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)} values 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 “azeitóninha-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′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 \(\delta\) values (ppm) with the residual solvent signal as the internal reference, with \(J\) in Hz. Deuterated methanol (CD\(_3\)OD) 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.

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.

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).
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 CC50(24h) by IC50(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 (Euslanov & Kusmartsev 2010).

Quantification of polar and neutral hydrophobic domains
Promastigotes (10⁶ 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. amazonensis, 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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...
oil presented anti-\textit{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 \textit{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 \textit{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 \textit{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. 

\textit{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 (∆Ψm) using the JC-1 assay. Our data indicated that 43.60 µg/mL TF-EpL increased 1.04-fold the ∆Ψm in comparison of untreated cells (Fig. 2e). Thus, TF-EpL affects parasite mitochondria, inducing its hyperpolarization, which is associated with apoptosis, as shown for \textit{L. amazonensis} after treated with the hexane fraction of \textit{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 \textit{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 ∆Ψm and increased of ROS and percentage of annexin-V-PI labeling after TF-EpL treatment, indicating incidental death of \textit{L. amazonensis} promastigotes (Proto et al. 2013). Corroborating our data, Ceole et al. (2017) demonstrated that nerolidol, the main constituent of \textit{Piper aduncum} essential oil, has anti-\textit{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.
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 $\delta_H$ 0.66 (s, H-16) and $\delta_H$ 0.99 (s, H-19), three methyl doublets at $\delta_H$ 0.79 (d, H-27), $\delta_H$ 0.81 (d, H-26) and $\delta_H$ 0.90 (d, H-21) and a methyl
triplet at δH 0.84 (t, H-24). Also, a signal referent to the olefinic proton appeared at δH 5.33 (d, H-5) and a triplet of double doublets at δH 3.51 (tdd, H-3) of a proton connected to the C-3 hydroxy group. The 13C-NMR spectrum of 1 exhibited typical twenty-nine signals of terpenes with a hydroxylated carbon at δC 71.83 (C-3), an olefinic carbons at δC 141.71 (C-5) and 121.71 (C-6), bound to the longer branch of the structure at δC 56.04 (C-17), a methyl carbons at δC 18.77 (C-28) and 11.85 (C-29) and terminal carbons of the main branch at δC 19.03 (C-19), 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]⁻ (C29H50O1). 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 1H-NMR spectrum of 2 showed as main signals, the presence of five methyl as singlets at
δH 1.26 (s, H-23), δH 1.25 (s, H-27), δH 1.08 (s, H-26), δH 1.04 (s, H-24) and δH 0.92 (s, H-25), a double doublet at δH 3.48 referent to H-3 of hydroxylated carbon and a triplet at δH 5.51 referent to H-12 bonded to sp² carbon. The combined two-dimensional ¹H-¹H data COSY showed important correlations as H-12 to H-11 (δH 5.51 and 1.99), H-3 to H-2 (δH 3.48 and 1.84), H-18 to H-19 (δH 2.66 and 1.49), H-15β to H-27 (δH 2.35 to 1.25), H-20 to H-21α (δH 1.07 and 1.40). The ¹³C-NMR spectrum of 2 showed thirty signals referents to a triterpenoid with the characteristic signals at δC 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]⁻ (C₃₀H₄₈O₃).

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 (IC₅₀(24h) = 6.4 µg/mL and IC₅₀(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 have 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 δH 5.24 (t, H-12), a double double-doublet at δH 3.63 (ddd, H-2) referent to H-2 and H-3, bonded to hydroxylated carbons, a triplet at δH 2.91 (d, H-3) and singlet methyl of H-23, H-24, H-29 and H-30 at δH 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 δC 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.](https://example.com/figure5.png)
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.

| Position | \( ^1 \)H | \( ^{13} \)C | \( ^1 \)H | \( ^{13} \)C | \( ^1 \)H | \( ^{13} \)C | \( ^1 \)H | \( ^{13} \)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    | H\( \alpha \) 1.59 (t) | 19.4     | 18.1     | 17.7     |
| 7        | 31.9     | H\( \alpha \) 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     | H\( \beta \) 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     | H\( \alpha \) 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. = 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 $\delta_H 5.26$, H-2 and H-3, bonded to hydroxylated carbons, as a double double-doublet at $\delta_H 3.69$ and as a triplet at $\delta_H 3.36$, respectively, and the singlet methyl of H-23, H-24, H-29 and H-30 at $\delta_H 3.27$, $\delta_H 0.70$, $\delta_H 0.89$ and $\delta_H 0.97$, respectively. The $^{13}$C-NMR spectrum of 4 also showed a typical thirty signals of triterpenoids such as at $\delta_C 68.2$ (C-2), 76.8 (C-3), 125.2 (C-12), 138.4 (C-13), 64.9 (C-23), at $\delta_C 12.5$ (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.