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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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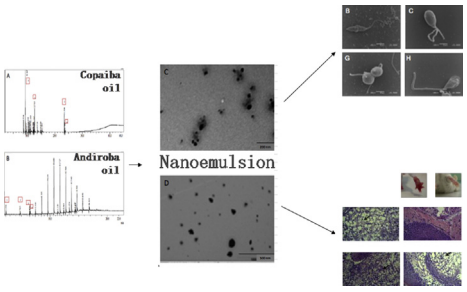
HIGHLIGHTS

- 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