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Effect of ethyl acetate extract from husk fiber water of *Cocos nucifera* in *Leishmania braziliensis* infected hamsters

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Abstract: The objective of this study was to evaluate the treatment with ethyl acetate extract (EAE) from husk fiber water of *Cocos nucifera* L., Arecaceae, in *L. braziliensis* (Lb) infected hamsters. Twelve male hamsters were randomly allocated in three groups (n=4): G1 received only EAE; G2 was infected with Lb only and G3 received EAE after Lb infection. The infection was carried 28 days prior to the treatment with EAE, which was administrated (0.2 mL, 300 mg.kg⁻¹) for 21 consecutive days. Infection was evaluated through skin lesions and infected footpad edema. Haematological evaluation was done on -28th, 0 and 21st days. Imprint footpad and lymph node weight were evaluated on 21st day. Lb infection significantly inhibited the peripheral leukocytes blood. However, neutrophils and lymphocytes values did not have significant alterations. G3 presented eosinophilia in relation to G2. The treatment with EAE did not reduce edema of infected footpad neither weight of drainage lymph node. Infected footpad imprints revealed amastigotes forms and cellular infiltration. Animals from G3 presented skin lesions on 7th day, shown a reduction of these lesions in day 14. Therefore, the treatment with EAE did not alter the etiological agent elimination in these conditions. However, EAE presents a healing activity in this experimental model.

Introduction

Leishmaniasis is a group of infectious diseases caused by different species of protozoan parasites of the genus *Leishmania*, which affect about two million people a year around the world. This infection has a broad clinical spectrum, ranging from subclinical disease to cutaneous and/or visceral infections, causing high morbidity and mortality. *Leishmania braziliensis* is one of the main agents of cutaneous leishmaniasis, which is usually unresponsive to treatment known (Mendonça-Filho et al., 2004). Many mammals, such as canines (Gramiccia & Gradoni, 2005) and felines (Simões-Mattos et al., 2005), may serve as reservoir and may develop the disease caused by *L. braziliensis*.

Leishmaniasis has been considered as an important zoonosis due to its ability to circulate among humans and animals that are present in domiciliary and peridomiciliar areas (Pereira & Alves, 2008). There is no treatment for animals and the seropositive dogs are being sacrificed as a way to control this disease. However, there is no study that has conclusively shown

that this kind of control is effective (Ashford et al., 1998; Ribeiro, 2007).

Cocos nucifera L., Arecaceae, is widely distributed in northeastern of Brazil. The products from its fruit have been used in popular medicine in this region as treatment for various diseases (Esquenazi et al., 2002). The liquid obtained from the husk fiber has antiproliferative (Kirszberg et al., 2003), antioxidant and analgesic activities (Alviano et al., 2004). Fractions of this liquid obtained with ethyl acetate solvent showed antibacterial, antiviral (Esquenazi et al., 2002) and leishmanicidal activities (Mendonça-Filho et al., 2004).

It has been reported by veterinarians in Ceará state the use of aqueous extract of the husk fiber of *C. nucifera* for the treatment of leishmaniasis infect dogs. However, there are no scientific data to validate this use.

Therefore, the aim of this work was to evaluate the ethyl acetate extract from *C. nucifera* treatment in *L. braziliensis* infected hamsters.

Materials and Methods

Extraction and fraction isolation

Husk fibers of *Cocos nucifera* L., Arecaceae, were dried in the sun, finely ground and the powder soaked for 3 h in 6 L of boiling distilled water. The extract obtained was filtered and lyophilized and partitioned with ethyl acetate. This fraction was named EAE (Mendonça-Filho et al., 2004). For use EAE was dissolved in saline with DMSO 5%.

Phytochemical study of EAE

EAE was yield to phytochemical study to evaluate the presence of cyanogenic glycosides, phenols, tannins, anthocyanidins, anthocyanins, flavonoids, catechins, flavanons, flavonols, flavanonols, xantons, steroids, triterpenoids, saponins, alkaloids compounds according to Matos (1997). It was used a HPLC column to evaluate the purification of EAE fraction. Spots were visualized by spraying vanillin-sulfuric acid reagent (Mendonça-Filho et al., 2004).

Animals

Twelve male hamsters (*Mesocricetus auratus*), 3-4 months old, weighing 120-140 g, obtained commercially, were used for the experiment. After acclimatization period, they were individually housed in clean polyethylene cages under standard experimental conditions of humidity (40-45%), temperature (23-25 °C), and 12 h light and dark cycle. The animals had free access to normal pellet diet and acidified water. All efforts were made to minimize the number of animals used and their suffering. The Ethics Committee for Use of Animals of State University of Ceará, approved (protocol number 07465010-6) the procedure in accordance to Brazilian laws and ethical principles.

Parasites

The *L. braziliensis* strain MHOM/BR/94/H-3227 was used after brief passages in culture medium. It was isolated from cutaneous ulcer of a leishmaniasis patient from Ceará state. The isolate was identified as *L. braziliensis* by monoclonal antibodies and PCR technique. Promastigotes were grown in Schneider medium (Sigma, St Louis, MO) at 25 °C supplemented with 10 % heat-inactivated fetal calf serum, 100 U.mL⁻¹ of penicillin and 100 µg.mL⁻¹ streptomycin (all from Gibco). Stationary phase promastigotes were used for infection. Before use, promastigotes were harvested from culture, washed in sterile saline and resuspended to get the right concentration. The parasites were given

by Federal University of Ceará Parasitology Laboratory. The animals were infected with 2x10⁷ of promastigotes forms.

Infection and experimental protocol

The animals were randomly allocated in three groups (n=4): G1 received only EAE (negative control); G2 was infected (positive control) and G3 was infected and treated with EAE. Promastigotes forms of *L. braziliensis* (2x10⁷) were inoculated in the right hind footpad of hamsters from G2 and G3, in the day -28. After 28 days of infection (Day 0 of the experiment), G1 and G3 received EAE 300 mg.kg⁻¹ (Silva et al., 2009) of *Cocos nucifera*, by gavage, during 21 consecutive days. The effect of EAE treatment was evaluated on 21st day by performing edema of footpad, skin lesions and leukocyte parameters. At the end of the experiment, drainage popliteal lymph nodes (PLN) were weighed and imprints of footpad were examined.

Measurement of footpad edema

Infection evolution was monitored weekly through measurement of infected footpad edema. Footpad thickness was measured with a dial gauge calliper (Mitutoyo, 0.01 mm sensitivity). Edema was represented by the difference in the thickness (mm) between the infected footpad and contralateral uninfected footpad. These measurements were initiated after inoculation of promastigotes (day-28) and followed weekly on days -21, -14, -7, 0 and 21.

Weight popliteal lymph node

At 21st day, all animals were sacrificed and the left and right popliteal lymph nodes (PLN) were collected and weighed (mg). The results were expressed as the difference between right and left PLN and compared among the groups.

Leukocyte counts

Peripheral blood samples of all animals were collected, in days -28, 0 and 21 of the experiment, by retro-orbital plexus, with a Pasteur pipette previously heparinised to prevent the coagulation. All samples were processed in a hematological analyzer to determine total and differential leukocytes counts.

Skin lesions evaluation

Skin lesions on infected animals were evaluated on days -7, 0, 7, 14 and 21. Ventral, dorsal and snout lesions were observed and had been described as present

or absent. The exam was realised in double blind.

Imprint of footpad

At the 21st day following the treatment with EAE, all animals were sacrificed and imprint of subcutaneous of the left and right footpad was obtained. These imprints were submitted to a routine of hematoxilin and eosin staining. Slides were analyzed at optical microscopy (400x) to detect the presence or absence of amastigotes forms of *L. braziliensis* and cellular infiltrate. The microscopic evaluation was realised in double blind.

Statistical analysis

Footpad edema (mm), weight of PLN (mg) and the leukocyte counts were expressed as the mean±SD. The data were submitted to analysis of variance (ANOVA) followed by the Tukey's test ($p<0.05$).

Results

The phytochemical study of EAE revealed the presence of condensed tannins, flavanonols, flavanols and flavonols. HPLC analysis indicated high concentrations of polyphenolic compounds such as catechins and epicatechins. Our results are according to others authors (Esquenazi et al., 2002; Mendonça-Filho et al., 2004).

The evolution of the footpad infection is demonstrated in Figure 1. There was a gradual increase in the thickness of infected footpad in animals of G2 and G3 when compared to G1, although there was no difference between them ($p<0.05$). Regarding the lymph node drainage (Figure 2), it was observed increase in weight of the infected PLN in G2 and G3 ($p<0.01$) when compared to G1. G2 differ significantly from G3 ($p<0.05$).

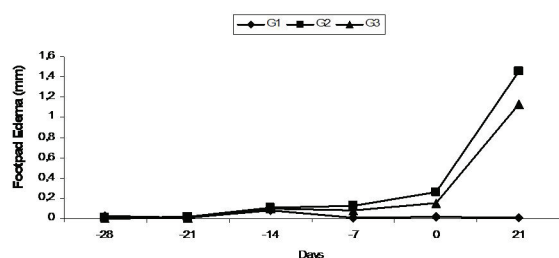


Figure 1. Effect of EAE of *Cocos nucifera* on the hind footpad edema of *L. braziliensis* infected hamsters. G1 negative control group, G2 positive control group and G3 infected and treated group.

The results of the leukocyte parameters are presented at Table 1. *Leishmania* infection (G2)

inhibited significantly the peripheral leukocytes blood, when compared to the -28th and zero days. However, neutrophils and lymphocytes values did not have significant alterations. The infected and treated group (G3) presented eosinophilia in relation to the infected and no treated group (G2).

On clinical evaluation, it was verified the presence of body lesions in the infected animals with *L. braziliensis* (Table 2). In G2 and G3 were verified alopecia areas (7th day) in dorsal and ventral regions and ulcers (7th and 14th day) in dorsal regions of the animals. However, the lesions remained in G2 until the end of the experiment, while in G3 the lesions disappeared on 21st day. Nails, eyes and ears were normal. The snout lesions were observed in the animals from G2 (n=3) and G3 (n=1). G1 did not present skin lesions during the experiment.

Table 3 presents the effect of EAE on subcutaneous imprints of *L. braziliensis* infected footpads that presented amastigotes forms and cellular infiltration. The inflammatory process was characterized by the presence of macrophages, lymphocytes and giant cells. Amastigotes forms of *L. braziliensis* were not found on opposite footpads. In most infected animals (G2 and G3) the amastigotes forms of *Leishmania* were not visualized in the imprints.

Regarding the lymph node drainage, it was observed a significant increase in weight of the infected PLN in G2 and G3 ($p<0.01$) when compared to G1. G2 differ from G3 ($p<0.05$).

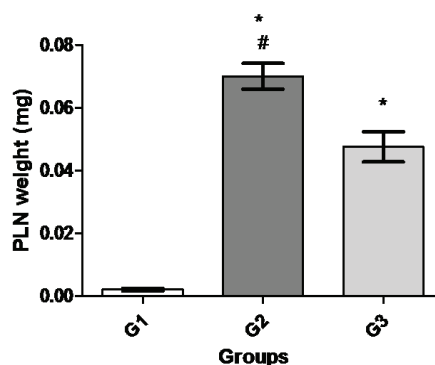


Figure 2. Effect of EAE of *Cocos nucifera* on PLN weight in *L. braziliensis* infected hamsters. The results were expressed as the difference in weight between the right and left PLN. G1 negative control group, G2 positive control group and G3 infected and treated group. * $p<0.01$ compared to G1; # $p<0.05$ compared to G3.

Discussion

There is an agreement that new drugs of herbal origin discovered through ethnopharmacological studies have shown interesting results for the growth

Table 1. Effect of EAE of *Cocos nucifera* on leukocyte counts from *L. braziliensis* infected hamsters. G1 negative control group, G2 positive control group and G3 infected and treated group.

Parameter	Day -28		
	G1	G2	G3
Leukocytes (x10 ³ /mm ³)	13.1±0.6	12.9±0.8	12.3±1.3
Neutrophils (%)	29.9±1.5	26.7±1.2	27.1±1.1
Lymphocytes (%)	73.5±2.3	74.2±3.1	73.1±2.6
Eosinophils (%)	1.1±0.8	1.4±0.6	1.2±0.7
Monocytes (%)	2.5±0.2	2.6±0.4	2.4±0.3
Basophils (%)	1.1±0.1	1.3±0.3	1.4±0.3
Parameter	Day 0		
	G1	G2	G3
Leukocytes (x10 ³ /mm ³)	8.4±2.3	4.9±1.3	5.3±1.8
Neutrophils (%)	25.6±4.3	21.3±8.8	14.9±9.3
Lymphocytes (%)	64.3±8.6	73.8±12.5	72.9±14.1
Eosinophils (%)	5.6±3.2	2.0±2.4	4.8±3.7
Monocytes (%)	2.4±1.2	1.6±1.4	5.2±1.7
Basophils (%)	1.9±1.1	1.2±1.8	2.2±2.1
Parameter	Day 21		
	G1	G2	G3
Leukocytes (x10 ³ /mm ³)	8.5±1.3	5.0±1.6	5.1±0.9
Neutrophils (%)	29.8±15.2	33.8±21.3	22.0±21.4
Lymphocytes (%)	63.2±20.2	54.9±23.5	58.7±21.5
Eosinophils (%)	3.8±6.1	0.6±0.7	9.1±12.1
Monocytes (%)	2.8±1.6	10.4±10.9	9.5±12.9
Basophils (%)	0.3±0.4	0.2±0.3	0.6±0.7

Table 2. Presence of lesions from *L. braziliensis* on different skin regions of hamsters.

Skin lesions	Day -7	Day 0	Day 7	Day 14	Day 21	Region (n)		
						D	V	S
Negative control group (G1)						D	V	S
Absence	4	4	4	4	4	4	4	4
Presence	0	0	0	0	0	0	0	0
Positive control group (G2)						D	V	S
Absence	4	4	1	1	1	4	1	1
Presence	0	0	3	3	3	0	3	3
Infected and treated group (G3)						D	V	S
Absence	4	4	1	1	4	4	1	3
Presence	0	0	3	3	0	0	3	1

Legend: D: dorsal; V: ventral; S: snout.

control of some microorganisms, including bacteria, fungi and protozoa (Esquenazi et al., 2002).

Estevez et al. (2007) studying the leishmanicidal activity *in vitro* of 27 plant extracts from the Amazon region, obtained results in only two species against the amastigote form of *L. amazonensis*, and in only one specie against the promastigote form. Ahua et al. (2007) studying 64 extracts of 21 plants used in traditional medicine in Africa, obtained significant activity in only seven extracts of six different plants, against both amastigotes and promastigotes of *L. major*. Lamidi et al. (2005) studying the leishmanicidal activity *in*

vitro of 67 crude extracts obtained from fifteen plants used in traditional medicine in Africa, observed that plants of the genus *Corynanthe*, which is rich in alkaloids, and fractions of *Polyalthia suaveolens* both have activity against promastigote forms of *L. major*.

Since the 1950s, it has been observed that many plant-derived chemical constituents were studied showing leishmanicidal effects. Some natural compounds with antileishmanial activity are being investigated (Lamidi et al., 2005; Estevez et al., 2007) corresponding to the following groups: alkaloids, terpenes, quinones, lactones, cumarins, chalcones, tetralones, lignins and

Table 3. Evaluation of the presence of *L. braziliensis* in footpad subcutaneous imprints.

Group	Right footpad	Left footpad
Negative control group (G1)		
Animal 1	absence	absence
Animal 2	absence	absence
Animal 3	absence	absence
Animal 4	absence	absence
Positive control group (G2)		
Animal 5	absence	absence
Animal 6	absence	absence
Animal 7	presence	absence
Animal 8	presence	absence
Infected and treated group (G3)		
Animal 9	presence	absence
Animal 10	absence	absence
Animal 11	absence	absence
Animal 12	presence	absence

saponins (Delorenzi et al., 2001) and limonene (Arruda et al., 2009). Our results show that EAE of *Cocos nucifera* L. presents high concentrations of catechins and epicatechins. These compounds are known to have immunomodulatory (Mendonça-Filho et al., 2004), anti-inflammatory and antioxidant (Silva et al., 2009) activities.

In the present study, hamsters were used as experimental model because these animals present great susceptibility to *L. braziliensis* infection. This information was confirmed by Neal & Hale (1983), that compared BALB/C, CBA/H, CDI mouse to hamster infections.

The infection with *L. braziliensis* promastigotes in hamsters footpad induced a progressive increase in the lesion size in all animals (G2 and G3). The treatment with EAE of *Cocos nucifera*, for 21 consecutive days, did not reduce neither the footpad edema nor the PLN weight. However, the animals from G3 (infected and treated animals) that presented skin lesions on 7th day, showed a reduction of these lesions on the 14th day. This fact suggests that the EAE may stimulate a healing activity in this infectious process. It has been reported that *L. braziliensis* infected animals present lesions like papules, nodules and ulcer, which can progress to a secondary bacterial infection (Herwaldt, 1999). The effects observed in our experiment may be mistaken as the heal of infected animals that present skin lesions after treatment with *C. nucifera* extract. However, the animals were not able to eliminate the parasite with the treatment.

C. nucifera L. has been largely used in popular medicine (Duke, 1992). Mendonça-Filho et al. (2004) demonstrated *in vitro* activity of EAE on macrophages

infected with *L. braziliensis* and showed inhibitory activity in the two development stages, promastigotes and amastigotes forms, of *L. amazonensis*. These authors suggested an immunomodulatory effect on infected macrophages. In our study, EAE did not modulate the immune response in *L. braziliensis* infected animals. This fact was verified by the presence of amastigotes forms of *L. braziliensis* and the cellular infiltrate in footpad, and an increase of drainage lymph nodes. On the other hand, the oral treatments with *Kalanchoe pinnata* (Da Silva et al., 1999), *Chenopodium ambrosioides* (Patrício et al., 2008), *Tinospora sinensis* (Singh et al., 2008) were able to control the dissemination of *Leishmania* sp. infection.

In this study, the infected and treated animals (G3) presented eosinophilia, monocytosis and accentuated lymphopenia, but the total leukocytes peripheral blood values and neutrophils remained unaltered when compared to G1 and to G2. It has been reported that in naturally infected patients with *Leishmania* species, it is possible to detect alterations in the haematological parameters. These alterations varied from leukopenia with neutropenia to a moderate lymphocytosis and monocytosis. It is also common to detect anemia in these patients (Herwaldt, 1999).

Our results suggest that the treatment with EAE of *Cocos nucifera* L. did not alter the parameters studied in *L. braziliensis* infected animals in these conditions. Therefore, the popular use has no scientific support, because it does not provide adequate stimulation of the immune system to eliminate the pathogen. But, the EAE seems to present a healing activity in this experimental model. However, the mechanism whereby EAE of *Cocos nucifera* L. modulated the healing response should be investigated further.

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