di- 
 methyl-2-thiazoly]-2,5-diphenyl-2H-tetrazolium bromide) (Roche®) was added at a 
 concentration of 10%. The macrophages were incubated for an additional 12 hours, after 
 which the formazan product was solubilized and read in a spectrophotometer (Epoch, 
 Bio-Tek®, USA) at 570 nm (Comandolli-Wyrepkowski et al., 2017).

In vitro biological assay with amastigote forms

RAW 264.7 macrophages were incubated in 6-well plates at a cell density of 105 
cells/mL, in RPMI-1640 medium (Himedia) supplemented with 10% HI FBSi, at 37 ºC 
for 48 hours. After this period, the macrophages were infected with 2 x 106 
 promastigotes/mL (1:10 cell/parasite) of Leishmania (Leishmania) amazonensis 
(MHOM/BR/77/LTB0016) for 24 hours at 37 ºC. The methanolic extract of L. ferrea 
(EXT); dichloromethane fraction of L. ferrea (DCM); Glucantime® (GL) and 
Pentacarinat® (PTM) – at 100, 50 and 1.6 μg.mL-1 were subsequently added to the 
infected cells. After 24 hours of incubation at 37 ºC, the cells were fixed. Nucleated cells 
were labeled with DAPI (Thermo Fisher Scientific) to determine the infection rate. 
Quantification of the infection was determined by enumerating infected macrophages in 
 at least 30 infected fields per group (Comandolli-Wyrepkowski et al., 2017).

Production of IL-6 cytokines

Macrophages were plated in 6-well plates at 5x105 cells/mL per well and, 
subsequently, cells were infected with stationary L. (L.) amazonensis promastigotes 
(5x107 promastigotes/mL) as described above. After 24 hours, the infected cells were 
treated with Glucantime® (GL) (50 and 100 μg.mL-1) or the methanolic extract of L. 
ferrea (EXT) (10 μg.mL) or the dichloromethane fraction of L. ferrea (DCM) (50 μg.mL- 
1) or Retro-2 (20 nm) or a combination of GL (100 μg.mL-1) + DCM (100 μg.mL-1) for 
24 hours in complete DMEM supplemented with LPS (100 ng.mL-1 or 500 ng.mL-1) and 
IFN-γ (100 ng.mL-1). Controls were treated with complete DMEM alone or LPS/IFN-γ 
without drugs or with drugs without LPS/IFN-γ stimulation. After 24 hours, the 
supernatants of each treatment were recovered and their IL-6 content determined in an
ELISA specific for IL-6. The cells in each well were processed for enumeration of the infection as described above (Croft; Coombs, 2003).

In vivo experiments

A total of 60 adult male golden hamsters (Mesocricetus auratus), with an average weight of 120 g and aged 60 days were used. These were obtained from the vivarium at INPA and kept in conditioned rooms with a photoperiod of 12/12 hours, free of pathogens and fed ad libitum. The animals were divided into ten experimental groups (six animals per group), of which nine groups were infected in the snout with a volume of 0.1 mL of promastigote forms of Leishmania (Leishmania) amazonensis (MHO/BR/2009/IM5584) (106 parasites/mL).

After the appearance of the lesions, the groups were treated according to the following experimental design: I) DCM Group: infected and treated topically with the microemulsion of the DCM fraction (DCM) of L. ferrea (50 mg/kg/day); II) placebo microemulsion (MPL) group: infected and treated topically (50 mg/kg/day); III) GLIM20 Group: infected and treated intramuscularly with 20 mg/SbV/kg/day; IV) GLIL20 group: infected and treated intralesionally with 20 mg/SbV/kg/day; V) DCM+GLIM10 group: infected and treated IM with 10 mg/SbV/kg/day and topically with DCM (50 mg/kg/day); VI) DCM+GLIM20 Group: infected and treated IM with 20 mg/SbV/kg/day and topically with DCM (mg/kg/day); VII) DCM+GLIL10 Group: infected and treated IL with 10 mg/SbV/kg/day and topically with DCM (mg/kg/day); VIII) DCM+GLIL20 Group: infected and treated IL with 20 mg/SbV/kg/day and topically with DCM (mg/kg/day); IX) IST group: infected and untreated and X) NIST group: uninfected and untreated.

The animals treated topically with DCM or intramuscular Glucantime® were treated daily for a period of 30 days. The animals that were treated with intralesional Glucantime® received a total of 3 doses, which was applied on the 1st, 15th and 30th days. After treatment, three animals from each experimental group were euthanized for the collection of biological material, and the other three animals from each group remained under observation until the 135th day. During the treatment period, the measurement of the total volume of the lesion (length, width and height) was performed on alternate days using a digital caliper (Zaas® Precision) to analyze the progression of
the lesion. Photo-documentation of the clinical evolution of the lesions was also performed.

At the end of the treatment, the animals were euthanized with Vetnil® (ketamine hydrochloride 10%) and Syntec® (xylazine 2%), as recommended by the INPA Animal Research Ethics Committee, and biological material was collected for parasitological and biochemical evaluation. For the parasitological evaluation, a tissue sample sectioned from the lesion area of each animal was used for printing on glass slides and, later, the biological material contained in the slides was fixed with methanol and stained with Giemsa (Sigma). The analysis was performed by quantification of the amastigotes and macrophages present in 25 fields of each slide. The biochemical evaluation was performed by collecting 2 mL of blood from each animal for subsequent analysis of liver and kidney function via the evaluation of urea (Ureia UV Liquid Stable, LaborLab, REF1770300) and creatinine (LaborLab, REF1770100) values and a cardiac marker test (CK Total, InterKit, batch 12213040) using an automatic biochemical and turbidimetric analyzer (MH Lab, URIT 8031).

Statistical analysis

The in vivo assays used an experimental design to verify the effects of the different treatments, and the statistical test was based on a population model to infer differences between treatments. The program used was the R Core Team (2016) version 4.1.1 and Excel 2019 (Microsoft Office Professional Plus). The tests used were the Kruskal-Wallis, the t-test and Mann–Whitney, and the significance level adopted for the tests was 5%.

Ethical aspects

This study was submitted to and approved by the Animal Research Ethics Committee of the National Institute for Amazonian Research (CEUA-INPA) under process No. 023/2021.

RESULTS
In order to begin the in vitro studies with the methanolic extract and the dichloromethane fraction of L. ferrea, their cytotoxicity to macrophages was evaluated. Cell viability was assessed in MTT assays after incubation of cells with a range of concentrations of the methanolic extract (EXT) and the dichloromethane fraction (DCM) of L. ferrea. The cytotoxicity of these compounds was compared to the toxicity of a range of concentrations of Glucantime® (GL) and pentamidine (PTM), which are well known drugs that are currently used to treat leishmaniasis. The results show that, at up to 100 µg/ml of Glucantime®, cell viability was equivalent to cell viability in the medium alone and in the medium supplemented with the diluent (DMSO) (Figure 1). Neither the EXT nor the DCM of L. ferrea at concentrations of up to 100 µg/ml were toxic to cells. No significant effect on cell viability was observed when Glucantime® was combined with EXT or when Glucantime® was combined with DCM. In contrast, only 20% of cells were viable after incubation in 25 µg/ml pentamidine.

**Figure 1.** Cytotoxicity in RAW cells at 24 hours. Glucantime ® (GL); Pentamidine (PTM); Libidibia ferrea dichloromethane fraction (DCM), association of Glucantime ® and L. ferrea dichloromethane fraction (GL+DCM), L. ferrea extract (EXT) and association of Glucantime ® and L. ferrea extract (GL+EXT), dimethylsulfoxide (DMSO). * indicates significant difference for the control (-) – untreated cells.

Source: Bacha (2023)
The antileishmanial activity of the drugs and extracts discussed above were evaluated via incubation with RAW264.7 macrophages infected with L. (L.) amazonensis. Pentamidine (PTM) reduced infection rates by over 50% (Figure 2). The Libidibia ferrea dichloromethane fraction (DCM) also effectively reduced the infection of RAW264.7 cells (Figure 3). Considering the cytotoxicity of pentamidine at the concentration tested, it is likely that the effects of pentamidine were non-specific. Infected cells treated with GL and also with a combination of treatments (GL + DCM and GL + EXT) were less effective, since these infected cells presented higher infection rates, with an average of over 35%, and showed no significant difference in relation to the negative control (p > 0.05).

Figure 2. Leishmania (Leishmania) amazonensis infection rate in RAW cells after 24 hours. Glucantime® (GL); Pentamidine (PTM); Libidibia ferrea dichloromethane fraction (DCM), association of Glucantime® and L. ferrea dichloromethane fraction (GL+DCM), L. ferrea extract (EXT) and association of Glucantime® and L. ferrea extract (GL+EXT), dimethylsulfoxide (DMSO). * indicates significant difference for control (-) – infected and untreated cells.

Source: Bacha (2023)
**Figure 3.** Leishmania (Leishmania) amazonensis infection rate in RAW cells after 24 hours. A - control (-) – infected and untreated cells; B – DMSO (1%); C – Glucantime® (100 µg. mL⁻¹); D – Pentamidine (50 µg. mL⁻¹); E – Libidibia ferrea dichloromethane fraction and association of Glucantime® (100 µg. mL⁻¹); F – L. ferrea dichloromethane fraction (100 µg. mL⁻¹); G – L. ferrea extract and association of Glucantime® (100 µg. mL⁻¹); H – L. ferrea extract (100 µg. mL⁻¹).

Source: Bacha (2023)

Leishmania infection is known to inhibit signal transduction pathways in infected cells, therefore a biological assay was performed to determine whether the L. ferrea extract and fraction exhibit the ability to reverse the inhibition of IL-6 secretion. RAW264.7 macrophages infected with L. (L.) amazonensis were incubated with LPS/IFNγ and the L. ferrea extract, fraction or Glucantime®.
As shown in Figure 4, levels of IL-6 in the infected cell medium were significantly lower when compared to levels of IL-6 in the uninfected cell medium treated with LPS/IFNγ, as shown previously. Incubation with increasing concentrations of L. ferrea extract and fraction reversed the suppression of IL-6 release. The L. ferrea fraction was deemed to be more potent than the extract since it was more effective at lower concentrations. Glucantime® had no effect on parasite-induced IL-6 secretion.

To evaluate the in vivo effects of these compounds, their effects were determined via a topical microemulsion formulation in combination with intralesional Glucantime®. Cutaneous lesions in hamsters were evaluated after 30 and 135 days of treatment (Figure 5). The DCM+GLIL20 group had the lowest mean lesion volume, which was significantly lower than the uninfected control group (Kruskal-Wallis test, p-value<0.001). This demonstrated that, during the entire period, the treatment performed on the DCM+GLIL20 group was more effective in reducing the volume of the lesions on the snouts of the animals infected with L. (L.) amazonensis (Figure 5A).

When evaluating the effects of the combined treatment with IM Glucantime® and the topical microemulsion of the L. ferrea DCM fraction, it was noted that, after the 30th day, the treatment group that presented the lowest mean lesion volume was the GLIM20
group when compared to the IST (infected and untreated) group (Kruskal-Wallis test, p-value<0.001). On the 135th day, there was a significant increase in the mean volume of the lesions in the GLIM20 group; however, in the same period, there was a significant reduction in the mean volume of the lesions in the DCM+GLIM10 group, which was significantly different when compared to the IST group (Kruskal-Wallis test, p-value<0.001). Thus, it can be inferred that treatment with DCM+GLIM10 was more effective in reducing the volume of the lesions on the snouts in the animals infected with L. (L.) amazonensis (Figure 5B).

**Figure 5.** Snout volume of uninfected hamsters infected with Leishmania (Leishmania) amazonensis. (A) Infected animals treated with the microemulsion containing the L. ferrea DCM fraction (topical route) and intralvesional Glucantime®. (B) infected animals treated with the microemulsion containing the DCM fraction of L. ferrea (topical route) and intramuscular Glucantime®. * indicates significant difference for IST group. NIST: uninfected and untreated; IST: infected and untreated; MPL: infected and treated with placebo microemulsion; GLIL20: infected and treated with Glucantime® (20 mg/SbV/kg/day) via IL; DCM: infected and treated with DCM microemulsion (50 mg/kg/day) topically; DCM+GLIL10: infected and treated with Glucantime® (10 mg/SbV/kg/day) via IL and DCM microemulsion (50 mg/kg/day) topically; DCM+GLIL20: infected and treated with Glucantime® (20 mg/SbV/kg/day) via IL and DCM microemulsion (50 mg/kg/day) topically; GLIM20: infected and treated with Glucantime® (20 mg/SbV/Kg/day) IM; DCM+GLIM10: infected and treated with Glucantime® (10 mg/SbV/kg/day) via IM and DCM microemulsion (50 mg/kg/day) topically; DCM+GLIM20: infected and treated with Glucantime® (20 mg/SbV/kg/day) via IM and DCM microemulsion (50 mg/kg/day) topically.

Source: Bacha (2023)
The clinical evaluation of the lesions on the snouts of the hamsters (Mesocricetus auratus) infected with L. (L.) amazonensis demonstrated a reduction in the edema in the DCM+GLIM10 group; however, there was no clinical cure in any of the treatment groups (Figure 6).
Figure 6. Clinical aspects of snout lesions of the hamsters (Mesocricetus auratus) infected with Leishmania (Leishmania) amazonensis. NIST: uninfected and untreated; IST: infected and untreated; MPL: infected and treated with placebo microemulsion; GLIL20: infected and treated with Glucantime® (20 mg/SbV/kg/day) via IL; DCM: infected and treated with DCM microemulsion (50 mg/kg/day) topically; DCM+GLIL10: infected and treated with Glucantime® (10 mg/SbV/kg/day) via IL and DCM microemulsion (50 mg/kg/day) topically; DCM+GLIL20: IL infected and treated with Glucantime® (20 mg/SbV/kg/day) via IL and DCM microemulsion (50 mg/kg/day) topically; GLIM20: infected and treated with Glucantime® (20 mg/SbV/kg/day) IM; DCM+GLIM10: infected and treated with Glucantime® (10 mg/SbV/kg/day) via IM and DCM microemulsion (50 mg/kg/day) topically; DCM+GLIM20: infected and treated with Glucantime® (20 mg/SbV/kg/day) via IM and DCM microemulsion (50 mg/kg/day) topically.

Source: Bacha (2023)
The infection rate in all the experimental groups was determined via the quantification of macrophages and amastigotes from the imprint of the lesion biopsy. This analysis revealed that, after the 30th day of treatment, the group that presented the lowest infection rate was DCM+GLIL20, which presented the lowest number of amastigotes per infected macrophages, and a significant difference was observed for the IST group (Figure 7A). The analysis performed after the 135th day of treatment revealed that the group that had the lowest infection rate in this period was DCM+GLIM10 (significant difference for the IST group \(p<0.001\)), which presented a lower number of amastigotes per infected macrophages.

**Figure 7.** Infection rate in hamsters on the 30\textsuperscript{th} day (A) and 135\textsuperscript{th} day (B) of treatment. * indicates significant difference for IST group. NIST: uninfected and untreated; IST: infected and untreated; MPL: infected and treated with placebo microemulsion; GLIL20: infected and treated with Glucantime\textsuperscript{®} (20 mg/SbV/kg/day) via IL; DCM: infected and treated with DCM microemulsion (50 mg/kg/day) topically; DCM+GLIL10: infected and treated with Glucantime\textsuperscript{®} (10 mg/SbV/kg/day) via IL and DCM microemulsion (50 mg/kg/day) topically; DCM+GLIL20: IL infected and treated with Glucantime\textsuperscript{®} (20 mg/SbV/kg/day) via IL and DCM microemulsion (50 mg/kg/day) topically; GLIM20: infected and treated with Glucantime\textsuperscript{®} (20 mg/SbV/kg/day) IM; DCM+GLIM10: infected and treated with Glucantime\textsuperscript{®} (10 mg/SbV/kg/day) via IM and DCM microemulsion (50 mg/kg/day) topically; DCM+GLIM20: infected and treated with Glucantime\textsuperscript{®} (20 mg/SbV/kg/day) via IM and DCM microemulsion (50 mg/kg/day) topically.

Source: Bacha (2023)
A biochemical analysis of the infected animals was performed, and the creatine kinase (CK) value for the NIST control group was 79.5 on the 30th and 135th day. The CK value for DCM+GLIL20 group was 83 on the 30th day and 234.5 on the 135th day (Figure 8). After performing the t-test, it was found that there was a significant difference (p<0.001) between the means of CK on the 30th and 135th day of treatment.

Urea analysis of the NIST control group revealed values of 45 and 42 on the 30th and 135th day of treatment, respectively. The treatment group with values closest to this group was the IST group with a value of 47 and 41 on the 30th and 135th day, respectively (Figure 8). After performing the t-test, it was found that there was a significant difference (p<0.001) between the mean urea values on the 30th and 135th day of treatment.

For creatinine analysis, the value for the NIST control group was 0.35 and 0.4 on the 30th and 135th day of treatment, respectively. The IST group had a value of 0.4 on the 30th day and the DCM+GLIL10, DCM+GLIL20 and DCM+GLIM20 groups had values of 0.4 on the 135th day (Figure 8). After performing the t-test, it was found that there was no significant difference (p<0.066) between the mean creatinine values on the 30th and 135th day of treatment.

**Figure 8.** Biochemical values of creatine kinase (CK), urea and creatinine in hamsters on the 30th and 135th day of treatment. * indicates significant difference for NIST group. NIST: uninfected and untreated; IST: infected and untreated; MPL: infected and treated with placebo microemulsion; GLIL20: infected and treated with Glucantime® (20 mg/SbV/kg/day) via IL; DCM: infected and treated with DCM microemulsion (50 mg/kg/day) topically; DCM+GLIL10: infected and treated with Glucantime® (10 mg/SbV/kg/day) via IL and DCM microemulsion (50 mg/kg/day) topically; DCM+GLIL20: IL infected and treated with Glucantime® (20 mg/SbV/kg/day) via IL and DCM microemulsion (50 mg/kg/day) topically; GLIM20: infected and treated with Glucantime® (20 mg/SbV/kg/day) IM; DCM+GLIM10: infected and treated with Glucantime® (10 mg/SbV/kg/day) via IM and DCM microemulsion (50 mg/kg/day) topically; DCM+GLIM20: infected and treated with Glucantime® (20 mg/SbV/kg/day) via IM and DCM microemulsion (50 mg/kg/day) topically.
Source: Bacha (2023)
DISCUSSION

In Brazil, the standard drug of choice for CL is meglumine antimoniate (Glucantime®). Several treatment regimens are offered according to the patient’s needs and their response to treatment; however, geographic location and the infecting species must be taken into account in order to determine the therapeutic regimen (Croft; Coombs, 2003).

In this study, the use of Glucantime® on its own and in association with the methanolic extract and the dichloromethane fraction of L. ferrea was evaluated in vitro and in vivo. In the in vitro assays, it was observed that Glucantime®, the methanolic extract and the dichloromethane fraction of L. ferrea presented low cytotoxicity in RAW264.7 cells in contrast to pentamidine, which was cytotoxic for RAW264.7 cells. These results corroborate the studies by Comandolli-Wyrepkowski et al. (2017) and Jensen et al. (2017), in which extracts of L. ferrea (>500 µg.mL-1) and fractions of L. ferrea (>74.1 µg.mL-1) were not found to be cytotoxic to J774 macrophages and murine peritoneal macrophages, respectively.

In the evaluation of the antileishmanial activity against amastigote forms of L. (L.) amazonensis, pentamidine and the dichloromethane fraction of L. ferrea were more effective in inhibiting the infection of RAW264.7 cells. These studies corroborate the observations of Comandolli-Wyrepkowski et al. (2017) and Jensen et al. (2017), who found that the extracts and fractions of L. ferrea reduced the infection rate of L. (L.) amazonensis amastigotes by 54% and 21.5%, respectively.

The occurrence of abnormality in the expression of the IL-6 gene may be associated with the risk of developing mucosal leishmaniasis, since this cytokine is related to a pro-inflammatory profile (Shehadeh et al., 2019). In this context, some studies have shown that there are substances that can act to suppress or increase the expression of the IL-6 cytokine, an illustration of which are the Retro-2 derivatives that reversed the inhibition of IL-6 production in infected cells treated with LPS (Craig et al., 2017).

Thus, this study sought to investigate the action of the substances on IL-6 expression and the infection rate, and observed that treatment with the methanolic extract of L. ferrea restored LPS responsiveness with increased IL-6 secretion. Intriguingly, the restoration of IL-6 secretion in response to LPS treatment appears to be independent of the cell’s parasite load. Although cells treated with the combination of the L. ferrea
fraction and Glucantime® had a lower rate of infection, the reversal of IL-6 expression was modest. These results are consistent with those of Craig et al. (2017), who showed that although treatment with miltefosine resulted in a significant reduction of cells infected by L. (L.) amazonensis, the suppression of IL-6 was not reversed.

In the evaluation of the in vivo treatments, Glucantime® was administered intramuscularly and intralesionally, as determined by the Brazilian Ministry of Health, whose recommendations include a 30-day treatment regime with adaptations according to the route of administration, and a daily dose of 20 mg/SbV/kg/day via the IM route and 20 mg/SbV/kg/day every 15 days via IL (Brasil, 2017). Glucantime® was evaluated as to its individual application and the topical application combined with the microemulsion with the DCM fraction of L. ferrea (50 mg/kg/day). It was observed that the treatment performed with only 20 mg/SbV/kg/day via the IL route individually showed less efficacy in reducing the leishmaniasis lesion and parasite viability when compared to the treatment with the combination of 20 mg/SbV/kg/day IL + the microemulsion. These results are similar to those observed when the treatment was performed with only 20 mg/SbV/kg/day via IM route individually. However, in the combination, the greatest efficacy was obtained with 10 mg/SbV/kg/day IM + the microemulsion.

The difference in the effectiveness of the tested drugs may have occurred due to the different routes of administration, association and dosage, since each route of administration has advantages and disadvantages. In addition, special circumstances and particular situations can determine which route is most appropriate, as well as the properties and dosage of the drug to be administered (Schellack, 2005). This demonstrates the need to continue the investigation of different therapeutic regimens in order to define a regimen that can provide the greatest efficacy and the least toxicity.

To the best of our knowledge, there are no published studies that have evaluated the combined use of Glucantime® with formulations containing bioactive substances of L. ferrea. However, in the literature, there are reports of in vivo antileishmanial activity via the topical use of hydrogels containing L. ferrea extract, and these have shown a significant reduction in leishmaniasis lesions (42.78%) and parasite loads in hamsters infected with L. (L.) amazonensis. This suggests that L. ferrea may become an alternative natural product in the treatment of CL (Comandolli-Wyrepkowski et al., 2017).

The study developed by Jensen (2020) also reported on the antileishmanial activity of L. ferrea. In this study, microemulsions containing the dichloromethane
fraction (DCM) of L. ferrea, used to evaluate its antileishmanial activity in hamsters infected with L. (L.) amazonensis, resulted in a similar efficacy of the microemulsion containing L. ferrea and Glucantime® via the IL and IM routes, with control of the evolution of the lesions, presence of a moderate inflammatory profile and reduction of the parasite load being observed.

As for the biochemical evaluation, the dosage and analysis of urea and creatinine concentrations can both serve as indicators of renal failure, especially when levels are very high, with concentrations ranging between 0.4-1 mg/dL of creatinine and 12-26 mg/dL of urea in blood serum being considered as a reference value for hamsters (Thrall et al., 2015).

In the biochemical evaluation, it was observed that the greatest increase in urea concentrations occurred in the group treated with 20 mg/SbV/kg/day IL in relation to the uninfected and untreated group. These data corroborate the study by Brustolin et al. (2022), who observed a significant increase in urea in mice infected with L. (L.) amazonensis that were treated intraperitoneally with different doses of Sbv (20, 100 and 200 mg/kg/day) for 30 consecutive days.

Regarding creatinine, the greatest increase in concentrations was observed in the groups treated with 20 mg/SbV/kg/day IL alone and associated with topical microemulsion with the DCM fraction of L. ferrea. Despite the increased values, there was no significant difference regarding the control group, unlike what was observed in the study by Brustolin et al. (2022), in which mice infected and treated with different doses of Sbv showed a reduction in creatinine.

Creatine kinase (CK) is an enzyme that is present in high concentrations in skeletal, cardiac and smooth muscle, as well as in the brain, with smaller amounts being present in several other organs, such as the intestine, liver and spleen (Carpenter; Marion, 2018). No reference values were found for golden hamsters; however, Melo et al. (2008) describe that the value of the reference interval for the CK index in mice is 20-220 (U/L), so it is possible to infer that, in general, for rodents, all groups presented increased values when compared to the reference values and the NIST group.

It is noteworthy that the groups that received treatment with Glucantime® alone or in combination, especially the GLIL20 and DCM+GLIL20 groups, showed a significant increase in CK. Glucantime® is known to cause cardiac disorders in a dose-dependent manner, though these are generally reversible, so it is necessary that new
studies reassess this association and that infected animals treated with Glucantime® and any possible associations remain under observation for a longer period after the end of the treatment in order to investigate the possible reversal of these CK levels.

The results obtained in this study with L. ferrea and in other studies described in the literature demonstrate that there is potential for the use of natural products. This is due to the presence of bioactive molecules in L. ferrea, which means that it can be used on its own or in combination with standard CL drugs. However, it is worth mentioning that there is still a need to investigate different formulations containing L. ferrea, as well as different therapeutic regimens, since these may present more effective results in terms of parasitological cure.

CONCLUSIONS

Based on the results obtained, it was determined that the extract and the fraction of L. ferrea were not cytotoxic. The L. ferrea fraction showed greater efficacy in inhibiting the infection of RAW cells by L. (L.) amazonensis. In the in vivo evaluation, the treatments performed in combination with Glucantime® and the microemulsion with the DCM fraction of L. ferrea were more effective in limiting the increase in lesions produced by L. (L.) amazonensis in golden hamsters. They also resulted in a reduction in the parasitic load, when compared to the groups that received treatment with the standard drugs alone.

Thus, we can conclude that the microemulsion with the combination of the DCM fraction of L. ferrea and Glucantime® is promising for a future treatment of CL caused by L. (L.) amazonensis. Nonetheless, it is recommended that further studies be carried out to improve the formulations containing bioactive substances of L. ferrea. In addition, the potential of different drug combinations should be evaluated in light of the limited toxicity of these compounds, as well as the evaluation of new therapeutic regimens on other species of the genus Leishmania.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.
ACKNOWLEDGEMENTS

We would like to express our gratitude to Fundação de Amparo à Pesquisa do Estado do Amazonas (FAPEM) for supporting the study via the call HORIZON/2020.

REFERENCES


