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Leishmania amazonensis: Effects of oral treatment with copaiba oil in mice

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ABSTRACT

Leishmaniasis is a severe public-health problem, with high rates of morbidity and mortality. Efforts to find new, effective and safe oral agents for the treatment of leishmaniasis have been ongoing for several decades, in order to avoid the problems with the currently used antimonials. In the present study, we found that a copaiba oil oral treatment (Group IV) caused a significant reduction in the average lesion size (1.1 ± 0.4 mm) against *Leishmania amazonensis* lesions compared with untreated mice (Group I) (4.4 ± 1.3 mm). To prove the safety of the oil, the toxicity and genotoxicity were also determined. Histopathological evaluation did not reveal changes in the copaiba oil-treated animals compared to the control animals. In the mutagenicity evaluation, (micronucleus test) the dose tested (2000 mg/kg) showed no genotoxic effects. Morphological and ultrastructural analyses demonstrated notable changes in parasite cells treated with this oleoresin. The main ultrastructural effect was mitochondrial swelling. We also demonstrated that in vitro copaiba oil treatment of *L. amazonensis* led to an increase in plasma membrane permeability, and depolarization in the mitochondrial membrane potential in parasite cells. Although the mechanism of action of the oleoresin is still unclear, these findings indicate that copaiba oil is a possible new drug, which would provide a safer, shorter, less-expensive, and more easily administered treatment for leishmaniasis.

1. Introduction

Leishmania is the protozoan parasite responsible for several pathologies known collectively as leishmaniasis (McConville and Handman, 2007). The worldwide prevalence is 12 million cases, and the estimated population at risk is about 350 million in 88 countries on four continents. Currently, the estimated global annual incidence of new cases is 2 million (Alvar et al., 2006). However, it is clear that official data frequently grossly underestimate the reality, because leishmaniasis is not a notifiable disease in all the countries where it is endemic. Therefore, a substantial number of cases are never recorded (Bustamante et al., 2009).

The clinical manifestations of this infection depend on the species of *Leishmania* and the immunological status of the host. The disease can be classified as cutaneous leishmaniasis, mucocutaneous leishmaniasis, and visceral leishmaniasis, also known as Kala-Azar (Clem, 2010). *Leishmania amazonensis* is a species that causes cutaneous leishmaniasis (CL), which ranges from small cutaneous nodules to gross mucosal tissue destruction (Reithinger et al., 2007).

The pentavalent antimonial compounds have been used since 1940 to treat visceral and cutaneous leishmaniasis. Although new drugs or drug formulations such as liposomal amphotericin B (AmBisome), miltefosine, and paromomycin should be available for treatment of leishmaniasis, they all have limitations of cost, specific toxicities, or the need for parenteral administration (Lee and Hasbun, 2003; Croft, 2008; Dujardin et al., 2010). Consequently there is an urgent need to discover new drugs effective against leishmaniasis.

The current use of herbal therapy in *Leishmania*-endemic regions has renewed interest in evaluation of plant remedies used in traditional medicine as sources of potential antileishmanials (Iwu et al., 1994; Gachet et al., 2010; Tiuman et al., 2011). Interestingly, the use of copaiba oils to treat leishmaniasis has been cited in several ethnopharmacological studies with data obtained from western Amazonia, in Peru (Kvist et al., 2006), eastern Amazonia, in the state of Maranhão, Brazil (Moreira et al., 2002), and northern Amazonia, in French Guiana (Fleury, 1997; Grenand and Moretti, 1987). Recently, Santos et al. (2008) reported that copaiba oils from different species of *Copaifera* show activity against promastigote forms of *L. amazonensis*. These results led us to investigate the

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in vivo antileishmanial activity of copaiba oil from *Copaifera martii*, together with in vitro studies by electron microscopy, biochemical analysis, and flow cytometry to determine the targets of copaiba oil in *L. amazonensis*.

2. Materials and methods

2.1. Copaiba oil

The copaiba oil was collected from the trunk of *Copaifera martii* tree at Tapará, Pará (DC 349), and the sample was deposited in the Herbarium Chico Mendes (Maricá, Rio de Janeiro). The chemical characterization was performed by high-resolution gas chromatography (HRGC) analyses with a Hewlett–Packard (HP) model 5890 instrument equipped with a flame ionization detector as published in Santos et al. (2008).

2.2. Preparation of copaiba oil formulations

The available formulations were prepared as follows: The topical cream (TE) was prepared using glycerin monostearate 6% (w/w), stearic acid 2% (w/w), beeswax 1.5% (w/w), cetiol 11% (w/w), ethoxylated lanolin 1% (w/w), triethanolamine 1% (w/w), methyl paraben 0.18% (w/w), propyl paraben 0.2% (w/w) and distilled water 50% (w/w). Copaiba oil was added in tween 80 at the proportions of 1:1 and mixed until to uniformity. So, this mixture was added in the

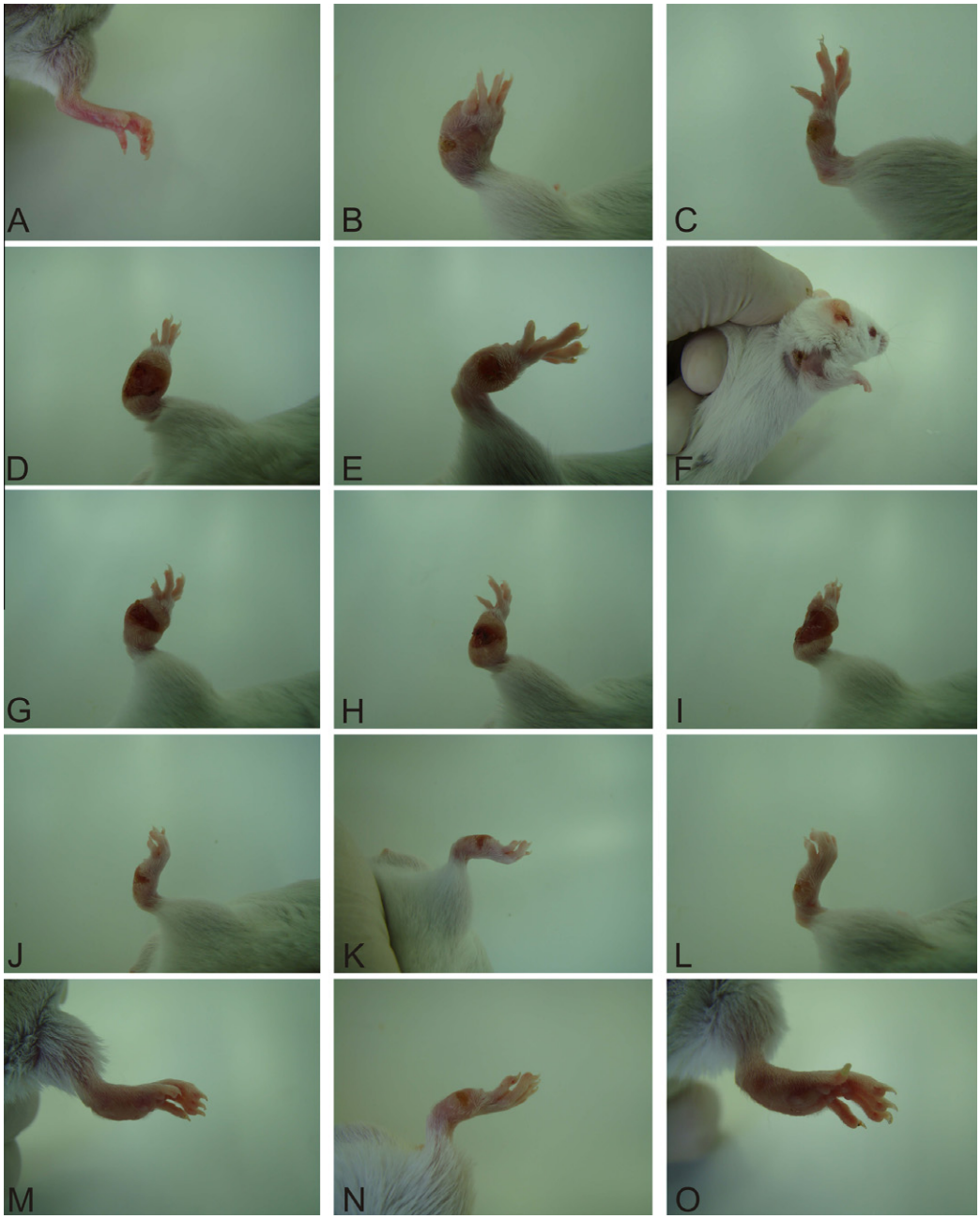

Fig. 1. Evaluation of cutaneous leishmaniasis development in mice treated with copaiba oil. (A) Group VII: Uninfected and untreated control; (B) Group I: infected and untreated control; (C) Group II: The reference drug Glucantime® (100 mg/kg/day) administered through intramuscular injection; (D–F) Group III: copaiba oil (100 mg/kg/day) by subcutaneous route; (G–I) Group V: Lesion treated topically with copaiba oil cream at a concentration of 4%, applied on the lesions in an amount of 1 mg/mm^2; (J–L) Group IV: copaiba oil emulsion at a dose of 100 mg/kg/day was administered orally by gavage. (M–O) Group VI: The animals received oral treatment by gavage (100 mg/kg/day) and also topical treatment with 4% copaiba oil cream applied on the lesions in an amount of 1 mg/mm^2.

emulsion at concentration of 100 μg of copaiba oil for each g of the cream.

For the oral formulation, the guar gum at concentration of 5% (w/w) was dispersed in water under stirring for 4 h. After, the remaining water and 5% (w/w) of tween 80 were added in order to complete the homogeneous polymer dispersion. The copaiba oil was mixed in tween 80 at proportion of 1:1 and added in the preparation in order to obtain the oral emulsion (OE). The oral dose of copaiba oil was 100 mg for kg of the evaluated animal. The intramuscular preparations were obtained by the mixture of copaiba oil in tween and PBS at proportions of 1:1:1.

2.3. Parasites

The promastigote forms of *L. amazonensis* (MHOM/BR/75/Josefa) were isolated from a human case of diffuse cutaneous leishmaniasis. This strain has been maintained by weekly transfers in Warren's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 25 °C in a tissue flask. The infective promastigote was maintained by inoculation into the footpads of BALB/c mouse every 4–6 weeks. After development of lesion, we performed the excised of lesions and the isolation of *L. amazonensis*. The infective promastigotes were cultured in Warren's medium supplemented with 20% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 28 °C. They were then used in the stationary phase of growth (day 6 of culture). Axenic amastigote forms were obtained as described by Ueda-Nakamura et al. (2001).

2.4. Animals

Male BALB/c mice between 4 and 6 weeks of age (20–25 g) and male and female (8–12 weeks) *Mus musculus* (Swiss) outbred mice, weighing 25–30 g were obtained from Universidade Estadual de Maringá (UEM, PR, Brazil). All animals were acclimatized to our laboratory conditions for 10 days before the beginning of the experiments. Animals were under standard laboratory conditions on a constant 12 h light/dark cycle with controlled temperature (22 ± 2 °C). Food (Nuvilab® Cr1) and water were given ad libitum. The Institutional Ethics Committee of Universidade Estadual de Maringá approved all procedures adopted in this study (Protocol 013/2010).

2.5. Infection of animals

BALB/c mice were divided into seven groups that consisted of at least 4 BALB/c mouse. Each mouse was inoculated subcutaneously with 1×10^7 metacyclic promastigotes using 27.5-gauge needle with a volume of 25 μl PBS into the left rear footpad of the mice. Lesion development was monitored weekly by measuring of the thickness of the infected footpad with a calliper (DIGIMESS® 150 mm) for a total period of 8 weeks. The treatments were started after development of lesions (30 days pos-infection) and continued for 4 weeks. Lesion size was expressed as the difference in thickness between parasite-inoculated footpad and the contralateral footpad of the same animal.

2.6. Treatments of infected animals and histological studies

The groups were distributed as follows: Group I: infected and untreated control; Group II: The reference drug Glucantime® (100 mg/kg/day) was dissolved in PBS and administered through intramuscular injection. Group III: copaiba oil (100 mg/kg/day) by subcutaneous route was made up in solution of Tween and PBS; Group IV: copaiba oil emulsion at a dose of 100 mg/kg/day was administered orally by gavage. The oil was suspended in Tween (Sigma Chemical Co. USA) and diluted in 5% Goma-Guar. Group V: Lesion received topical treatment. The copaiba oil cream at concentration of 4% was applied on the lesions in an amount of 1 mg/mm^2. Group VI: The animals received oral treatment by gavage (100 mg/kg/day) and also topical treatment with copaiba oil cream 4% being applied on the lesions in an amount of 1 mg/mm^2. Group VII: Uninfected and untreated control. The solutions were made up daily. All treatments were realized by 30 days after development of lesion. At the end of the 30-day-treatment period the animals were killed in a CO_2 chamber. Some selected organs (esophagus, stomach, duodenum, heart, kidney, liver, spleen, lung, and testiculum) were removed and processed for histological studies. Thus, the organs were fixed in Bouin' fluid, embedded in paraffin wax. Blocks were sectioned at 7 μm thickness in a microtome (Leica Microsystems Inc, Germany), mounted in a electrostatic slides and stained with hematoxylin-eosin.

2.7. Micronucleus test

Ten-animal groups (*Mus musculus* five males and five females) were treated orally by gavage with 2000 mg copaiba oil/kg body weight (bw), and were killed 24 h after the copaiba oil administration. The positive control (cyclophosphamide = 40 mg/kg bw) and negative control (distilled water) groups were also included (Costa et al., 2010). After the treatment periods, the femurs were exposed and sectioned, and the bone marrow was gently flushed out using fetal calf serum. After centrifugation (3000*g*, 5 min) the bone marrow cells were smeared on glass slides, coded for blind analysis, and air-dried. The smears were stained with May-Grunwald-Giemsa to detect micronucleated polychromatic erythrocytes (MNPCE) (Schmid, 1975). For each animal, three slides were prepared and 2000 polychromatic erythrocytes (PCE – polychromatic erythrocytes) were counted to determine the frequency of MNPCE. The slides were analyzed with the use of an Olympus BH-2 microscope (1000×).

Table 1
Effects of copaiba oil on lesion development in mice infected with *L. amazonensis* promastigotes. Results are shown as the mean ± SD for the four mice in each group.

Treatment	Average lesion size (mm) (mean ± SD)
Group I C(−): Infected and untreated	4.4 ± 1.3
Group II (C+): Glucantime®	0.9 ± 0.3[a]
Group III: C.O. Subcutaneous route	3.0 ± 1.0[b]
Group IV: C.O. Oral route	1.1 ± 0.4[a]
Group V: C.O. Topical	4.9 ± 0.3[b]
Group VI: C.O. Oral route and topical	1.2 ± 0.2[a]

C(−): negative control; C(+): positive control; C.O.: copaiba oil; SD: standard deviation.

[a] No significant difference when compared to positive control and significant difference when compared to negative control (ANOVA test; $p < 0.05$).

[b] Significant difference when compared to positive control (ANOVA test; $p < 0.05$).

Table 2
Micronucleus test – frequency of micronuclei in 2000 polychromatic erythrocytes (MNPCE) in bone marrow of mice after 24 h of oral treatment with copaiba oil (2000 mg copaiba oil/kg body weight – bw); positive control (cyclophosphamide = 40 mg/kg bw); and negative control (distilled water).

Treatment	MNPCE (mean ± SD)
Copaiba oil	9.0 ± 1.4[a]
Cyclophosphamide	27.0 ± 4.0[b]
Distilled water	7.0 ± 1.8

MNPCE: micronucleated polychromatic erythrocytes; SD: standard deviation.

[a] No significant difference when compared to negative control and significant difference when compared to positive control (ANOVA test; $p < 0.05$).

[b] Significant difference when compared to negative control (ANOVA test; $p < 0.05$).

2.8. Study of parasite morphology and ultrastructure by electron microscopy

Promastigote forms were treated with copaiba oil obtained from *C. martii* and then processed for scanning and transmission electron microscopy as described previously in Santos et al. (2008).

2.9. Flow cytometry

L. amazonensis axenic amastigotes (5×10^6 parasites/mL) were treated with copaiba oil from *C. martii* (100 μg/mL for 3 h at 32 °C), or untreated. After that were harvested and washed with PBS. The integrity of the plasma membrane was assessed by the entrance of the propidium iodide (PI) (20 μg/mL for 5 min). To analyses of mitochondrial membrane potential ($\Delta\Psi$m) parasites were stained with Rhodamine 123 (Rh 123) (5 mg/mL for 30 min at 37 °C) reagents. The compounds Amphotericin B (5 μM) and, Carbonyl Cyanide m-chlorophenylhydrazone (CCCP) (200 μM) were used as a positive control. The material was kept on ice until analysis. The mean of fluorescence intensity of the cells were analyzed by flow cytometry FACSCalibur and CellQuest software. A total of 10,000 events were acquired in the region previously established as that corresponding to the parasites.

2.10. Statistical analysis

Data are presented as mean ± SD. The statistical significance of differences in percentage between treated and untreated was ana-

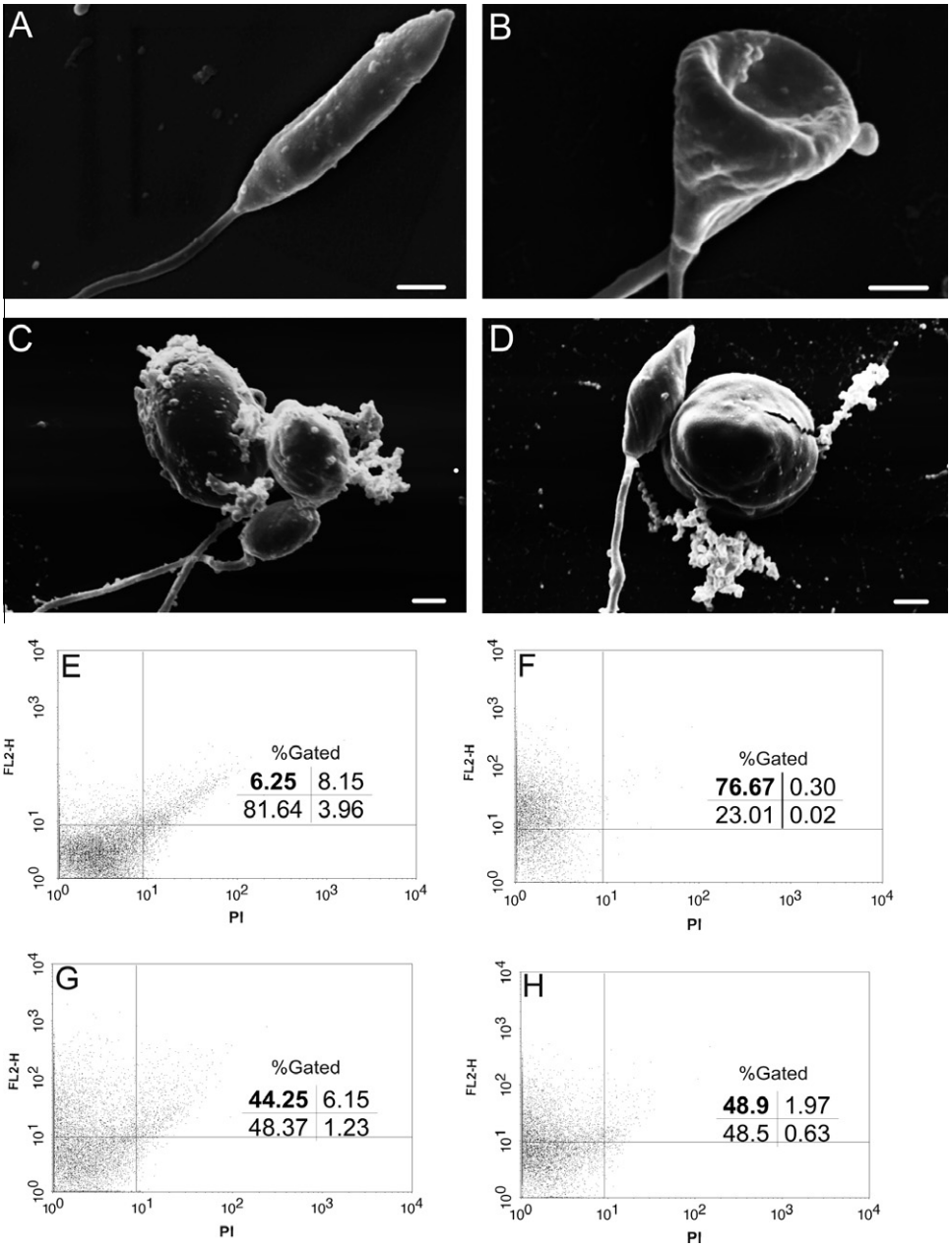

Fig. 2. Scanning electron micrographs of promastigote forms of *L. amazonensis* treated with copaiba oil (A) Promastigotes, control; (B) Promastigotes after treatment with IC_{50} of copaiba oil; (C and D) Promastigotes after treatment with IC_{90} of copaiba oil (Bars = 1 μm) and flow cytometry analysis of *L. amazonensis* axenic amastigotes treated with copaiba oil stained with propidium iodide (PI) (D) Untreated cells; (F) Amphotericin-B; (G) Amastigotes treated with 100 μg/mL; (H) Amastigotes treated with 200 μg/mL. The numbers in bold show the percentage of PI-stained positive cells in the upper left quadrant.

lysed by Statistics 8.0®. The statistical analyses were performed by Anova one-way test followed by Dunnet test. Differences were considered significant at p value of less than 0.05.

3. Results and discussion

There is no single optimal treatment for cutaneous leishmaniasis (Hepburn, 2000; Markle and Makhoul, 2004; Palumbo, 2010). Natural products have made and are continuing to make important contributions to the search for new antileishmanial drugs (Tiuman et al., 2005; Khaliq et al., 2009; Singh et al., 2009; Vendrametto et al., 2010). Recently, Santos et al. (2008) reported that copaiba oils from different species of *Copaifera* show activity against promastigote forms of *L. amazonensis*. Taken together, these facts encouraged us to evaluate the treatment of experimentally infected animals with copaiba oil. For the in vivo tests, BALB/c mice were infected subcutaneously with *L. amazonensis* (1×10^7 cells/ml) in the left rear footpad. The treatment was started on the 4th week post-infection. Mice were treated subcutaneously (Group III), orally (Group IV), topically (Group V), or orally and topically (Group VI). Treatment with Glucantime® (Group II) was used as a positive control. The lesion size was measured with a caliper each week for one month of infection. Fig. 1 and Table 1 present the results of the treatments. Interestingly, the oral treatment (Fig. 1J, K, and L), and the oral and topical treatments (Fig. 1M, N, and O) caused significant ($p < 0.05$) reductions in the average lesion size (1.1 ± 0.4 mm), and (1.2 ± 0.2 mm) compared with untreated mice (Group I) (4.4 ± 1.3 mm) (Fig. 1B), and showed no significant difference compared to the positive control Glucantime® (Fig. 1C) (0.9 ± 1.3 mm). The topical (Fig. 1G, H, and I) (4.9 ± 0.3 mm) and subcutaneous (Fig. 1D and F) (3.0 ± 1.0 mm) treatments showed no significant reduction in the average lesion size ($p < 0.05$). Moreover, the subcutaneous treatment caused injuries in the animals during the applications, as shown in Fig. 1G.

The main chemical constituents of *C. martii* are sesquiterpenes (37.7%) and diterpenes (62.3%). The most common sesquiterpenes are β-bisabolene (10.7%) and α-zingiberene (7.2%). Kaurenoic (7.9%) and kovalenic (29.0%) acids are the main diterpenes (Santos et al., 2008). Several reports have demonstrated that the biological effect of copaiba oils may be explained by the complex nature of these sesquiterpene and diterpene mixtures, which might affect

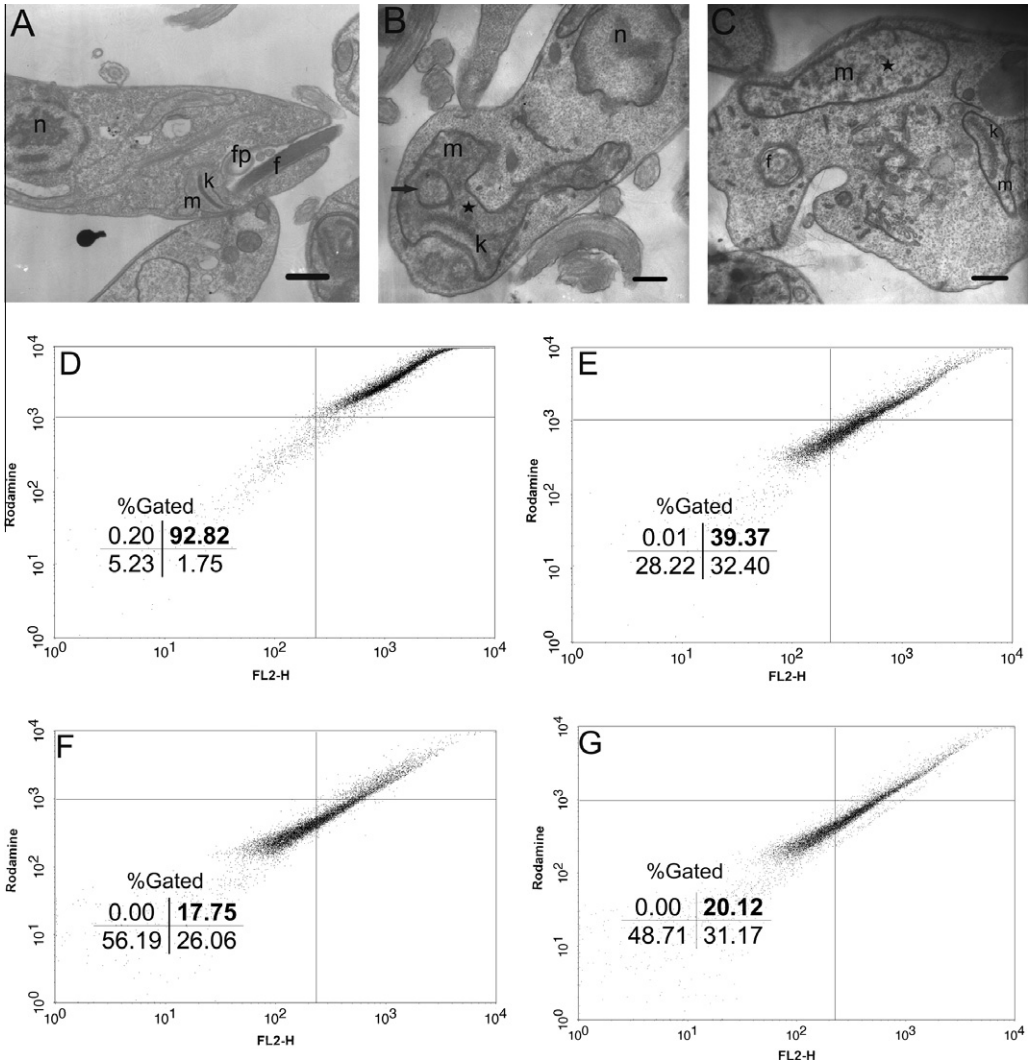

Fig. 3. Ultrastructural effect of copaiba oil on promastigote forms of *L. amazonensis*. (A) Promastigote control; (B) Promastigote treated with IC_{50} of copaiba oil; (C) Promastigote treated with IC_{90} of copaiba oil; The treatment with copaiba oil led to changes in the mitochondria (m) including mitochondrial swelling (stars) and the appearance of concentric membrane structures inside the organelle (arrow). n, nucleus; f, flagellum; fp, flagellar pocket; k, kinetoplast; m, mitochondrion. Bars = 1 μm. Flow Cytometry analysis of Rh123-labeled axenic amastigotes of *L. amazonensis*. (D) Untreated cells; (E) CCCP 200 μM; (F), Amastigotes treated with 100 μg/mL; (G) Amastigotes treated with 200 μg/mL. The numbers in bold represent the percentage of collapsed $\Delta\Psi m$ cells in the upper right quadrant.

the active component by a synergistic effect (Fernandes and Freitas, 2007). Lima et al. (2003) reported that fractionation of copaiba oils results in fractions that are less active than the crude copaiba oil.

An important criterion in the search of active compounds with antiprotozoal activity is their toxicity to mammalian host cells. For this purpose, the toxicity and genotoxicity of the copaiba oil were determined. Histopathological evaluation did not reveal changes in the esophagus, stomach, duodenum, heart, kidney, liver, spleen, lung, or testicles of animals treated with copaiba oil compared to control animals. No clinical or behavioral changes were observed in the animals treated with the oleoresin. In the mutagenicity evaluation, the dose tested (Schmid, 1975) showed no genotoxic effects. The copaiba oil dose of 2000 mg/kg showed a frequency of MNPCE of 9.0 ± 1.4 (Mean ± SD) in 2000 polychromatic erythrocytes. These levels were significantly lower than the positive control (27.0 ± 4.0), and did not differ from the negative control (7.0 ± 1.8) (Table 2). However, are still further testing is needed to prove the total absence of genotoxicity.

To investigate which organelles might be the initial targets of copaiba oil, SEM and TEM techniques were employed. Figs. 2 and 3 show morphological alterations in promastigotes treated with concentrations corresponding to the IC_{50} (14.0 μg/mL) and IC_{90} (70.0 μg/mL) values of copaiba oil. Untreated control promastigotes (Fig. 2A) showed the typical elongated shape. In contrast, the parasites treated with copaiba oil showed notable morphological changes, such as the appearance of aberrant-shaped cells (Fig. 2B–D). Ultrastructural changes in promastigotes treated with copaiba are illustrated in Fig. 3. Untreated promastigotes showed no plasma membrane alterations, and organelles with normal morphology (Fig. 3A). The most prominent effects observed in treated parasites were swollen mitochondria in all the treated cells (Fig. 3B and C).

To improve drug therapies for leishmanial infections and for the development of new drugs, it is necessary to identify the targets of parasite cells that have the potential to be safe and effective treatments. The cell viability of axenic amastigotes of *L. amazonensis* was checked by staining the cells with propidium iodide (PI), a fluorescent dye that binds specifically to DNA. As shown in Fig. 2, the gated percentage (upper-left quadrant) of PI-stained cells after treatment with copaiba oil (44.25% in parasites treated with 100 μg/mL and 48.9% treated with 200 μg/mL) (Fig. 2G and H) was higher than the number in non-treated parasites (6.25% Fig. 2E) and similar to the positive control treated with Amphotericin B (76.67%, treated with 5 μg/mL) (Fig. 2F). All treatments were administered for 3 h at 32 °C. These data might indicate that copaiba oil induces in axenic amastigotes a considerable increase in plasma membrane permeability. This is in agreement with the observations of Santa-Rita et al. (2004) who demonstrated that treatment with edelfosine at concentrations above 1 mM in PBS for 10 min led to a gradual increase in membrane permeability.

The mitochondrial transmembrane potential was investigated by using the fluorescent probe Rh 123, which accumulates within energized mitochondria, in conjunction with flow cytometry analysis. Data obtained showed a marked decrease in the percentage population of the upper right gated (17.75% and 20.12%), indicating depolarization of the mitochondrial membrane potential in the cells following treatment with copaiba oil at 100 μg/mL and 200 μg/mL, respectively (Fig. 3F and G). Similarly, a decrease in membrane potentials was also observed following treatment with the standard drug Carbonyl Cyanide m-chlorophenylhydrazone (CCCP) (39.37%) at 200 μM for 3 h at 32 °C (Fig. 3E). In contrast, untreated cells maintained the membrane potential (92.82%) (Fig. 3D). Several antileishmanial drugs have produced a depolarization in the mitochondrial membrane potential. Investigations of the mitochondrial potential have reported that a main functional impact of mitochondrial alterations is programmed cell death by apoptosis (Singh et al., 2009; Santa-Rita et al., 2006; Sen et al., 2007; Misra et al., 2008; Kaur et al., 2010).

Copaiba oil from *C. martii* was identified as an orally active antileishmanial drug, through evaluation of BALB/c mice infected with *L. amazonensis*. Exposure of the parasites to copaiba oil induces an increase in plasma membrane permeability, and depolarization in the mitochondrial membrane potential. Although the mechanism of action of the oleoresin is still unclear, these findings indicate that copaiba oil might be a new drug, which would constitute a safer, shorter, less-expensive, and more easily administered treatment for leishmaniasis. Given the promising results obtained with copaiba oil (oral treatment) our next goals are to test new oral formulations containing copaiba oil, and to verify the effects of these oral formulations distribution (different sites of infection), providing an evidence about the pathologic lesion pre-and post-treatment, immuno-modulators studies, and tests of acute and chronic toxicity to provide information about the safety of the use of copaiba oil.

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