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HEALTH SCIENCES

Anti-*Leishmania amazonensis* activity of the terpenoid fraction from *Eugenia pruniformis* leaves

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Abstract: Leishmaniasis is caused by protozoan parasites belonging to the genus *Leishmania* and includes cutaneous, mucocutaneous and visceral clinical forms. Drugs currently available for leishmaniasis treatment present high toxicity, and development of parasite resistance. Plants constitute an important source of compounds with leishmanicidal potential. This study aimed to evaluate the anti-*Leishmania amazonensis* activity of the terpenoid fraction of *Eugenia pruniformis* leaves (TF-EpL). TF-EpL was active against the promastigote and intracellular amastigote forms of *L. amazonensis* with $IC_{50(24h)}$ value of 43.60µg/mL and 44.77µg/mL, respectively. TF-EpL altered the cell cycle of the parasite, increasing 2.32-fold the cells in the Sub-G0/G1 phase. TF-EpL also changed the ΔΨm and increased ROS and the number of annexin-V-PI positive promastigotes, which suggests incidental death. β-sitosterol, ursolic acid, corosolic acid and asiatic acid were isolated from TF-EpL. The results showed the antileishmanial activity of *E. pruniformis* terpenoids and its potential for further studies as a source of new drugs for leishmaniasis.

Key words: *Eugenia pruniformis*, *Leishmania amazonensis*, terpenoids, Leishmaniases.

INTRODUCTION

Leishmaniasis is a neglected tropical disease caused by unicellular protozoan parasites of the genus *Leishmania*, that affect 98 countries on 5 continents, with approximately 0.7 to 1 million cases of cutaneous leishmaniasis and 50.000 to 90.000 cases of visceral leishmaniasis occur every year. It causes several types of clinical manifestations and is classified as visceral, cutaneous and mucocutaneous forms. The pentavalent antimonials are the first-line drugs for leishmaniasis treatment, amphotericin B deoxycholate, liposomal amphotericin B and paromomycin, are secondary options for resistant cases (Burza et al. 2018). All these compounds have limitations in their use because of side effects, high cost, induction of parasite resistance and in-patient administration (Andrews et al. 2014). Miltefosine is the first oral treatment approved for leishmaniasis in India. However, it has a low efficacy against cutaneous leishmaniasis, besides being teratogenic (Kevric et al. 2015). Therefore, researches for new compounds are required.

Plants are an important source of biologically active compounds such as terpenoids that could be studied in the treatment of the neglected tropical diseases as leishmaniasis (Duarte et al. 2019, Ogungbe & Setzer 2013). Terpenoids

as β-sitosterol and ursolic acid have shown antileishmanial activity and were found in *Eugenia* genus (Myrtaceae) (Santos et al. 2008, Yamamoto et al. 2015, Frighetto et al. 2005), which in turn is represented by several species that have been used in folk medicine and are known for their antidiabetic, antirheumatic, antipyretic, antidiarrheal, anti-inflammatory, antifungal, antibacterial, antioxidant and cytotoxic activities (Reynertson et al. 2005, de Souza et al. 2018). Thus, species of this genus can be considered promising in order to obtain products to combat leishmaniasis and associate diseases.

Eugenia pruniformis, popularly known as "azeitoninha-da-praia", is distributed in sandbank vegetation along the Brazilian coast. The essential oil from the leaves of *E. pruniformis* has shown anticholinesterasic and antioxidant activities and β-caryophyllene as a major compound (Albuquerque et al. 2012). *E. pruniformis* has also shown wound healing activity in a skin rat model and the main flavonoid compounds present in the ethyl acetate extract are quercetin, kaempferol, and hyperoside (Albuquerque et al. 2016). Considering the anti-*Leishmania* potential of terpenes and the chemical diversity of the genus *Eugenia*, the present study aims to evaluate the anti-*Leishmania amazonensis* activity of the terpenoid fraction from *E. pruniformis* leaves (TF-EpL), as well as analyze its chemical composition.

MATERIALS AND METHODS

Plant material

Leaves and flowers of *Eugenia pruniformis* Cambess (Myrtaceae) were collected from four specimens in Restinga de Jurubatiba National Park, Rio de Janeiro State, Brazil, in open Clusia scrub vegetation (S22°12'40.85''–W41°35'14.61''; S22°12'40.85''–W41°35'14.61''; S22°12'36.36''–W41°35'20.18''; S22°12'34.90''–W41°35'21.04'') (Authorization number for scientific activities: 13659-3 SISGEN). Plant material was identified by the botanist Dr. Marcelo Guerra and a voucher specimen (M.G. Santos 2206) was deposited at the herbarium of the Faculdade de Formação de Professores (Universidade Estadual do Rio de Janeiro, Brazil).

Extraction and isolation of terpenoids

The leaves were subjected to drying in a forced ventilation oven, with a temperature of approximately 35°C, for 48 hours. Then, dried and powdered leaves (1200 g) were exhaustively extracted by maceration in 96 % (v/v) ethanol and the filtrate was concentrated under reduced pressure (35°C). The resulting hydroethanolic extract (143.7 g) was resuspended in 2.0 L of ethanol 90 % (v/v) and then partitioned with n-hexane (2 x 1.0 L) to obtain 35.0 g of n-hexane extract, with the yield of 2.92 % w/w. For preparative isolation, the n-hexane extract (20.0 g) was purified with acetone to afford the terpenoid fraction (TF-EpL), which was chromatographed on a Silica Gel 60 column eluted with n-hexane, ethyl acetate and methanol, using an increasing polarity gradient to yield 81 fractions (1-89) that were combined with the aid of Thin Layer Chromatography (TLC) analysis. Further purification with n-hexane: ethyl acetate (9:1) solution of Fraction 14 gave compound 1 (20.0 mg). Fraction 40 was finally purified with ethyl acetate to yield compound 2 (43.0 mg). Fractions 54-59 were purified with ethyl acetate to afford compound 3 (25.0 mg). Final purification with ethyl acetate of Fractions 74-77 gave compound 4 (25.0 mg). Isolated compounds 1, 2, 3 and 4 were in the form of a white powder.

Structural elucidation of the terpenoids

All isolated compounds were identified by ^{1}H and ^{13}C NMR spectral data analysis, including 1D and 2D NMR experiments and mass spectrometry. The NMR spectra were recorded on a Varian VNMRS 500 MHz spectrometer operating at 500 (^{1}H) and 125 (^{13}C) MHz. Chemical shifts are reported as δ values (ppm) with the residual solvent signal as the internal reference, with *J* in Hz. Deuterated methanol (CD_3OD) was obtained from the Cambridge Isotope Laboratories (USA) and used for solubilization.

HPLC-Q-TOF/MS analyses were carried out by Infinit 1200 system hyphenated to a 6530 Accurate mass spectrometer (Agilent) equipped with a quadrupole-time-of-flight (Q-TOF). TF-EpL sample was dissolved in methanol at a concentration of 5 µg/mL. Separations were performed on a reverse-phase (C_{18}, 30 mm, 2.1 mm x 3.5 µm; Zorbax). Water (A) and acetonitrile (B) were used as mobile phases as follows: 0-30 min, 40 to 95 % B. The flow rate was 0.3 mL/min and the injection volume was 5 µL. Mass spectra were recorded in negative ion mode. The Q-TOF/MS data were acquired in negative mode over a *m/z* range of 100–600, at a rate of 2 spectra/sec. The MS profile was performed in full scan mode and displayed in TIC (Total Ion Current) chromatogram. The conditions were as follows: gas temperature 325 °C; gas flow: 8 L/min; nebulizer: 40 psig. The raw data were acquired and processed with software from Agilent Technologies.

Parasites

Leishmania amazonensis (WHOM/BR/75/Josefa) promastigotes were cultured at 26°C in Schneider insect medium (Sigma), 10 % fetal calf serum (Gibco, MD, US) and 20 µg/mL of gentamicin (Schering-Plough, RJ, Brazil).

Ethics Statement

All animal experiments were performed in strict accordance with the Brazilian animal protection law (Lei Arouca number 11.794/08) of the National Council for the Control of Animal Experimentation (CONCEA, Brazil). The protocol was approved by the Committee for Animal Use Ethics of the Universidade Federal do Rio de Janeiro (Permit Number: 128/15).

Antipromastigotes activity

Stationary-phase promastigotes were treated with 40, 20, 10 and 5 µg/mL of TF-EpL for 24 h at 26°C and then were added 20 µL/well of MTT solution (5 µg/mL) (Sigma) and incubated for 4 hours at 37°C with 5 % CO_2. The culture was centrifuged for 15 minutes at 4000 rpm 4°C. Then, the supernatant was removed, and 0.5 mL DMSO was added to each well to dissolve the resulting formazan crystals. The absorbance was measured at a wavelength of 595 nm. The results are expressed in the percentage of viable cells compared to untreated control, as reported by (Ceole et al. 2017).

Antiamastigotes activity

Infected mice peritoneal macrophage cultures were treated with different concentrations TF-EpL during 24 h at 35°C, 5 % CO_2. Then, cultures were washed and incubated with 0.01 % sodium dodecyl sulfate for 10 min followed by 1.0 mL of Schneider's medium supplemented with 10 % FCS, and maintained at 26°C for 2 days. The relative intracellular load of viable *L. amazonensis* amastigotes was measured, after promastigote transformation, using Alamar Blue (Invitrogen), as previously described (Alves Passos et al. 2017). Mean values were calculated in 3 independent experiments using 5 wells per condition.

Cytotoxicity for host macrophages

Mice peritoneal macrophages adhered to 96-well plates were treated with the TF-EpL for 24 h and cell viability was determined by XTT (Sigma), as previously described (Ferreira et al. 2017). The selectivity index (SI) was calculated through the division of the $CC_{50(24h)}$ by $IC_{50(24h)}$ values.

Cell Cycle Analysis

Promastigotes were treated or not with 43.60 µg/mL TF-EpL, 0.1 µg/mL amphotericin B and 1 % DMSO for 24 h. The cells were fixed in 70 % ice-cold methanol for 1 h at 4°C. Then, the cells were incubated in phosphate buffered saline (PBS) supplemented with 10 µg/mL propidium iodide (PI) and 20 µg/mL RNAse at 37°C, 45 min. Next, were measured using BD FACScalibur (Becton and Dickson) and analyzed using CellQuest software, as reported (Ferreira et al. 2017).

Measurement of mitochondrial membrane potential (ΔΨm)

Promastigotes were treated or not with 43.60 µg/mL TF-EpL for 24 h and then incubated with 5 µg/mL JC-1 staining solution (Sigma) for 10 min at 37°C. ΔΨm was measured on a BD FACScalibur (Becton and Dickson) and analyzed using CellQuest software, as described previously (Alves Passos et al. 2017).

Detection of Reactive Oxygen Species (ROS)

Promastigotes were treated or not with 43.60 µg/mL TF-EpL for 24 h at 26°C and then stained with 50 µM DCFDA (Sigma) for 30 min, and ROS was measured immediately using a BD FACScalibur (Becton and Dickson) and analyzed using CellQuest software, as reported (Eruslanov & Kusmartsev 2010).

Quantification of polar and neutral hydrophobic domains

Promastigotes (10^6 cells/mL) were treated with 43.60 µg/mL TF-EpL for 24 h at 26°C. Cells were centrifuged and resuspended in PBS. Nile Red (1 µg/mL, Sigma) was added and the cells were incubated at room temperature for 7 min. After three wash with PBS, the red and yellow fluorescence were measured immediately using BD FACScalibur (Becton and Dickson) and analyzed using CellQuest software, as reported (Greenspan et al. 1985).

Statistical analysis

Data were analyzed using Student's t-test when comparing two groups or one-way ANOVA for more than two groups using the software GraphPad Prism. *P* values of less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Leishmaniasis is a neglected parasitic disease for which the current antileishmania therapies are complicated by drug toxicity, need for parenteral administration, high cost, increasing treatment failure rates, and emergence of drug resistance (Burza et al. 2018). Natural products extracted from a crude extract of plants or fractions are a good source of diverse chemical structures that show potent biological profile and pharmacological activities (Duarte et al. 2019, Ogungbe & Setzer 2013). In a search for new antileishmanial drugs and improve the chemical knowledge of *E. pruniformis*, the effect os TF-EpL on *L. amazonesis*, a Brazilian endemic species, was studied. In humans, this species may cause cutaneous leishmaniasis, a severe anergic diffuse cutaneous leishmaniasis and the visceral form of this disease (Burza et al. 2018).

The terpenoid fraction was obtained from the hydroethanolic extract of *E. pruniformis* leaves. In this study, the effect of this fraction TF-EpL on promastigotes and amastigotes forms of *L. amazonensis* was investigated, as well the cytotoxicity for murine macrophages. TF-EpL in the promastigote forms, which are present in the invertebrate host, presented an IC_{50} value of 43.60 ± 4.272 µg/mL (Fig. 1a), while in the intracellular amastigote forms, which belong to the vertebrate host, TF-EpL presented an IC_{50} of 44.77 ± 13.96 µg/mL (Fig. 1b).

The leishmanicidal effect of plant extracts is already described in the literature. Our group demonstrated that the crude extract of branches from *Guatteria latifolia* presents an IC_{50} value of 51.7 and 30.5 µg/mL against *L. amazonensis* promastigotas and amastigotes, respectively (Ferreira et al. 2017). Rodrigues et al. (2013) demonstrated that *E. uniflora L.* essential

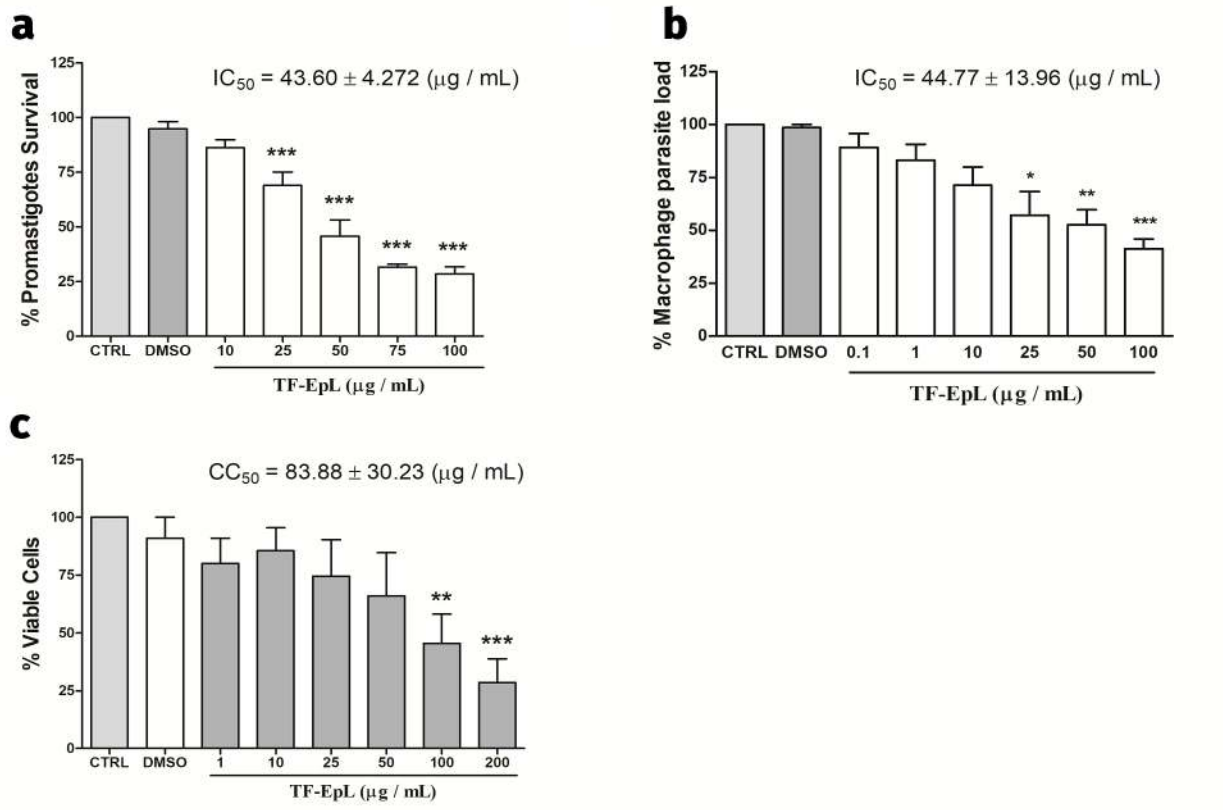

Figure 1. Inhibition of *L. amazonensis* proliferation by terpenoid fraction of *E. pruniformis* leaves (TF-EpL) and cytotoxicity against murine peritoneal macrophages *in vitro*. (a) Promastigotes (10^7 / mL) were grown in the presence or absence of TF-EpL, or diluent (DMSO), at the indicated concentrations, during 24 h. Parasite viability was checked by the dehydrogenases activity using the MTT assay. The results were expressed as the percentage of viable promastigotes in relation to the untreated control (100 %) and are shown as the mean ± SEM of 3 independent experiments. (b) Peritoneal macrophages (10^5) from BALB/c mice were infected with promastigotes at a ratio of 10 parasites to 1 macrophage for 24 h and were either left untreated or were treated with the indicated concentrations of TF-EpL for 24 h, washed, fed with Schneider's complete medium, and cultured at 26 °C. The macrophage parasite load was evaluated after 48 h using Alamar Blue. (c) Cells were cultured with 1, 10, 25, 50, 100 and 200 µg/mL TF-EpL for 24 h at 37 °C in 5 % CO_2. Viability was measured by the XTT method. The results represent the means ± SEM from 3 experiments performed in triplicate. $p < 0.05$ (*), $p < 0.001$ (**) and $p < 0.0001$ (***) compared to the control.

oil presented anti-*L. amazonensis* activity with IC_{50} of 1.75 µg/mL after 72h of treatment. However, the viability of the macrophages was altered, presenting significant toxicity at the concentration of 6.25 µg/mL, with an CC_{50} value of 45.3 µg/mL. TF-EpL also affects *L. amazonensis* strain MHOM/BR/77LTB0016, with an IC_{50} of 8.50 µg/mL after 24 hours of treatment (data not shown).

Analysis of results from the cytotoxicity assay, performed on mice peritoneal macrophages, revealed that TF-EpL was less toxic to the host cells than to parasite with the $CC_{50(24h)}$ value of 83.88 µg/mL (Fig. 1c). Thus, the TF-EpL showed SI values of 1.92 for promastigotes and 1.87 for intracellular amastigotes forms.

The effects of TF-EpL on the parasite and the cell cycle of *L. amazonensis* promastigotes were analyzed (Fig. 2). Untreated promastigotes (Fig. 2a), 1 % DMSO (Fig. 2b), 43.60 µg/mL TF-EpL (Fig. 2c) or 0.1 µg/mL amphotericin B (Fig. 2d), were labeled with propidium iodide in cell cycle solution and analyzed via flow cytometry. Our results showed that TF-EpL arrested *L. amazonensis* promastigote cycle, increasing 2.32-fold the number of cells in the sub-G0/G1 phase. Amphotericin B, used as a control, increased 2.18-fold the number of cells in the sub-G0/G1 phase and decreased 2.05-fold the number of cells in G0/G1 phase.

Leishmania presents a single mitochondria, an organelle that plays an important role in energy metabolism, and the dysfunction of this organelle can lead the parasite death. Thus, the parasite mitochondrial toxicity of TF-EpL was investigated by the reduction of the mitochondrial membrane potential (ΔΨm) using the JC-1 assay. Our data indicated that 43.60 µg/mL TF-EpL increased 1.04-fold the ΔΨm in comparison of untreated cells (Fig. 2e). Thus, TF-EpL affects parasite mitochondria, inducing its hyperpolarization, which is associated with apoptosis, as shown for *L. amazonensis* after treated with the hexane fraction of *Serjania lethalis* leaves (Alves Passos et al. 2017).

To elucidate the possible type of cell death was involved in the TF-EpL-treated promastigotes, the effects of treatment on ROS production and phosphatidylserine exposure were tested, by labeling with DCFDA probe and Annexin-V-PI, respectively (Fig. 2f and Fig. 3). Our results show that after 24 hours of treatment with 43.60 µg/mL TF-EpL, the ROS production increase 1.53-fold in a relation of control (Fig. 2f). TF-EpL treated promastigote increase 7.20-fold PI labeling (Fig. 3d), however, Annexin-V binding to TF of *E. pruniformis* TF-EpL treated promastigote was similar to the controls (Fig. 3e). The association between hyperpolarization and increased ROS generation may also result in the initiation and occurrence of necrotic cell death (Van Den Berghe et al. 2010). Moreover, TF-EpL increased 6.46-fold annexin-V-PI binding to the promastigotes (Fig. 3f).

In this paper, the changes in the parasite cell cycle were demonstrated, alterations in the ΔΨm and increased of ROS and percentage of annexin-V-PI labeling after TF-EpL treatment, indicating incidental death of *L. amazonensis* promastigotes (Proto et al. 2013). Corroborating our data, Ceole et al. (2017) demonstrated that nerolidol, the main constituent of *Piper aduncum* essential oil, has anti-*Leishmania braziliensis* activity, such as mitochondrial membrane depolarization, phosphatidylserine exposure, and cell size reduction, indicating incidental death process.

The effect of TF-EpL on the accumulation of lipid bodies with Nile Red was evaluated by flow cytometry for quantification of polar hydrophobic domains (red fluorescence) and neutral hydrophobic domains (yellow fluorescence). The treatment with 43.60 µg/mL of TF-EpL decreases 1.26-fold the accumulation

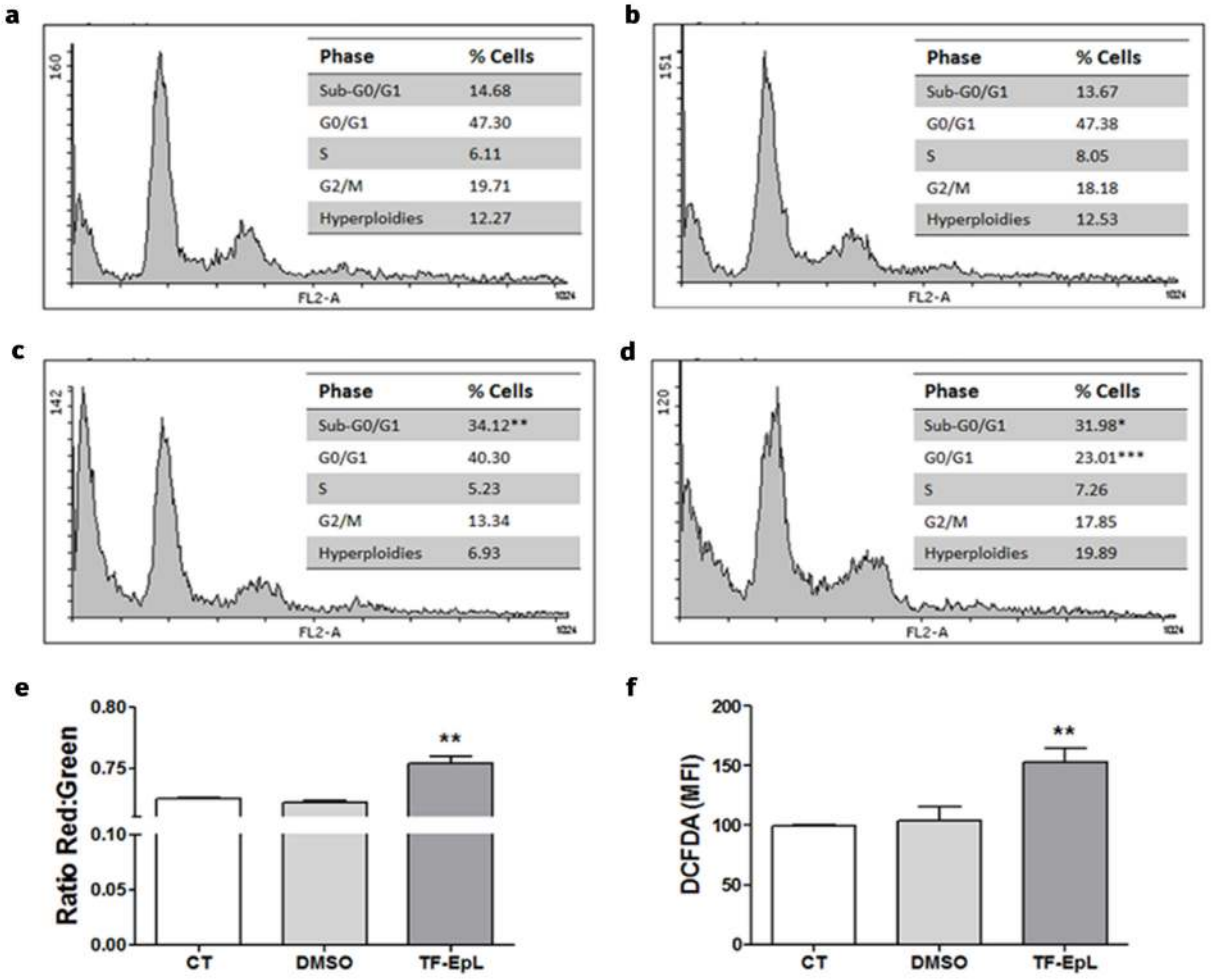

Figure 2. Evaluation of cell cycle, mitochondrial membrane potential (ΔΨm) and reactive oxygen species (ROS) production by *Leishmania amazonensis* promastigotes. Promastigotes were treated or not with TF-EpL and cell cycle was evaluated after 48h after PI staining by flow cytometry analysis. Untreated parasites control (a), 1 % DMSO (b), 43.60 µg / mL TF-EpL (c) and 0.1 µg/mL Amphotericin B (d). Representative plots of at least three independent experiments with similar results. (e) Promastigotes treated or not with 43.60 µg/mL TF-EpL was evaluated using a JC-1 assay. The results are expressed as red / green fluorescence ratios and represent the averages ± SEM from 3 independent experiments. (f) Promastigotes treated or not with 43.60 µg/mL TF-EpL or 1 % DMSO were then stained with 50 µM DCFDA and ROS was measured by flow cytometer. The results represent the means ± SEM from 3 experiments performed in triplicate. $p < 0.05$ (*), $p < 0.001$ (**), and $p < 0.0001$ (***) compared to the untreated control.

of neutral hydrophobic domains (Fig. 4a) but do not alter the polar hydrophobic domains or phospholipids (Fig. 4b). The reduction of neutral lipid in TF-EpL treated parasites suggested either inhibition or a deviation in the lipidic synthesis pathway (Ferreira et al. 2011).

The fractionation of TF-EpL led to the isolation of four compounds (1 – 4), identified by NMR (Table I) and HPLC-Q-TOF/MS. ^{1}H-NMR spectrum of 1 showed six methyl signals as two methyl singlets at δ_H 0.66 (s, H-16) and δ_H 0.99 (s, H-19), three methyl doublets at δ_H 0.79 (d, H-27), δ_H 0.81 (d, H-26) and δ_H 0.90 (d, H-21) and a methyl

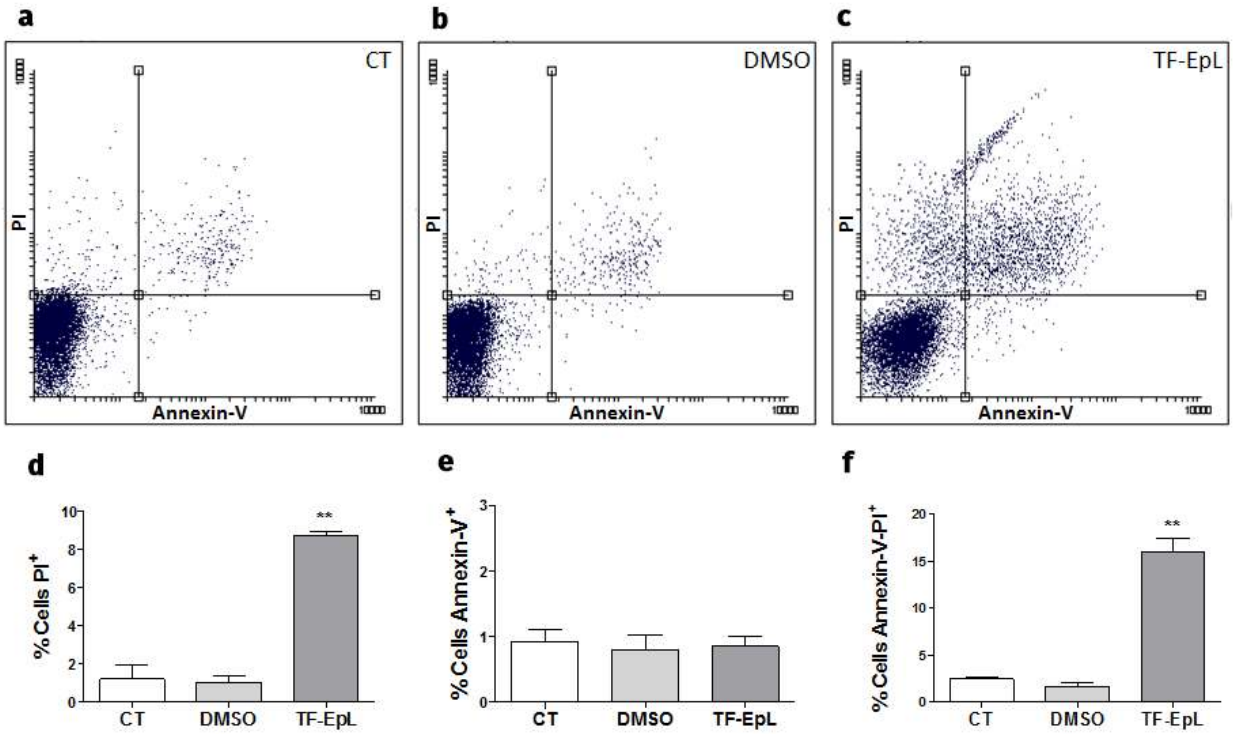

Figure 3. Assessment of Annexin-V expression on *Leishmania* induced by terpenoid fraction of *E. pruniformis* leaves (TF-EpL) treatment. Promastigotes were treated or not with TF-EpL and the expression of Annexin-V / Propidium Iodide (PI) measured. Untreated parasites control (a), parasites treated with 1 % DMSO (b) and 43.60 µg/mL TF-EpL (c) are shown as a representative result out of 3 independent experiments with similar results. The percentage of PI positive (d), Annexin-V positive (e) and Annexin-V-PI positive promastigotes (f), treated as above, shown as mean ± SEM of 3 independent experiments. p < 0.001 (**) compared to the control.

triplet at δ_H 0.84 (t, H-24). Also, a signal referent to the olefinic proton appeared at δ_H 5.33 (d, H-5) and a triplet of double doublets at δ_H 3.51 (tdd, H-3) of a proton connected to the C-3 hydroxy group. The ^{13}C-NMR spectrum of 1 exhibited typical twenty-nine signals of terpenes with a hydroxylated carbon at δ_C 71.83 (C-3), an olefinic carbons at δ_C 141.71 (C-5) and 121.71 (C-6), bound to the longer branch of the structure at δ_C 56.04 (C-17), a methyl carbons at δ_C 18.77 (C-28) and 11.85 (C-29) and terminal carbons of the main branch at δ_C 19.03 (C-19), 11.97 (C-24), 19.80 (C-26) and 19.38 (C-27). Afterward, the identification of compound 1 was followed using accurate mass measurements by HPLC-Q-TOF/MS analysis that presented deprotonated molecular ion peaks at *m/z*: 413.17091 $[M-H]^-$ ($C_{29}H_{50}O_1$). Analysis of data obtained associated with literature data (Aguirre et al. 2006) allowed the identification of 1 as β-sitosterol (Fig. 5).

β-sitosterol was found in different species from *Eugenia* genus (Frighetto et al. 2005) and was active against *Leishmania infantum* in promastigotes and amastigotes forms (Santos et al. 2008) and against of *Leishmania tropica* promastigotes (Majid Shah et al. 2019). Besides, this phytosterol exhibited analgesic and anti-inflammatory activities that could be helpful for the treatment of secondary infections in leishmaniasis (Bin Sayeed et al. 2016).

The ^{1}H-NMR spectrum of 2 showed as main signals, the presence of five methyl as singlets at

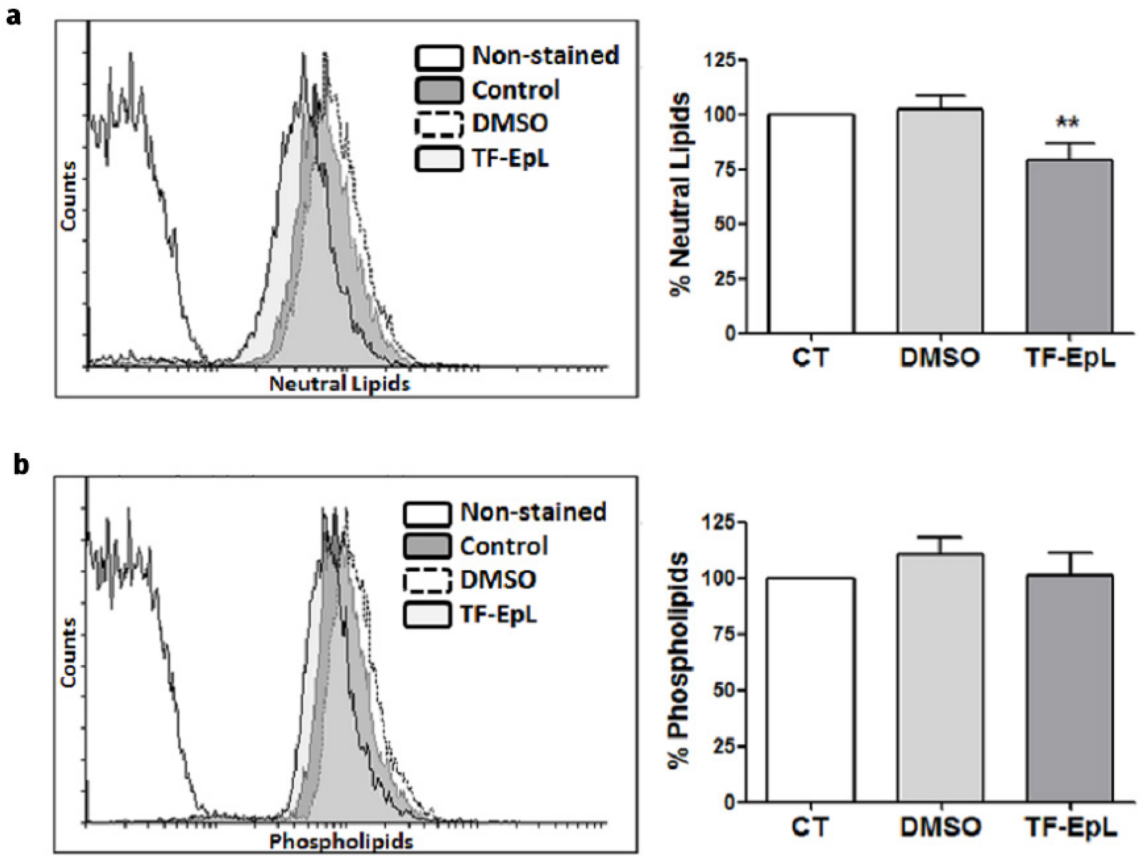

Figure 4. Effect of terpenoid fraction of *E. pruniformis* leaves (TF-EpL) on the synthesis of total lipids on *Leishmania*. Promastigotes were incubated in the presence or absence of 43.60 µg/mL TF-EpL for 24 h. Cells were labeled with Nile Red (1 µg/mL) and the percentage of neutral lipids (a) and phospholipids (b) were estimated in flow cytometry. Histograms are representative of 3 independent experiments with similar results. p < 0.001 (**), compared to control.

δ_H 1.26 (s, H-23), δ_H 1.25 (s, H-27), δ_H 1.08 (s, H-26), δ_H 1.04 (s, H-24) and δ_H 0.92 (s, H-25), a double doublet at δ_H 3.48 referent to H-3 of hydroxylated carbon and a triplet at δ_H 5.51 referent to H-12 bonded to sp^2 carbon. The combined two-dimensional ^{1}H-^{1}H data COSY showed important correlations as H-12 to H-11 (δ_H 5.51 and1.99), H-3 to H-2 (δ_H 3.48 and 1.84), H-18 to H-19 (δ_H 2.66 and 1.49), H-15β to H-27 (δ_H 2.35 to 1.25), H-20 to H-21α (δ_H 1.07 and 1.40). The ^{13}C-NMR spectrum of 2 showed thirty signals referents to a triterpenoid with the characteristic signals at δ_C 78.6 (C-3), 126.1 (C-12), 139.7 (C-13), 40.1 (C-19 and C-20) and 180.0 (C-28). Compound 2 was analyzed by HPLC-Q-TOF/MS and presented a deprotonated molecular ion at *m/z* 455.32534 $[M-H]^-$ ($C_{30}H_{48}O_3$). Comparing these spectral data to those reported in the literature (Gnoatto et al. 2008), compound 2 was identified as ursolic acid (Fig. 5).

Ursolic acid was identified in some *Eugenia* species as *E. gustavoides*, *E. florida* and *E. brasiliensis* and was reported to show anti-*Leishmania amazonensis* activity against promastigotes and amastigotes forms of the parasite ($IC_{50(24h)}$ = 6.4 µg/mL and $IC_{50(24h)}$ = 27.0 µg/mL, respectively) (Frighetto et al. 2005, Yamamoto

et al. 2015, Torres-Santos et al. 2004). Moreover, ursolic acid was also described to has activity against the visceral form of *Leishmania* (Jesus et al. 2017) and also anti-inflammatory property by suppression of NF-κB, AP-1, and NF-AT (Checker et al. 2012). Thus, β-sitosterol and ursolic acid may be responsible for the anti-parasitic activity of the TF-EpL which these compounds were isolated as main constituents. Furthermore, the anti-inflammatory activity is described in the literature for these isolated triterpenes, which are also known as antibacterial agents (Singh & Sharma 2015) and maybe useful to avoid secondary infections caused by leishmaniasis.

The ^{1}H-NMR spectrum of 3 showed as main signals, the presence of a triplet at δ_H 5.24 (t, H-12), a double double-doublet at δ_H 3.63 (ddd, H-2) referent to H-2 and H-3, bonded to hydroxylated carbons, a triplet at δ_H 2.91 (d, H-3) and singlet methyl of H-23, H-24, H-29 and H-30 at δ_H 1.02, 0.81, 0.97 and 0.87, respectively. The ^{13}C-NMR spectrum of 3 exhibited thirty signals characteristic of triterpenoids with typical signals at δ_C 68.1 (C-2), 86.1(C-3), 126.9 (C-12), 139.9 (C-13), 31.0 (C-23), 19.1 (C-24), 19.3 (C-29), 23.2 (C-30). Compound 3 was analyzed by HPLC-Q-TOF/MS and presented a deprotonated molecular ion peak at *m/z*: 471.14000 $[M-H]^-$ ($C_{30}H_{48}O_4$). Analysis of these spectral data compared to those reported in the literature (Aguirre et al. 2006) allowed the identification of compound 3 as corosolic acid (Fig. 5).

Corosolic acid was reported in *E. gustavoides* and asiatic acid was found in *E. gustavoides* and *E. crebinervis* (Frighetto et al. 2015). According to the literature, corosolic acid presented anti-inflammatory activity by different mechanisms such as attenuating of apoptotic and oxidative stress. Corosolic acid inhibits ethanol-induced apoptosis and increases the levels of tumor necrosis factor-α (TNF-α) and reactive oxygen species (ROS) *in vitro* (Guo et al. 2016). Furthermore, corosolic acid was effective as an anti-inflammatory agent through the arachidonic acid cascade inhibition, with similar potency to nimesulide (Aguirre et al. 2006).

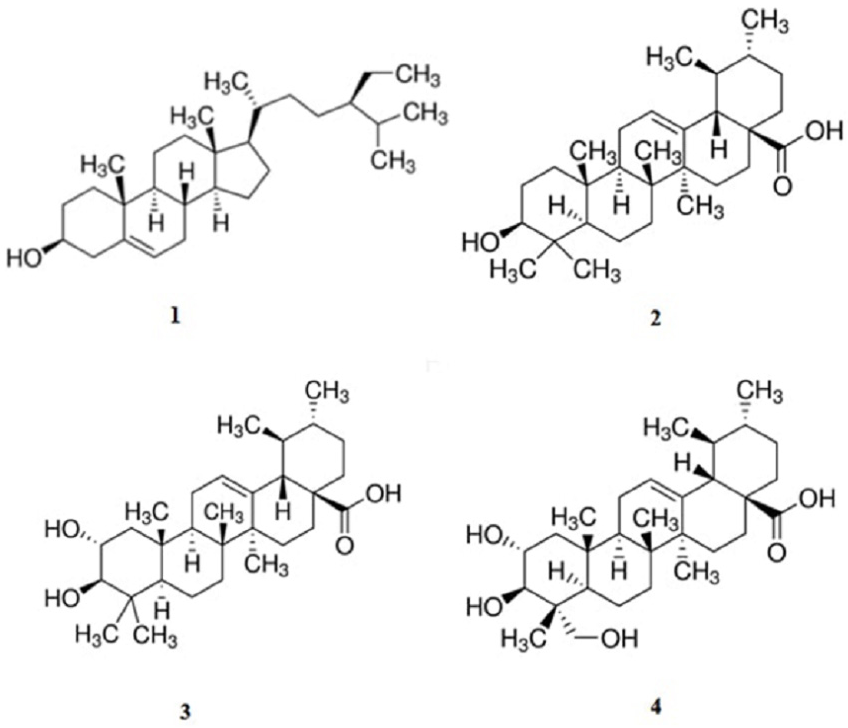

Figure 5. Triterpenoids from *E. pruniformis* leaves. 1 – β-Sitosterol; 2 – Ursolic acid; 3 – Corosolic acid; 4 – Asiatic acid.

Table I. ^{1}H. and ^{13}C-NMR Data of Compounds 1, 2, 3 and 4, in ppm. Assignments of ^{13}C-NMR data were accomplished with the aid of DEPT, HSQC and HMBC experiments.

	β-sitosterol		Ursolic Acid		Corosolic Acid		Asiatic Acid	
Position	**δ H**	**δ C**	**δ H**	**δ C**	**δ H**	**δ C**	**δ H**	**δ C**
1		37.2		40.0		46.9		46.6
2		31.6	1.84	28.8	3.63 (ddd)	68.2	3.69 (ddd)	68.2
3	3.51 (m)	71.8	3.48 (dd)	78.8	2.91 (d)	83.1	3.36 (dd)	76.8
4		42.3		39.7		39.1		42.7
5	5.33 (d)	140.7		56.5		55.3		46.8
6		121.7	Hα 1.59 (t)	19.4		18.1		17.7
7		31.9	Hα 1.60 (t)	34.2		32.8		32.2
8		31.9		40.6		39.4		39.4
9		50.1	1.65 (t)	48.7		47.6		47.4
10		36.5		37.9		37.8		37.6
11		21.1	1.99 (m)	24.3		23.0		23.2
12		39.8	5.51 (t)	126.3	5.24 (t)	125.3	5.26 (t)	125.2
13		42.3		139.9		138.3		138.4
14		56.8		43.2		41.9		41.9
15		26.1	Hβ 2.35 (td)	29.3		27.8		27.7
16	0.66 (s)	28.2	2.15 (td)	25.6		23.9		23.9
17		56.0		48.7		47.6		48.4
18		36.1	2.66 (d)	54.2	2.21 (d)	53.0	2.21 (d)	52.8
19	0.99 (s)	19.0	1.49 (t)	40.1		39.0		39.0
20		34.0	1.07	40.1		39.0		39.0
21	0.90 (d)	26.1	Hα 1.40 (t)	31.7		30.4		30.3
22		45.8	1.96 (m)	37.9		36.7		36.7
23		23.1	1.26 (s)	29.5	1.02 (s)	27.9	3.50 (d)	64.9
24	0.84 (t)	12.0	1.04 (s)	17.2	0.81 (s)	16.1	0.70 (s)	12.5
25		29.2		16.3	1.01 (s)	15.8	1.03 (s)	16.2
26	0.81 (d)	19.8	1.08 (s)	18.1	0.85 (s)	16.4	0.85 (s)	16.4
27	0.79 (d)	19.4	1.25 (s)	24.6		22.7	1.14 (s)	22.7
28		18.8		180.2		180.2		180.2
29		11.9	1.03 (d)	18.2	0.89 (d)	16.2	0.89 (d)	16.2
30	-	-	0.98 (d)	22.1	0.97 (s)	20.1	0.97 (s)	20.1
U.S.	1.12-2.32 (m)							

U.S. = Undefined NMR Signals. Data were recorded at 500 (^{1}H) and 125 (^{13}C) MHz. *Some multiplicities are omitted due to overlapping events.

The ^{1}H-NMR spectrum of asiatic acid (4) exhibited also characteristic triterpenoids signals with the presence of H-12 as a triplet at δ_H 5.26, H-2 and H-3, bonded to hydroxylated carbons, as a double double-doublet at δ_H 3.69 and as a triplet at δ_H 3.36, respectively, and the singlet methyl of H-23, H-24, H-29 and H-30 at δ_H 3.27, δ_H 0.70, δ_H 0.89 and δ_H 0.97, respectively. The ^{13}C-NMR spectrum of 4 also showed a typical thirty signals of triterpenoids such as at δ_C 68.2 (C-2), 76.8 (C-3), 125.2 (C-12), 138.4 (C-13), 64.9 (C-23), at δ_C 12.5 (C-24), 16.2 (C-29), 20.1(C-30). These data, associated to that obtained from HPLC-Q-TOF with the quasi-molecular ion peak $[M-H]^-$ of 487.48385 ($C_{30}H_{48}O_5$) and those found in the literature (Aguirre et al. 2006), allowed the structural identification of asiatic acid (Fig. 5).

Asiatic acid possesses a wide spectrum of biological activities, notably anti-inflammatory (Hao et al. 2017) and antimicrobial effects (Harnvoravongchai et al. 2018). Hao and collaborators (Hao et al. 2017) show that asiatic acid significantly inhibited LPS-induced IL-6 and IL-8 expression levels in gingival tissues and significantly attenuated LPS-induced PGE2, NO, IL-6, and IL-8 production *in vivo*. Besides, asiatic acid displayed substantial inhibitory effects on *Clostridium difficile*, the causative agent of antibiotic-associated diarrhea, with the minimal inhibitory concentrations (MIC) value of 10.0 µg/mL, and also displayed an inhibitory effect on cell motility (Harnvoravongchai et al. 2018).

In conclusion, the results of our study show that TF-EpL have anti-*Leishmania amazonensis* activity of *in vitro*, which might be associated with promastigotes' incidental death, and its potential for further studies as a source of new drugs for leishmaniasis.

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RDDGA (PhD. student) contributed to collecting plant samples, running the laboratory work, analysis of the data and drafted the paper. MGS contributed to plant identification and herbarium confection. RSE contributed to the phytochemical analysis. VFA and GBB contributed to anti-promastigote assay in strain MHOM / BR / 77LTB0016. DCS contributed to anti-amastigote studies. CF, CLAP, and EF designed the leishmanicidal studies in strain WHOM / BR / 75 / Josefa analysis of the data and drafted the paper. ALMM contributed to the chromatographic analysis. APO and LMR designed the study, supervised the laboratory work and contributed to the critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.