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Effects of nanoemulsions prepared with essential oils of copaiba- and andiroba against *Leishmania infantum* and *Leishmania amazonensis* infections

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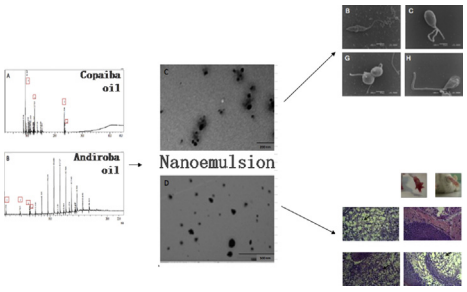
HIGHLIGHTS

- Copaiba- and andiroba-based nanoemulsions exhibit activities against *Leishmania infantum* and *L. amazonensis* in vitro.
- Copaiba- and andiroba-based nanoemulsions exhibit activities against *Leishmania infantum* and *L. amazonensis* in vivo.

GRAPHICAL ABSTRACT



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ABSTRACT

Plant products are an important source of bioactive agents against parasitic diseases, including leishmaniasis. Among these products, vegetable oils have gained ground in the pharmaceutical field. Here we report the development of nanoemulsions as a delivery system for copaiba and andiroba oils (nanocopa and nanoandi) in order to test their effects on *Leishmania infantum* and *L. amazonensis*. The nanocopa and nanoandi had an average particle size of 76.1 and 88.1, respectively with polydispersity index 0.14 to 0.16 and potential zeta −2.54 to −3.9. The data indicated toxic activity of nanocopa and nanoandi against promastigotes of both *Leishmania* species ultrastructural analyses by scanning electron microscopy revealed that exposition to nanoemulsions induced oval cell shape and retracted flagella. The treatment with nanocopa and nanoandi led to a reduction in *L. infantum* and *L. amazonensis* infection levels in macrophage cultures. The nanoemulsions treatment have significant beneficial effects on all the parameters evaluated in lesions induced by *L. amazonensis* (lesion size, parasite burden and histopathology) on BALB/c mice. The treatment of *L. infantum*-infected BALB/c mice with nanoemulsions also showed promising results reducing parasite burden in spleen and liver and improving histopathological features.

1. Introduction

Leishmaniasis is a widespread parasitic disease throughout the

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world, caused by the protozoan *Leishmania*, an obligate intracellular parasite of humans that resides and multiplies in macrophages (Kaye and Scott, 2011; Pace, 2014). The disease encompasses multiple clinical forms: cutaneous, diffuse cutaneous, mucosal and visceral (Okwor and Uzonna, 2016). *Leishmania infantum* causes the visceral form and *L. amazonensis* causes the cutaneous and diffuse cutaneous forms in Latin American countries (Pace, 2014). There is no vaccine: most of drugs have side effects, and resistance to classical chemotherapy has become a threat (Okwor and Uzonna, 2016; No, 2016). Therefore efforts are required to develop newer drug therapies.

Plant products are a good source of bioactive agents against parasitic diseases, including leishmaniasis (Rocha et al., 2005; Sen and Chatterjee, 2011). Copaiba (*Copaifera* sp. Linnaeu) oil used as cream (Santos et al., 2011) or dissolved in dimethyl sulfoxide (DMSO) (Santos et al., 2013) has shown experimentally anti-*L. amazonensis* activities (Albuquerque et al., 2017). In traditional herbal medicine from the Amazon, crude andiroba (*Carapa guianensis* Aublet) oil is recommended for the treatment of skin diseases due to anti-microbial and anti-inflammatory properties (Nayak et al., 2011). Recently, Baldissera and co-workers have shown that andiroba oil can be more effective against *Trypanosoma evansi* an important aetiological agent of trypanosomiasis in livestock when encapsulated in nanostructure (Baldissera et al., 2013). The encapsulation of vegetable oils is considered a promising strategy to facilitate the application of these natural products and to potentiate the actions (Bajerski et al., 2016). In fact, the nanoemulsions have shown promise as a carrier system for the delivery of poor water-soluble and poor membrane-permeable drugs due to their ability to dissolve lipophilic drugs (Gupta et al., 2016). Also, this nanometric carrier system is able to increase the stability, efficacy and safety of these oils (Lucca et al., 2017).

Here we report the development copaiba and andiroba oils nanoemulsions in view to test their effects on *L. infantum* and *L. amazonensis* promastigotes and intracellular amastigotes, in addition to macrophage viability. The effects of oral treatments with copaiba- and andiroba-based nanoemulsions on *L. infantum* and *L. amazonensis* infected mice are also reported here.

2. Materials and methods

2.1. Oils and reagents

The copaiba (*Copaifera* sp.) oil was obtained from a producer cooperative (Cooperativa Agroextrativista dos Produtos Rurais do Vale do Rio Iaco, Sena Madureira, Acre, Brazil), and andiroba (*C. guianensis*) oil was purchased from the producer cooperative Jurua Ecoextrativismo Eireli EPP, Cruzeiro do Sul, Acre, Brazil. The reagents were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise noted.

2.2. Analysis of copaiba and andiroba oils

The oils were tested for purity and physical and chemical characteristics using refractometry (Biobrix refractometer, São Paulo, Brazil), densitometry (Mettler Toledo 30PX) and viscometry (Ford viscosity cup). The oils were analyzed for standardized constituents by gas chromatography mass spectrometry (Agilent 6890N gas chromatograph coupled to a quadripolar mass spectrometer Agilent 5973N) following the instructions of Ramos et al. (2015).

2.3. Preparation of oils-nanoemulsions

Emulsions were prepared by adding water (10 ml) to Tween 80 (0.4 g) under stirring using a mechanical stirrer at 500 rpm, 70 °C for 10 min (aqueous phase). The organic phase was prepared using Span-80 (0.4 g) and copaiba or andiroba oil (1 g) under agitation at 70 °C for 10 min. Final homogenization was achieved using an Ultra-Turrax homogenizer (IKA T18 basic) for 3 min (8000 rpm). The emulsification was further sonicated by a tip sonicator (Vibra Cell Sonic & Materials Inc.) under 60 W and 40 Khz for 25 min (Barbosa et al., 2013a,b; Baldissera et al., 2013). As a negative control for cell culture tests, “emulsions” prepared without oils were developed using the same methodology.

2.4. Particle size, polydispersity and zeta potential

The size, polydispersity and zeta potential of the nanoemulsion particles were analyzed by dynamic light scattering (DLS) using a Zetasizer® Nano ZS90 (Malvern Instruments, Malvern, UK). For size and polydispersity measurements the samples were diluted with deionized water. For determination of the zeta potential the samples were diluted with 1 mM NaCl and placed in the proper electrophoretic cells. The analyses were performed at 25 °C, and the results were expressed as the average of three determinations. The measurements were conducted on the same day of preparation and 90 days after preparation for stability purposes. The nanotracking analysis (NTA) was used in order to determine the size distribution of the freshly prepared samples in real time (Filipe et al., 2010).

2.5. Ultrastructural analyses of nanoemulsions

The nanoparticle morphology was analyzed using a Zeiss LEO-906 60 kV transmission electron microscope. A drop of each nanoparticle sample was placed in a 200 mesh copper grid and a drop of a 2% aqueous uranyl solution was added; excess volumes were removed with filter paper. Samples were incubated for 4 h to dry at room temperature prior to transmission electron microscopy (TEM) analysis (Barbosa et al., 2013a,b).

2.6. Parasites, cells and animals

Leishmania infantum (MHM/BR/1972/LD) promastigotes were cultured in Schneider's medium supplemented with 50 μg/ml gentamicin, 10% inactivated fetal bovine serum (FBS) and 5% filtrated human urine at 26 °C. *Leishmania amazonensis* (MHOM/BR/M2269) promastigotes were cultured in RPMI medium supplemented with 50 μg/ml gentamicin and 10% FBS at 26 °C, and amastigotes were isolated from active lesions of BALB/c mice as described earlier (Giorgio et al., 1998). Peritoneal mouse macrophages were obtained from normal BALB/c mice by peritoneal lavage as previously described (Barbiéri et al., 1993). The cells were cultured in RPMI medium supplemented with 50 μg/ml gentamicin and 10% FBS at 37 °C in 5% CO_2, 21% O_2 and balanced N_2. Six-week-old female BALB/c mice were purchased from the Animal Center of Campinas State University (Unicamp), Campinas, São Paulo, Brazil. The protocols used were approved by the Animal Care Committee of Unicamp under project license number 3669-1.

2.7. Assessment of the in vitro effects of nanoemulsions on *L. infantum*, *L. amazonensis* and macrophages

Promastigotes cultured in 24-well plates at 26 °C (1×10^6/well) were treated with different concentrations nanoemulsions ranging from 1.25 μl/ml to 10 μl/ml i.e 125 μg/ml to 1000 μg/ml for nanoandi and 0.1 μl/ml to 2 μl/ml i.e. 10 μg/ml to 200 μg/ml for nanocopa during 24 h and 48 h. Their numbers were determined using a Neubauer hemocytometer.

Mouse peritoneal macrophages were cultivated on 24-well

plates (5×10^5/well) containing 13 mm glass cover slips as previously described (Barbiéri et al., 1993) and infected with *L. infantum* or *L. amazonensis* promastigotes in stationary phase of growth (2.5×10^6/well) for 24 h. The cultures were then washed to remove extracellular parasites. Fresh medium and nanoemulsions (concentrations ranging from 0.1 μl/ml to 0.9 μl/ml i.e. 10 μg/ml to 90 μg/ml for nanocopa and 1 μl/ml to 3 μl/ml i.e 100 μg/ml to 300 μg/ml for nanoandi) were added to the wells and maintained at 37 °C in 5% CO_2, 21% O_2 and balanced N_2 for an additional 24 h. For the evaluation of the infection index (IF) (percentage of infected macrophages X number of amastigotes per macrophage) cells on coverslips were stained with Giemsa and examined microscopically at 1000x magnification. At least 600 macrophages were counted on triplicate coverslips (Barbosa et al., 2015). The drug concentration that caused a 50% reduction in IF (IC50) was estimated by nonlinear regression analyses using GraphPad Prism, Sigma Plot and OriginLab OriginPro 8.5. Macrophage adherence as a direct measurement of the cell's viability and integrity (Kidd et al., 1997) was assessed by counting the cells in 20 random fields per coverslip of infected and uninfected macrophage cultures stained with Giemsa. The experiments were performed in triplicate wells and were repeated independently at least three times.

2.8. Ultrastructural analyses of parasites

The promastigotes (1×10^6 cells/ml) were fixed using 2.5% glutaraldehyde and 1% tannic acid (Electron Microscope Science, Hatfield, PA) in 0.1 M sodium cacodylate buffer (Electron Microscope Science) at pH 7.4 and prepared for scanning electron microscopy as previously described (Codonho et al., 2016), then observed using a JEOL 5800 LV (Leica, Wetzlar, Germany) scanning electron microscope operated at 10 kV.

2.9. Assessment of the effects of nanoemulsions on BALB/c mice infected with L. infantum and L. amazonensis

Experimental groups of BALB/c mice were intraperitoneally inoculated with 1×10^7 *L. infantum* promastigotes. Other groups of mice were subcutaneously inoculated with 1×10^6 *L. amazonensis* promastigotes (footpad injection). Four days after infection mice were treated with nanoemulsions (25–40 μl = 100–160 mg oil/kg of body weight), which were delivered to the mouth with a pipette tip (Butchbach et al., 2007) every day (one administration per day) during 8 weeks. For miltefosine (Cayman Chemical Company, Ann Arbour, MI, USA) treatment, three weeks after infection groups of mice were treated orally (20 mg/kg of body weight) every day during 5 weeks (Costa-Filho et al., 2008). The lesion size of *L. amazonensis* infected mice was measured weekly using a caliper and compared to the contralateral uninfected footpad (Araújo et al., 2012). To estimate the parasite burden at the end of experimental period, footpad lesions from *L. amazonensis*-infected mice as well as spleen and liver from *L. infantum*-infected mice were extracted and weighed, and amastigotes were collected after mechanical rupture of tissues. Estimation of the yield of amastigotes was by haemocytometer counting (adapted from Barbiéri et al., 1993 and Arrais-Silva et al., 2005). For histopatological preparation, tissue samples were fixed by immersion in 4% paraformaldehyde in 0.1 M PBS and processed for standard paraffin embedding (Arrais-Silva et al., 2006). Tissue sections were stained with haematoxylin and eosin (HE) and checked for pathological changes under an optical microscope (Eclipse E800-Nikon). The images were captured with a digital imaging system using the microscope, a CoolSNAP-Pro color

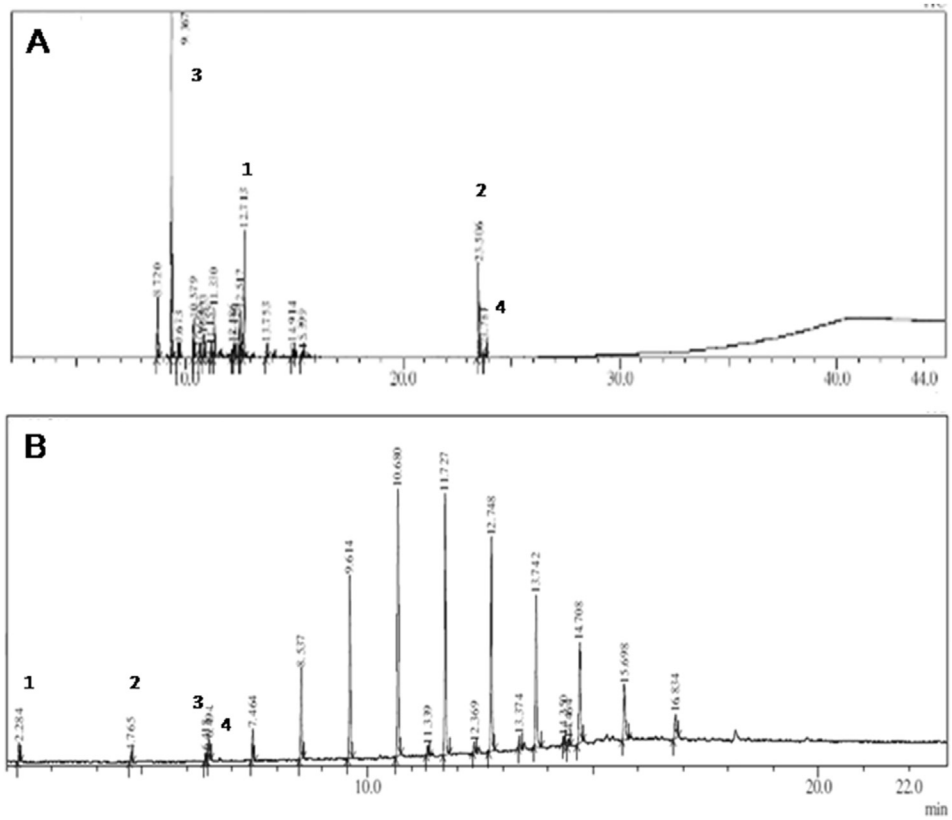

Fig. 1. Gas chromatography mass spectrometry chromatograms of oils. (A) Copaiba oil. The numbers indicate the main constituents of copaiba oil. 1. serquiterpenes, 2. diterpenes, 3. copaene and 4. kairenic acid. (B) Andiroba oil. The numbers indicate the main constituents of andiroba oil. 1. limonoids, 2. palmitic oil, 3. oleic acid and 4. linoleic acid.

camera (Media Cybernetics, Silver Spring, MD) and Image-Pro Plus capture software (Media Cybernetics).

3. Results

3.1. Composition of oils and nanoemulsion forms

Chromatographic analysis of copaiba oil sample allowed the detection of several sesquiterpenes and a high proportion of α-copaene (Veiga-Junior and Pinto, 2002; Almeida et al., 2012). Others compounds detected were diterpenes, among them kaurenoic acid – as already reported for oleoresin of copaiba (Veiga-Junior and Pinto, 2002; Almeida et al., 2012; Barbosa et al., 2013a,b) (Fig. 1). The chemical profile of the andiroba oil sample agrees with that described in the literature (Ambrozin et al., 2006; Novello et al., 2015). It was mainly composed of fatty acids – palmitic and linoleic acids –, limonoids and phenolic compounds (Fig. 1).

Table 1
Average size, polydispersity index and zeta potential of the copaiba- and andiroba-based nanoemulsions.[a]

	Time (days)	Size (nm)	Polydispersity Index	Zeta potential (mV)
Nanocopa	0	76.10 ± 0.32	0.14 ± 0.01	- 2.5 ± 0.4
	90	80.01 ± 0.57	0.14 ± 0.01	- 2.0 ± 0.5
Nanoandi	0	88.17 ± 0.88	0.16 ± 0.01	- 3.9 ± 1.0
	90	92.20 ± 0.39	0.19 ± 0.01	- 4.6 ± 2.7

[a] Nanocopa and nanoandi nanoparticulated systems, as analyzed by DLS in fresh samples (0 day) and after 90 days of storage at room temperature. Each result represents the mean ± SD of three experiments.

In order to prepare a nanoemulsion system, some surfactants and adjuvants were tested: Tween 80, Span 80, propylene glycol, methylparaben, Emulgin B2, propylparaben, butylated hydroxytoluene, cetyl and stearyl alcohols, and cetone. The mixture of Tween 80, Span 80 and copaiba or andiroba oils submitted to tip sonication provided nano-sized emulsions with optimal parameters, as analyzed by DLS (Table 1), NTA and TEM (Fig. 2). Table 1 shows that, among the nanosize dimensions of the produced emulsions, the average diameters of the particles were 90 nm (nanoandi) and 80 nm (nanocopa). As desired, the polydispersivity index (PDI) was very low (<0.2), attesting the homogeneity of the nanoparticle sizes (Silva et al., 2016) (Table 1). The DLS data (Table 1) also disclose the stability of the copaiba- and andiroba-based nanoemulsions, since there is no significant change in size, PDI and zeta potential during 90 days of storage at room temperature. To confirm the DLS data, NTA analysis which provides particle-by-particle evaluation was used to determine the size distribution of the freshly prepared samples in real time (Filipe et al., 2010). As shown in Fig. 2 NTA data corroborated the DLS measurements, showing main peak populations of particles in the range of 80–90 nm for nanocopa and nanoandi (Fig. 2A). Accordingly, TEM images revealed dark round-shape particles with bright surrounding, and no signs of aggregate formation were detected (Fig. 2B).

3.2. Leishmanicidal activity in vitro

Next, the effects of nanocopa and nanoandi on *L. infantum* and *L. amazonensis* promastigotes were analyzed by direct observation under optical microscopy. The assays were performed at 24 h and 48 h and revealed toxic activity for parasites, i.e. the number of

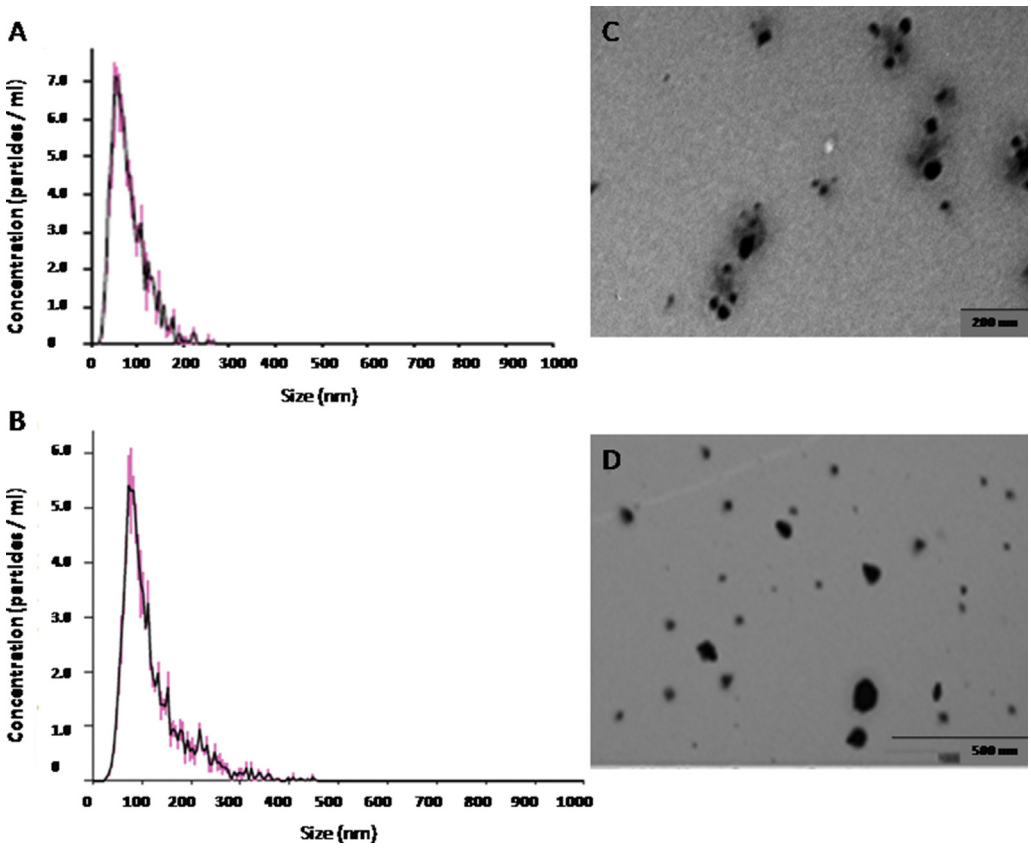

Fig. 2. Particle size, as determined by NTA in nanocopa (A) and nanoandi (B) samples. Morphology, as determined by TEM in nanocopa (C) and nanoandi (D).

Table 2
Activity of nanoemulsions against *Leishmania* promastigotes.

	Time (h)	IC50[a] *L. infantum*	IC50 *L. amazonensis*
Nanocopa	24	16 ± 0.9	18 ± 0.16
	48	18 ± 0.2	30 ± 0.7
Nanoandi	24	366 ± 21	590 ± 23
	48	320 ± 28	260 ± 29

[a] Nanoemulsion concentration (μg/ml) that inhibits 50% of promastigotes proliferation at 24 h and 48 h incubation time. A positive control (amphotericin B IC50 0.13 ± 0.02 μg/ml for *L. amazonensis* promastigotes) and a negative control (nanoemulsions without oils IC50 > 20 μl for *L. infantum* and *L. amazonensis*) were included in the assay. Results are mean ± standard deviation of triplicate values from one of three representative experiments.

promastigotes in cultures was reduced when compared with the promastigotes of untreated cultures. Table 2 shows the estimated IC50 values. The data indicated that nanocopa is more toxic to promastigotes of both *Leishmania* species than nanoandi and that higher doses of nanoemulsions are necessary to kill *L. amazonensis* promastigotes than *L. infantum* ones. As a control *L. amazonensis* promastigotes were treated with a standard anti-*Leishmania* drug, amphotericin B the IC50 value was 0.13 ± 0.02 μg/ml, however a complete reduction of parasites was not obtained. The ultrastructural analyses of promastigotes by scanning electron microscopy revealed that nanoandi and nanocopa treatments with doses near the IC50 values (Table 2) induced ultrastructural alterations, oval cell shape and retracted flagella as early as 1 h after treatment (Fig. 3). In contrast, *L. infantum* and *L. amazonensis* promastigotes exposed to nanoemulsions without oils or no treated are fully viable as judged by the growth rate and motility and by morphology both parasites are splinde-shaped with long flagellum (data not shown and Fig. 3).

Promastigotes are the insect vector stage of *Leishmania* whereas amastigotes are the intracellular form of the mammalian stage that replicates in macrophages. Both nanoemulsions nanoandi and nanocopa were tested against *Leishmania* infected macrophages. In these assays (Fig. 4) macrophages were efficiently infected with *L. infantum* and *L. amazonensis* (75–90% of infected macrophages and 5–6 intracellular parasites per macrophage). Cells infected with *L. infantum* or *L. amazonensis* and treated with nanocopa or nanoandi (doses above the IC50 values against promastigotes, Table 2) during 24 h showed significant reduction of IF (Fig. 4A and B). The treatment with nanocopa (30 and 90 μg/ml) led to a reduction in around 50% of *L. infantum* IF whereas the same treatment reduced around 50% and 90% *L. amazonensis* IF, respectively

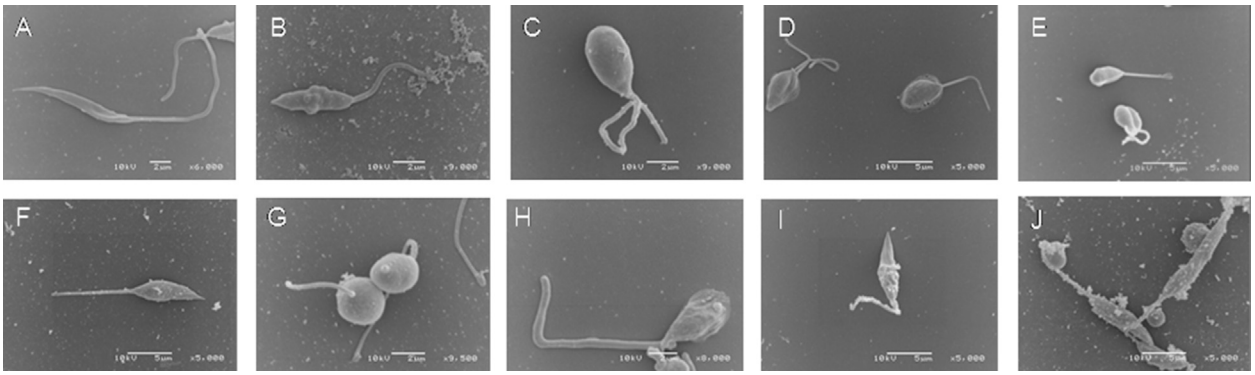

Fig. 3. Ultrastructural analyses of *L. infantum* and *L. amazonensis* promastigotes treated with nanoemulsions. Electron microscopy scanning of *L. infantum* promastigotes untreated (A), treated with nanocopa 20 μg/ml for 1 h (B) or 24 h (C), and treated with nanoandi 200 μg/ml for 1 h (D) or 24 h (E); and *L. amazonensis* promastigotes untreated (F), treated with nanocopa 20 μg/ml for 1 h (G) or 24 h (H), and treated with nanoandi 200 μg/ml for 1 h (I) or 24 h (J).

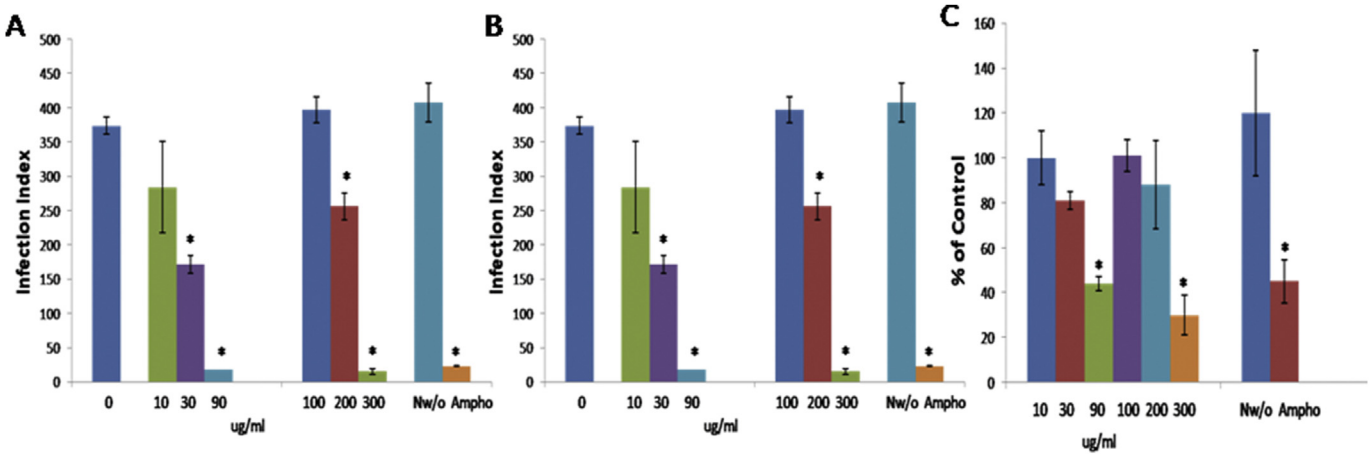

Fig. 4. Effects of nanoemulsions on *Leishmania* infected macrophages and cytotoxicity. Peritoneal mouse macrophages were infected with *L. infantum* (A) or *L. amazonensis* (B) and left untreated (0) or treated with nanocopa (10, 30 and 90 μg/ml), nanoandi (100, 200 and 300 μg/ml), nanoemulsion prepared without oils (N w/o) or amphotericin B (ampho) (3 μg/ml); after 24 h IF was determined as described in Materials and methods section. (C) Peritoneal mouse macrophages were treated with nanocopa (10, 30 and 90 μg/ml), nanoandi (100, 200 and 300 μg/ml), N w/o or ampho (3 μg/ml). Data are the percentages of macrophages adhered to the coverslips compared with those from untreated macrophage cultures set as 100%. * indicates a significant difference ($P < 0.01$) compared with untreated macrophages.

(Fig. 4). The nanoandi treatment (200–300 μg/ml) was also capable to significantly reduce the infection levels in *L. infantum*- (36% and 89%, respectively) and *L. amazonensis*- (54% and 96%, respectively) infected macrophage cultures (Fig. 4). The macrophages were treated with a standard anti-*Leishmania* drug, amphotericin B. The drug caused a diminution of IF (around 90%), but a complete reduction of parasites was not obtained (Fig. 4). The control treatment (nanoemulsion prepared without oils) did not alter infection rates as compared with untreated cell cultures (Fig. 4). In general, the viability of untreated macrophages was maintained at 90–95% during the cell culture period, but decay of macrophage number was observed in cell cultures treated with high doses of nanoemulsions (Fig. 4C). The compound amphotericin B is toxic to macrophages, and nanoemulsions without oils did not alter their viability (Fig. 4C). It should be note that essential oils of copaiba and andiroba were toxic for parasites and cells although a good reproducibility of the data was not obtained (data not shown), probably due to incomplete dispersal of the oils in the culture medium.

3.3. Leishmanicidal activity in vivo

Next, experiments were performed in order to determine whether nanoemulsions could alter the course of infection in visceral and cutaneous leishmaniasis models. Fig. 5 demonstrates that cutaneous lesions progressively increase in size in *L. amazonensis* infected mice, and animals treated for 8 weeks with nanoemulsions showed delay in lesion development. The mice treated with nanocopa had lesions that were significantly smaller

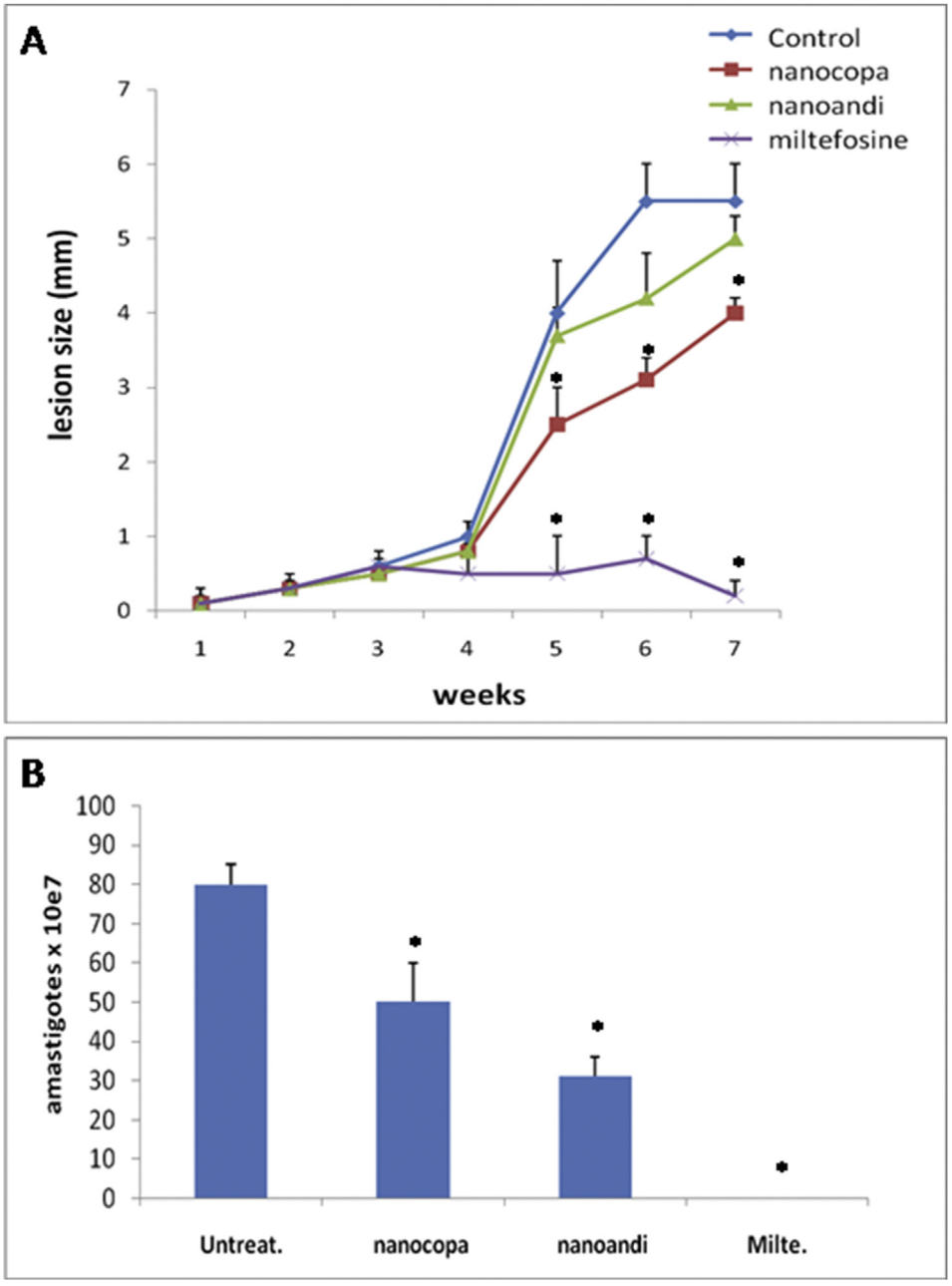

Fig. 5. Effect of nanoemulsions on *L. amazonensis* infected mice. BALB/c mice were infected with 1×10^6 promastigotes left untreated or treated with nanoemulsions (nanocopa and nanoandi) and miltefosine as described in Materials and methods section. The lesion size is expressed as the difference in size between the infected and contralateral non-infected footpads (A). Parasite burden of lesion was determined after 8 weeks of infection (B). $P < 0.05$.

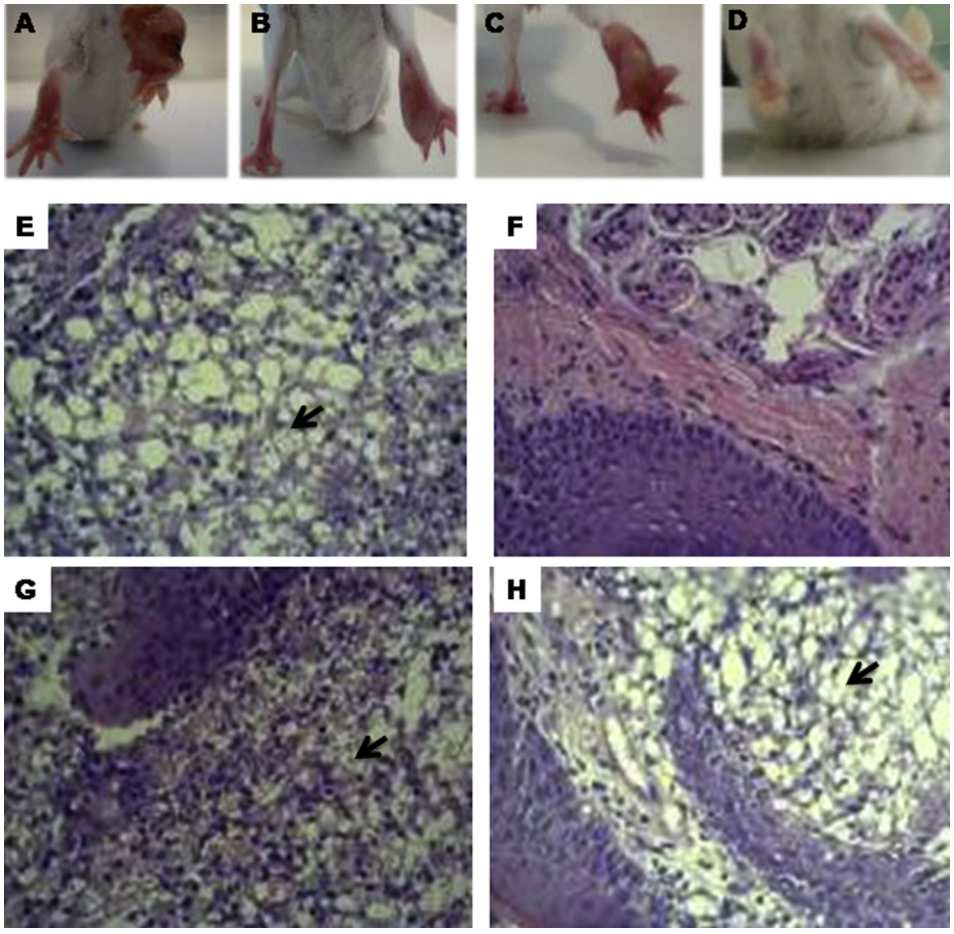

Fig. 6. Effect of nanoemulsions on *L. amazonensis* infected mice. BALB/c mice were infected with 1×10^6 promastigotes and treated with nanoemulsions and miltefosine as described in Materials and methods section. Evolution of footpad lesion in mice infected and left untreated (A), treated with nanocopa (B), nanoandi (C) and miltefosine (D). Histological patterns of footpad lesions from mouse untreated (E), treated with miltefosine (F), nanocopa (G) and nanoandi (H). The arrows indicate vacuolized and infected macrophages. HE (1000x).

than those on untreated mice from 5 weeks until the end of experiment (Fig. 5A). The reduction of lesion size was related to a reduction of parasite burden; amastigotes were less abundant in lesions of nanoemulsions treated-mice (Fig. 5B). The results showed a decrease in the size of the lesions after treatment with miltefosine the only oral anti-*Leishmania* drug used in clinical treatment (Sundar and Olliaro, 2007), and no parasite was detected after 8 weeks (Fig. 5). It should be noted that animals treated with essential oils of copaiba or andiroba lost body weight, were less active and gasping occurred after few days of treatment. These mice were removed from study.

Significant differences in tissue pathology were observed between groups of mice. In the footpads of untreated mice a large infiltration of vacuolated macrophages within amastigotes and inflammatory cells were observed, and adypocytes and muscle cells in the subcutaneous tissues were noticed (Fig. 6). The lesions of nanocopa-treated mice showed inflammatory cells and infected macrophages infiltration, but in a lesser extention than those observed in the lesions of untreated mice. In the lesions of animals treated with miltefosine few inflammatory cells were observed (Fig. 6).

The results obtained from the *in vivo* therapy of mice infected with *L. infantum* showed that both nanoemulsions were effective in reducing the parasite burden (around 50% reduction in parasites within livers and spleens of mice treated with nanocopa and nanoandi as compared with untreated animals) (Fig. 7). Histological data revealed that spleens from untreated mice showed granulomas, inflammatory infiltrates, fibrous connective tissues and cellular disorganization. The same spleen histological pattern was observed in treated mice, although a predominance of inflammatory infiltrates was observed (Fig. 7).

We also evaluate different organs from mice upon different treatments by histological examination (Fig. 8). The histological findings of liver, spleen, stomach, intestine and kidney of nanoemulsion-treated mice indicated no morphological changes in comparison with control (untreated mice). However, the stomach of mice treated with miltefosine showed reduction of villi and enhancement of gastric wall mucus and glandular tissue (Fig. 8).

4. Discussion

In the present study we have developed copaiba- and andiroba-based nanoemulsions and evaluated its potential leishmanicidal activity. The chemical profile of andiroba and copaiba oil samples used in this study was analyzed by gas chromatography and agrees with that described in the literature (Veiga-Junior and Pinto, 2002; Almeida et al., 2012; Novello et al., 2015; Ambrozin et al., 2006). The emulsions prepared with non-ionic detergents (Tween 80 and Span 80) and andiroba or copaiba oil resulted in nanoemulsions with particles which average diameter is in the range of 80–90 nm; these results are appropriate since the low particle size increases the surface area favoring drug-delivery. The low PDI (<0.2)

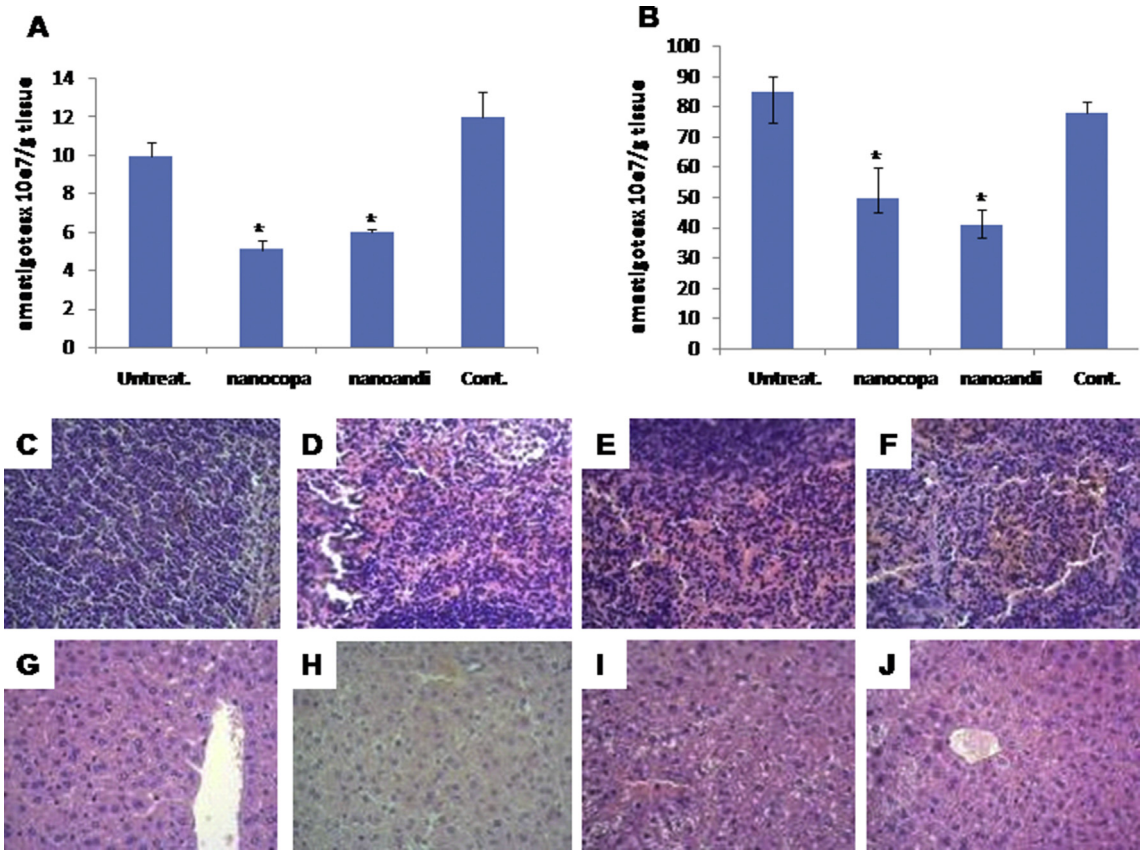

Fig. 7. Effect of nanoemulsions on *L. infantum* infected mice. BALB/c mice were infected with 1×10^6 promastigotes. Parasite burden of the liver (A) and spleen (B) of mice left untreated or treated with nanocopa, nanoandi or nanoemulsion without oil (Cont.). Histological patterns from liver of normal mouse (C), infected and untreated mouse (D), treated with nanocopa (E), and treated with nanoandi (F), spleen of normal mouse (G), infected and untreated mouse (H), treated with nanocopa (I) and treated with nanoandi (J). HE (1000x). $P < 0.05$.

indicates the achievement of monodisperse (homogenous) systems (Attama et al., 2012). It should be noted that even the zeta potential values were close to neutrality, nanoandi and nanocopa were found to be stable during the time as a result of the steric stabilization provided by the bulky surface groups of the surfactants Tween 80 and Span 80; such effect is desirable and prevents or minimizes the occurrence of particle aggregation (Attama et al., 2012; Ribeiro et al., 2017).

Nanocopa is more toxic to *Leishmania* promastigotes and intracellular amastigotes than nanoandi since IC50 values of nanocopa are lower than those of nanoandi (IC50 16–30 μg/ml *versus* IC50 260–590 μg/ml). The previous evaluation of the leishmanicidal activity of copaiba oil was carried out mainly in *L. amazonensis* (Santos et al., 2008; Estevez et al., 2007; Albuquerque et al., 2017). In vitro assays were performed with oils of different species of copaiba solubilized in DMSO or ethanol (<0.1%) and have been shown to be active against promastigotes, axenic amastigotes and intracellular amastigotes (IC50 10–30 μg/ml) (Estevez et al., 2007; Santos et al., 2008). Similar to our observations, promastigotes were more sensitive to treatment than amastigotes (Estevez et al., 2007; Santos et al., 2008; Albuquerque et al., 2017). In another report, Rondon and coworkers found IC50 values of copaiba oil diluted in water around 8 μg/ml for *L. chagasi* promastigotes and low IC50 value for amastigotes (0.52 μg/ml) (Rondon et al., 2012).

In general the changes in the morphology of *Leishmania* treated with nanocopa are similar to previous findings using crude oil aberrant shaped cells and retracted flagella (Santos et al., 2012). The main chemical constituents of copaiba oil are sesquiterpenes, which are less active than the crude oil (Santos et al., 2011; Albuquerque et al., 2017; Lima et al., 2003). Further studies with oil-based nanoemulsions are needed to confirm these previous findings.

The nanoandi had leishmanicidal effect in promastigotes and intracellular forms. No reports of leishmanicidal activity of andiroba oil are available for comparison with our results. However, andiroba oil in nanostructured form was tested in *T. evansi*, a parasite belonging to same family of *Leishmania* (Trypanosomatidae) (Baldissera et al., 2013). The authors reported a significant reduction in the number of live parasites after 1–6 h treatment with 0.5% nanoemulsion of andiroba, with no morphological alterations in the flagellates (Baldissera et al., 2013). In our study, oval cell shape and retracted flagella in *L. amazonensis* and *L. infantum* promastigotes were observed as early as 1 h, suggesting that different structures of *Leishmania* and *Trypanosoma* should be targets of andiroba. Although the mechanisms of toxicity are not known, it could be suggested that the surfactant coating of the nanoparticles surface may facilitate the penetration of the loaded drug into the parasite (Lherm et al., 1987). We did not identify the constituents of andiroba oil involved in leshmanicidal effect, although a series of chemical elements present in andiroba oil, such as fatty acids and phenolic compounds, obtained from other plants had already proved leishmanicidal activity (Cunningham et al., 1972; Carballeira et al., 2012; Rodrigues et al., 2014). Further studies so as to identify andiroba oil components with leishmanicidal activities will need to be performed.

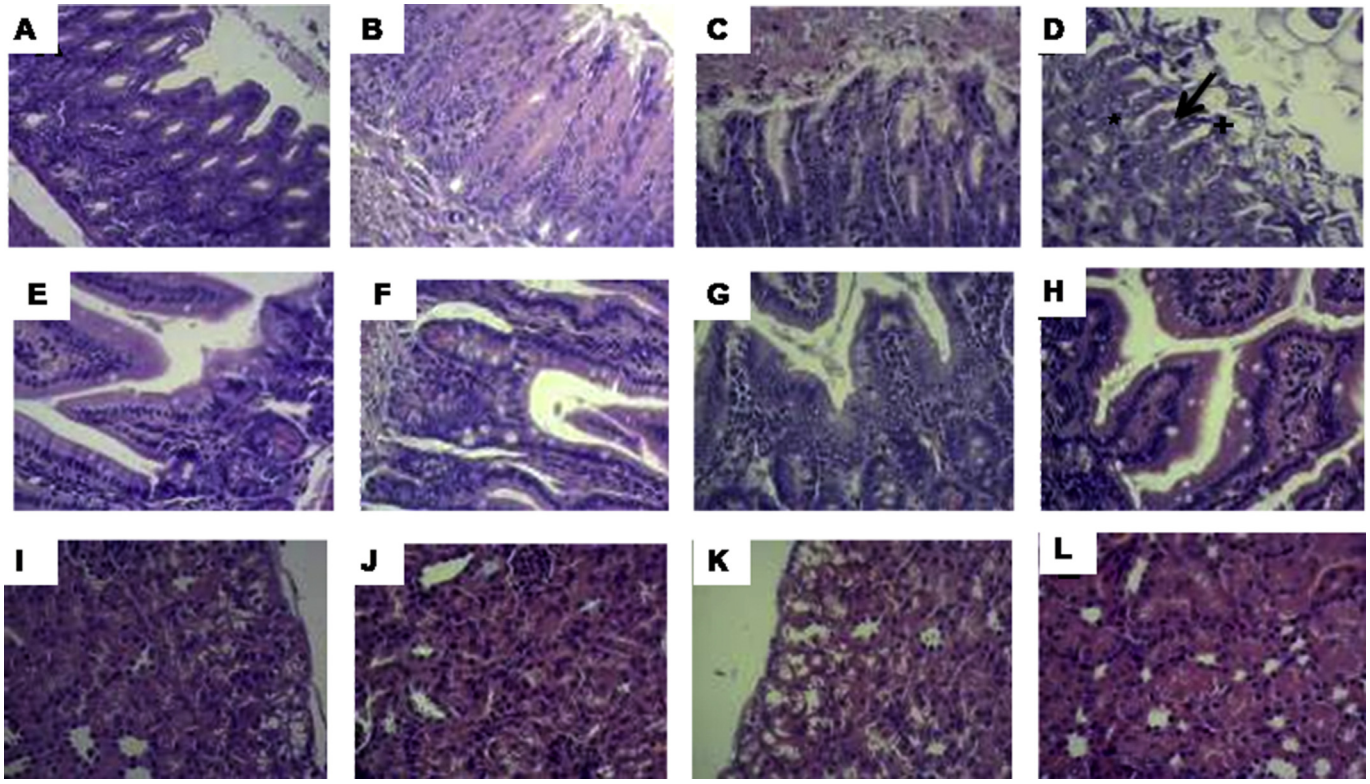

Fig. 8. Effect of nanoemulsions on mice. BALB/c mice were left untreated (A, E and I) or treated with nanoandi (B, F and J), nanocopa (C, G and K) or miltefosine (D, H and L). Histological patterns of the stomach (first row), intestine (middle row) and kidney (last row). Arrow and asterisk in (D) indicate enhanced gland and reduced microvilosities, respectively. HE (1000x).

Features such high physical stability, lack of toxicity and solubilization of lipophilic molecules make nanoemulsions excellent vehicle for vegetable oils encapsulation and a promising system in the pharmaceutical field (Bajerski et al., 2016). Regarding cytotoxicity, our data indicated that nanoemulsions with or without andiroba and copaiba oils had low *in vitro* toxicity against macrophages. Also as expected, when administred orally in BALB/c mice, nanocopa and nanoandi did not cause gastrointestinal, hepatic, splenic or renal histological abnormalities. Santos and coworkers reported no histological changes in various organs of mice treated with copaiba oil resin compared to control animals (Santos et al., 2011).

Our data showed that both oils nanoemulsions have significant effects on the reduction of BALB/c mice lesions induced by *L. amazonensis* a susceptible model of cutaneous leishmaniasis (Giorgio et al., 1998). While a complete cure did not occur, all the parameters evaluated (lesion size, parasite burden and histopathology) changed significantly after treatment, in a fashion indicative of a reduced pathology. The treatment of mice with miltefosine, the only orally active anti-*Leishmania* drug (Sundar and Olliaro, 2007), was effective. Although drugs such miltefosine, amphotericin b, paromycin and pentavalent antimonials are effective to treat human leishmaniasis, an increasing number of treatment failures and development of primary resistance to these drugs have been reported (Hendrickx et al., 2016; Okwor and Uzonna, 2016; No, 2016). In a previous report Santos and coworkers demonstrated that *L. amazonensis*-infected BALB/c mice treated with an oral emulsion of copaiba reduced the average lesion size after 1 month of infection (Santos et al., 2011). The treatment of *L. infantum*-infected BALB/c mice under a susceptible model of visceral leishmaniasis with oil nanoemulsions also showed promising results reducing parasite burden in spleen and liver and improving histopathological features. We are not aware of any previous reports describing the effects of andiroba on leishmaniasis.

Taken together our results indicate that different oils nanoemulsions treatment regimens might be further tested to a level that prevent and cure leishmaniasis.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.exppara.2018.03.005.

References

Albuquerque, K.C., da Veiga, A.D., Silva, J.V., Brigido, H.P., Ferreira, E.P., Costa, E.V., Marinho, A.M., Percário, S., Dolabela, M.F., 2017. Brazilian Amazon traditional medicine and the treatment of difficult to heal leishmaniasis wounds with *Copaifera*. 2017. Evid. Compl. Alt. Med., 8350320, 9 pages.

Almeida, M.R., Darin, J.D., Hernandes, L.C., de Souza Ramos, M.F., Antunes, L.M., de Freitas, O., 2012. Genotoxicity assessment of Copaiba oil and its fractions in Swiss mice. Genet. Mol. Biol. 35, 664–672.

Ambrozin, A.R.P., Leite, A.C., Bueno, F.C., Vieira, P.C., Fernandes, J.B., Bueno, O.C., Fernandes da Silva, F.G., Pagnocca, F.C., Hebling, M.J.A., Bacci Jr., M., 2006.

Limonoids from *Cipadessa fruticosa* and *Cedrela fissilis* and their insecticidal activity. J. Braz. Chem. Soc. 17, 542–547.
Araújo, A.P., Arrais-Silva, W.W., Giorgio, S., 2012. Infection by *Leishmania amazonensis* in mice: a potential model for chronic hypoxia. Acta Histochem. 114, 797–804.
Arrais-Silva, W.W., Arrais-Silva, W.W., Paffaro Jr., V.A., Yamada, A.T., Giorgio, S., 2005. Expression of hypoxia-inducible factor-1alpha in the cutaneous lesions of BALB/c mice infected with *Leishmania amazonensis*. Exp. Mol. Pathol. 78, 49–54.
Arrais-Silva, W.W., Pinto, E.F., Rossi-Bergmann, B., Giorgio, S., 2006. Hyperbaric oxygen therapy reduces the size of Leishmania amazonensis-induced soft tissue lesions in mice. Acta Trop. 98, 130–136.
Attama, A.A., Momoh, M., Builders, A., Philip, F., 2012. Lipid nanoparticulate drug delivery systems: a revolution in dosage form design and development. In: Recent Advances in Drug Delivery Systems, pp. 107–140.
Bajerski, L., Michels, M.R., Colomé, L.M., Bender, E.A., Freddo, R.J., Bruxel, F., Haas, S.E., 2016. The use of Brazilian vegetable oils in nanoemulsions: an update on preparation and biological applications. Brazilian J. Pharm. Sci. 52, 347–363.
Baldissera, M.D., Da Silva, A.S., Oliveira, C.B., Zimmermann, C.E., Vaucher, R.A., Santos, R.C., Rech, V.C., Tonin, A.A., Giongo, J.L., Mattos, C.B., Koester, L., Santurio, J.M., Monteiro, S.G., 2013. Trypanocidal activity of the essential oils in their conventional and nanoemulsion forms: in vitro tests. Exp. Parasitol. 134, 356–361.
Barbiéri, C.L., Giorgio, S., Merjan, A.J.C., Figueiredo, E.N., 1993. Glycosphingolipid antigens of *Leishmania* (*Leishmania*) *amazonensis* amastigotes identified by use of a monoclonal antibody. Infect. Immun. 61, 2131–2137.
Barbosa, P.C., Moreira Wiedemann, L.S., da Silva Medeiros, R., Sampaio, T.B.P., Vieira, G., Veiga-Junior, F.C.V., 2013a. Phytochemical fingerprints of copaiba oils (*Copaifera multijuga* Hayne) determined by multivariate analysis. Chem. Biodivers. 10, 1350–1360.
Barbosa, A.M., Costa, S.S., Rocha, J.R., Montanari, C.A., Giorgio, S., 2015. Evaluation of the leishmanicidal and cytotoxic effects of inhibitors for microorganism metabolic pathway enzymes. Biomed. Pharmacother. 74, 95–100.
Barbosa, R.M., da Silva, G.M.C., Bell, T.S., de Araújo, D.R., Marcato, P.D., Durán, N., Paula, E., 2013b. Cytotoxicity of solid lipid nanoparticles and nanostructures lipid carriers containing the local anesthetic dibucaine be designed for topical application. J. Phys. Conf. 429, 012035.
Butchbach, M.E., Edwards, J.D., Schussler, K.R., Burghes, A.H., 2007. A novel method for oral delivery of drug compounds to the neonatal SMNDelta7 mouse model of spinal muscular atrophy. J. Neurosci. Meth. 161, 285–290.
Carballeira, N.M., Cartagena, M., Li, F., Chen, Z., Prada, C.F., Calvo-Alvarez, E., Reguera, R.M., Balaña-Fouce, R., 2012. First total synthesis of the (±)-2-methoxy-6-heptadecynoic acid and related 2-methoxylated analogs as effective inhibitors of the leishmania topoisomerase IB enzyme. Pure Appl. Chem. 84, 1867–1875.
Codonho, B.S., Costa, S.S., Peloso, E.F., Joazeiro, P.P., Gadelha, F.R., Giorgio, S., 2016. HSP70 of *Leishmania amazonensis* alters resistance to different stresses and mitochondrial bioenergetics. Mem. Inst. Oswaldo Cruz 111, 460–468.
Costa-Filho, A.V., Lucas, I.C., Sampaio, R.N., 2008. Estudo comparativo entre miltefosina oral a antimoniato de N-metil glucamina parenteral no tratamento da leishmaniose experimental causada por *Leishmania (Leishmania)* amazonensis. Rev. Soc. Bras. Med. Trop. 41, 424–427.
Cunningham, L.V., Kazan, B.H., Kuwahara, S.S., 1972. Effect of long-chain fatty acids on some trypanosomatids flagellates. J. Gen. Microbiol. 70, 491–496.
Estevez, Y., Castillo, D., Pisango, M.T., Arevalo, J., Rojas, R., Alban, J., Deharo, E., Bourdy, G., Sauvain, M., 2007. Evaluation of the leishmanicidal activity of plants used by Peruvian Chayahuita ethnic group. J. Ethnopharmacol. 114, 254–259.
Filipe, V., Hawe, A., Jiskoot, W., 2010. Critical evaluation of Nanoparticle Tracking Analysis (NTA) by nanosight for the measurement of nanoparticles and protein aggregates. Pharm. Res. (N. Y.) 27, 796–810.
Giorgio, S., Linares, E., Ischiropoulos, H., Von Zuben, F.J., Yamada, A., Augusto, O., 1998. In vivo formation of electron paramagnetic resonance-detectable nitric oxide and of nitrotyrosine is not impaired during murine leishmaniasis. Infect. Immun. 66, 807–814.
Gupta, A., Eral, H.B., Hatton, T.A., Doyle, P.S., 2016. Nanoemulsions: formation, properties and applications. Soft Matter 12, 2826–2841.
Hendrickx, S., Guerin, P.J., Caljon, G., Croft, S.L., Maes, L., 2016. Evaluating drug resistance in visceral leishmaniasis: the challenges. Parasitology 21, 1–11.
Kaye, P., Scott, P., 2011. Leishmaniasis: complexity at the host-pathogen interface. Nat. Rev. Microbiol. 9, 604–615.
Kidd, M.T., Qureshi, M.A., Hagler, J.R., Ali, R., 1997. T-2 tetraol is cytotoxic to a chicken macrophage cell line. Poultry Sci. 76, 311–313.
Lherm, C., Couvreur, P., Loiseau, P., Bories, C., Gayral, P., 1987. Unloaded poly-isobutylcyanoacrylate nanoparticles: efficiency against bloodstream trypanosomes. J. Pharm. Pharmacol. 39, 650–652.
Lima, S.R., Junior, V.F., Christo, H.B., Pintom, A.C., Fernandesm, P.D., 2003. In vivo and in vitro studies on the anticancer activity of *Copaifera multijuga* Hayne and its fractions. Phytother Res. 17, 1048–1053.
Lucca, L.G., de Matos, S.P., Kreutz, T., Teixeira, H.F., Veiga Jr., V.F., de Araújo, B.V., Limberger, R.P., Koester, L.S., 2018. Anti-inflammatory effect from a hydrogel containing nanoemulsified Copaiba oil (Copaifera multijuga Hayne). Am. Ass. Pharm. Sci. Pharm. Sci. Tech. 19, 522–530. https://doi.org/10.1208/s12249-017-0862-6.
Nayak, B.S., Kanhai, J., Milne, D.M., Pinto Pereira, L., Swanston, W.H., 2011. Experimental evaluation of ethanolic extract of Carapa guianensis L. Leaf for its wound healing activity using three wound models. Evid. Compl. Alt. Med. 2011, 419612.
No, J.H., 2016. Visceral leishmaniasis: revisiting current treatments and approaches for future discoveries. Acta Trop. 155, 113–123.
Novello, Z., Scapinello, J., Magro, J.D., Zin, G., Luccio, M.D., Tres, M.V., Oliveira, J.V., 2015. Extraction, chemical characterization and antioxidant activity of andiroba deeeds oil obtained from pressurized n-butane. Ind. Crop. Prod. 76, 697–701.
Okwor, I., Uzonna, J., 2016. Social and economic burden of human leishmaniasis. Am. J. Trop. Med. Hyg. 94, 489–493.
Pace, D., 2014. Leishmaniasis. J. Infect. 69, S10–S18.
Ramos, S.A., Silva, J.R.A., Oliveira, A.A., Mpalantinos, M.A., Basso, S.L., Ferreira, J.L.P., Amaral, A.C.F., 2015. Fingerprint by gas chromatography-mass spectometry of two *Himatanthus* species of Brazilian north region. Chem. Nat. Comp. 51, 1149–1151.
Ribeiro, L.M.N., Breitkreitz, M.C., Guilherme, A.V., Silva, G.H.R., Couto, V.M., Castro, S.R., de Paula, B.O., Machado, D., de Paula, E., 2017. Natural lipids-based NLC containing lidocaine: from pre-formulation to *in vivo* studies. Eur. J. Pharmaceut. Sci. 106, 102–122.
Rocha, L.G., Almeida, J.R., Macêdo, R.O., Barbosa-Filho, J.M., 2005. A review of natural products with antileishmanial activity. Phytomedicine 12, 514–535.
Rodrigues, I.A., Azevedo, M.M., Chaves, F.C., Alviano, C.S., Alviano, D.S., Vermelho, A.B., 2014. Arrabidaea chica hexanic extract induces mitochondrion damage and peptidase inhibition on *Leishmania* spp. Biomed. Res. Inter. 2014, 985171.
Rondon, F.C., Bevilaqua, C.M., Accioly, M.P., Morais, S.M., Andrade-Júnior, H.F., Carvalho, C.A., Lima, J.C., Magalhães, H.C., 2012. In vitro efficacy of *Coriandrum sativum*, *Lippia sidoides* and *Copaifera reticulata* against *Leishmania chagasi*. Rev. Bras. Parasitol. Vet. 21, 185–191.
Santos, A.O., Costa, M.A., Ueda-Nakamura, T., Dias-Filho, B.P., da Veiga-Júnior, V.F., de Souza Lima, M.M., Nakamura, C.V., 2011. *Leishmania amazonensis*: effects of oral treatment with copaiba oil in mice. Exp. Parasitol. 129, 145–151.
Santos, A.O., Ueda-Nakamura, T., Dias Filho, B.P., da Veiga Junior, V.F., Nakamura, C.V., 2012. Copaiba Oil: an alternative to development of new drugs against leishmaniasis. Evid. Compl. Alt. Med. 2012, 898419.
Santos, A.O., Izumi, E., Ueda-Nakamura, T., Dias-Filho, B.P., Veiga-Júnior, V.F., Nakamura, C.V., 2013. Antileishmanial activity of diterpene acids in copaiba oil. Memórias. do Instuto. Oswaldo. Cruz. 108, 59–64.
Santos, A.O., Ueda-Nakamura, T., Dias Filho, B.P., Veiga Junior, V.F., Pinto, A.C., Nakamura, C.V., 2008. Effect of Brazilian copaiba oils on *Leishmania amazonensis*. J. Ethnopharmacol. 120, 204–208.
Sen, R., Chatterjee, M., 2011. Plant derived therapeutics for the treatment of leishmaniasis. Phytomedicine 18, 1056–1069.
Silva, C.M.G., Fraceto, L.F., Franz-Montan, M., Couto, V.M., Casadei, B.R., Cereda, C.M.S., de Paula, E., 2016. Development of egg PC/cholesterol/α-tocopherol liposomes with ionic gradients to deliver ropivacaine. J. Liposome Res. 26, 1–10.
Sundar, S., Olliaro, P.L., 2007. Miltefosine in the treatment of leishmaniasis: clinical evidence for informed clinical risk management. Therapeut. Clin. Risk Manag. 3, 733–740.
Veiga-Junior, V.F., Pinto, A.C., 2002. The *Copaifera* L. genus. Quim. Nova 25, 273–286.