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Efficacy of nanoemulsion with *Pterodon emarginatus* Vogel oleoresin for topical treatment of cutaneous leishmaniasis

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ABSTRACT

Cutaneous leishmaniasis (CL) is a neglected tropical skin disease caused by the protozoan genus *Leishmania.* The treatment is restricted to a handful number of drugs that exhibit toxic effects, limited efficacy, and drug resistance. Additionally, developing an effective topical treatment is still an enormous unmet medical challenge. Natural oils, *e.g.* the oleoresin from *P. emarginatus* fruits (SO), contain various bioactive molecules, especially terpenoid compounds such as diterpenes and sesquiterpenes. However, its use in topical formulations can be impaired due to the natural barrier of the skin for low water solubility compounds. Nanoemulsions (NE) are drug delivery systems able to increase penetration of lipophilic compounds throughout the skin, improving their topical effect. In this context, we propose the use of SO-containing NE (SO-NE) for CL treatment. The SO-NE was produced by a low energy method and presented suitable physicochemical characteristic: average diameter and polydispersity index lower than 180 nm and 0.2, respectively. *Leishmania (Leishmania) amazonensis*-infected BALB/c mice were given topical doses of SO or SO-NE. The topical use of a combination of SO-NE and intraperitoneal meglumine antimoniate reduced lesion size by 41 % and tissue regeneration was proven by histopathological analyses. In addition, a reduction in the parasitic load and decreased in the level of IFN-γ in the lesion may be associated, as well as a lower level of the cytokine IL-10 may be associated with a less intense inflammatory process. The present study suggests that SO-NE in combination meglumine antimoniate represents a promising alternative for the topical treatment of CL caused by *L. (L.) amazonensis*.

1. Introduction

Leishmaniasis are anthroponotic and zoonotic diseases of global public health significance caused by obligatory intracellular digenetic parasites of the genus *Leishmania*. Listed by the World Health Organization (WHO) as one of the major neglected diseases, its clinical features include visceral and cutaneous forms [1]. Cutaneous leishmaniasis (CL) is the most common form and is characterized by painless skin lesions. However, when ulcerative lesions are infected or if they are near to joint areas they can be painful [2]. This disease is considered endemic in about 98 countries, and generally affect mostly low-income population living in peripheries or rural areas [3,4]. According to the WHO, approximately 350 million people live in risk areas for this disease and approximately 600,000 to 1 million new cases occur worldwide annually for the cutaneous form and 50,000 to 90,000 new cases for the visceral form [1,5]. In Brazil, one of the main etiologic agents for CL is *Leishmania (Leishmania) amazonensis*.

Parenteral administration of pentavalent antimony compounds is the treatment of choice for leishmaniasis therapy. Other alternative drugs are amphotericin B, pentamidine, and paromomycin however, their use

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is limited by high toxicity, low efficacy, or cost [6]. Additionally, the prevalence of parasite resistance requires higher drug doses and/or increase treatment time [7]. The high incidence of the disease, the absence of a vaccine, and the scarcity of therapeutic agents motivate the search for new molecules for the treatment [8].

Natural resources of plant origin are promising sources of bioactive substances. Brazil has one of the largest biodiversity in the world, with thousands of cataloged species and unlimited sources of molecules that can be exploited for the search of new agents for the treatment of diseases, including those caused by protozoa [9]. The genus *Pterodon* (Fabaceae) comprises four Brazilian native species: *P. abruptus* Benth., *P. pubescens* Benth., *P. apparicioi* Pedersoli, and *P. polygalaeflorus* Benth. (synonym: *P. emarginatus* Vogel.) [10]. *P. emarginatus* Vogel is popularly known as "Sucupira", "Sucupira-branca" or "Faveira". From the fruits of Sucupira it is extracted an oleoresin with a viscous aspect, light brown coloration, and rich in bioactive substances, mainly furanoditerpenes, sesquiterpenes and, vouacapan skeleton diterpenes [11–13]. Several studies have proven these terpenes to be the main responsible compounds for the biological activity attributed to the species as a larvicide against *Aedes aegypti* [14], anti-inflammatory and analgesic activities [15,16], antimicrobial against *Staphylococcus aureus* and leishmanicidal activity against promastigotes of *L. amazonensis* [17].

Despite the biological activities attributed to *P. emarginatus* Vogel, the high viscosity and intrinsic low solubility of its oleoresin in water are obstacles to the development of a product for *in vivo* application [18]. The use of nanotechnology could be an alternative by improving the water-dispersibility of substances with low polarity, increasing drug stability, and even controlling the release of bioactive compounds [19,20]. Nanoformulations composed of natural products, such as nanoemulsions, have been reported as potential agents for the delivery of bioactive substances against blood or intracellular forms of mammalian parasites [21–23]. The small size of the oil droplets (20−200 nm) allows nanoemulsions to reach kinetic stability. These colloidal drug delivery systems can be obtained even by low-energy methods, using the system chemical energy [24]. Moreover, the small particle size is associated with better cell interaction that can lead to bioactive compound action and pathogen death [25,26]. Just as these systems are capable of protecting herbal oils from temperature variations and oxidative processes minimizing loss and decomposition of bioactive compounds [27,28].

Despite its great biological potential application, currently there is no data supporting leishmanicidal activity of the oleoresin of *P. emarginatus*. Therefore, this study aimed to prepare a nanoemulsion containing Sucupira oleoresin (SO) and to evaluate its *in vivo* efficacy in the topical treatment of CL caused by *L. (L.) amazonensis*.

2. Materials and methods

2.1. Materials

Sorbitan monooleate and polysorbate 80 were purchased from Praid Produtos Químicos Ltda (São Paulo, Brazil) and Meglumine antimoniate (Glucantime® - 300 mg/mL, 81 mg/mL Sb+5) from Sanofi Aventis (São Paulo, Brazil). Medium-chain triglyceride (MCT) was provided by Lipoid GmbH (Ludwigshafen, Germany). For the quantification of cytokines by Elisa, the BD OptEIA kit (BD Biosciences, San Diego, CA) was used. All other chemicals were of analytical reagent grade.

2.2. Animals

Female BALB/c mice and Golden Hamsters were obtained from the breeding unit of the Institute of Biological Sciences of the Federal University of Minas Gerais (Belo Horizonte, Brazil). Animals were housed under standard conditions in an area with a standardized light/dark cycle and received water and food *ad libitum*. The procedures involving animals were under in accordance with the National Council on Animal Experiments and Control (Ministry of Science and Technology, Brazil) guidelines. All described procedures had prior approval from the Animal Ethics Committee of the Federal University of Minas Gerais, Brazil (CEUA/UFMG) with protocol numbers 191/2018 and 392/2018.

2.3. Plant material

Fruits from *P. emarginatus* Vogel (Fabaceae) were obtained at the Central Market of Goiânia – GO (Brazil). Identification of plant material was performed by Dr. José Realino de Paula and a voucher specimen was deposited at the Herbarium of Goiás Federal University (GO, Brazil) under the register number 41714. SO was obtained by cold pressing using a mini mechanical press (MPE-40 ECIRTEC), weighed, and hermetically stored at −20 °C until utilization in an amber glass flask. The extraction yield was 30 % in the weight of SO.

2.3.1. Gas-chromatography coupled to mass spectrometry (GC–MS) analysis

The SO was esterified according to Bannon et al., 1982 [29] before GC injection to remove free fatty acids. The analysis was performed using a GC–MS-QP2010 Ultra (Shimadzu Corporation, Tokyo, Japan) gas chromatography under the following conditions: fused silica capillary column (Rxi-5ms™ 30 m length x 0.25 mm inner diameter, 0.25 μm phase thickness); temperature program (100–260 °C at 4 °C/5 min, then 5 °C/min to 260 °C, ending with a 20 min isothermal at 280 °C); carrier gas (helium); flow rate (0.96 mL min-1); split ratio (1:40); injection port (250 °C); ion source temperature (200 °C); interface temperature (250 °C); electron impact ionization (70 eV); scan mass range (35–400 *m/z*) and sampling rate (1.0 scans/s).

2.4. Preparation and characterization of the SO—NE

The nanoemulsion was carried out according to the method used by Oliveira et al. [18], with some modifications. The oily phase was constituted by the non-ionic surfactants (polysorbate 80/sorbitan monooleate) and the SO, while the aqueous phase was deionized water. Prior to the nanoemulsification, the components of the oily phase were gently mixed for 1 min and then, ethanol 96 % (v/v) was added. After this step, the aqueous phase was titrated to the oily phase under vigorous vortex stirring. The nanoemulsion had a final composition as follows: polysorbate 80 (6.3 %) sorbitan monooleate (3.7 %), ethanol 96 % (v/v) aqueous solution (2 %), SO (20 %), and water (68 %).

The SO-NE was characterized for droplet size distribution and polydispersity index (PdI) using a Zetasizer Nano ZS (Malvern, UK) with a 10-mW red laser (λ = 632.8 nm) at an angle of 173°. The NE-SO were diluted 50-times with deionized water and measured at room temperature. Droplet size distribution and PdI were evaluated at different times (0 – initial day, 1, 7, and 14 days after preparation and stored at room temperature). All measurements were performed in triplicate.

For the preparation of cryogenic transmission electron microscopy (Cryo-TEM), the samples were mounted on a copper screen with lacey carbon film, with a blotting time of 3 s. The acquisition was carried out using the MET Tecnai Spirit G2-12 BioTwin equipment. The Image J software was used for image analysis. The sizes were delimited by the line tool previously calibrated using the scale bar printed on the TEM images.

2.5. In vivo anti-leishmanial activity

2.5.1. L. (L.) amazonensis topical infection

L. (*L.*) *amazonensis* (strain IFLA/BR/1967/PH8) amastigotes were isolated from dorsal nodules from Golden hamsters. The nodules were homogenized with an Ultra-Turrax homogenizer (IKA, Germany) in Schneider's modified medium supplemented with 10 % bovine fetal serum and 1% 100-U/mL penicillin–100 μg/mL streptomycin solution. The tissue was centrifuged at 50 × *g* for 2 min for sedimentation (Thermo Scientific Haraeus). The supernatant was separated,

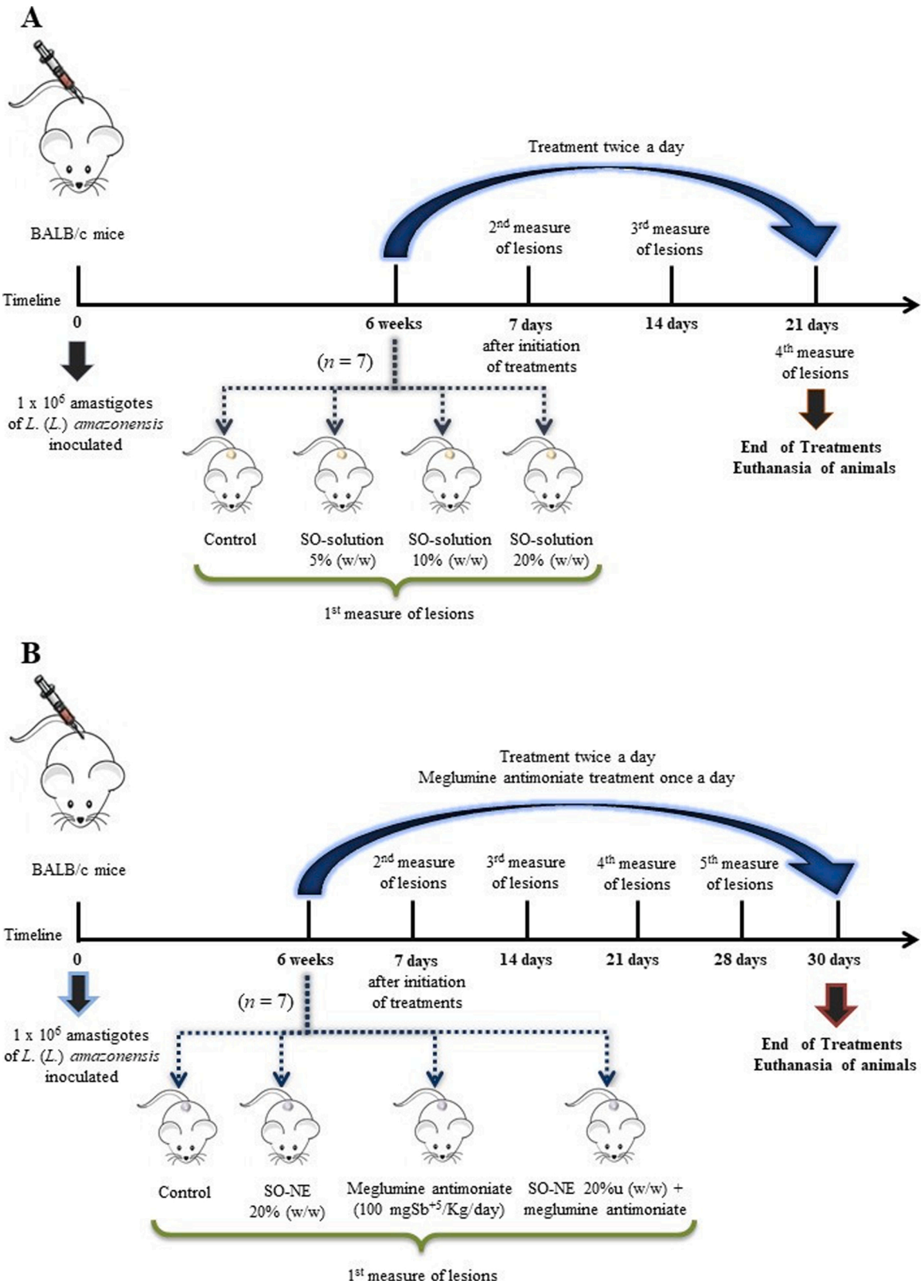

Fig. 1. Timeline course and treatment regimen for groups. (A) In the dose-effect study, Female BALB/c mice were infected with *L. (L.) amazonensis* amastigotes, and different topical treatments with SO (5; 10 or 20 % w/w) were evaluated during 21 consecutive days. (B) At the second moment, Female BALB/c mice were infected with *L. (L.) amazonensis* amastigotes and the evaluation of the topical efficacy SO—NE and its association with injectable meglumine antimony for 30 consecutive days was evaluated.

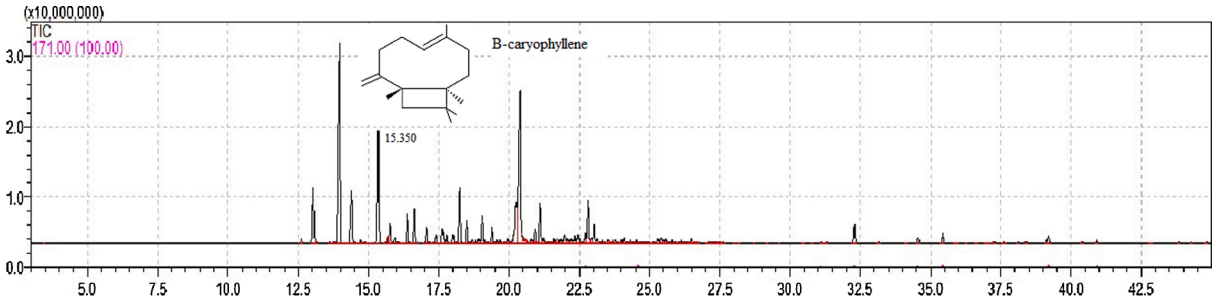

Fig. 2. Chromatogram (GC–MS) of SO with a peak relating to the sesquiterpene β-caryophyllene - Retention time (RT) = 15.350.

centrifuged at 1,700 × *g* for 15 min (Thermo Scientific Haraeus), and resuspended in Schneider's modified medium at 5.0×10^7 amastigotes/mL. BALB/c mice (females, 5 weeks old) were inoculated with approximately 1×10^6 amastigotes of *L.* (*L.*) *amazonensis* through subcutaneous injections at the base of the tail after trichotomy.

2.5.2. Treatment of infected animals

The course of the timeline and treatment regimen for the groups is schematized in Fig. 1. Initially, a dose-effect study using the SO-solution was conducted. To prepare the SO-solution, the SO was solubilized under agitation in MTC at 5, 10, or 20 % w/w (Fig. 1A). After the development of ulcerated lesions (average diameter, 7–9 mm), BALB/c mice were divided into four groups ($n = 7$) according to lesion diameter to ensure similar average lesion sizes among all groups. All the animals had ulcerated lesions at the beginning of treatment. The lesions were covered with 50 µL SO-solution (5; 10 or 20 % w/w) twice a day for consecutive 21 days. After the topical treatments, the animals were manually restrained for about three minutes, until the presence of the formulation on the surface of the lesions was no longer observed. For the first study, the treatment efficacy was evaluated by measuring the sizes of the lesions.

After obtaining the best SO dose-response, another study was performed to evaluate the topical efficacy SO—NE and its association with antimoniate meglumine for consecutive 30 days (Fig. 1B). After the development of ulcerated lesions, BALB/c mice were divided into four groups ($n = 7$). For the SO—NE group, lesions were covered with 50 µL SO—NE (20 % w/w) twice a day. For the meglumine antimoniate group the animals were treated intraperitoneally with antimoniate meglumine at 100 $mgSb^{+5}$/Kg/day once a day. For SO—NE topical and antimoniate meglumine combination group the lesions were covered with 50 µL twice a day (SO—NE 20 %w/w) and the animals were treated by intraperitoneal *via* with antimoniate meglumine at 100 $mgSb^{+5}$/Kg/day once a day.

The animals that received topical treatment were manually immobilized for about three minutes, immediately after the application of SO—NE on the lesion, until the presence of the formulation on the surface was no longer observed. For the control group, the animals did not receive treatment. Treatment efficacy was evaluated by measuring the sizes of the lesions, as well as by histopathological analysis and parasite quantification in the skin after the interruption of treatment. The cytokines (IL-10 and IFN-γ) were dosed in CL lesions and lymph nodes by the Enzyme-Linked Immunosorbent Assay (ELISA) (Fig. A1).

The toxicity parameters of the treatment such as body weight, piloerection, and survival were evaluated.

2.5.3. Lesion size measurements and weight of the animals

During and after treatment, lesion sizes were measured weekly using a digital caliper (Mitutoyo, Brazil). The lesion size was determined by the average value obtained between the longest line that could be traced from one border of the lesion to another and the line that bisected this distance at a 90° angle [30]. Careful observation of tails evaluated the appearance of metastasis in other locations on the animal's skin. The determination of the animal's body weight was evaluated up to 21 and 30 days of treatment with SO-solution and SO-NE, respectively. Other signs such as piloerection were used as indicators of treatment toxicity.

2.5.4. Parasite quantification in the lesion

Three days after the interruption of treatment, the number of viable parasites in the lesion was determined by a limiting dilution assay. The animals were euthanized in a CO_2 chamber and the cutaneous lesion was removed. The tissue was weighed and homogenized with an Ultra-Turrax homogenizer (IKA, Germany) in Schneider's medium containing 100 U/mL penicillin and 100 µg/mL streptomycin solution. Next, the tissue was centrifuged at 1620 × *g* for two minutes for sedimentation. The supernatant was separated and centrifuged at 1700 × *g* for 15 min. The pellets were resuspended in 1 mL of Schneider's medium containing 20 % bovine fetal serum, penicillin (100 U/mL) and streptomycin (100 µg/mL). The homogenate was submitted to serial dilutions in triplicate (1:10) in successive sterile 96-well culture plates and incubated at 23 °C. Each well was examined in an inverted microscope for the presence of parasites, and the parasite load was determined by the highest dilution at which parasites could grow over a 7 days [30]. The results were expressed as the mean obtained from the triplicate.

2.5.5. Histological analysis

After the euthanasia procedure, the skin samples of the lesions of mice were collected and fixed in 10 % neutral buffered formalin (pH 7.2), for a minimum period of 48 h. After processing, the fragments of the lesions were embedded in paraffin, submitted to microtomy with 5 µm, and stained with H&E. The images were obtained using a micro-camera (Q-Color5, Olympus) coupled to the BX53 microscope (Olympus) with Q-Capture Pro 7.0 software. The inflammatory reaction was evaluated using a semiquantitative procedure assessing the presence of leukocytes in the dermis and hypodermis [31]. The inflammatory infiltrate score system was adapted as follows: 0, absent; 1, slight; 2, moderate (moderate diffuse or focal inflammatory infiltrate, including areas in the deep dermis or hypodermis); 3, intense (severe diffuse or focal inflammatory infiltrate around the vessels, glands, hair follicles in deep dermis and/or hypodermis); 4, much intense (widespread inflammatory infiltrate through the dermis). The extension of necrotic areas was graded from 0 (absent) to 3 (severe) [32]. The morphological evaluation of epidermis comprised the following parameters: acanthosis, dyskeratosis, papillomatosis, exocytosis, and hyperkeratosis, which were graded as absent (0) or present (1). The total score ranged from 0 to 12. Histopathological analysis was performed using a single-blinded model.

2.5.6. Cytokines quantification

The lymph nodes and skin lesions were homogenized in cytokine extraction solution. After, in culture supernatants, the cytokines were quantified by ELISA as proposed by Oliveira et al., 2017 [33]. BD OptEIA (BD Biosciences, San Diego, CA) kits were used for the measurement of IL-10 and IFN-γ according to the manufacturer's guidelines. A spectrophotometric reading at 492 nm was performed using the reader Emax

Table 1
Physicochemical characteristics of SO-NE.

Samples[a]	Parameters evaluated	
	Diameter ± SD	Polydispersity index ± SD
0	158.10 ± 0.26	0.136 ± 0.004
1	145.60 ± 0.60	0.102 ± 0.014
7	147.63 ± 1.85	0.098 ± 0.013
14	147.56 ± 0.83	0.108 ± 0.013

[a] Data expressed as mean ± standard deviation (SD). Measurements were performed in triplicate.

Molecular Devices and software SoftMax Pro 5.2 (Molecular Devices Corporation, Sunnyvale, CA, USA). The result of cytokine dosage in lymph node cells is presented in Appendices 1.

2.6. Statistical analysis

Normality and homogeneity of variance were assessed using the Kolmogorov–Smirnov and Bartlett's tests, respectively. The comparison of lesion sizes and weights of animals among the groups was performed using one-way analysis of variance followed by Tukey's test. The parasite quantification was performed using t test. For histological analysis, group comparison was performed by Kruskal-Wallis test. The difference was considered significant when the p value was <0.05.

3. Results

3.1. SO-NE characterization

The oleoresin from *P. emarginatus* used in the preparation of SO-NE was previously analyzed by GC–MS. The obtained chromatogram (Fig. 2) revealed the presence of some chemical constituents of the SO, such as the sesquiterpenes β-caryophyllene, β-elemene, α-copaene, and α-humulene.

The SO-NE were prepared by a low-energy method and presented a thin appearance and bluish reflex. The diameter of the nanoemulsion was measured by the dynamic light scattering (DLS) technique and showed droplets of 158.1 nm and 147.6 nm on the day of preparation and after 14 days, respectively. The PdI obtained was around 0.1 throughout the analysis (Table 1). The size and PdI remained without major alteration during the storage at room temperature for 14 days. The superposed graphs of the size distribution of SO-NE during the different days are shown in Fig. 3 and confirm the homogeneity of the droplet size distribution throughout the evaluated period. Furthermore, the evaluation of SO-NE morphology by Cryo-TEM image (Fig. 4) showed spherical droplets and diameter less than 180 nm, in accordance with DLS results. Altogether the results demonstrate the stability of SO-NE in terms of size and PdI, which are characteristics suitable for topical administration.

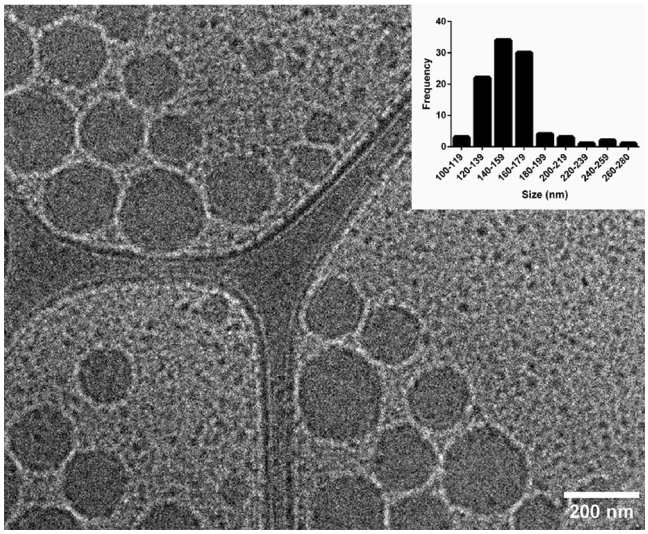

Fig. 4. Cryogenic transmission electron microscopy (cryo-TEM) image of Sucupira oil nanoemulsion, prepared by a low energy consumption method. The formulation was visualized on a copper screen with lacey carbon film. The scale bars correspond to 200 nm and the frequency of droplet size (inset figure).

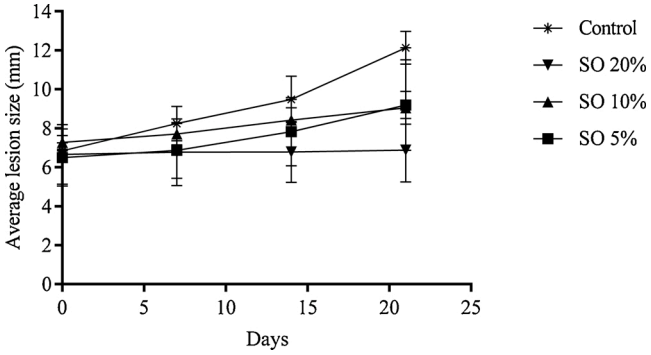

Fig. 5. Monitoring of average lesion size in response to different topical treatments with SO. Female BALB/c mice were infected with *L. (L.) amazonensis* amastigotes in the base of the tail (n = 7). The animals were treated topically with OS-solutions 5 %, 10 %, 20 % w/w, and the control group was treated with MHC for 21 days twice a day. Lesion size is shown as average and standard deviation. A significant difference was observed for topical SO-solution 20 % w/w on the 14th day after initiation of treatment compared with the control group ($p < 0.05$). For the SO-solutions 5% and 10 % w/w a significant difference was observed on the 21 st day after initiation of treatment compared with the control group ($p < 0.05$).

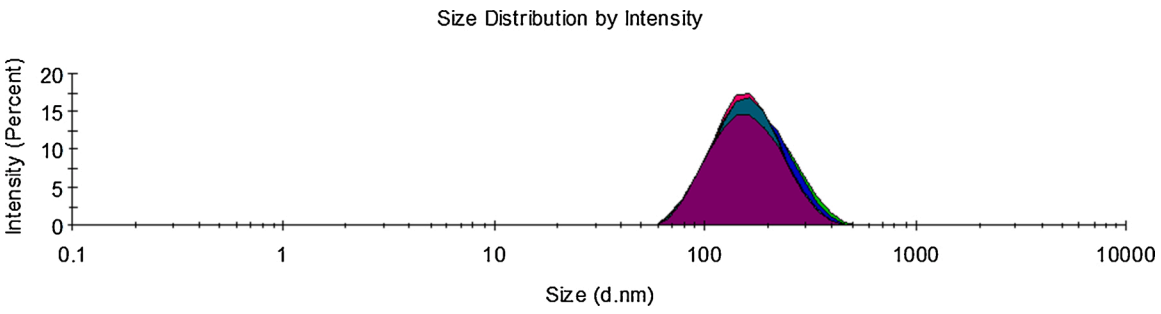

Fig. 3. Droplet size distribution of SO-NE.

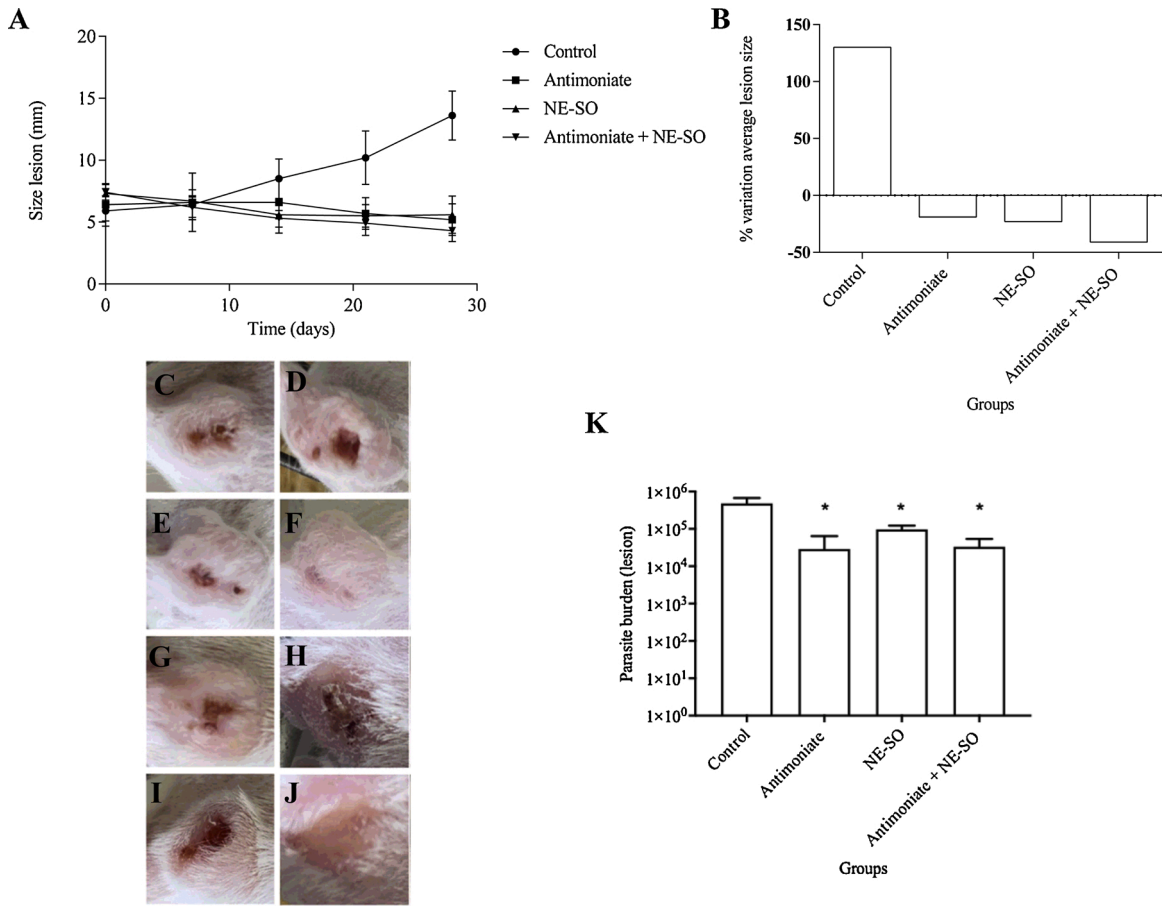

Fig. 6. Efficacy of the combination of topical SO—NE and antimoniate meglumine. Female BALB/c mice were infected with L. (L.) *amazonensis* amastigotes in the base of the tail (n = 7). The animals were treated topically with SO—NE, meglumine antimoniate administered by injectable *via* (Glucantime® 100 mgSb+5/Kg/day), SO—NE meglumine antimoniate combination, and control group no treated for 30 days. Lesion size is shown as average and standard deviation (A). A significant difference was observed for topical SO—NE and SO—NE plus meglumine antimoniate combination on the 14th day after initiation of treatment compared with the control group ($p < 0.05$). For meglumine antimoniate a significant difference was observed on the 21 st day after initiation of treatment compared with the control group ($p < 0.05$). % variation in average lesion size determined by the difference between the average lesion size at the beginning of treatment and the end (B). Clinical aspect of macroscopic lesions (C—J). Control group: animal infected and untreated day 1 (C) and day 30 (D). Animal treated with injectable meglumine antimoniate day 1 (E) and day 30 (F). Animal treated with topical NE—SO day 1 (G) and day 30 (H). Animal treated with SO-NE meglumine antimoniate combination day 1 (I) and day 30 (J). Parasite burden quantified in lesions (K). All data are presented as average and standard deviation. * significant difference when compared with the control group ($p < 0.05$).

3.2. *In vivo anti-leishmanial activity*

3.2.1. *Dose-effect study of topical SO-solutions*

The lesion sizes assessment was used to select the best SO concentration to be further used in the preparation of the SO—NE. The lesions in BALB/c mice infected with *L. (L.) amazonensis* was followed by ulcers progression in size and shape. The treatment started 6 weeks post-inoculation. Fig. 5 shows the evolution of the lesion sizes, after the beginning of the treatment with SO, as a function of time. At the beginning of the treatment, the animals treated with topical control (MTC), SO-solution 5, 10, and 20 % presented lesions with an average diameter and the standard deviation of 6.8 ± 0.41, 6.5 ± 1.46, 7.3 ± 0.35, and 6.7 ± 1.53 mm, respectively, without significant differences among all the groups ($p > 0.05$). The lesion size of the animals treated with topical SO-solutions showed stabilization at the time interval evaluated. In contrast, the control group showed a gradual increase in the average lesion size. The topical treatment with 20 % w/w SO-solution resulted in a significantly smaller mean lesion size compared to the control group on the 14th day after the beginning of the treatment ($p < 0.05$). At the end of the treatment, the groups presented no significant body weight loss ($p > 0.05$) (data not shown). Based on these results, the concentration of 20 % w/w of the SO was selected for the preparation of the SO-loaded NE for further studies in the treatment of *L. (L.) amazonensis*-infected mice.

3.2.2. *Efficacy of the combination of topical SO-NE and intraperitoneal antimoniate meglumine*

The second study was carried out to evaluate the efficacy of the topical SO-loaded NE combined with injectable antimoniate meglumine. Fig. 6A shows the evolution of the lesion size, after the beginning of the treatment, as a function of time. At the beginning of the treatment, the animals without treatment (control group), treated with injectable meglumine antimoniate, topical SO—NE, and animals SO—NE plus injectable meglumine antimoniate combination presented lesions with an average diameter of 5.9 ± 1.2 mm, 6.4 ± 1.3, 7.3 ± 0.8, and 7.4 ± 0.6, respectively. The lesion sizes of animals treated decreased during the evaluation period, which could be observed 14 days after the onset of therapy for both SO—NE and SO—NE plus meglumine antimoniate compared to the control group ($p < 0.05$) (Fig. 6B).

The lesion size in the control group had an increase of 130 % during the period analyzed (5.9 ± 1.2 and 13.6 ± 1.9 mm, initial and end-time of evaluated, respectively) (Fig. 6B). In addition, the worsening of the lesion aspect was visibly noted (Fig. 6C and D). All treated groups showed size lesion reduction, with the greatest reduction of 41 % in the

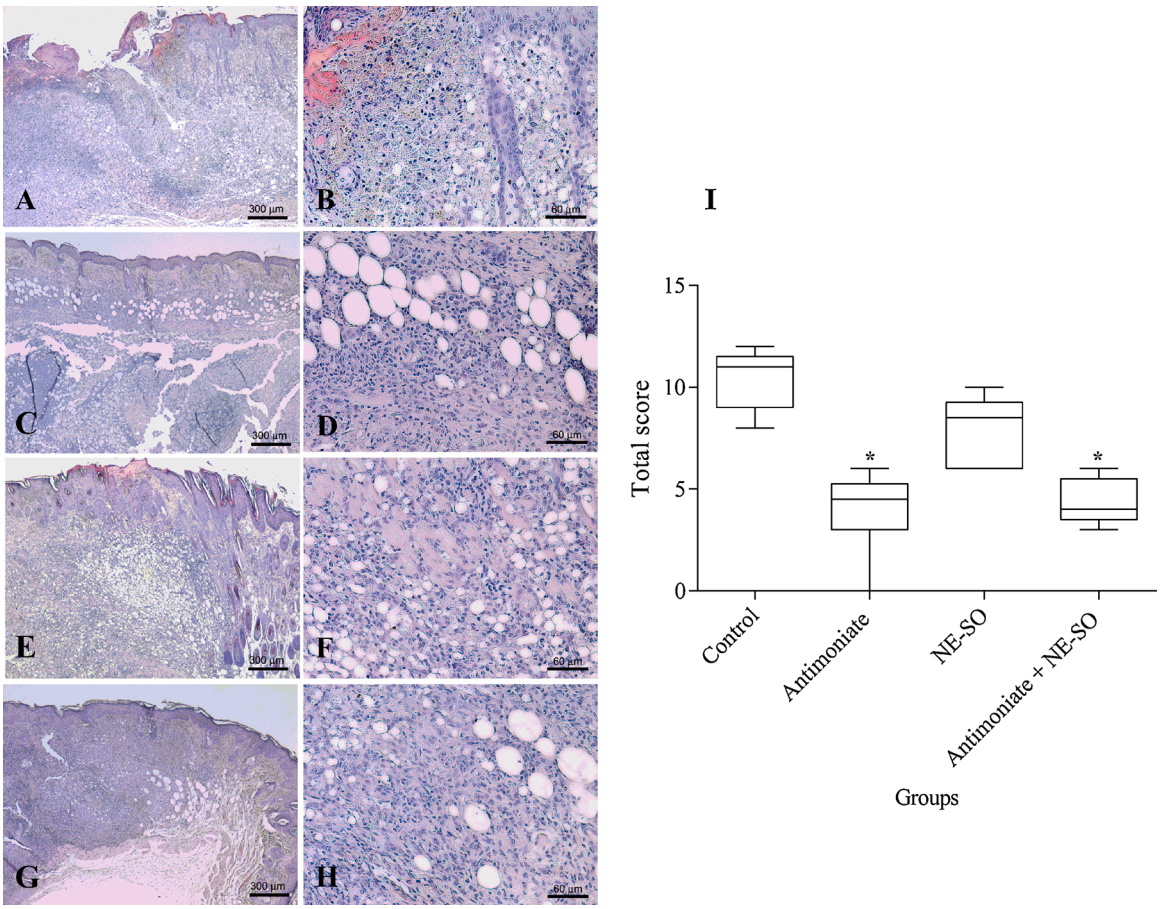

Fig. 7. Histopathological analysis (H&E) were performed in the control group no treated (A and B), group treated with meglumine antimoniate administered by injectable *via* (Glucantime® 100 mgSb+5/Kg/day) (C and D), 30 days post-treatment topically with SO—NE (E and F) and group that received SO-NE meglumine antimoniate combination (G and H). Original magnification x300 (AD) and x60 (EH———), (I) Total score of the histopathological analysis. Data are presented as mean median ± min to max.

group treated with the combination of SO—NE and injectable meglumine antimoniate (7.4 ± 0.6–4.3 ± 0.9 mm). The lesions of the animals of this group also presented a better healing aspect (Fig. 6I and J) compared to the groups treated only with injectable meglumine antimoniate (Fig. 6E and F) or topical NE—SO (Fig. 6G and H). It is also interesting to note that the SO—NE 20 % (7.3 ± 0.8–5.6 ± 1.5 mm) led to a greater reduction in lesion sizes when compared to the SO-solution 20 % (6.7 ± 1.5–6.8 ± 1.6 mm) (Fig. 5). Additionally, it could be seen that the treatment of infected animals with the topical SO—NE and combination of topical SO—NE and injectable meglumine antimoniate also reduced the parasite load in the lesion, compared to the control group ($p < 0.05$) (Fig. 6K). At the end of the treatment, animals from all groups did not present significant body weight loss ($p < 0.05$).

Histopathological analysis of biopsies skin lesions of mice (Fig. 7) revealed significant inflammatory alterations, especially in the control group (Fig. 7A and B). In this group, during the whole experiment the animals did not receive any treatment and the cutaneous lesion caused by *L. (L.) amazonensis* was quite evolved. The histological analysis revealed extensive lesions with intense alteration of the tissue architecture. The epidermis presented frequent evidence of ulceration, exocytosis and dyskeratosis. In the exposed connective tissue of the dermis, there was an intense and mixed inflammatory infiltrate with a predominance of polymorphonuclear neutrophils, and mononuclear cells, as in an acute inflammatory process. Additionally, in some samples, there were areas of hemorrhage, numerous macrophages with parasitophorous vacuoles, richly parasitic, and areas of necrosis.

In the group that received the injectable meglumine antimoniate the epidermis lesion and ulceration areas were not apparent (Fig. 7C and D). There was still the presence of inflammatory infiltrate with the polymorphonuclear neutrophils and mononuclear cells, however in smaller numbers and less frequency of intracellular parasite than in the control group. In some areas, the dermis presented scarce leukocytes and evident fibroblasts, as healthy connective tissue, suggesting a more advanced process of conclusion of the inflammation compared to the control group.

The group that received the SO—NE topically (Fig. 7E and F), also presented an active inflammatory process. The presence of intense leukocyte infiltration and a significant number of vacuolized macrophages was seen, however with less evident parasites than the control group. Areas of ulceration with exposure of connective tissue were smaller than in the control group and less frequently seen. The dermis presented areas with dense fibers, which may suggest a regeneration of the tissue.

The group treated with the combination of SO—NE and injectable meglumine antimoniate presented no ulceration or epithelial damage, and a scenario of resolving the inflammatory process was noticed (Fig. 7G and H). The inflammatory infiltrate was restricted to the lower layers of the dermis, however in smaller quantities than in other groups. A greater presence of connective tissue, without necrotic areas, were also observed. Parasites were rarely detected inside macrophages indicating that the process had not finished yet but tended to resolution. These results confirm the score presented in Fig. 7I where injectable meglumine antimoniate and its association with topical SO—NE presented the lowest scores and no statistical difference between them. The significant reduction of parasite load in the lesions of these groups, when compared to the untreated group ($p < 0.05$), also corroborates this

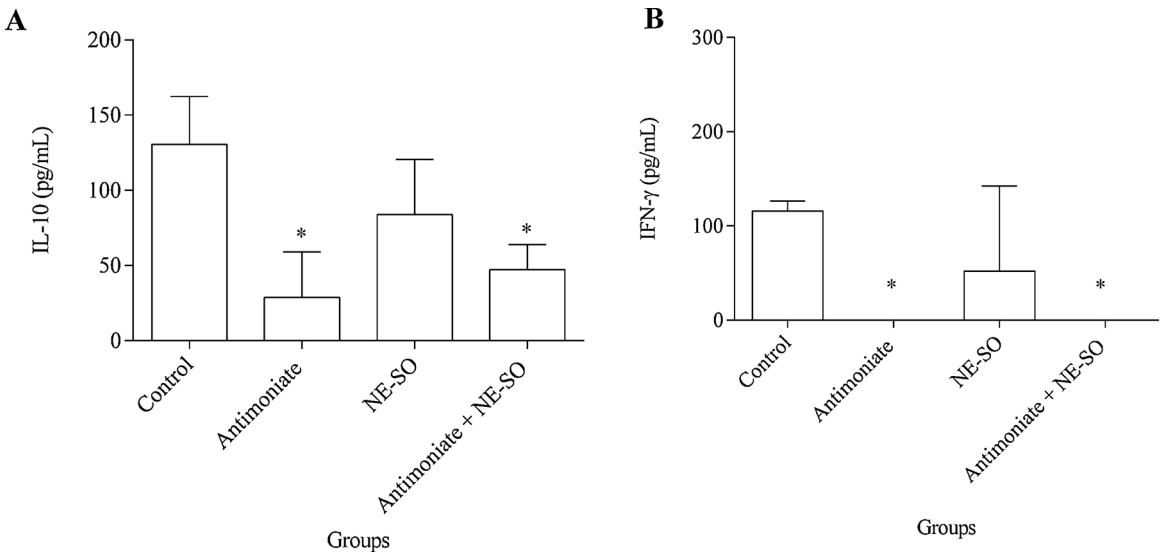

Fig. 8. Cytokine levels in skin lesions of CL treated animals (A) IL-10, (B) IFN-γ. Female BALB/c mice were infected with *L. (L.) amazonensis* amastigotes in the base of the tail (n = 7). The animals were treated topically for 30 days with SO—NE and meglumine antimoniate administered by injectable *via* (Glucantime® 100 mg Sb^{+5}/Kg/day). The cytokine levels were determined after the end of treatment. All data are presented as average and standard deviation. * significant difference when compared with the control group ($p < 0.05$).

finding (Fig. 6K).

To evaluate the resolution of the inflammatory response, activation macrophages leishmanicidal mechanisms, and control of *L. (L.) amazonensis* infection the influence of some essential cytokines was investigated. The levels of IL-10 (Fig. 8A) IFN-γ (Fig. 8B) produced by CL were significantly reduced in animals treated with a combination of SO—NE and injectable meglumine antimoniate when compared to the control group ($p < 0.05$), presenting values below the detection limit. These findings may indicate that the cytokine reduction level was associated with minor inflammatory response, parasite burden at the infection site, and lesions size reduction.

4. Discussion

The use of medicinal plants against parasitic diseases has been studied for a long time. Many natural product groups have revealed antiparasitic properties of efficacy and selectivity [34]. Secondary metabolites from natural products such as alkaloids [35], aldehydes [36], chalcones [37], flavonoids [38], terpenoid compounds [39] among others, have demonstrated considerable antiparasitic activity against the *Leishmania* species. However, most studies are usually conducted *in vitro*. There are few *in vivo* studies related to leishmaniasis and vegetable derivatives. This was the first study to evaluate the *in vivo* efficacy of SO in the topical treatment of CL caused by *L. (L.) amazonensis* a specie causing New World CL.

The oil obtained from the genus *Pterodon* has several bioactive compounds, especially from the terpenes class such as diterpenes and sesquiterpenes [13]. The cold-pressing process was chosen to obtain the SO once it offers an oleoresin rich in terpenes. The identified compounds, including the main volatile of the species (β-caryophyllene), were previously described as phytochemicals of the fruits of this species, as part of our ongoing studies with bioactive nanoemulsions of Sucupira-branca herbal derivatives [40,41]. The GC–MS of the SO profile carried out proved the presence of important compounds of the species, including the β-caryophyllene, a sesquiterpene widely studied for its anti-inflammatory activities [42], antiparasitic against *Trypanossoma cruzi* [43] and *L. (L.) amazonensis* [44]. The presence of β-caryophyllene has been observed in sucupira fruit derivatives [14,40,45]. However, the β-caryophyllene leishmanicidal mechanism of action remains to be elucidated, as well as its effect on *in vivo* models. *In vitro* studies have suggested β-caryophyllene responsible for the leishmanicidal activity of some plant species. In the evaluation of the copaiba oil (*Copaifera spp*), also belonging to the Fabaceae family, it was demonstrated that the amount of β-caryophyllene in the species was directly related to the activity against *L. (L.) amazonensis* observed [46].

The activity of the genus *Pterodon* against different species of *Leishmania*, which causes visceral and CL, has been shown *in vitro*. Arrais et al., 2014 showed the activity of the ethanolic extract of *P. pubescens* against *L. (L.) amazonensis* amastigotes [47]. In another study, Dutra et al. (2009) evaluated the activity of vegetable derivatives obtained from seeds of *P. emarginatus Vogel* against promastigotes forms of *L. (L.) amazonensis* (isolated from patients with diffuse CL) and *L. (L.) infantum chagasi* (isolated from patients with visceral leishmaniasis). The hexane (IC_{50} = 50.06 μg/mL) and butanol (IC_{50} = 46.65 μg/mL) fractions showed activity against the promastigote forms of *L. (L.) amazonensis*, but not against the promastigote forms of *L.(L.) infantum chagasi* [17].

Since there were no previous reports on the activity of SO against *L. (L.) amazonensis* in animal models, the first step of this study was to evaluate its efficacy in experimentally infected mice through a dose-effect analysis. The dose-response study performed *in vivo* demonstrated that the topical use of SO at 20 % was able to prevent the lesion size growth during the evaluated period, while the animals in the control group showed a progressive increase. This stabilization in the lesion size may be related to the anti-inflammatory property of SO [15,16,48]. It is known that the main treatment for all forms of leishmaniasis, including the cutaneous, is still parenteral administration of pentavalent antimonial, even with its limitations [6].

Topical treatment, although quite challenging, is an attractive alternative, offering significant advantages over systemic therapy such as less adverse effects, maximizing the action of the drug on the target tissue, bypassing first-pass metabolism, ease of administration and, lower costs [49]. However, in this case, the high intrinsic hydrophobicity of phytocomplexes and the variety of compounds with different molecular weights in their composition hinders the permeation of the oil through the hydrophilic barrier of the skin. This may lead to less absorption of bioactive antileishmaniotic compounds and, consequently preventing the action on site. One way to improve the cutaneous absorption of lipophilic agents present in the SO is to develop a nanoemulsion and consequently improve the pharmacological activity [50–52].

Nanoemulsions are kinetically stable colloidal systems of two immiscible liquids eventually stabilized by surfactants. Thus, a topical nanoemulsion loaded with SO was successfully obtained in the present study using a low-energy method, which does not require specialized and powerful equipment since it uses energy from the system itself [24,53,54]. Considering the result of the dose-response study, SO-NE at 20 % was developed for *in vivo* studies once it was the most effective concentration.

Due to the high complexity of natural oils rich in secondary metabolites, with a wide range of distinct physical and chemical properties (*e.g.* log P, water-solubility, polarizability), difficulties would be expected in the production of fine and stable nano-drops using these natural products. However, despite a large amount of SO required for the development of this system, it was possible to obtain nanoemulsions by a low-energy method with desirable characteristics. The SO-NE showed no signs of instability such as cremation or phase separation. In addition, it presented spherical and well-delimited droplets observed by Cryo-TEM image, and size within the range considered adequate for nanoemulsions (20−200 nm) [55]. The PdI values were lower than 0.2, indicating the homogeneity of particle size [56]. The droplet size distribution graph evidenced a suitable narrow distribution in accordance with monomodal nanodispersion systems [57].

Nanoemulsions from fruit derivatives of the genus *Pterodon* had already been proposed by other methodologies [17,41,56,58]. Dos Santos et al., 2016 [59] demonstrated higher *in vitro* leishmanicidal activity against amastigotes and promastigotes of *L. (L.) amazonensis* for nanoemulsions prepared with extract of the fruits of *P. pubescens* when compared to the extract. It was suggested that the small droplet size facilitated the penetration of the active compound and destruction of the pathogen [60]. In our study, the effectiveness of SO-NE alone and SO-NE plus meglumine antimoniate combination in the treatment of BALB/c mice infected with *L. (L.) amazonensis* was evaluated. It was found that the topical treatment of CL with SO-NE reduced lesion sizes in animals.

It is important to note that BALB/c mice are considered a rigorous non-cure model in which only the most active drugs are effective. Thus, any improvement can be attributed to the effects of chemotherapy [61]. The clinical aspect of CL therapeutic efficacy is very important and must consider aspects related to anti-inflammatory reactions and healing capacity. Nanoemulsions present penetration-enhancing ability and have already proved to be advantageous for the administration of anti-inflammatory drugs [62].

Measuring the size of the lesion is an excellent clinical indicator of disease progression. In the first two weeks of treatment, topical SO—NE administration was able to significantly inhibit lesion growth when compared to the control group. Similar evolution was seen in the group that received treatment in combination. According to clinical evaluations, the presence of the plant derivative in the treatment of these animals seems to have prevented the formation of ulcers in the lesions. These results are in accordance with Santos et al., 2008 that tested the action of copaiba oleoresin (*C. martii*) in mice infected with *L. (L.) amazonensis*. The paw lesion of the animals that received treatment with oral and topical copaiba showed a decrease in the size of the lesion with a significant difference when compared to control [63]. Topical application of hydrogels containing extract of *Libidibia ferrea*, a species also belonging to the Fabaceae family and rich in terpenoid compounds, contained the development of CL lesions caused in Golden Hamsters (*Mesocricetus auratus*) by *L. (L.) amazonesis* when compared to the control group [64].

Nanoscale products using plant derivatives for the treatment of leishmaniasis has emerged as an alternative approach. Silver nanoparticles containing *Cuminum cyminum* L (Cumin) seed extract showed higher leishmanicidal activity *in vitro* against *L. (L.)tropica* life forms when compared to *C. cyminum* seed extract [65]. Another study demonstrated the efficacy of green nanoparticles biosynthesized by *Moringa oleifera* leaf extract against cutaneous lesions induced by *L. (L.) major* infection in BALB/c mice. Also, its use decreased CL size lesion average and induced complete healing after 14 days compared to the standard drug (which needed more than 28 days for complete healing) [66].

In our study, it was observed that treatment with SO—NE showed greater lesion reduction compared to SO-solution. The results suggest that the penetrability and the diffusion rate of active compounds were enhanced with the use of the nanostructured product. The treatment of experimentally infected animals with the topical SO—NE and intraperitoneal meglumine led to a significant reduction in the parasite load of the lesion when compared to the untreated group, suggesting that the topical treatment collaborated both in the inflammatory process and in the elimination of amastigotes from the protozoan. A similar result was noted when evaluating the topical use of biogenic antimony sulfide nanoparticles in *L. (L.) major* lesions after 4 weeks in BALB/c mice as susceptible animal models for CL. It was evidenced that, besides the considerable decrease in the size of the lesions, the nanoparticle was able to cause a reduction of the parasite load when evaluating the number of amastigotes in macrophages [67]. This is an important finding for CL treatment. As the amastigotes form of the protozoan multiplies intracellularly in macrophages it is essential that the drug can reach the cells of the polymorphonuclear system, in the deepest tissues of the skin, and thus cause the destruction of the parasite [68].

Histopathological analysis of mice lesion (skin) biopsies demonstrated for the group topically treated with SO—NE that although macrophages and amastigotes were still observed, they were less numerous. In addition, the areas with exposed connective tissue were smaller than in control. Possibly the treatment applied in the evaluated period caused a proliferative stimulus in the epithelial basal layer cells. The regeneration of the dermis with recovery of ulcerated areas was noted. In the group treated with the SO—NE and meglumine antimoniate combination, it was possible to observe that the macrophages were abandoning the process. Rare parasites were found suggesting a tendency to finalize the infectious process. The CL produces lesions that, when ulcerated, can be extensive, severe and disfiguring, because they lead to secondary infections. The reduction of the local inflammatory process and the improvement of the appearance of the lesions can favor the improvement of the clinical aspects related to the disease [69].

The cytokines levels reduction in lesions confirms these results. The ability of *Leishmania* to resist into cells is related to innate or adaptive response such as macrophages as they help the parasite to remain multiplying and infecting new cells. However, effective immune response by the phagocytic cells can destroy the pathogen [70]. IFN-γ levels seem to be involved both in controlling parasite multiplication during the early stages of *Leishmania* infection and in mediating tissue injury [71,72]. The low IFN-γ dosage in the lesion of treated groups showed compared to the control group points to an end of the process. The IL-10 has been associated with worse CL prognosis. Its blockage has been demonstrated decrease disease progression in leishmaniasis [73,74]. Here, topical SO—NE with meglumine antimoniate decreased IL-10 lesion levels that could have favored the best reduction of CL lesion sizes.

Moreover, the dosage of IFN-γ and IL-10 in the lymph nodes was also performed. However, as expected, the results showed no significant difference between the systemic levels of both groups treated with SO—NE topically and injectable meglumine antimonate combination compared to the untreated group (control) (Fig. A1). On the other hand, the local action of SO—NE in these groups was very clear and evidenced together by histological analyses, cytokine levels, and parasite load. Taking into account that in New World *Leishmania* infections the protozoan can spread to other distant organs of the body, the combined therapy can be a promising alternative [75].

The pathology of CL is complex, so when proposing a possible treatment for the disease it is necessary to evaluate jointly both the question of elimination of the causative agent and issues related to the intense inflammatory process present. Therefore, the overall results obtained in this study suggest that the topical use of SO-NE in

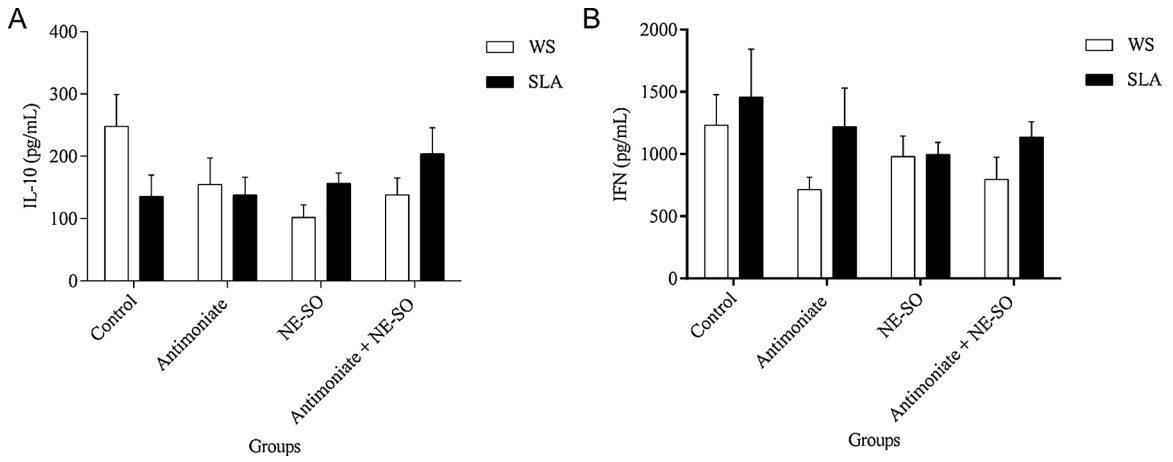

Fig. A1. Cytokines levels in lymph node cells obtained from Female BALB/c mice infected with *L. (L.) amazonensis* amastigotes (n = 7) and stimulated with Soluble *Leishmania* Antigens (SLA) (10 g/mL) alone or without stimulus (WS). Supernatants were evaluated for IL-10 (A) and IFN-γ levels by ELISA. All data are presented as average and standard deviation. *significant difference when compared with the control group ($p < 0.05$).

combination with meglumine antimoniate can be a new strategy for the treatment of CL. This is the first report on the utilization of topical SO-NE for CL treatment caused by *L. (L.) amazonensis*. Therefore, further studies are necessary to investigate the mechanisms by which SO operates on CL.

Legal aspects

Access to genetic resources and associated traditional knowledge was authorized by Genetic Heritage Management Council (CGen) with the number A037650. Animal use was authorized by Animal Ethics Committee of the Federal University of Minas Gerais, Brazil (CEUA/UFMG) with protocol numbers 191/2018 and 392/2018.

Declaration of Competing Interest

The authors declare they have no conflict of interest.

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Appendix A

Cytokines quantification in the lymph nodes

The lymph nodes were removed at the end of the treatment, homogenized in cytokine extraction solution, and centrifuged at 10,000 rpm for 10 min (4 °C). After, in culture supernatants, the cytokines IL-10 and IFN-γ were quantified by ELISA as proposed by Oliveira et al., 2017 [33]. BD OptEIA (BD Biosciences, San Diego, CA) kits were used for cytokines measurements according to the manufacturer's guidelines. A spectrophotometric reading at 492 nm was performed using the reader Emax Molecular Devices and software SoftMax Pro 5.2 (Molecular Devices Corporation, Sunnyvale, CA, USA) Fig. A1.

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