

Therapeutic effect of ursolic acid in experimental visceral leishmaniasis



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ABSTRACT

Leishmaniasis is an important neglected tropical disease, affecting more than 12 million people worldwide. The available treatments are not well tolerated and present diverse side effects in patients, justifying the search for new therapeutic compounds. In the present study, the therapeutic potential and toxicity of ursolic acid (UA), isolated from the leaves of *Baccharis uncinella* C. DC. (Asteraceae), were evaluated in experimental visceral leishmaniasis. To evaluate the therapeutic potential of UA, hamsters infected with *L. (L.) infantum* were treated daily during 15 days with 1.0 or 2.0 mg UA/kg body weight, or with 5.0 mg amphotericin B/kg body weight by intraperitoneal route. Fifteen days after the last dose, the parasitism of the spleen and liver was stimulated and the main histopathological alterations were recorded. The proliferation of splenic mononuclear cells was evaluated and IFN- γ , IL-4, and IL-10 gene expressions were analyzed in spleen fragments. The toxicity of UA and amphotericin B were evaluated in healthy golden hamsters by histological analysis and biochemical parameters. Animals treated with UA had less parasites in the spleen and liver when compared with the infected control group, and they also showed preservation of white and red pulps, which correlate with a high rate of proliferation of splenic mononuclear cells, IFN- γ mRNA and iNOS production. Moreover, animals treated with UA did not present alterations in the levels of AST, ALT, creatinine and urea. Taken together, these findings indicate that UA is an interesting natural compound that should be considered for the development of prototype drugs against visceral leishmaniasis.

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1. Introduction

Leishmaniasis are infectious parasitic diseases caused by protozoans belonging to the Trypanosomatidae family, Kinetoplastida order, *Leishmania* genus. These diseases affect humans, several wild and domestic mammal species, as well as invertebrates belonging to the Diptera order, Psychodidae family, *Lutzomyia* genus in the New World, as well as *Phlebotomus* genus in the Old World (WHO,

2010).

Leishmaniasis consist of a complex of diseases with important clinical spectrum and epidemiological diversity. Depending on the infecting species and the intrinsic characteristics of the host (Lana et al., 2015), cutaneous leishmaniasis or visceral leishmaniasis (VL) can be clinically characterized. So far, VL has been recognized as the most serious clinical form of this group of diseases (Monge-Maillo and López-Vélez, 2013).

In the New World, the only species causing VL is *L. (Leishmania) infantum* (syn. *L. (L.) chagasi*) (Silveira and Corbett, 2010), with an incidence of 1.9 cases per 100,000 inhabitants and a 90% mortality

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rate if not treated properly (Gomes et al., 2016). VL is considered a generalized chronic disease, the first symptom of visceralization being a frequent and relapsing low fever with two or three daily peaks. Fever is the most notable symptom due to its irregular or remitting feature (Van Griensven and Diro, 2012). Splenomegaly and hepatomegaly are also important clinical signs that persist during the course of the disease. The chronicity of VL is marked by progressive weight loss and general weakening, hence increasing the risk of acquiring secondary infections. It might progress quickly, though, leading the patient to cachexia or death within a few weeks or months (Van Griensven and Diro, 2012).

Although different groups have made efforts to characterize affordable and safe vaccines for human VL, they are currently still under characterization (Passero et al., 2012; Duthie et al., 2016). Thus, chemotherapy remains the only possible method that can be used to eliminate parasites from tissues. Antimonial and amphotericin B are the standard drugs used in human therapy (Rath et al., 2003). Pentavalent antimony has been used for more than seven decades, and nowadays it is still used as the first choice of treatment for all clinical forms of leishmaniasis (Tempone et al., 2011). While effective, patients treated with this drug present local and systemic side effects, such as nausea, vomiting, weakness, myalgia, abdominal pain, skin rash, liver and heart toxicities (McGwire and Satoskar, 2014). Moreover, some reports suggest that in the New World *L. (Viannia) braziliensis*, *L. (V.) guyanensis* and *L. (L.) infantum* have acquired increased resistance against antimonial drugs (Tessarollo et al., 2015; de Moura et al., 2016; Moreira et al., 2015; Monte-Neto et al., 2015).

In cases of unsuccessful treatment with antimonial or disease relapse, amphotericin B is chosen as the second-line drug, being effective against amastigote forms. It has, however, a number of side effects, including nephrotoxicity and cardiotoxicity, which limit its use. Besides resistance against amphotericin B have been suggested for some *Leishmania* species (Chattopadhyay and Jafurulla, 2011). In more detail, resistance development seems to be related with a replacement of ergosterol, the main target for this drug, by cholesta-5,7,24-trien-3-ol in the parasite membrane, an increase in the levels of MDR1 protein, and an upregulation in the cascade of the trypanedoxin pathway, among other functional changes, which all together make the parasite more resistant to amphotericin B (Purkait et al., 2012).

In view of the serious side effects of drugs commonly used in leishmaniasis chemotherapy and the emergence of drug-resistant parasites, it is urgent to search for new compounds that require few cycles of administration and that are more effective and less toxic to patients or animals. An interesting alternative for the discovery of new therapeutic agents for the treatment of leishmaniasis is prospecting natural products from different sources, such as plants, which possess a wide range of secondary metabolites, including triterpenes (Passero et al., 2014; Duarte et al., 2016).

Triterpenoids are the most representative group of phytochemicals, comprising more than 20,000 known compounds that can be classified into groups based on their structural skeletons, such as cycloartanes, dammaranes, euphanes, friedelanes, lanostanes, lupanes, oleananes, tirucallanes, and ursanes, among others (Hill and Connolly, 2012). The diversity of triterpenes is highly associated with their broad range of pharmacological effects, and different studies have already shown that these compounds present multispecies action against *Leishmania* sp. (Gnoatto et al., 2008; Bero et al., 2011; Begum et al., 2014). Recently, it was demonstrated that ursolic acid (UA) displayed activity against promastigote and amastigote forms of *L. (L.) amazonensis*, *L. (V.) braziliensis*, *L. (L.) chagasi*, and *L. (L.) major* (Passero et al., 2011; Begum et al., 2014; Yamamoto et al., 2014, 2015), suggesting that UA presents multispectral action. In spite of that, few works have

performed *in vivo* studies in order to evaluate the therapeutic potential of this triterpene and, more importantly, up to now there has not been any report analyzing the therapeutic action of this compound on VL, one of the most important medical conditions caused by *Leishmania* parasites.

Based on the multispecies action of UA in *Leishmania* parasites, and considering the absence of reports on the therapeutic potential of UA in experimental VL, the present work aimed to analyze the therapeutic effect of UA isolated from the leaves of the Brazilian plant *Baccharis uncinella* C. DC. (Asteraceae) using the experimental model of VL.

2. Material and methods

2.1. General experimental procedures

The ^1H and ^{13}C NMR spectra were recorded at 300 MHz and 75 MHz, respectively, in a Bruker Advance III spectrometer using DMSO- d_6 (Sigma-Aldrich Co., St Louis, MO, USA) as solvent and internal standard. Silica gel (230–400 mesh; Merck & Co., Kenilworth, NJ, USA) and Sephadex LH-20 (Sigma-Aldrich Co.) were used for column chromatographic separation, while silica gel 60 PF $_{254}$ (Merck & Co.) was used for analytical TLC (0.25 mm). High-performance liquid chromatography (HPLC) purification was performed in a Dionex Ultimate 3000 chromatograph, using a Luna Phenomenex RP-18 column (3 μm ; 150 \times 5 mm) and an ultraviolet (UV)-diode array detector (DAD). All chemicals employed were of analytical reagent grade. Amphotericin B was purchased from Cristalia (Brazil) and solubilized in sodium chloride 0.9% (w/v).

2.2. Plant material

The leaves of *B. uncinella* were collected in Campos do Jordão, São Paulo, Brazil, in June 2005. The plant was authenticated by Dr. Oriana A. Fávero and the voucher specimen was deposited at the Herbarium of the Prefeitura Municipal de São Paulo with the reference number PMSP8983.

2.3. Extraction and isolation of UA

Dried and powdered leaves of *B. uncinella* (207 g) were exhaustively extracted using EtOH 95% at room temperature for 7 days. The crude extract (9.8 g), obtained after evaporation of solvent under reduced pressure, was dissolved in MeOH:H $_2$ O 7:3 (v/v) and partitioned using CH $_2$ Cl $_2$. The obtained CH $_2$ Cl $_2$ phase (5.3 g) was subjected to column chromatography over silica gel and eluted with different mixtures of n-hexane:EtOAc in gradient form to yield five groups (A–E). Group C (232 mg) was purified using semi-preparative RP-18 HPLC, eluted with MeOH:H $_2$ O 95:5 (flow rate: 5 mL/min; detector: $\lambda = 218$ nm) to afford 91 mg of a white amorphous solid. The isolated compound was identified as UA following NMR spectral analysis and comparison with the literature data (Olea and Roque, 1990), (UA: 99.8% purity, as determined by HPLC). ^1H NMR (300 MHz, DMSO- d_6) δ_{H} : 4.89 (br s, H-12), 4.06 (br s, H-3), 1.87 (d, $J = 3.4$ Hz, H-18), 1.68 (s, H-23), 1.61 (s, H-27), 1.52 (s, H-25), 1.31 (s, H-26), 0.80 (br s, H-30), 0.63 (br s, H-29), 0.58 (s, H-24). ^{13}C NMR (75 MHz, DMSO- d_6) δ_{C} : 178.7 (C-28), 138.6 (C-13), 125.0 (C-12), 77.3 (C-3), 55.4 (C-5), 53.0 (C-18), 47.3 (C-9), 47.5 (C-17), 42.1 (C-14), 40.5 (C-8), 40.2 (C-19), 40.0 (C-10), 39.7 (C-22), 39.6 (C-7), 39.5 (C-21), 39.4 (C-23), 38.8 (C-15), 36.8 (C-1), 30.7 (C-4), 33.2 (C-20), 28.7 (C-2), 28.0 (C-29), 27.4 (C-16), 24.3 (C-27), 23.7 (C-11), 21.6 (C-30), 17.5 (C-6), 17.3 (C-26), 16.5 (C-25), 15.7 (C-24). The structure of UA is showed in Fig. 1.

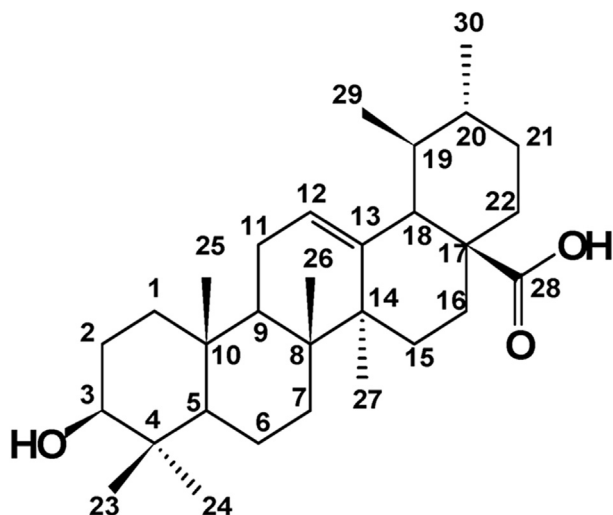


Fig. 1. The chemical structure of ursolic acid (UA) isolated from the leaves of *B. uncinella*.

2.4. Parasites and antigen production

L. infantum (syn. *L. chagasi*) parasite (MHOM/BR/72/46) was kindly provided by Prof. Dr. Fernando T. Silveira from the cryobank of the “Leishmaniasis Laboratory Prof. Dr. Ralph Laison”, Department of Parasitology, Evandro Chagas Institute, Ministry of Health, Belém, Pará State, Brazil. Species confirmation was accomplished using monoclonal antibodies and isoenzyme electrophoretic profiles at the Leishmaniasis Laboratory of the Evandro Chagas Institute. *L. infantum* was grown in M199 medium (Sigma-Aldrich Co.) supplemented with 10% fetal calf serum (FCS), 50,000 IU/mL penicillin, 50 µg/mL streptomycin, and 2% human urine at 25 °C. Stationary phase promastigotes were used throughout the entire study. To produce total antigen, *L. infantum* promastigotes were recovered by centrifugation at 1200 g for 10 min at 4 °C, washed three times with phosphate buffered saline (PBS), before resuspension in PBS with 1% protease inhibitors (Sigma-Aldrich Co.). Parasites were then lysed by successive freeze-thaw cycles.

2.5. Animals and ethical considerations

Golden hamsters (*Mesocricetus auratus*), 8 weeks old, were obtained from the Medical School of the University of São Paulo, Brazil. This study was carried out in strict accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (<http://www.cobea.org.br>). The protocol was approved by the Ethics Committee of Animal Experiments of the Institutional Committee of Animal Care and Use at the Medical School of São Paulo University (CEP 259/13), and also by the Federal University of São Paulo (955422). For all experimental procedures, the animals were anaesthetized with tiopental (1 mg/200 µL).

2.6. Infection and experimental treatment

Twenty female golden hamsters were intraperitoneally infected with 2×10^7 promastigote forms of *L. infantum*, and 5 gold hamsters received only phosphate buffered saline (PBS) plus 1% DMSO under the same route (PBS group). Sixty days after infection, *L. infantum*-infected hamsters were divided into four groups: group 1 was injected with 1.0 mg of UA per kg of body weight (mg/kg); group 2 was injected with 2.0 mg/kg of UA; group 3 was injected with 5.0 mg/kg of amphotericin B (Corral et al., 2014), group 4 was

injected with PBS plus 1% DMSO solution (infected control group). Group 5 was constituted by non-infected animals that received only the vehicle solution (PBS control). UA, amphotericin B, and the vehicle solution were injected intraperitoneally daily, once a day, over the course of 15 consecutive days. Fifteen days after the last injection, the animals were sacrificed in a CO₂ chamber; their blood, spleen, kidney, lungs, heart, and liver were collected to analyze different parameters. Sera were collected and immediately stored at –80 °C and used for the evaluation of biochemical parameters. Before treatment, a few hamsters were euthanized in order to verify the presence of parasites in spleen and liver. Animals showed high parasitism and the organs presented macro- and microscopic alterations, as previously reported by our group (Duarte et al., 1988; Laurenti et al., 1990, 1996; Corbett et al., 1992; Corbett and Laurenti, 1998). UA was solubilized in DMSO and further PBS (never exceeding 1% DMSO); and amphotericin B was solubilized in sterile water for injection plus 1% DMSO.

2.7. Determination of parasite load

The parasite load was estimated using the quantitative limiting-dilution assay, as described by Campos et al. (2015), with minor modifications. Briefly, fragments of spleen and liver from the different groups were aseptically excised, weighted, and homogenized in M199 medium (Sigma-Aldrich Co.). The suspensions of organs were subjected to 12 serial dilutions with four replicate wells. The number of viable parasites was determined based on the highest dilution rate where promastigote forms could be observed after 15 days of cultivation at 25 °C.

In addition to the limiting-dilution assay, parasitism in the spleen and liver was evaluated by immunohistochemistry according to Laurenti and collaborators (Laurenti et al., 2014). Briefly, slides with histological sections were deparaffinized and hydrated. Antigenic recovery was developed in citric acid solution (10 mM, pH 6.0) for 3 min in a pressure cooker. Next, the slides were washed six times with 3% hydrogen peroxide (H₂O₂) to block endogenous peroxidase and to avoid nonspecific ionic binding; the sections were also incubated in a solution of powdered skim milk (10%), diluted in PBS, pH 7.4, at room temperature for 30 min. The immunolabeling reaction was performed with polyclonal mouse anti-*Leishmania* antibody at 1:1000 (produced in the Laboratory of Pathology of Infectious Diseases), diluted in PBS and 1% bovine serum albumin (BSA). This polyclonal antibody was raised against *Leishmania infantum* crude antigen in BALB/c mice and was standardized to be used in immunohistochemistry. In order to detect the enzyme nitric oxide synthase 2 the polyclonal antibody anti-NOS2 (Santa Cruz, USA) was used at 1:200 in PBS plus 1% Bovine serum albumin, and add in histological section of spleen and liver for 60min, 37 °C. To develop the reaction, the LSAB kit (Dako Denmark A/S, Glostrup, Denmark) and diaminobenzidine (Sigma-Aldrich Co.) in PBS containing 3% hydrogen peroxide were used. Histological sections were counterstained in Harris's hematoxylin, dehydrated and mounted in resin with cover slides.

The main histopathological changes in the red and white pulps of the spleen, as well as in the portal regions and parenchyma of the liver were visualized in histological sections stained with hematoxylin and eosin (HE).

2.8. Analysis of cell proliferation

Spleens were individually homogenized in Roswell Park Memorial Institute (RPMI) 1640 medium and erythrocytes were lysed using lysis buffer (150 mM NH₄Cl, 7 mM K₂HCO₃ and 0.01 mM EDTA). Cells were adjusted to 5×10^5 cells/well and cultured in sterile 96-well plates under specific stimulation with the total

antigen (T-AG) of *L. infantum* promastigotes (10 µg/well) or under unspecific stimulation with concanavalin A (1 µg/well) as a positive control of proliferation. In addition, cells from all groups were cultured only with RPMI 1640 medium as negative controls. Plates were cultured in a humidified incubator at 37 °C under 5% CO₂. Following 48 h of incubation, the plates were washed with PBS three times at 1000 rpm for 10 min, at 4 °C. Then, PrestoBlue reagent (Life Technologies, Carlsbad, CA, USA) was added to measure cellular proliferation (Sá-Nunes et al., 2009; Hamalainen-Laanaya and Orloff, 2012). After 2 h, fluorescence was read with the excitation and emission wavelengths at 570 nm and 620 nm, respectively. The fluorescent levels of negative controls were subtracted from all samples. The PBS control group (uninfected, untreated) did not proliferate under stimulation with the T-AG of *L. (L.) infantum*.

2.9. Cytokine determination by real-time polymerase chain reaction (PCR)

RNA from spleen fragments (~10 mg) was extracted using the commercial RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized with the SuperScript[®] VILO™ cDNA Synthesis Kit (Life Technologies). Amplification conditions consisted of an initial denaturation phase at 95 °C for 10 min, followed by 40 amplification cycles consisting of 95 °C for 15 s, 61 °C for 90 s, and 72 °C for 30 s, using a thermocycler (Eppendorf, Hamburg, Germany). Prior to quantification, the efficiency of each reaction was verified using cDNA from spleens of healthy animal; it was always above 95%. Expression levels of genes of interest were normalized to β-actin (endogenous control). qPCR reaction was carried out using the GoTaq[®] qPCR Master Mix kit (Promega Corporation, Madison, WI, USA) and 25 nM of specific primers. The primer sequences were as follows (5' to 3'): IFN-γ forward: GACAACCAGGCCATCC and reverse: CAAAA-CAGCACCAGCT; IL-10 forward: TGGACAACATACTACTACTG and reverse: GATGTCAAATTCATTCATGGC; IL-4 forward: CCACGGA-GAAAGACCTCATCTG and reverse: GGGTCACCTCATGTTGGAATAA; β-actin forward: TCCTGTGGCATCCACGAACTACA and reverse: ACAGCACTGTGTTGGCATAGAGGT. Quantification results are expressed in fold changes of 2^{-ΔCt} over the infected control group.

Prior to performing the qPCR, standard PCR reactions were performed to assess the specificity of the primers; one single amplification product of predicted size, according to Lafuse et al. (2013), was always obtained for such reactions.

2.10. Evaluation of the toxicity of UA

Healthy golden hamsters were divided into 4 groups containing 5 animals/group. Group 1 was treated intraperitoneally with 1.0 mg/kg of UA; group 2 was treated with 2.0 mg/kg of UA; group 3 was treated with 5.0 mg/kg of amphotericin B; and group 4 was injected with the vehicle solution (PBS). Animals were subjected to the same treatment described in 2.6. Five days after the last injection, animals were anaesthetized with thiopental and sacrificed by cardiac puncture. Sera were collected and immediately stored at -80 °C. Fragments of spleen, liver, kidney, lung, and heart were collected, processed histologically, and stained with HE. The biochemical parameters associated with hepatic and renal functions were evaluated through the quantification of seric alanine transaminase (ALT), aspartate aminotransferase (AST), urea (Sigma-Aldrich Co.) and creatinine (Labtest, Brazil).

3. Statistical analysis

The experiments were repeated four times and all parameters were assayed in triplicate; each experiment contained 25 golden

hamsters. Experiments pertaining to toxicity contained 20 golden hamsters. The results were expressed by the arithmetic mean ± standard deviation. Statistical analyses were performed using GraphPad Prism 5.0, and the nonparametric Mann–Whitney *U* test was used to evaluate the differences between treated and infected control groups. Differences were considered statistically significant at a 5% significance level (*P* < 0.05).

4. Results

4.1. Determination of splenic and liver parasitism

The parasite load in the spleen of infected hamsters treated with 1.0 or 2.0 mg/kg of UA, isolated from *B. uncinella*, was established at 2.3×10^9 (reduction of 92.7%) and 2.1×10^9 (reduction of 93.3%) parasite/g of spleen, respectively, when compared to the infected control group (31.5×10^9 parasite/g of spleen), as detailed in Fig. 2A (*P* < 0.05). In addition, amastigote reduction during UA treatment was evidenced by immunohistochemistry (Fig. 2D and E). Animals treated with 5.0 mg/kg of amphotericin B also showed reduction in splenic parasitism (8.5×10^9 parasite/g of spleen; reduction of 73%) when compared to the infected control group (*P* < 0.05) (Fig. 2A–F).

Similarly, a significant reduction in parasitism was verified in the liver of animals treated with 1.0 mg/kg (2.2×10^8 parasite/g of liver; reduction of 96.9%) or 2.0 mg/kg (2.4×10^8 parasite/g of liver; reduction of 96.7%) of UA (*P* < 0.05) when compared with the infected control group, which presented liver parasitism of 72.9×10^8 parasite/g of liver (Fig. 2B, H and I). Animals treated with 5.0 mg/kg of amphotericin B presented a decrease in liver parasitism (2.5×10^8 parasite/g of liver; reduction of 96.5%) as well. Fig. 2J shows an immunolabeled histological section of liver from amphotericin B-treated animals to evidence amastigote forms.

4.2. Histopathological changes

In the spleen of hamsters from the infected control group, a replacement of lymphoid follicles by infected macrophages was observed, suggesting immunosuppression caused by *L. (L.) infantum* (Fig. 3A). Furthermore, in the red pulp, proliferation of often-parasitized macrophages was observed and the presence of infected giant cells was also noted (Fig. 3F), indicating high disease severity evidenced by the histological sections stained by immunohistochemistry (Fig. 2C). Infected animals treated with 1.0 mg/kg and 2.0 mg/kg of UA showed preservation of the white pulp, suggesting a less severe condition and a better immune response (Fig. 3B–C). For these groups, nodules of macrophages were also detected in the red pulp, but they were fewer and exhibited less parasitism when compared to the infected control group (Fig. 3G–H). Animals treated with amphotericin B showed preservation of the white and red pulps (Fig. 3D) with few infected macrophages in the red pulp (Fig. 3I). Splenic from healthy animals exhibited white and red pulps with normal characteristics as shown in Fig. 3E–J.

In the liver of all animal groups that were infected with *L. (L.) infantum*, independently of treatment, parasites were detected in portal areas that presented inflammatory foci characterized by the occurrence of lymphocytes and macrophages (white arrow in Fig. 3K–N). In addition, macrophage granulomas were also visualized in the liver parenchyma (white arrow in Fig. 3P–S). Treatment with UA and amphotericin B resulted, however, in lesser parasitism and fewer areas of portal inflammation.

4.3. Proliferation of splenic cells, expression of cytokines and nitric oxide synthase 2 (NOS2) immunostaining

Splenic cells stimulated with T-AG from animals treated with

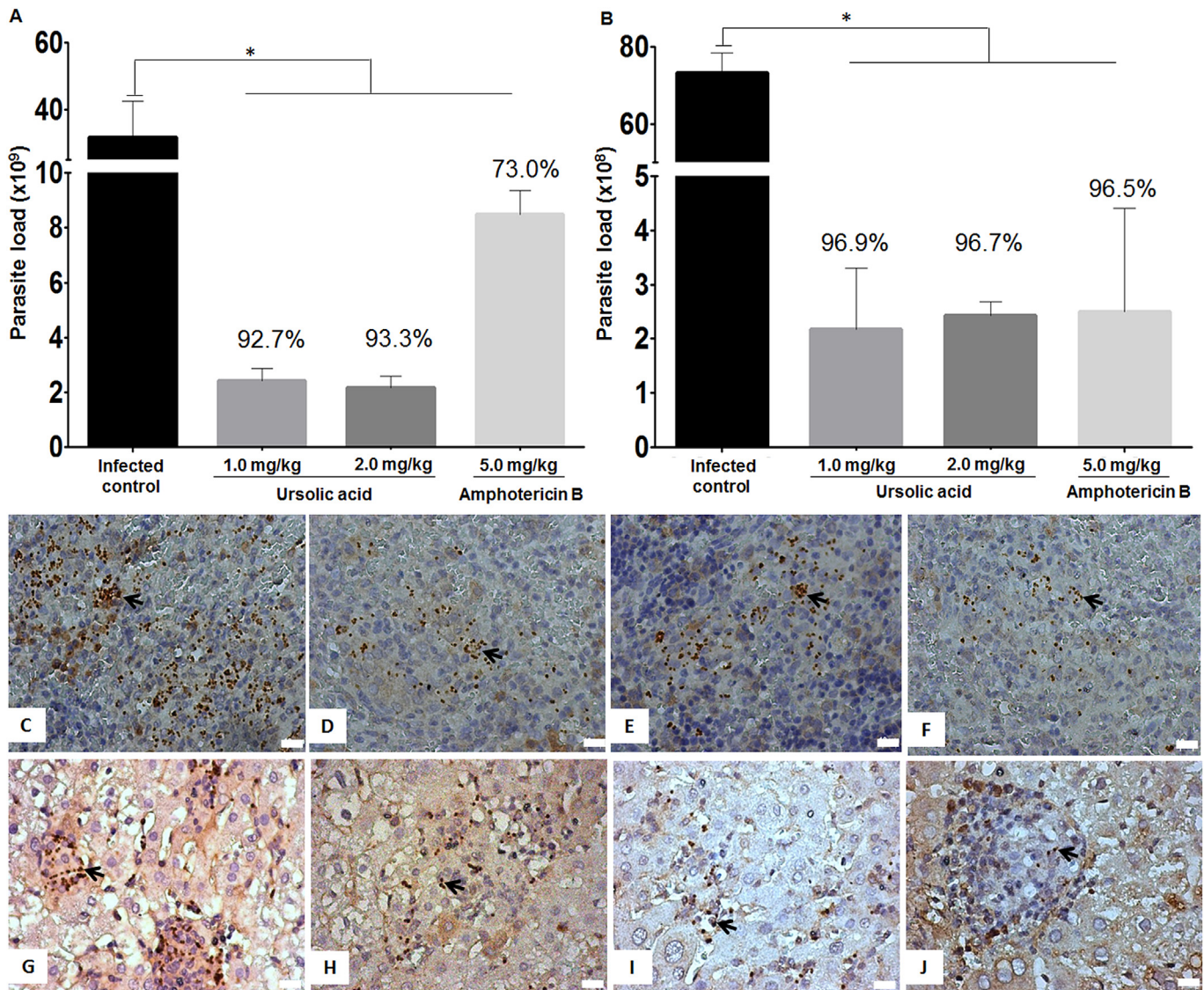


Fig. 2. Parasite load in the spleen (A) and liver (B) of animals treated with UA or amphotericin B. Photomicrographs of histological sections stained by immunohistochemistry show amastigote forms (stained in dark brown) in the spleen (C, D, E and F) and liver (G, H, I and J) of the infected control group (C and G), as well as in the UA- (D, E, H, and I) or amphotericin B- (F and J) treated hamsters (400 \times magnification; scale bar: 10 μ m). * $P < 0.05$ indicates statistical significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1.0 mg/kg or 2.0 mg/kg of UA proliferated significantly more in comparison with the cells from the infected controls ($P < 0.05$). Cells from animals treated with 5.0 mg/kg of amphotericin B and stimulated with T-Ag did not proliferate (Fig. 4).

The spleen of infected golden hamsters treated with 1.0 mg/kg or 2.0 mg/kg of UA expressed higher levels of IFN- γ mRNA when compared to the infected control group ($P < 0.05$) (Fig. 5A). Infected animals treated with either concentration of UA or amphotericin B expressed significantly less IL-4 mRNA in the spleen than the infected control group ($P < 0.05$), as illustrated in Fig. 5B. Conversely, IL-10 gene expression was shown to be elevated in the spleens of animals treated with either of UA or amphotericin B (Fig. 5C).

Infected hamsters treated with 1.0 mg/kg or 2.0 mg/kg of UA presented NOS2 positive areas diffusely detected throughout spleen histological sections, as illustrated in Fig. 6B–C. In addition, NOS2 positive areas could also be detected in the animals treated with amphotericin B, however it was in less amount and focally (Fig. 6D) when compared to histological section of animals treated

with UA. The infected control group presented only basal positivity for NOS2 while the non-infected PBS control did not present positive areas for NOS2 enzyme (Fig. 6A–E, respectively).

In the infected control group, few positive NOS2 cells were detected in inflammatory foci of periportal areas from the liver (Fig. 6F), while in infected animals treated with 1.0 and 2.0 mg/kg of UA NOS2 positive cells were detected in high number in the inflammatory areas of liver parenchyma (Fig. 6G–H, respectively). Infected animals treated with amphotericin B (Fig. 6I) presented few NOS2 positive cells, and the non-infected PBS control group did not show NOS2 positive cells (Fig. 6J).

4.4. Toxicity evaluation

Healthy hamsters treated with 1.0 mg/kg and 2.0 mg/kg of UA or 5.0 mg/kg of amphotericin B did not show significant changes in the spleen, liver, lung, or heart (data not shown). Although UA did not alter the histology of the kidney, treatment with amphotericin B changes kidney medullary region. In this case, it was observed that

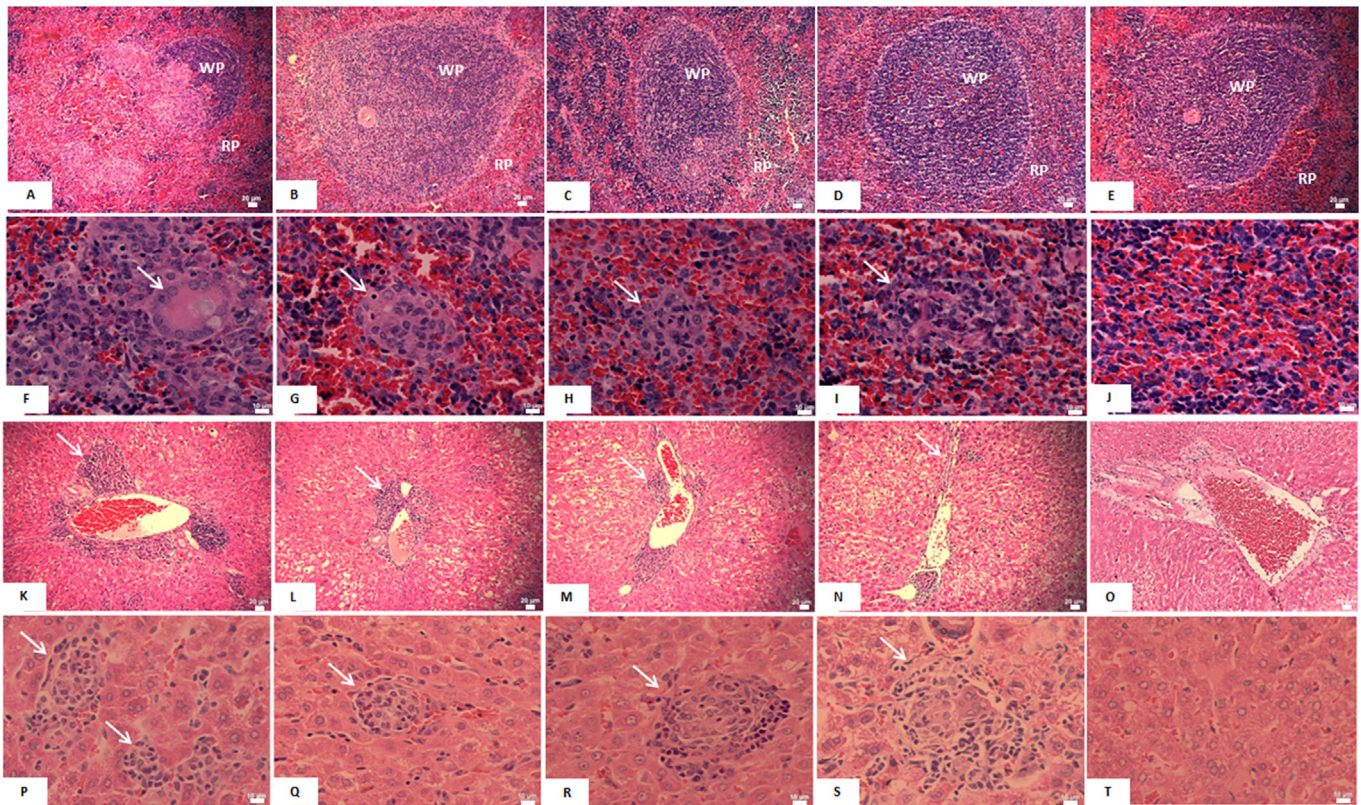


Fig. 3. Photomicrographs of histological sections of the spleen and liver from golden hamsters infected with *L. (L.) infantum* and treated with 1.0 mg/kg or 2.0 mg/kg of ursolic acid (UA) and 5.0 mg/kg of amphotericin B. Macrophagic invasion in the white pulp of the spleen (WP) was observed in the infected control group (A), and to a lesser degree in the animals treated with 1.0 mg/kg (B) or 2.0 mg/kg (C) of UA and amphotericin B (D). In the non-infected control group (E), no alterations were found. In the red pulp of the spleen (RP), macrophagic nodules (arrow) were found in all infected groups (F–I), whereas non-infected control animals did not present such alterations (J). Inflammation foci (arrow) were verified in liver portal areas of the infected control group (K), as well as of the UA- (L and M) or amphotericin B- (N) treated hamsters. Non-infected animals (O) showed normal histological characteristics. In the liver parenchyma, macrophagic granulomas (arrow) were observed in the infected control group (P) and in animals treated with UA (Q and R) or amphotericin B (S), while the non-infected control group did not exhibit such alterations (T). Spleen histological sections (A–J), 400× magnification; scale bars: 20 μm (A–E) and 10 μm (F–J). Liver histological sections (K–T), 100× magnification; scale bars: 20 μm (K–O) and 10 μm (P–T).

the cuboidal epithelium of the distal tubule was necrotic (Fig. 7K). Histological changes were not detected in the cortical region of kidney of all animals analyzed (Fig. 7E and H).

To confirm renal alterations induced by amphotericin B in golden hamsters, biochemical evaluations were carried out. In this regard, it was verified that animals treated with 1.0 mg/kg or 2.0 mg/kg of UA did not present seric alterations in creatinine or urea (Fig. 7A–B, respectively). In contrast, animals treated with 5.0 mg/kg of amphotericin B presented high levels of seric creatinine ($P < 0.05$) in comparison to the untreated control group (Fig. 7A). The levels of seric urea in all treated animals were similar to that of the control (Fig. 7B). Changes in the levels of either AST or ALT were not verified between treated and control animals (Fig. 7C–D, respectively).

5. Discussion

Currently, the main therapeutic arsenal available for treating leishmaniasis has been considered outdated. Moreover, patients face diverse side effects, and the drugs employed in present therapy can lead to the emergence of drug-resistant parasites. Therefore, it is urgent to characterize the leishmanicidal action of new compounds *in vivo* to increase the collection of effective prototype drugs. In this regard, UA seems to be an interesting target to develop new formulations to be used in human health, because different reports demonstrated its multivalent activities against

pathological conditions. UA was demonstrated to be active against different tumor cell lines (Chuang et al., 2016; Aguiriano-Moser et al., 2015; Kim and Moon, 2015), its anti-tumoral effect being associated to apoptosis induction by intrinsic and extrinsic pathways of death (Li et al., 2014; Meng et al., 2015; Zhang et al., 2016; Villar et al., 2016).

In addition, *in vivo* experiments demonstrated the efficacy of UA, alone or in association with standard drugs, in colorectal and pancreatic tumors therapy (Shan et al., 2016; Prasad et al., 2016). Other studies also demonstrated the protective potential of UA in models of liver and endothelial cells injuries (Li et al., 2016), suggesting that the administration of high doses of UA trigger some antioxidant effects in experimental animals. Furthermore, UA was confirmed to possess antiprotozoal activities. Similarly to its effect on tumor cells, this triterpene induced programmed cell death in *L. (L.) amazonensis*, and presented therapeutic efficacy in the model of American Tegumentar Leishmaniasis (Yamamoto et al., 2015).

Despite the collection of related evidences, studies dealing with the antileishmanial effect of UA on experimental visceral leishmaniasis are rare. Thus, the present work aimed to characterize the therapeutic action of UA isolated from *B. uncinella* in experimental VL. In this regard, a reduction in splenic and liver parasitism was observed following treatment with UA, suggesting that the course of infection in treated animals was controlled when compared to the infected control group. Moreover, in spleen, UA showed to be more effective than amphotericin B; in contrast, livers of animals

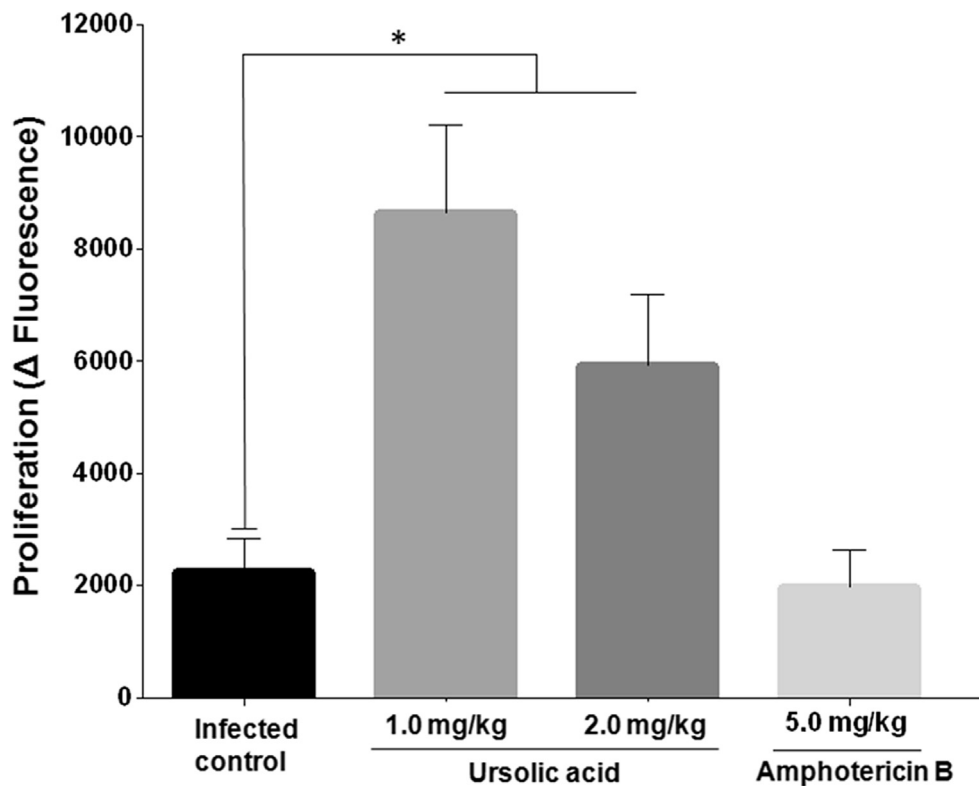


Fig. 4. Proliferation of mononuclear cells in the spleen stimulated with 10 μ g of total parasite antigen during 48 h * $P < 0.05$ indicates statistical significance when comparing the infected control group versus the treated groups.

treated with UA or amphotericin B showed similar parasitism, which was in both cases significantly less in comparison with the infected control group. Of note, in this study, amphotericin B did not totally cure infected hamster, possibly by the low number of injections, since it was not possible to continue the experimental treatment, because the infected control group was suffering from chronic and systemic infection that precluded the end of the experiment. Moreover, the therapeutic effect of UA impacted the histological architecture of the spleen and liver in treated animals. While the infected control group showed macrophagic invasion and disruption of the white and red pulps, suggesting immune suppression (Kaye et al., 2004; Rodrigues et al., 2016), animals treated with UA or amphotericin B demonstrated that the areas of lymphoid follicles, as well as the T-cell zones, remained intact, suggesting a better immune response pattern as confirmed by the reduction of viable parasites and by the decrease of parasitized areas.

The liver has been considered an acute site of infection by viscerotropic species of *Leishmania*, as it features less damage than the spleen (Stanley and Engwerda, 2007). Still, the infected control group presented chronification of the inflammatory process in the portal regions, as well as granulomas in the liver parenchyma, while hamsters in the UA- and amphotericin B-treated groups exhibited a decrease in the inflammatory process with the presence of well-organized granulomas. According to several studies, the resolution of acute infection is associated with the development of inflammatory granulomas around Kupffer cells, although at the same time, the presence of granulomas can be considered a marker for parasite persistence (Engwerda et al., 2004; Stanley and Engwerda, 2007; Rodrigues et al., 2016). Giunchetti et al. (2008) showed that the liver of symptomatic dogs infected with *L. infantum* displayed portal inflammation and intralobular

granulomas, among other histological changes, suggesting that these features could be associated with disease progression. Considering these findings, the UA triterpene showed a therapeutic effect on the liver of golden hamsters infected with *L. infantum*. Even though the increase in UA concentration did not improve its efficacy in the experimental model of visceral leishmaniasis, a fact that may have a direct association with UA bioavailability. Liao et al. (2005) as well as Chen et al. (2011) showed that after administration in animals UA could be rapidly detected in high levels in plasma, spleen and liver [55,56] – the main organs affected by *L. infantum*. However, it was quickly excreted, impacting the distribution or accumulation of this triterpene in the animal body; and causing a constant availability of UA independently of the administered dose. Therefore, similar number of parasites detected in the treatment with 1.0 or 2.0 mg/kg of UA may be related with the bioavailability of this compound.

Although the leishmanicidal action of UA (*in vitro*) was already evaluated (Passero et al., 2011; Yamamoto et al., 2015), its action in experimental models of leishmaniasis was poorly demonstrated until now. In this regard, Yamamoto and collaborators (Yamamoto et al., 2014) showed that UA treatment of *L. (L.) amazonensis*-infected BALB/c mice decreased skin parasitism, which was associated with the preservation of the epidermis and dermis as a possible consequence of IFN- γ production. Other studies showed that the astrakururone triterpene from the mushroom *Astraeus hygrometricus* was able to restrain parasitism in a murine model of VL, and this therapeutic action was associated with the upregulation of IFN- γ and IL-17 cytokines (Chen et al., 2011). Other terpenes were also shown to be active in VL, such as the clerodane diterpene 16 α -hidroxicleroda clerodane-3,13Z-dien-15,16-olide from the leaves of *Polyalthia longifolia*, since hamsters treated with the diterpene exhibited splenic, liver, and bone marrow parasitism that

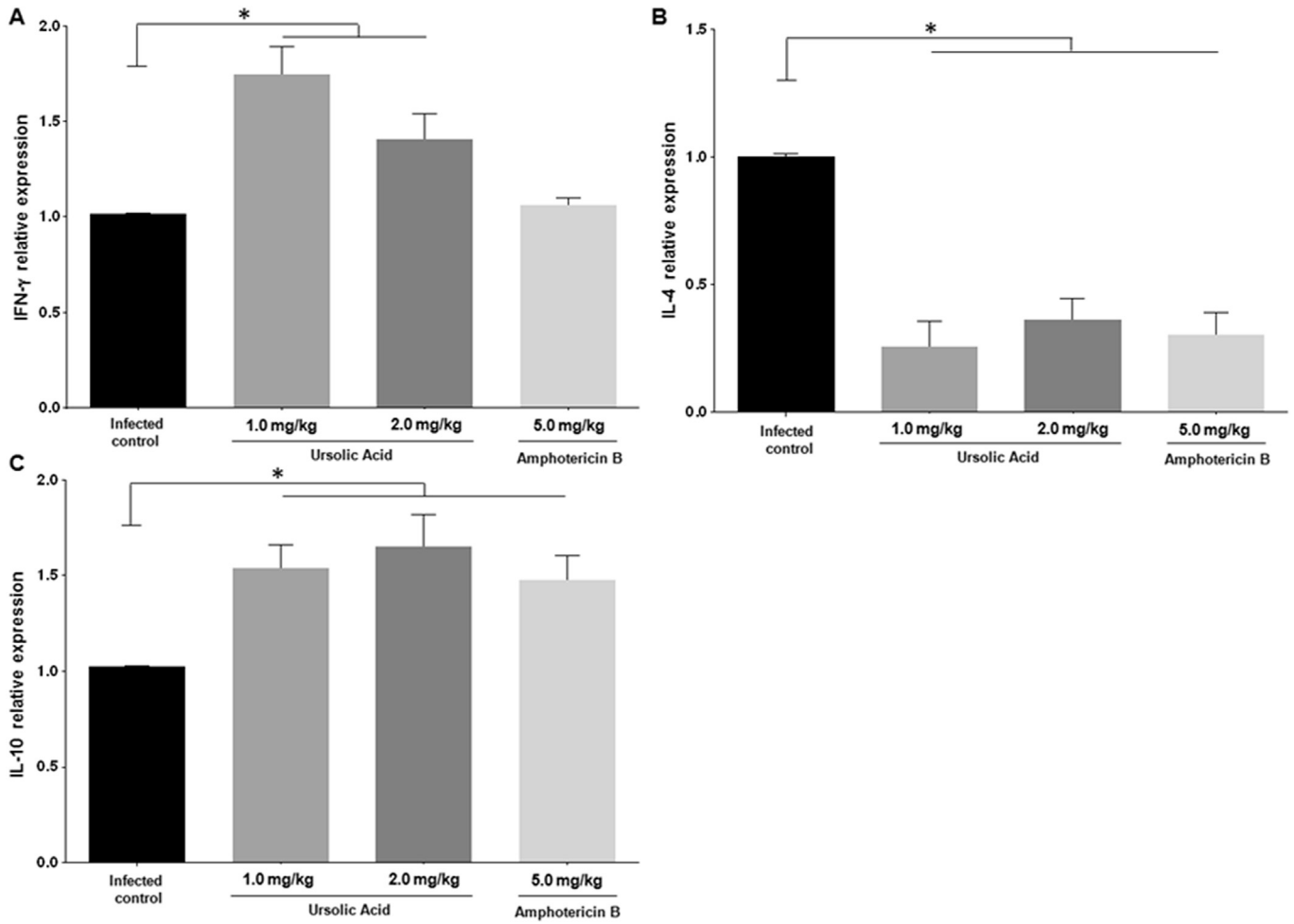


Fig. 5. Relative RNA expression of IFN- γ , IL-4, and IL-10. Cytokine gene expression was evaluated in the spleen of the treated and infected control groups. * $P < 0.05$ indicates significant differences when comparing the treated versus infected control group (control).

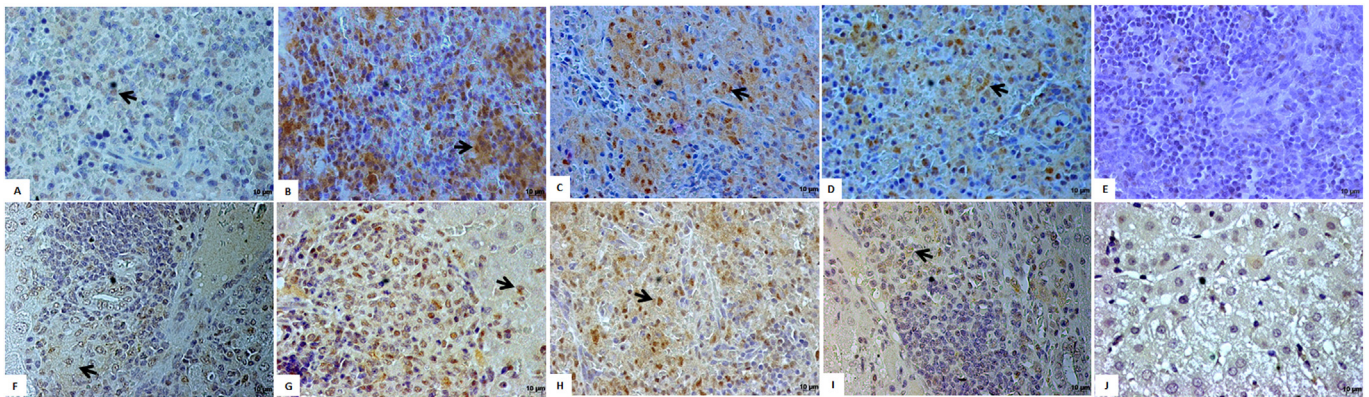


Fig. 6. Histological sections of spleen and liver of groups of hamsters were immunolabeled by immunohistochemistry in order to detect NOS2 enzyme, the precursor of nitric oxide metabolite. Spleen sections of Infected Control, Animals treated with 1.0 mg/kg and 2.0 mg/kg of UA, animals treated with 5.0 mg/kg of amphotericin B and non-infected PBS control are represented in A, B, C, D and E, respectively. Similarly, histological sections of liver from Infected Control, Animals treated with 1.0 mg/kg and 2.0 mg/kg of UA, animals treated with 5.0 mg/kg of amphotericin B and non-infected PBS control are represented in F, G, H, I and J, respectively.

was similar to that of animals treated with miltefosine (Misra et al., 2010). Additionally, an oleanane triterpenoid derivative (maesabalide III) isolated from *Maesa balansae* showed *in vivo* activity against *L. donovani* following administration of a single subcutaneous dose on either day 1 (prophylactic treatment) or day 28

(curative treatment) after infection (Maes et al., 2004). These data suggest that in addition to their therapeutic effects, some terpenes can also exert modulatory immune effects on animals (Yamamoto et al., 2014; Mallick et al., 2016).

Indeed, in the present study, it was demonstrated that golden

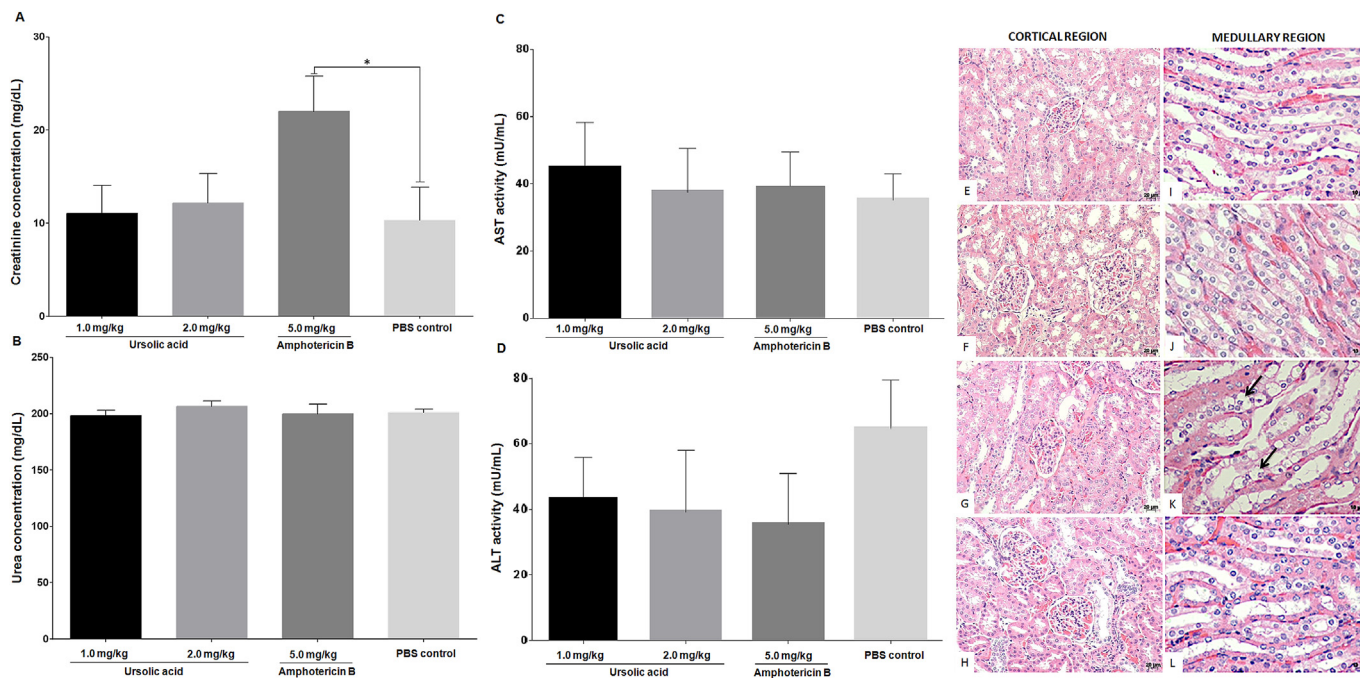


Fig. 7. Toxicity of UA and amphotericin B. Levels of serum creatinine (A), urea (B), AST (C) and ALT (D) were evaluated, and histological alterations were also recorded in the cortical and medullary regions of the kidney from healthy golden hamsters treated with 1.0 mg/kg (E and I) or 2.0 mg/kg of UA (F and J), or with 5.0 mg/kg of amphotericin B (G and K). The regions of the control group are shown in H and L. The arrows indicate necrotic areas (K). Cortical region: 100 \times magnification; scale bar: 20 μ m. Medullary region; 400 \times magnification; scale bar: 10 μ m.

hamsters infected with *L. infantum* and treated with UA exhibited preservation of important areas of the spleen, and that mononuclear cells stimulated with parasite T-Ag proliferated significantly more in comparison with the mononuclear cells of the infected control group and amphotericin B-treated animals. In VL, it is widely recognized that mononuclear cells have low, or even absent, proliferative potential to parasite antigens, indicating the immunosuppressive status of the cellular immune response (Fazzani et al., 2011). Therefore, the ability of mononuclear cells to proliferate under specific antigens suggests that the animals in the present study developed a beneficial immune response following treatment. Although animals treated with amphotericin B presented low parasitism and preservation of the white and red pulps, the mononuclear cells of these animals did not proliferate under specific antigens, a fact that point out to some specific immune suppression. However, this effect should not be understood as immunosuppression, since mononuclear cells of this group proliferated under stimulation with concanavalin A (data not shown). Previous studies also indicated that spleen cells from golden hamsters treated with free or encapsulated amphotericin B did not proliferate under specific stimuli (Dea-Ayuela et al., 2004, 2007), but such findings should not be considered as immunosuppression, since concanavalin A-stimulated cells proliferated.

The dichotomy of the specific cellular immune response is one of the main factors that determine disease development or control, particularly because IFN- γ is an important marker of resistance (Lykens et al., 2010). On the contrary, IL-4 and IL-10 are recognized markers of susceptibility to infection (Osorio et al., 2014). In the present study, this dichotomy was evaluated by the relative expression levels of IFN- γ , IL-4, and IL-10 genes in the spleen. Infected hamsters treated with UA showed a polarized Th1 immune response that was related to protection (Kima and Soong, 2013). However, the increase in IL-10 expression verified in the UA- and amphotericin B-treated animals may regulate the inflammatory

effect of IFN- γ . Studies have shown that although IL-10 may prevent tissue injuries caused by an exacerbated inflammatory response (Banchereau et al., 2012), this cytokine is also responsible for parasite maintenance at the site of infection, especially since IL-10 limits the generation of IFN- γ , as well as the microbicidal activity of macrophages (Belkaid et al., 2001), thus allowing parasites to survive inside host cells. Even in the group treated with amphotericin B, a drug used in leishmaniasis therapeutics, IL-10 exhibited increased gene expression and low amounts of IFN- γ mRNA. Moreover, parasites were visualized in histological sections and in the limiting-dilution assay, suggesting that the presence of IL-10 can be a crucial factor for maintaining tissue parasites, even in conditions that are adverse for them.

On the other hand, all treated groups presented decreased IL-4 expression, and although its role is not entirely clear in leishmaniasis, the IL-4 cytokine may be associated with pathogenic effects in experimental VL, as it was able to activate the STAT-6 transcription factor. This led to a strong expression of the gene encoding for L-arginase, an important enzyme that inhibits NO production (Osorio et al., 2014), which is essential for parasite elimination. Therefore, decreased expression of this cytokine following experimental treatments may be associated with a beneficial response against experimental VL. In fact, the infected control group expressed high amounts of IL-4 gene and produced only marginal levels of NOS2 in spleen or liver suggesting that IL-4 can be a factor associated with the pathogenesis of VL. Yet the spleen of UA-treated animals presented low IL-4 and high IFN- γ expression, inducing the Th1 immune response and macrophage activation by NOS2 mechanism, as verified in the spleen and liver, and culminating with parasite reduction. In histological section of the spleen from animals treated with amphotericin B, NOS2 positive areas were also detected, but the treatment with UA seems to induce a stronger reaction associated with the NOS2, the final enzyme responsible to trigger nitric oxide production. It is still important to note that the majority of

immunological studies were carried out with qPCR because immunological reagents for hamster are rare.

Considering the therapeutic effect of UA in experimental VL, toxicity studies in golden hamsters were conducted. Histopathological analysis verified that the treated animals did not develop tissue injuries in the liver, spleen, heart, lung or kidney. Conversely, animals treated with amphotericin B displayed injuries in the medullary area of the kidney. In this case, tubular epithelial cells suffered an extensive process of necrosis. A study conducted by [Berdichevski et al. \(2006\)](#) demonstrated that patients treated with amphotericin B showed nephrotoxicity, which was characterized by a reduced glomerular filtration rate and tubular dysfunction. Furthermore, creatinine levels were higher and acute renal failure occurred in 31% of patients. In our study, the process of tubular necrosis found in animals treated with amphotericin B was associated with increased levels of serum creatinine, as already described by a previous study ([Deray, 2002](#)). Taken together, these findings demonstrated that UA is not a toxic drug for golden hamsters, while amphotericin B, although recognized as a leishmanicidal drug, can induce critical injuries. Moreover, a recent study demonstrated that UA had a nephroprotective effect by attenuating renal damage induced by toxic compounds ([Pai et al., 2012](#)), suggesting that this natural product has the potential to be considered as a prototype drug. Remarkably, UA triterpene was active in lower doses compared to the standard drug amphotericin B, and considering the molecular mass of both compounds, it is possible to assume that animals were treated with pharmaceutical dosage of UA, allowing its formulation as a medicament.

Different natural products had already been tested in viscerotropic *Leishmania* species, and the results had demonstrated that in some cases these natural compounds could be classified as promising antileishmanial drugs. Our present work evidenced that UA was an effective drug in the treatment of experimental VL without inducing toxicity; at the same time, it avoided immunosuppression by inducing a Th1 immune response leading to parasite elimination. Taken together, these findings support the conclusion that UA has an important leishmanicidal potential that is comparable to that of the standard drug, amphotericin B. UA can thus be considered an interesting candidate for further testing as a prototype drug for the treatment of VL.

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