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BIOLOGICAL SCIENCES

Ethanolic extract of *Croton blanchetianus* Ball induces mitochondrial defects in *Leishmania amazonensis* promastigotes

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Abstract: Leishmaniasis is a neglected disease caused by *Leishmania*. Chemotherapy remains the mainstay for leishmaniasis control; however, available drugs fail to provide a parasitological cure, and are associated with high toxicity. Natural products are promising leads for the development of novel chemotherapeutics against leishmaniasis. This work investigated the leishmanicidal properties of ethanolic extract of *Croton blanchetianus* (EECb) on *Leishmania infantum* and *Leishmania amazonensis*, and found that EECb, rich in terpenic compounds, was active against promastigote and amastigote forms of both *Leishmania* species. *Leishmania infantum* promastigotes and amastigotes presented IC_{50} values of 208.6 and 8.8 µg/mL, respectively, whereas *Leishmania amazonensis* promastigotes and amastigotes presented IC_{50} values of 73.6 and 3.1 µg/mL, respectively. Promastigotes exposed to EECb (100 µg/mL) had their body cellular volume reduced and altered to a round shape, and the flagellum was duplicated, suggesting that EECb may interfere with the process of cytokinesis, which could be the cause of the decline in the parasite multiplication rate. Regarding possible EECb targets, a marked depolarization of the mitochondrial membrane potential was observed. No cytotoxic effects of EECb were observed in murine macrophages at concentrations below 60 µg/mL, and the CC_{50} obtained was 83.8 µg/mL. Thus, the present results indicated that EECb had effective and selective effects against *Leishmania infantum* and *Leishmania amazonensis*, and that these effects appeared to be mediated by mitochondrial dysfunction.

Key words: *Leishmania amazonensi*, *Leishmania infantum*, mitochondrial metabolism, natural products.

INTRODUCTION

Leishmaniasis is a disease caused by protozoan parasites from more than 20 *Leishmania* species (WHO 2016). The interplay between the type of infecting species and the host's immunity results in a spectrum of clinical manifestations that includes four main forms: visceral leishmaniasis (VL, also known as kala-azar), post-kala-azar dermal leishmaniasis (PKDL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL) (Pace 2014, WHO 2016). While cutaneous leishmaniasis is the most common form of the disease, the visceral form is the most serious, and can be fatal if untreated. Endemic leishmaniasis transmission has been reported in 98 countries and three territories in five continents (Alvar et al. 2012). Approximately 556 million people are at risk of VL in VL high-burden countries, and almost 399 million people are at risk of CL in CL high-burden countries. Among these countries, Brazil

is the only one that has high burden of both VL and CL (WHO 2016).

The first-line drug currently used in leishmaniasis treatment is the pentavalent antimony Sb (V). Besides its high toxicity, an increase in clinical resistance to this drug has been reported (Lira et al. 1999, Mittal et al. 2007, Sen & Chatterjee 2011).

Second-line treatments include pentamidine and amphotericin B, which are also toxic and too expensive for routine use in developing countries (Croft & Olliaro 2011, Murray et al. 2005, Singh & Sundar 2012). A promising alternative drug is miltefosine, which is the only oral drug that can be used to treat leishmaniasis. Miltefosine proved to be active in the treatment of mucosal leishmaniasis in Bolivia (Soto et al. 2007), and of visceral leishmaniasis in the Indian subcontinent (Dorlo et al. 2012, Sundar et al. 2002). However, studies suggest that miltefosine is not equally effective for all forms of leishmaniasis, and that the same species of *Leishmania* may have different levels of susceptibility to miltefosine in different endemic areas (Soto et al. 2008). Therefore, further studies are required to find safe, less expensive, and more effective treatments against leishmaniasis.

Plant-derived natural products represent potential sources of new therapeutic agents for the treatment of various diseases. During recent decades, studies have demonstrated leishmanicidal effects of several plants used in traditional medicine in Brazil, including some of the *Croton* genus, such as *C. macrostachyus* (Tariku et al. 2010), *C. pullei* (Guimaraes et al. 2010), *C. caudatus* (Dey et al. 2015), and *C. cajucara* (Lima et al. 2015, Rodrigues et al. 2013, Rosa et al. 2003). This has encouraged the study of *C. blanchetianus*, a shrub widely disseminated in northeast Brazil, where it is known as "black marmeleiro". Its leaves and bark have multiple uses in popular medicine, including the treatment of gastrointestinal disturbances, rheumatism, and cephalalgia (Melo et al. 2013). Furthermore, the essential oil of *C. blanchetianus* is rich in terpenes (Angélico et al. 2014), and its antimicrobial and antiparasitic properties have already been demonstrated (Angélico et al. 2014, Melo et al. 2013, Silva et al. 2014). Therefore, this work presents, for the first time, the leishmanicidal activity of *C. blanchetianus* bark extract, and its effect on mitochondrial metabolism in *L. amazonensis* promastigotes.

MATERIALS AND METHODS

Plant material

The bark of *C. blanchetianus* was collected in the village Fazenda Serrote, in the Municipality of Piranhas, state of Alagoas, Brazil. Botanical identification was performed by Dr. Ana Paula Silveira, botanist of the Department of Biology, Universidade Federal de Sergipe, Brazil (DB-UFS). A voucher specimen was deposited in the DB-UFS herbarium under the registration number ASE 24664.

Preparation of the *C. blanchetianus* extract and phytochemical analysis

A total of 1.2 kg of *C. blanchetianus* bark was dried at room temperature and pulverized in a blade mill to obtain the powder, which was extracted by maceration in 90% ethanol for five days. Afterwards, the material was filtered and concentrated in a rotatory evaporator under reduced pressure at 45°C, to give 114.35 g of the ethanolic extract (HEE, yield of 9.53%).

To obtain a chromatographic profile of the extract, High Performance Liquid Chromatography (HPLC) analyses were performed on a series of liquid chromatograph Shimadzu Prominence LC comprised of two pumps 6AD, photodiode array detector (DAD) and a SPD M20A analytical

C18 column (25.0 × 0.46 cm, 5 µm particles). The solvents used were HPLC grade H_2O and CH_3OH. From hydroethanolic extract was made a solution with final concentration of 1 mg/ml subjected to elution through gradient 5 to 100% CH_3OH exploration, exploratory gradient: 20 to 100% CH_3OH up to 40 min followed by isocratic gradient of 40 to 60 min 100% CH_3OH to investigate the chromatographic profile. The substances were detected using a photodiode array detector (DAD - UV/Vis) using a 254 nm wavelength (Queiroz et al. 2002). For the acquisition and processing of chromatographic data was used LC Solution software.

Parasites

For the assays, *L. amazonensis* (MHOM/BR/77/LTB0016) and *L. infantum* (MHOM/MA67ITNAB263) were maintained as promastigotes at 26°C in Schneider's insect medium (Sigma-Aldrich), supplemented with 10% and 20% heat-inactivated fetal calf serum (HIFCS) for *L. amazonensis* and *L. infantum*, respectively, in addition to 100 µg/mL streptomycin and 100 U/mL penicillin.

Antipromastigote activity

L. amazonensis and *L. infantum* promastigotes (5×10^6 cells/mL) were cultivated in Schneider's insect medium supplemented with HIFCS, as previously described. Parasites were incubated at 26°C for 24 h, in either the absence or presence of *C. blanchetianus* extract dissolved in DMSO (50 to 500 µg/mL). The final concentration of DMSO never exceeded 0.4%, a concentration which is not toxic for the protozoa (Oliveira et al. 2009). The viability of promastigotes was assessed by the AlamarBlue® assay (Fonseca-Silva et al. 2011). Pentamidine was used as reference drug. The vehicle of extract (DMSO 0.4%) was added to the control group.

Antiamastigote activity

Peritoneal macrophages were collected from BALB/c mice (6–8 weeks old) and plated in cold RPMI medium supplemented with 10% HIFCS, 100 µg/mL streptomycin, and 100 U/mL penicillin, at a concentration of 2×10^6 cells/mL (0.4 mL/well), in Lab-Tek eight-chamber slides. Cells were then incubated for 3 h at 37°C in a 5% CO_2 atmosphere, for cell attachment. Non-adherent cells were removed by phosphate buffered saline (PBS) washing, and *L. amazonensis* or *L. infantum* promastigotes were added to the peritoneal macrophages at a parasite ratio of 3:1 and 5:1, respectively. After 5 h incubation, free parasites were removed by successive washes with warmed RPMI 1640, and *Leishmania*-infected macrophages were then incubated in either the absence or presence of *C. blanchetianus* extract (3.0 to 30.0 µg/mL) for 72 h. The percentage of infected macrophages was determined by light microscopy and random counts of a minimum of 300 cells in each treatment, in duplicate. Results were expressed as an infection index (% of infected macrophages X number of amastigotes/total number of macrophages). Pentamidine was used as a reference drug.

Macrophage viability assay and NO production

Peritoneal macrophages (2×10^6 cell/mL) collected from BALB/c mice (6–8 weeks old) were allowed to adhere in 96-well tissue culture plates for 1 h, at 37°C, in a 5% CO_2 atmosphere, for cell attachment. Nonadherent cells were removed by washes with RPMI 1640 medium. Serial dilutions of *C. blachetianus* extract in concentrations from 120 to 1.875 µg/mL were added to the wells containing adherent macrophages. After 72 h incubation, the medium was discharged, and the macrophages were washed with RPMI 1640 medium; cell viability was assessed after 12 h incubation with AlamarBlue® (Fonseca-Silva et al. 2013).

The selectivity index (SI) was determined using the following equation: macrophage CC_{50}/ intracellular amastigote IC_{50} (Weniger et al. 2001). Peritoneal macrophages were lysed with 0.1% Triton X-100 and used as positive controls. For the estimation of nitric oxide (NO) in EECb treated macrophages, the cell supernatants were collected and distributed (50 µL/well) in 96-well plates, and equal volume of Griess reagent was added to each well; these were then incubated for 10 min at 25° C, and the absorbance was taken at the wavelength 540 nm. The concentration of NO was given in µM.

Analysis of promastigote cellular morphology

Morphological changes in parasites as a result of *C. blanchetianus* extract treatment were identified microscopically. Briefly, *L. amazonensis* and *L. infantum* promastigotes in exponential growth phase were incubated in either the absence or presence of *C. blanchetianus* extract (100 µg/mL) for 24 h. Cytospin smears from cell suspensions were stained with 10% Giemsa staining solution and examined under an optical microscope (Olympus). At least 20 microscope fields were observed for each sample.

Determination of promastigotes growth rate and doubling time

L. amazonensis promastigotes were cultivated in Schneider's insect medium in the absence or presence of 70.0 µg/mL or 140.0 µg/mL EECb (starting from 5 x 10^5 parasites/mL). The parasites growing were determined by direct counting with a Neubauer chamber either every 24 h for five days for growth curve determination, or every 6 h for 48 h for doubling time determination. The doubling time was calculated by the Least Squares Fitting-Exponential method accessed in the online platform Doubling Time, available at http://www.doubling-time.com/compute_more.php.

Cellular membrane integrity assessment

L. amazonensis promastigotes (5 x 10^5 cells/mL) were cultivated in Schneider's insect medium supplemented with HIFCS, as previously described, in either the absence or presence of 70.0 µg/mL and 140.0 µg/mL of *C. blanchetianus* extract, at 26° C for 24 h. Promastigotes were then harvested, washed twice with PBS (pH 7.2), and stained with propidium iodide (PI) (40 µg/mL) for 15 min, in the dark, at room temperature. PI-positive cells were evaluated by flow cytometry using an Attune® Acoustic Focusing (Life Technologies) cytometer. A total of 10,000 events were obtained and analyzed using Attune® Cytometer Software.

Analysis of the cell cycle

L. amazonensis promastigotes (1 x 10^5 cells/mL) were incubated in either the absence or presence of *C. blanchetianus* extract (70.0 µg/mL or 140.0 µg/mL), for 24 h, and then washed twice with PBS (pH 7.2). Cells were fixed in chilled 70% ethanol and incubated for 1 h on ice. Parasites were again washed twice with PBS and resuspended in 0.5 mL PBS containing PI (40 µg/mL) and RNase A (Sigma: R464; 200 µg/mL). The mixture was incubated for 1 h at room temperature in the dark. Data was obtained using an Attune® Acoustic Focusing (Life Technologies) cytometer and analyzed using Attune® Cytometer Software.

Determination of mitochondrial membrane potential (Δψm)

The variation in mitochondrial membrane potential ($\Delta\psi_m$) was monitored using JC-1 dye. *L. amazonensis* promastigotes (0.5 to 1 x 10^6 cells/mL) were incubated in either the absence or presence of *C. blanchetianus* extract (70.0 µg/mL and 140.0 µg/mL) for 24 h, and then washed twice with PBS (pH 7.2). Afterwards, promastigotes were incubated with JC-1 (10 µg/mL in HBBS) for 20 min, at room temperature, in

the dark. JC-1 stained cells were washed twice with HBSS, and fluorescence was measured spectrofluorometrically at both 530 nm and 590 nm, using an excitation wavelength of 480 nm. The ratio of values obtained at 590 nm and 530 nm was plotted as the relative $\Delta\Psi_m$. The mitochondrial uncoupling agent carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP; 20 µM; 20 min) was used as positive control (Fonseca-Silva et al. 2016).

Statistical analysis

All experiments were performed in triplicate, except the antiamastigote activity, which was performed in duplicate. The minimum EECb concentration that caused 50% reduction in the survival of the amastigote and promastigote *Leishmania* forms (IC_{50}), and in macrophages (CC_{50}), was obtained by regression analysis using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Data were analyzed statistically using two-way analysis of variance (ANOVA) and Student's t-test.

Ethical Approval

Macrophages from BALB/c mice were obtained following protocols approved by the Ethics Committee for Animal Use of the Instituto Oswaldo Cruz (L026/2015).

RESULTS AND DISCUSSION

During the last few decades, studies have demonstrated leishmanicidal effects of some *Croton* species. Dey et al. (2015) showed that the hexanic extract of *C. caudatus* is an effective growth inhibitor of *L. donovani* promastigotes and amastigotes. Also, extracts significantly altered the biochemical parameters (protein, lipids, and carbohydrates) in promastigotes, and caused morphological changes, DNA condensation, and subsequent apoptosis. Previously, Rosa et al. (2003) showed that at the nanogram level, the essential oil of *C. cajucara* caused gross morphological changes in *L. amazonensis* promastigotes. Moreover, after 30 min in the presence of the essential oil, the parasites were completely destroyed. These data show that the genus *Croton* is a source of promising leads for novel leishmaniasis chemotherapeutics. These findings encouraged our study of the antileishmanial activity of ethanolic extract of *C. blanchetianus* (EECb) on *L. amazonensis* and *L. infantum*.

Phytochemical characterization

The chemistry of the *Croton* genus is very diverse. Terpenoids are the predominant metabolites, especially diterpenes. Triterpenoids, pentacyclic or steroids, are also often reported. These compounds may be associated with the antibacterial, antifungal, antiviral, antimalarial, anti-inflammatory and anticancer activities, corroborating with the traditional therapeutic uses of plants of this genus (Salatino et al. 2007).

The chromatographic profile of ethanolic bark extract of *C. blanchetianus* was focused from 22 to 55 min of chromatographic running in which the most prevalent compounds or groups of compounds were eluted between 37 and 55 min, that is characteristic of low polarity compounds (Fig. 1a). Also, UV scan for the selected chromatographic peaks with retention time of 37.63, 41.08, 50.81 and 54.33 min are showed on Fig. 1b, which complies with a chemical profile of terpenic compounds, whose absorption spectra typically range between 200 and 270 nm. Besides, a plain I Band, characteristic of terpenes (Kong et al. 2001) could be observed. Based on the compound absorption profile, we can observe that the main groups of terpenes found in the ethanolic bark extract of *C. blanchetianus* are unsaturated, whose absorption ranges between

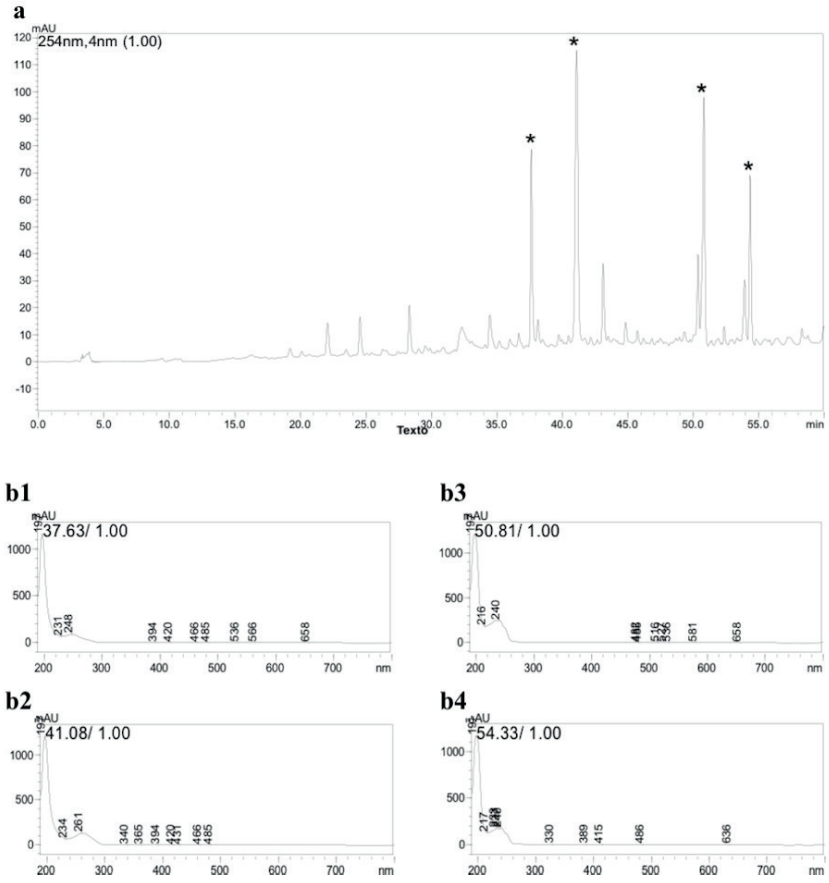

Figure 1. Phytochemical characterization of ethanolic bark extract of *C. blanchetianus*. a: Chromatographic profile at 60 min of chromatographic running; b: UV scan (190–800 nm) for the chromatographic peaks with retention time of 37.63 min (B1); 41.08 min (B2); 50.81 min (B3) and 54.33 min (B4) complying with compounds of terpene nature. (*) Chromatographic peaks analyzed in B1; B2; B3 and B4.

215 and 250 nm and aromatic terpenes, whose absorption ranges between 250 and 270 (Ugaz 1994).

One of the mechanisms of action of terpenes is the attack on the plasma membrane. Mendanha & Alonso (2015) demonstrated that terpenes act as lipid spacers, increasing the amount of the most mobile component. Thus, the insertion of terpenes in the lipid bilayer, increase the membrane fluidity. This is consistent with the reported broad spectrum of terpene activity against protozoa, pathogenic fungi and tumor cells (Camargos et al. 2014).

Plant extracts have been traditionally used in the treatment of protozoan diseases (Cock et al. 2018, Da Silva et al. 2018) and several scientific studies with the chemical characterization of the extracts and/or terpenoids compounds and their leishmanicidal potential have been developed in the last years (Teles et al. 2015, de Almeida et al. 2016, Garcia et al. 2017).

The trypanocide activity of the methanolic extract of the stem bark of *Croton cajucara* and of the isolated terpenes, transdehydrocrotonin and acetyl aleuritolic acid, were investigated on *Trypanosoma cruzi*. The extract and the terpenes isolated inhibited *T. cruzi* for cause inhibition of the trypanothione reductase pathway, alterations of the plasma membrane and mitochondrial damage (Campos et al. 2010). Diterpenes from *Croton cajucara* displayed *in vitro* antileishmanial effects against promastigotes and amastigotes of *L. amazonensis*. The possible mechanism of action of *C. cajucara* diterpenes was the inhibition of the trypanothione reductase enzyme (Lima et al. 2015).

Effect on parasite and macrophage survival

The *in vitro* leishmanicidal activity of EECb was evaluated in both axenic promastigote and intracellular amastigote forms of *L. amazonensis* and *L. infantum*. The effect of different concentrations of EECb on the parasite survival and the 50% inhibitory concentrations (IC_{50}) are shown in Figure 2 and Table I, respectively. Besides the IC_{50} value for *L. amazonensis* promastigotes being nearly three times lower than that for *L. infantum*, at 100 µg/mL EECb, a reduction of 70% in the *L. amazonensis* promastigote survival was observed (Figure 2a). This level of reduction was only observed in promastigotes of *L. infantum* at an EECb concentration of 300.0 µg/mL. Likewise, an 80% reduction in *L. amazonensis* amastigote survival was detected in macrophages treated with EECb concentrations between 5.0 and 10.0 µg/mL, whereas in *L. infantum* amastigotes, this level of survival reduction was detected in concentrations between 20 and 30 µg/mL (Figure 2b). Based on these results, we can notice that promastigote and amastigote forms of *L. amazonensis* are more sensitive to EECb, when compared to *L. infantum*.

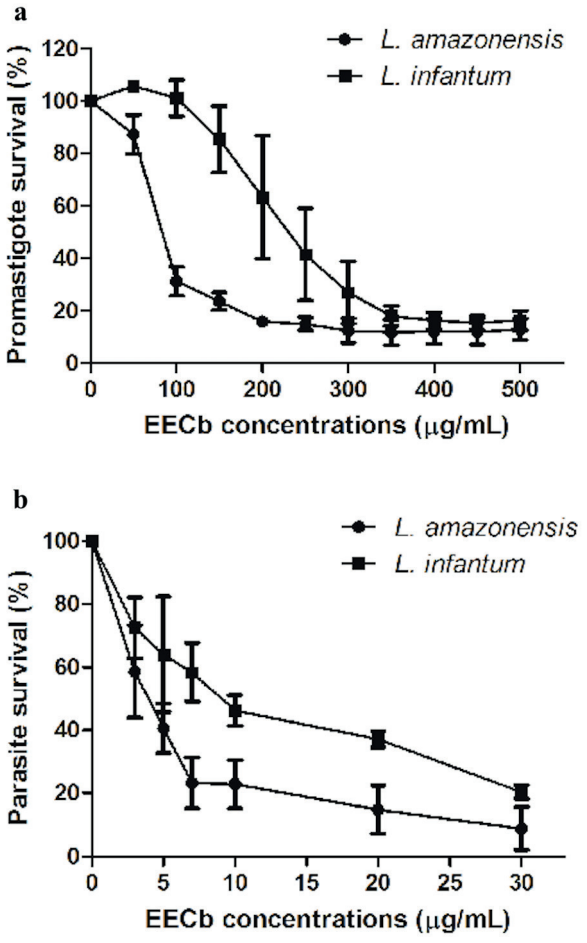

Figure 2. Effect of EECb on *L. amazonensis* and *L. infantum* survival. a: Effect of EECb on *L. amazonensis* and *L. infantum* promastigotes survival after 24 h of treatment, and b: effect of EECb on *L. amazonensis* and *L. infantum* amastigotes survival after 72 h of treatment. Results from three experiments in duplicate (a), and from two experiments in duplicate (b), are shown as percentages and standard errors of survival inhibition, compared to the untreated control.

Rosa *et al.* (Rosa et al. 2003) showed that the linalool-rich essential oil from the leaves of *C. cajucara* has a strong effect on the viability of *L. amazonensis* promastigotes (LD_{50} = 8.3 ng/mL) and amastigotes (LD_{50} = 22.0 ng/mL). The effect of *C. cajucara* essential oil included gross morphological changes in *L. amazonensis* promastigotes, such as disruption of flagellar membranes, mitochondrial swelling, and nuclear and kinetoplast chromatins destruction. More recently, Rodrigues et al. (2013) showed similar effects of *C. cajucara* essential oil on the morphology of *L. infantum* promastigotes. However, the IC_{50} obtained was 67.0 µg/mL, considerably higher than that previously observed for *L. amazonensis*. These observations corroborate the findings in the present study, since *L. amazonensis* promastigotes are more sensitive to the action of *Croton* products than *L. infantum*.

Even though antileishmanial drugs currently in use have strong *in vitro* activity against amastigote forms, they usually present high toxicity to mammalian host cells. On the other hand, several studies have shown that, at effective concentrations, essential oils of

Table I. ***In vitro*** **antileishmanial activity, cytotoxicity, specificity, and selectivity index for EECb.**

Treatment	Murine Macrophage	*L. amazonensis*				*L. infantum*			
		Promastigote	Amastigote	SI[a]	SPI[b]	Promastigote	Amastigote	SI[a]	SPI[b]
	CC_{50} (µg/mL)	IC_{50} (µg/mL)				IC_{50} (µg/mL)			
EECb	83.8 (64.0-101.4)	73.6 (63.0–86.0)	3.1 (2.4–3.8)	27.0	23.7	208.7 (176.7-246.5)	8.8 (7.4–10.2)	9.5	23.6
Pentamidine	2.8 (2.5-3.3)	1.63 (1.60-1.66)	0.64 (0.61-0.67)	4.3	2.5	1.94 (1.90-1.98)	0.14 (0.007-0.21)	20.0	13.8

[a] **SI: selectivity index (macrophage CC_{50}/amastigote IC_{50} ratio).**
[b] **SPI: specificity index (promastigote/amastigote IC_{50} ratio).**
95% confidence intervals are shown in brackets.

Croton, and their major compounds, present low toxicity to host cells, when compared with parasites (Guimaraes et al. 2010, Lima et al. 2015, Rodrigues et al. 2013, Rosa et al. 2003). Thus, to test the selectivity of EECb for the parasite, murine macrophages were treated with increasing concentrations of EECb; after 72 hours treatment, no cytotoxic effects were observed at concentrations below 60 µg/mL, and the CC_{50} obtained was 83.8 µg/mL.

Despite the difference in the sensitivity to EECb observed between *L. amazonensis* and *L. infantum*, the effect of the extract on parasite survival of both species was higher on amastigotes, when compared with promastigotes. The specificity index (SPI), corresponding to the ratio between the IC_{50} for promastigote and the IC_{50} for intracellular amastigotes, indicates the specificity of antileishmanial activity of a given compound. De Muylder et al. (2011) considered an SPI value greater than 2.0 as the cut-off point to define a compound as more active against intracellular amastigotes. The SPI obtained for EECb was higher than 20.0 for both *L. amazonensis* and *L. infantun* (Table I).

This is a relevant finding, as the targets for new chemotherapy are the intracellular amastigotes, i.e., the parasite form that survives and multiplies inside human macrophages (Croft & Coombs 2003). According to Guimarães et al. (2010) one of the possible explanations for the higher activity of a compound on intracellular amastigotes is that this compound may be responsible for increasing the effectiveness of the macrophage's microbicide response by producing NO. However, this seems not to be the case, since EECb did not cause macrophages to increase their NO production (data not shown). Alternatively, macrophages might accumulate higher levels of EECb, and this could be the possible explanation for the distinct action of EECb on promastigotes alone, and on amastigotes in an intracellular environment (Inacio et al. 2014).

The ratio between the CC_{50} of EECb for macrophages, and the IC_{50} of EECb for amastigotes, which corresponds to the SI, was 27.0 for *L. amazonensis* and 9.5 for *L. infantum*. Therefore, at concentrations lower than 10.0 µg/mL, EECb effectively reduced the survival of both *L. infantum* and *L. amazonensis* intracellular amastigotes. Besides, the extract appeared to be acting directly on the parasite, with no cytotoxic or immunomodulatory effect on mammalian macrophages.

Effect on parasite morphology and growth rate

Although *C. blanchetianus* extract showed leishmanicidal activity, its mechanism of action on the parasites remains unknown. Optical microscopic analysis of promastigotes treated with EECb at 100 µg/mL revealed a volume reduction and rounding of the cellular body, as well as flagellum duplication, as shown in Figure 3. Similar morphological changes were observed by da Silva et al. (da Silva et al. 2015b) in *L. amazonensis* promastigotes exposed to 100 µg/mL of *Physalis angulata* aqueous extract. Atypical flagella duplication has already been shown in *L. amazonensis* promastigotes treated with essential oil from *Cymbopogon citratus* (Santin et al. 2009). Although *P. angulata* and *C. citratus* are representative of genera different from that studied in this work, and certainly contain chemical compounds different from that present in EECb composition, morphological alterations such as flagella duplication seem to be a recurrent result of interference of plant extracts in the process of cell division, as suggested by Adade & Souto-Padrón (2010). Therefore, the present results suggest that EECb may be interfering with the process of cytokinesis, which could be causing a decline in the parasite multiplication rate.

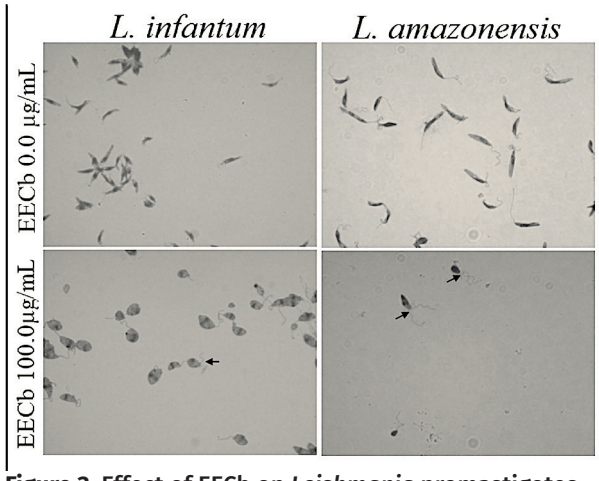

Figure 3. Effect of EECb on *Leishmania* promastigotes morphology as observed by microscopy. Untreated *L. amazonensis* and *L. infantum* promastigotes showing the typical elongated shape. *L. amazonensis* and *L. infantum* promastigotes treated with 100 µg/mL of EECb showed body rounding, cellular volume reduction, and flagellum duplication (arrowed). Magnification: 1000X.

In order to verify this hypothesis, the effect of EECb on promastigotes growth was monitored over five days. Considering that the same morphological changes were detected in both *L. amazonensis* and *L. infantum*, but since the results obtained so far have shown that *L. amazonensis* survival is more strongly affected by EECb action, *L. amazonensis* promastigotes were chosen to investigate the mechanism of action on *Leishmania*. The growth curves presented in Figure 4a show that, compared to the control, treatment with 70.0 and 140.0 µg/mL (concentration corresponding to nearly one and two times the IC_{50}) produced reductions of around 60% and 70%, respectively, in parasite count from 24 to 72 hours of growth. This caused the treated parasites to reach the stationary phase after only 96 hours, whereas the untreated reached this phase after 72 hours of cultivation. Indeed, a 35% reduction in the growth rate of the parasites was observed, as evidenced by the comparison of parasite doubling time, which increased from 6.9 hours in untreated parasites, to around 10.0 hours for parasites exposed to EECb (Figure 4b).

A possible explanation for the growth rate decrease could be the delay in the cell cycle caused by EECb. However, no significant changes in the cell cycle progression were observed in *L. amazonensis* promatigotes exposed to EECb, when compared with the control cells (data not shown). These results indicate that the effect of the extract on the growth kinetics is not due to an arrest on the cell cycle of the parasites. A similar scenario was observed by Scher et al. (2012) in response to overexpression of the gene anti-silencing factor 1 (ASF1) in *L. major*. The authors

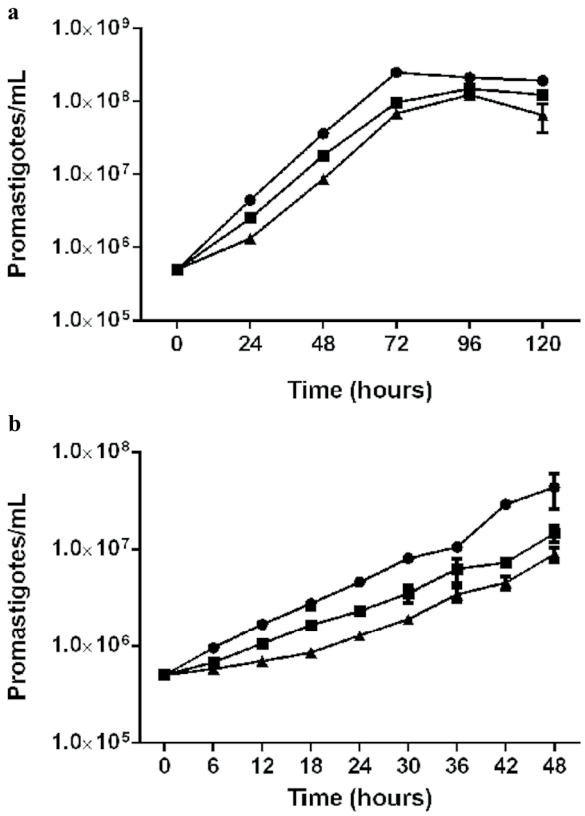

Figure 4. Effect of EECb on *L. amazonensis* promastigotes proliferation. *L. amazonensis* was cultivated in Schneider's insect medium in the absence (circle) or presence of 70.0 µg/mL (square) or 140.0µg/mL EECb (triangle), and the number of parasites was determined by direct counting with a Neubauer chamber, every 24 h for 5 days (a), and every 6 h for 48 h (b). The doubling time was calculated by the Least Squares Fitting-Exponential method accessed in the online platform Doubling Time, available at http://www.doubling-time.com/compute_more.php. Values are presented as the mean ± standard error of three different experiments in triplicate.

suggest that the delay in the growth rate could be a strategy used by the parasite to recover the cellular homeostasis compromised by adverse conditions. Therefore, it can be considered that, at the evaluated conditions, EECb affects the metabolism of *L amazonensis* promastigotes, so that it reduces parasite proliferation.

Effect on cell membrane integrity and mitochondrial membrane potential

Mitochondrial metabolism could be a relevant target for EECb action. Mitochondria are essential organelles for any cell survival, since they play an important role in energy metabolism. Maintaining the proper mitochondrial membrane potential is vital to the ATP production process, and consequently, to energy production by cells (Joshi & Bakowska 2011, Machado et al. 2014). In kinetoplastids parasites, such as *Trypanosoma* and *Leishmania*, mitochondrion is a single organelle essential for survival, and its proper functioning is extremely important, when compared with organisms that have multiple mitochondria, in which viable organelles can compensate for malfunctioning of the damaged ones (Shaha 2006). Thus, the mitochondria of trypanosomatides are considered one of the most fascinating target for drugs within protozoan organisms (Fidalgo & Gille 2011). Indeed, several studies have shown an association between trypanocidal action of some compounds and disorders in mitochondrial membrane potential, in *Leishmania* (Antinarelli et al. 2016, Britta et al. 2014, Corral et al. 2016, Fonseca-Silva et al. 2015, Kathuria et al. 2014), *Trypanosoma cruzi* (Martins et al. 2016, Menna-Barreto et al. 2009, Volpato et al. 2015), and *T. brucei* (Alkhaldi et al. 2016).

Thus, to verify if the reduction detected in the growth rate was related to the effect of EECb on mitochondrial metabolism, the mitochondrial membrane potential ($\Delta\Psi m$) was assessed using JC-1. This dye is lipophilic and concentrates in mitochondria in proportion to the membrane potential; increased JC-1 aggregate (red fluorescent) accumulation is observed inside mitochondria with greater $\Delta\Psi m$, whereas JC-1 monomers (green fluorescent) accumulate in the cytoplasm when $\Delta\Psi m$ decreases. The histograms of the fluorescence (Figure 5) demonstrated that the treatment with

70.0 and 140.0 µg/mL of EECb decreased the parasite mitochondrial membrane potential by 30.0% and 43.0%, respectively. In this case, the level of mitochondrial membrane depolarization of *L. amazonensis* promastigotes treated with 140 µg/mL of EECb did not differ from that observed following treatment with the standard drug Carbonylcyanide-4(trifluoromethoxy) phenylhydrazone (FCCP) (20µM).

These results suggest that the leishmanicidal activity of EECb is related to a marked depolarization of the mitochondrial membrane of parasites. Similar results were obtained by Da Silva et al. (2015) concernig the antipromastigote activity of phytol rich hexane fraction (PRF)

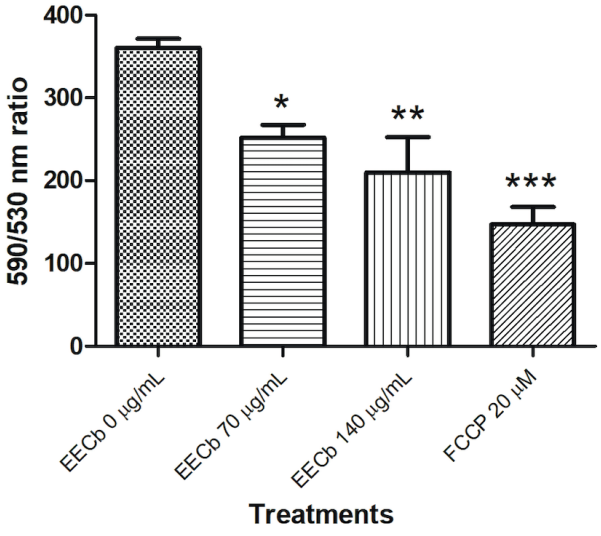

Figure 5. The effect of EECb on mitochondrial membrane potential (ΔΨm) in *Leishmania amazonensis*. *L. amazonensis* promastigotes were cultivated in Schneider's insect medium in the absence or presence of 70.0 µg/mL or 140.0µg/mL EECb for 24 h. Positive control was treated with FCCP (20 µM) for 20 min, and in the negative control (absence of EECb), the same volume of vehicle (DMSO) was added to the growth medium. Dose-dependent alterations in relative ΔΨm values are expressed as the ratio of the JC-1 fluorescence measurements at 590 nm (for J-aggregate) versus 530 nm (for J-monomer). Data are expressed as the means ± standard errors of three different experiments. Significant differences, relative to the control group, are indicated by * ($p<0.05$), ** ($p<0.01$), and * ($p<0.001$).**

from leaves of *Lacistema pubescens* on *L. amazonensis*. Sifaoui et al. (Sifaoui et al. 2014) demonstrated that treatment with maslinic acid extracted from Limouni olive leaf decreased the *L. amazonensis* and *L. infantum* mitochondrial membrane potential by 22.0% and 9.7%, respectively. Also, 81.7% reduction in Δ*Ψm* of *L. amazonensis* promastigotes was induced by Neem (*Azadirachta indica*) leaf extract (Dayakar et al. 2015). Further, mitochondrial dysfunction associated with ultrastructural changes on the mitochondrial matrix, with the appearance of complex structures and swelling of this organelle, was detected in *L. amazonensis* (Rosa et al. 2003) and *L. chagasi* promastigotes (Rodrigues et al. 2013) treated with linalool-rich essential oil from *Croton cajucara*. The fact that previous studies have shown that secondary products from plants from a range of genera, including *Croton,* can induce mitochondrial damage, supports our finding that the antiproliferative effect of EECb on *Leishmania* promastigotes is due to the generation of a defect in mitochondrial metabolism.

To better understand the effects of EECb on parasite viability, the membrane integrity was evaluated with PI staining. PI is a DNA and RNA intercalating dye that diffuses selectively through plasma membranes whose integrity has been disrupted (Britta et al. 2014). No changes in the PI fluorescence intensity were detected in parasites exposed to 70.0 and 140.0 µg/mL EECb after 24 hours (data not shown).

Taken together, the results showed that EECb presents activity against the promastigote and intracellular amastigote forms of *L. amazonensis* and *L. infantum,* with no effect upon mammalian cells. Moreover, mitochondrial dysfunction with no change in plasma membrane permeability appear to be the main effects involved in the delay in the growth rate caused by EECb on *L. amazonensis* promastigotes.

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