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In vitro evaluation of immunomodulatory activity of the copper(I) complex and *Libidibia ferrea* in cutaneous leishmaniasis

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Abstract
Leishmaniasis is considered by the World Health Organization (WHO) to be one of the most neglected tropical diseases in the world. The host´s immune response is crucial for parasite elimination and, although the Th1 profile is associated with control of infections, if not modulated, it can cause tissue damage. The treatment of cutaneous leishmaniasis (CL) is still a challenge because it is not adapted to the context of the patients, and is long, toxic and invasive. Thus, this study aimed to evaluate the lymphoproliferation and dosage of cytokines induced by bioactive compounds of plant and inorganic origin, with antileishmanial action, through peripheral blood mononuclear cells (PBMCs) of patients with CL. Lymphoproliferation was evaluated against the stimuli from the methanolic extract and fraction of Libidibia ferrea, copper complex and phytohemagglutinin using a BrdU cell proliferation ELISA kit after 72 hours of incubation. The dosage of IL-6, IL-8 and IL-1β was determined using a BD™ cytometric bead array (CBA) human Th1/Th2/Th17 cytokine kit. Our results indicate that the bioactive substances significantly stimulated in vitro lymphoproliferation of PBMCs (Cu(I) $p<0.000$; LFME $p<0.02$) and patients showed higher levels of IL-6 and IL-8 before treatment. It is therefore suggested that these bioactive compounds can enhance the cellular immune response.

Keywords: Cutaneous leishmaniasis; *Libidibia ferrea*; Copper complex; Therapeutic alternatives.

Avaliação in vitro da atividade imunomoduladora do complexo de cobre(I) e *Libidibia ferrea* na leishmaniose cutânea

Resumo
As leishmanioses são consideradas pela Organização Mundial de Saúde (OMS) uma das doenças tropicais mais negligenciadas do mundo. A resposta imunológica do hospedeiro é determinante para eliminação do parasito e apesar do perfil Th1 estar associado ao controle da infecção, se não modulado, pode ocasionar danos teciduais. O tratamento da leishmaniose cutânea (LC) ainda é um desafio pois não é adaptado ao contexto dos pacientes, são longos, tóxicos e invasivos. Desse modo, este estudo teve como objetivo avaliar a linfoproliferação e dosagem de citocinas induzidas por bioativos de origem vegetal e inorgânica, com ação antileishmania, através de células mononucleares do sangue periférico (PBMC) de pacientes com LC. A linfoproliferação foi avaliada frente a estímulos do extrato metanólico e Fração de Libidibia ferrea, complexo de cobre e Phytohemagglutinin utilizando BrdU Cell Proliferation ELISA Kit após 72h de incubação. A dosagem das citocinas IL-6, IL-8 e IL-1β foi determinada por BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit. Nossos resultados indicam que os bioativos estimularam significativamente a linfoproliferação in vitro de PBMC (Cu(I) $p<0,000$; LFME $p<0,02$) e pacientes apresentaram maiores níveis de IL-6 e IL-8 antes do tratamento. Sugere-se então que estes bioativos podem potencializar a resposta imune celular.

Palavras-chave: Leishmaniose tegumentar; *Libidibia ferrea;* Complexo de cobre; Alternativa terapêutica.

Introduction

Cutaneous leishmaniasis (CL) comprises a group of diseases with different clinical forms due to its great multiplicity of elements involved in transmission, in which each species of *Leishmania* presents a diversity of vectors, reservoirs, epidemiological patterns, geographical distribution and even different therapeutic responses (Brasil, 2017; Sato, 2017). Cutaneous leishmaniasis (CL) is considered the most common form of *Leishmania* sp. infection. In Brazil, the main species involved in the etiology of CL are *Leishmania (V.) braziliensis, Leishmania (V.) guyanensis, Leishmania (V.) lainsoni, Leishmania (L.) amazonensis, Leishmania (V.) shawi, Leishmania (V.) naifi* and *Leishmania (V.) lindenbergi* (Brasil, 2017).

The immunopathogenesis of leishmaniasis depends on the magnitude of the immune system's response, which is associated with an appropriate balance of T cell subsets that define the evolution and severity of the infection. The regulated response of the Th1 type has been considered as a key mediator for cure, with the production of proinflammatory cytokines (IL-12, IL-8, IFN-γ and TNF-α) and susceptibility to the Th2 profile and the production of cytokines such as IL-4, IL-5, and IL-13 (Oliveira *et al.*, 2021). Another cell subtype, Th17, has been pointed out as a crucial modulator of adaptive immunity, and acts in the regulation of pro- and anti-inflammatory cytokines (Albuquerque *et al.*, 2017) and, through the production of IL-17, Th17 increases the production of multiple inflammatory mediators, such as IL-1, IL-6, TNF-α and NOS2 (Rocha, Silveira & Quixabeira, 2019).

Leishmania sp. interacts dynamically with cells and evolves to adapt to the hostile environment of host cells, and is even able to modulate its gene expression for better adaptation. This fact favors the development of resistance to some of the therapeutic agents that are currently available (Ikeogu *et al.*, 2020). In Brazil, the drugs currently recommended by the Ministry of Health for the treatment of CL are exclusively parenteral and have hepato-, cardio- and nephrotoxic potential. In addition, most cases of CL occur in rural and difficult to access areas, which hinders both the application of the drug and the monitoring of its side and adverse effects (Brasil, 2018).

Given the continuing and urgent need for new drugs for CL, plants are being increasingly explored in the pharmaceutical area due to their numerous therapeutic properties, as well as their low cost, low incidence of adverse effects, and reduced toxicity (Mushtaq *et al.*, 2018). In addition, phytopharmaceuticals with immunomodulatory action may be better strategies for alternative treatment or may be combined with standard medications in order to cope with leishmaniasis (Silva *et al.*, 2021).

Libidibia ferrea, popularly known as "juca" or "pau-ferro" in Brazil, is a tree that is native to Brazil. It is popularly used for therapeutic purposes, mainly in the acceleration of wound healing (KOBAYASHI et al., 2015). The therapeutic effect of *L. ferrea* has already been demonstrated by Commandoli-Wyrepkowski *et al.* (2017), with promising results against *Leishmania* sp.

Another therapeutic possibility is metal ions, which play crucial roles in several biological processes, such as electrolyte balance and oxygen transport, and act as cofactors in the biological function of proteins, among others (Varol, 2016), as well as having shown promising results regarding antileishmanial activity. Work carried out by Chagas *et al.* (2021) demonstrated that copper (Cu+) showed activity against promastigote and amastigote forms of *L. amazonensis* and showed low toxicity to macrophages.

In this perspective, this study aimed to evaluate the immunomodulatory potential of three bioactive substances on lymphoproliferation and cytokine production, in order to contribute to the improvement and innovation of treatment for cutaneous leishmaniasis.

Materials and Methods

Acquisition of the bioactive substances

Copper complex*:* For the *in vitro* immunological assays performed in this study, the copper(I) complex $[HB(pz)_3]Cu(PCN)$ was used, which was synthesized at Istituto per l´Energetica and Le Interphasi, IENI, CNR in Padua, Italy (Marzano *et al.*, 2008; Porchia *et al.*, 2009; Gandin *et al.*, 2014). To carry out the *in vitro* assays, Cu(I) was diluted in 1% dimethylsulfoxide (DMSO), filtered using a membrane with pores of 0.22 μm and then resuspended in complete RPMI (Roswell Park Memorial Institute) medium in order to obtain the concentration of 281.4 μg mL^{-1}, which is the concentration previously evaluated by Chagas *et al.* (2021) for antileishmanial effects.

Plant material: In this study, the methanolic extract (LFME) and the DCM fraction (FDCM) of *L. ferrea* were used. Samples of the plant were obtained in the city of Manaus, Amazonas, Brazil. The preparation of the methanolic extract of *L. ferrea* (LFME) is described in Comandolli-Wyrepkowski *et al.* (2017). The LFME and FDCM were dissolved in DMSO (1%), filtered using a membrane with pores of 0.22 μm and then resuspended in complete RPMI medium, adjusting the concentrations to 600 μg mL^{-1}.

Ethical aspects

All the participants treated in this project agreed to participate voluntarily in the study and signed an informed consent form before blood collection. This work was approved by the Ethics Committee for Research with Human Beings (CEP) at the Federal University of Amazonas (UFAM) - CAAE / UFAM: 29406319.2.0000.5020.

Sample population

The volunteers evaluated in this study were treated at a basic health unit located in the municipality of Rio Preto da Eva (3° 07' 06" S, 59° W), Amazonas, Brazil, which is part of the metropolitan region of the state capital, Manaus. A total of 20 people with a positive diagnosis for CL were treated. These were divided into 2 groups: 1) before treatment – 10 patients diagnosed with CL primoinfection, with an active lesion, with no history of previous infection or treatment with antimonials (18 ± 36 years, 90% males); 2)

after treatment – 10 patients with healed lesions, with treatment completed with Glucantime® (18 ± 58 years, 80% males, between 30 days and 12 months post-treatment). The patients were invited to participate in the project voluntarily and were selected via a search of the health unit's medical records. The negative control group was formed of 10 healthy individuals with no clinical history of CL infection and residents of non-endemic areas of the metropolitan region of Manaus (18 ± 50 years 70% female gender). The complete epidemiological data of the evaluated patients are presented in (Silva *et al.*, 2021).

Blood collection and extraction of PBMC

The isolation of peripheral blood mononuclear cells (PBMC) is described in more detail in Silva *et al.* (2021). In summary, 8 mL of peripheral venous blood from patients with CL and from healthy individuals was collected in heparinized polypropylene tubes. Blood was diluted in complete RPMI-1640 medium, and Ficoll-Histopaque (Histopaque-1077, Sigma-Aldrich®) was added. After centrifugation at 2,200 rpm for 30 minutes, the PBMCs were washed twice with a half complete RPMI-1640 and centrifuged at 1,800 rpm for 10 minutes at 4 ºC. Then, the PBMCs were resuspended in 1 mL of supplemented RPMI medium, and the PBMC concentration was adjusted to 2 x 10^5 cells mL^{-1}. The PBMCs were transferred to cryotubes and stored at -80 ºC.

Cell proliferation assay

For the *in vitro* assays, the PBMCs were transferred to flat-bottomed 96-well culture microplates, and organized in triplicate. A final volume of 200 µL per well was achieved by adding 100 µL/PBMC [2 x 10^5] and 100 µL of each bioactive LFME and Cu(I), which were tested separately. Sensitization of cell cultures with the DCM fraction was performed in another study (Silva *et al.*, 2021). Unstimulated and cultures stimulated with phytohemagglutinin (PHA) [10 µg mL^{-1}] were used as negative and positive controls, respectively. Then, the PBMCs were incubated in a humidified atmosphere (95%) with 5% CO_2, for 72 h at 37 °C and, after 18 h of incubation, BrdU (5-bromo-2'-deoxyuridine) was added [10 mM mL^{-1}].

The ELISA colorimetric assay was performed with the BrdU cell proliferation ELISA kit (Biotrak, Amersham, UK), following the manufacturer's guidelines. Finally, the incorporation of BrdU was evaluated, which was based on the average increase in optical density using spectrophotometry (absorbance at 450 nm). The stimulation of lymphoproliferation of each bioactive substance was determined based on the relative value between the arithmetic mean of the triplicates of the positive control and the negative control, in relation to wells with stimulated cells.

Cytokine dosage using flow cytometry

After incubation of the *in vitro* cell stimulation plates, the plates were centrifuged at 2,200 rpm for 15 minutes to collect the supernatants of the culture. The supernatants from each well were transferred individually to cryopreservation tubes and stored at -80 ºC until cytokine dosing using flow cytometry.

The levels of the cytokines IL-6, IL-8 and IL-1β were quantified using the BD™ cytometric bead array (CBA) human Th1/Th2/Th17 cytokine kit, following the manufacturer's protocol. The samples were acquired in a flow cytometer (FACS Canto II, BD Biosciences, San Jose, CA, USA), and the software FCAP-Array v3 (BD Biosciences, San Jose, CA, USA) was used for data analysis. Data were reported in picograms per milliliter (pg mL^{-1}), according to the standard curves provided in the kits.

Statistical analysis

The Shapiro-Wilk normality test was performed, then Kruskall-Wallis analyses were performed for comparisons between the groups of patients, and Mann-Whitney tests for comparisons between the groups of bioactive substances used, using the statistical software PAST version 4.0 (Hammer, Harper & Ryan, 2001). Significance was defined based on values of $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

Results and Discussion

About 20 trace elements essential for the proper functioning of the organism have been described, and it was found that, of these 20, ten are metals. Metal components have been widely studied and applied in the diagnosis and therapy of different diseases (Mjos & Orvig, 2014; Zoroddu *et al.*, 2019). Among the known metals, it is known that copper [Cu^+] plays a fundamental role in several biological processes and maintenance of body homeostasis; thus, this metal has been investigated in regards to its therapeutic application and actuation in the immune system (Taghipour *et al.*, 2021).

Another approach of therapeutic interest concerns the use of medicinal plants, since many of them have been included in the scope of the Brazilian Unified Health System (SUS) due to the identification of bioactive substances that are derived from the secondary metabolism of plants, and which are capable of preventing and treating some diseases (Cunha *et al.*, 2016; Zeni *et al.*, 2017). Among the plants used, constituent parts of *L. ferrea* have presented numerous proven therapeutic properties, and are used in folk medicine for the treatment of liver and respiratory problems, gastrointestinal disorders and as a healing agent (Almeida *et al.*, 2021).

The bioactive substances LFME and Cu(I) showed significant effects (≥80%; $p<0.0001$) on the increase in the proliferation in PBMCs in the 20 patients evaluated in the groups before and after treatment, when compared to the positive control PHA and the negative control (Figure 1). Cu(I) induced higher levels of lymphoproliferation (≥93%) in patients before ($p=0.001$) and after ($p=0.0006$) treatment (Figure 1A), when compared with LFME (≥81,3%) (Figure 1B), although they are discrete differences.

The levels of the cytokines IL-6, IL-8 and IL-1β quantified after stimulation of PBMCs with the bioactive Cu(I), FDCM and LFME for the groups before and after treatment differed significantly from the negative control (Figure 2A-I).

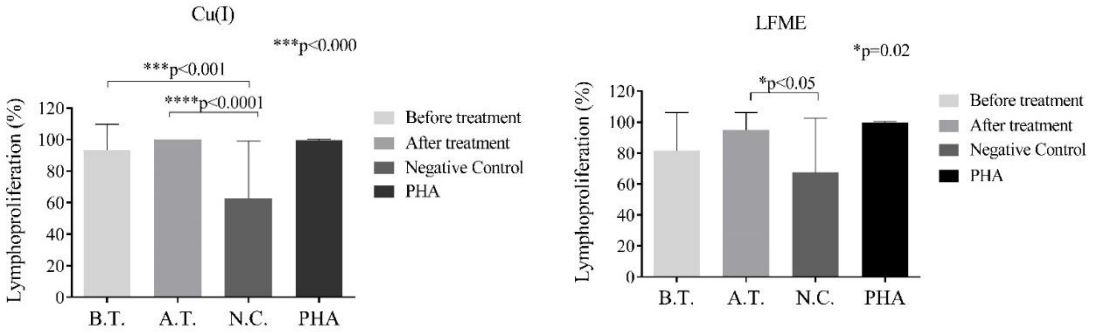

Figure 1. PBMC lymphoproliferation of volunteers treated in Rio Preto da Eva, AM, Brazil, during March and May 2020. (A) PBMCs treated with Cu(I); (B) PBMCs treated with LFME. Cu(I): copper complex; LFME: *Libidibia ferrea* methanol extract; BT: before treatment; AT: after treatment; NC: negative control; PHA: phytohemagglutinin. Vertical lines represent the standard deviation; horizontal lines represent statistical significance between treatments ($p<0.05$).

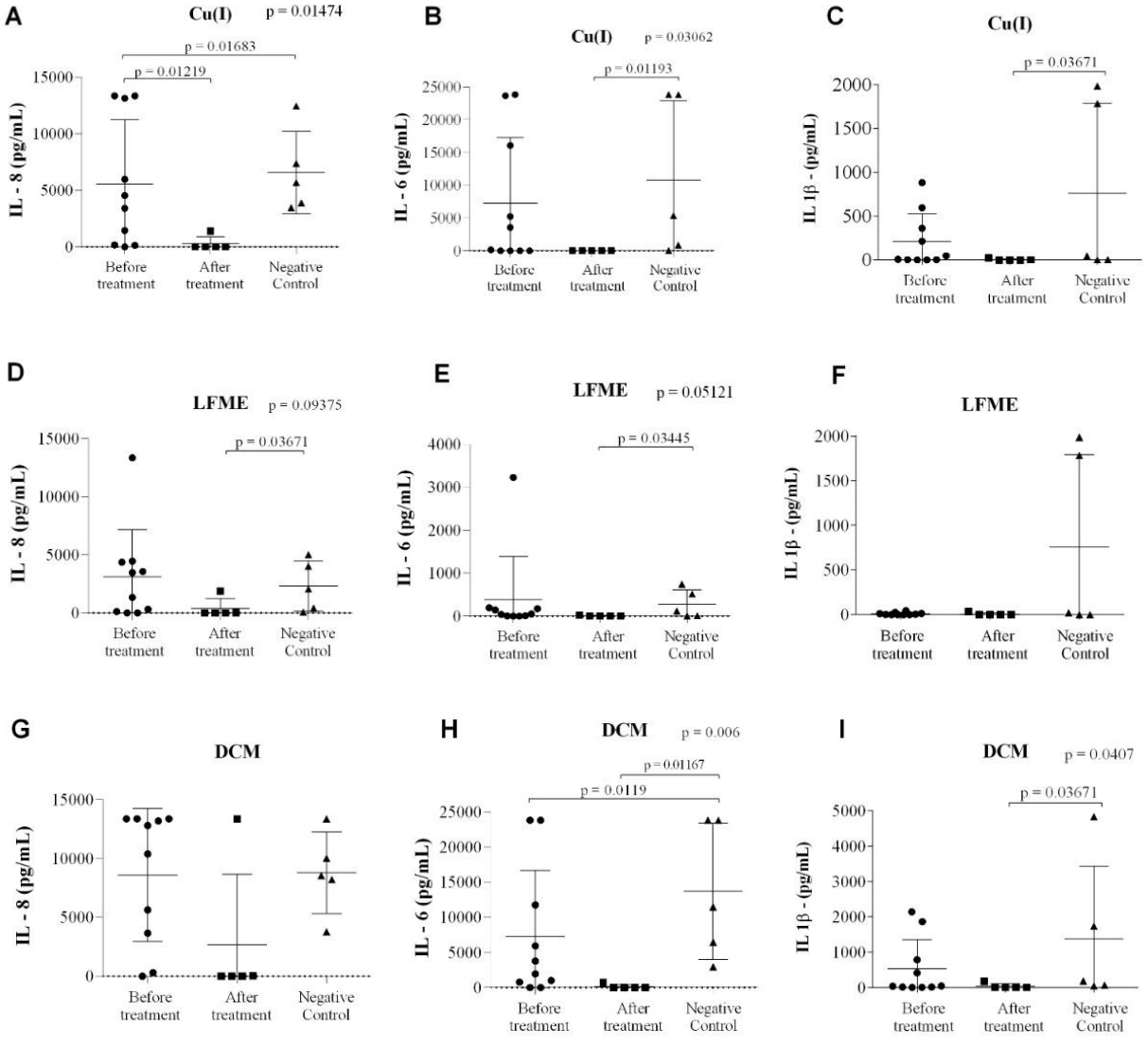

Figure 2. Levels of IL-8, IL-6, IL-1β (pg mL^{-1}) in the supernatants of cultures of PBMCs extracted from patients before and after conventional treatment for CL and from healthy individuals (negative control). (A-C) PBMCs sensitized by treatment with Cu(I), (D-F) LFME and (G-I) FDCM. The means (horizontal lines) are shown for each group of patients. CL: Cutaneous leishmaniasis; Cu(I): copper complex; FDCM: *Libidibia ferrea* fraction; LFME: *Libidibia ferrea* methanol extract.

The PBMCs that received treatment with the three bioactive substances showed that the production of the inflammatory cytokine IL-8 was higher in CL primoinfection patients (Cu(I) – p=0.01; LFME – p=0.09) in relation to the other groups (Figure 2A-I). There was no statistical significance in IL-8 production using FDCM treatment, despite demonstrating increased levels (Figure 2G).

Regarding IL-6, it can be observed that there was greater production of this inflammatory cytokine in primoinfected cells treated with the Cu(I) complex (p=0.03) (Figure 2B) and FDCM (p=0.06) (Figure 2H). In contrast, cell cultures treated with LFME (p=0.05) showed baseline levels of IL-6 production (Figure 2E). Similar results were observed when dosing IL-1, with baseline production levels in LFME-treated cells (Figure 2F) and expressive levels in PBMCs treated with Cu(I) (Figure 2C) and FDCM (Figure 2I). Statistically significant difference was only observed in the treatment with FDCM (p=0.04) (Figure 2I).

Thus, this study investigated the lymphoproliferation of PBMCs (Figure 1A-B) and the production of the cytokines IL-6, IL-8 and IL-1β in cell cultures exposed to the Cu(I) complex (Figure 2A-C), since this metallic complex has shown significant antileishmanial activity in the treatment of hamsters (*Mesocricetus auratus*) infected with *L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (V). naiffi* and *L. (L.) amazonensis* (Chagas *et al.*, 2021a; Chagas *et al.*, 2021b).

Regarding the immunomodulatory effects, interleukin-6 (IL-6) is an inflammatory cytokine that mediates a series of physiological functions, including lymphocyte differentiation, cell proliferation and survival stimulation, as well as potentializing apoptotic signals (Klafke, 2015). Regarding IL-8, this is considered to be an important factor for inflammatory diseases due to its pro-inflammatory properties that promote the coordinated and directional migration of cells of the immune system such as neutrophils, basophils and T lymphocytes (Rossi & Zlotnik, 2000).

There are currently no studies that have investigated the production of cytokines in cells that have been treated with copper(I) complexes. However, our study showed that cells exposed to Cu(I) presented a strong induction of cell proliferation of the PBMCs of patients before and after treatment (≥93%) (Figure 1A) and increased production of inflammatory cytokines (IL-6 and IL-8) (Figure 2A-B). These data corroborate the studies by Kocyigit *et al.* (2002) and Van Weyenbergh *et al.* (2004), who demonstrated that patients with CL who present a higher concentration of copper in plasma and, at the same time, present a higher serum production of IL-6 and IL-8.

IL-6 has a therapeutic role in CL caused by *L. (V.) donovani*, and plays a crucial role in the ability of dendritic cells to regulate the development of $CD4^+$ T cells (Bankoti & Stager, 2005). The production levels of IL-6 and IL-8 were expressive (p=0.03022; p=0.01474) in the supernatants of Cu(I)-sensitized cell cultures of primoinfected patients, in relation to patients after treatment, who presented baseline levels of cytokine production, and it is possible to observe that Cu(I) has nflammatory potential against infection by *Leishmania* sp.

In the serum of pre-treatment patients, the levels of IL-8, MCP-1, and nitric oxide (NO) are higher than in patients in the post-treatment phase, suggesting that IL-8 is an immunodeterminant effector in the progression of CL (Kumar, Bumb & Salotra, 2010). In this context, the study conducted by Taghipour *et al.* (2021) emphasizes the importance of trace elements in the regression or progression of cutaneous leishmaniasis, since these are associated with the activity of the immune system, which could be prescribed as adjuvants for the treatment of cutaneous leishmaniasis.

Regarding the influence of copper on the immune response of patients with leishmaniasis, the study by Van Weyenbergh *et al.* (2004) demonstrates that there was an increased concentration of Cu after Glucantime® treatment. According to Kocyigit *et al.* (2002), copper concentrations and levels of IL-1β, IL-8, IL-6 and TNF-α were significantly higher in CL patients than in the healthy controls, which demonstrates that plasma trace element content is altered in CL patients and that these changes are likely defense strategies of an organism that is regulated by immunoregulatory cytokines.

Thus, it is suggested that the copper(I) complex has a stimulating action on the activation and proliferation of T lymphocytes, in addition to inducing production of pro-inflammatory cytokines, and there is the possibility of this complex presenting potential antileishmanial activity.

It is important to emphasize that there are appropriate amounts of trace elements for the correct functioning of the organism (Zoroddu *et al.*, 2019), and Cu deficiency or excess is implicated in a variety of pathological conditions such as Menkes disease (MD) and Wilson's disease (WD), which represent the most well recognized and understood disorders of copper homeostasis (Shim & Harris, 2003) and neurodegeneration processes (Tisato, 2010; Duncan & White, 2011). Nonetheless, the studies of Chagas *et al.* (2021a) and Chagas *et al.* (2021b) demonstrated that the Cu(I) complex did not present cytotoxicity in J774 lineage cells, murine peritoneal macrophages and human monocytes at the concentrations tested.

As for the results with *L. ferrea* compounds, this study analyzed the levels of IL-8, IL-6 and IL-1β in the culture supernatant when confronted with the bioactives LFME (Figure 2D-F) and FDCM (Figure 2G-I), extracted from *L. ferrea*. These compounds were previously validated through *in vivo* pre-linear assays and presented antileishmanial activity (Comandolli-Wyrepkowski *et al.*, 2017; Jensen, 2020).

With regard to the various bioactive substances isolated from this plant, a preliminary phytochemical study conducted by Gonzalez *et al.* (2004) identified the presence of flavonoids, tannins, coumarins, steroids and anthracene derivatives in the hydroalcoholic extract of the stem of *L. ferrea*. Among the phenolic compounds isolated from *L. ferrea*, gallic acid is a polyphenol that has shown anti-inflammatory, antitumor, and antimutagenic activity and strong antioxidant action (Lima *et al.*, 2016).

Such phenolic compounds are widely distributed in nature and are derived from benzoic and cinnamic acids, as well as flavonoids, and are associated with the inhibition of

chronic degenerative diseases such as atherosclerosis and cancer (Moreira & Filho, 2004), hepatoprotective and antioxidant action (Barros *et al.*, 2014) and also possess analgesic and anti-inflammatory properties (Carvalho *et al.*, 1996), in addition to healing activity in goat skin lesions (Oliveira *et al.*, 2010).

Furthermore, in relation to these properties of the secondary metabolites of this plant, an *in vitro* study using the methanol extract obtained from *L. ferrea* caused a reduction in the size of the lesions (42.78%), low parasitic load and significant reduction of viable promastigotes of *L. amazonensis* and potential inhibition of amastigotes of *L. amazonensis* and *L. guyanensis* (Comandolli-Wyrepkowski *et al.*, 2017).

Subsequently, these dichloromethane fractions of the methanol extract of *L. ferrea* were incorporated into a micro-emulsified system for topical evaluation *in vivo*. Three substances were isolated from the DCM fraction, and methyl gallate and a phenylpropanoic acid derivative were identified. The substances showed antileishmanial activity against promastigote forms and effectiveness in topical treatment *in vivo* (Jensen, 2020).

The DCM fraction is a bioactive substance used for the stimulation of cell proliferation of PBMCs from patients before and after treatment for CL in the study by Silva *et al.* (2021). The authors observed that the FDCM had stimulatory action on the activation and lymphoproliferation of PBMCs, thus characterizing this bioactive substance as having an immunomodulatory response to infections by *Leishmania* sp., which causes CL. From the cytokine dosage data evaluated in this study, it was found that FDCM is a bioactive substance that induces the production of inflammatory cytokines (Figure 2B). In this sense, this corroborates the findings of Comandolli-Wyrepkowski *et al.* (2017) and Jensen (2020), and therefore *L. ferrea* should be considered a phytopharmaceutical candidate for the alternative treatment of cutaneous leishmaniasis (Silva *et al.*, 2021).

In the lymphoproliferation results analyzed in this study, LFME also positively induced (≥81%) the proliferation of PBMCs extracted from patients before and after treatment for CL (Figure 1B). It is worth remembering that lymphocyte proliferation is crucial to the control of *Leishmania* sp. infection. (Brasil, 2017). In relation to cytokine dosage, the DCM fraction showed induction of IL-1β in patients with primoinfection compared to the healthy individuals (Figure 2I), which is a result that was similarly found by Silva *et al.* (2019) in which plasma levels of IL-1β were higher in patients compared to the controls and it was observed that low levels of cytokine IL-1Ra and genotype rs16944 C/C seem to confer susceptibility to infection by *L. guyanensis* in populations of the Amazon.

Dendritic cell-derived IL-1α/β plays a critical role in inducing Th1-dependent immunity against *Leishmania*. Thus, a complex regulation of several members of the IL-1 cytokine family mediated by effects in dendritic cells and T cells contributes critically to the failure of the vaccine against this important human pathogen (Kautz-Neu *et al.*, 2011).

Interleukin 6 (IL-6) is a pleiotropic cytokine, which, when produced under normal conditions, helps in the response to infections and tissue lesions, and contributes to host defense (Jones, Maerz & Bucker, 2018). In response to infection or tissue damage, IL-6 is readily synthesized and activates acute immune response, inducing differentiation of active B cells into antibody-producing plasma cells and also immature CD4+ T cells into effectors (Klafke, 2015). In our analyses, PBMCs of primoinfection patients showed elevated levels of IL-6 from the treatment using the DCM fraction (Figure 2H). The significant production of this cytokine that is induced by the Th17 profile may favor clinical cure in patients with CL (Almeida, 2013).

As such, our results suggest that the substances derived from *L. ferrea* can induce strong lymphoproliferative action and stimulate the production of inflammatory cytokines, as well as having potential antileishmanial activity. Therefore, we suggest the continuity of studies with the DCM fraction, given its most expressive results already shown in a previous study (Silva *et al.*, 2021) in order to better delineate its effect on the immune system.

Conclusion

In this study, the bioactive substance evaluated herein demonstrated strong induction (80%) of lymphoproliferative activity compared to the positive control PHA. Likewise, it is suggested that there was stimulation of the proliferation of T lymphocytes *in vitro*, potentiating, in this way, the cellular immune response. In addition to these findings, the evaluation of cytokine levels may indicate that patients produced acute-phase inflammatory cytokines, as well as cellular immune response. It is suggested that the stimulation caused by these bioactive substances in the clinical evolution of these patients may be related to diverse mechanisms of interaction between two different response profiles, for example, Th1 and Th17. Further studies should evaluate the potential effect of these bioactive substances on immunoregulation by quantifying other cytokines for a broader analysis. Our work generates perspectives for new studies with these bioactive substances considering their potential as future leishmanicidal candidates for alternative treatments.

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