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Antileishmanial activity of essential oil and 6,7-dehydroroyleanone isolated from *Tetradenia riparia*

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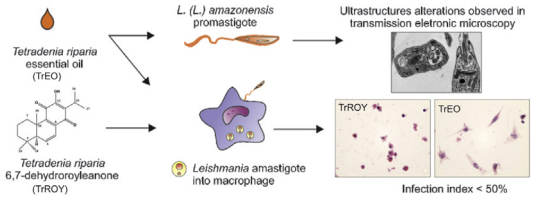
HIGHLIGHTS

- *Tetradenia riparia* essential oil induces the ultrastructures changes on *Leishmania*.
- *T. riparia* 6,7-dehydroroyleanone acts against *L. (L.) amazonensis* infection.
- *T. riparia* essential oil did not induce iNOS mRNA expression in *Leishmania* infection.

GRAPHICAL ABSTRACT



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ABSTRACT

Tetradenia riparia plant is used as a traditional medicine in Africa for the treatment of inflammatory and infectious diseases as like parasitic. Therapy for leishmaniasis caused by *Leishmania (Leishmania) amazonensis* specie often fails, and the conventional drugs are toxic, expensive, require a long period of treatment, and adverse effects are common. The alternative therapies using natural products are inexpensive and have few or any adverse reaction. These reasons are sufficient to investigate the new natural therapeutic for leishmaniasis. We evaluated the potential of the essential oil (TrEO) and 6,7-dehydroroyleanone (TrROY) isolated from *T. riparia* on *L. (L.) amazonensis* promastigote and amastigote forms, cytotoxicity on human erythrocytes and murine macrophages, nitric production and inducible nitric oxide synthase (iNOS) mRNA expression. TrEO was the most effective to promote the *Leishmania* promastigote death. After 72 h incubation, the lethal dose of TrEO and TrROY that promoted 50% *Leishmania* death (LD_{50}) were 0.8 μg/mL and 3 μg/mL, respectively. TrEO and TrROY were not cytotoxic to human erythrocytes, but TrROY was toxic to murine macrophages resulting in a low selectivity index. The transmission electronic microscopy showed that TrEO (0.03 μg/mL) was able to modify the promastigote ultrastructures suggesting autophagy as chromatin condensation, blebbing, membranous profiles and nuclear fragmentation. Infected-macrophages treated with TrEO (0.03 μg/mL) or TrROY (10 μg/mL) had an infection index decreased in 65 and 48%. TrEO did not induce iNOS mRNA expression or nitrite production in macrophages infected with *Leishmania*. TrROY and mainly TrEO promoted the *Leishmania*

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death, and TrROY showed loss toxicity to erythrocytes cells. Other compounds derived from *T. riparia* and the essential oil could be explored to develop a new alternative treatment for leishmaniasis.

1. Introduction

Tetradenia riparia (Hochstetter) Codd plant belongs to the Lamiaceae family, also known as *Iboza riparia* and *Moschosma riparium*. It is used as a traditional medicine in Africa for the treatment of inflammatory and infectious diseases. The plant is an herbaceous shrub that occurs throughout tropical Africa and other regions of the world (Polya, 2003; Shale et al., 1999; van Puyvelde et al., 1988; York et al., 2011, York et al., 2012). In Brazil, it is known as false myrrh, and it is mainly used as an ornamental plant and incense (Gazim et al., 2010; Martins, 2008). Its leaves and essential oil have been used for the treatment of malaria, cryptococcosis, candidiasis, and respiratory infections (Campbell et al., 1997; Okem et al., 2012; van Puyvelde et al., 1986; York et al., 2012). Natural products are relatively inexpensive, accessible, and sustainable, and many of them do not have a high likelihood of causing serious adverse effects (Adebayo, 2013).

Since 1940, leishmaniasis has been treated with antimonials such as glucantime, but therapy often fails, and adverse effects are frequently observed. *Leishmania (Leishmania) amazonensis* is the etiologic agent of cutaneous and diffuse cutaneous leishmaniasis, the most severe and destructive clinical form of the disease. This species of parasite is also associated with therapeutic failure and disease recurrence (WHO, 2010). For these reasons, researchers have investigated natural products to discover new therapeutic strategies for leishmaniasis.

The essential oil from *T. riparia* (TrEO) is a complex mixture of terpenoids, including monoterpenes, sesquiterpenes, and diterpenes (hydrocarbons or oxygenated), the most representative class (Gazim et al., 2010). *T. riparia* is known as false myrrh and has been used as traditional medicine also known as indigenous or folk medicine used by lay people, but few studies have evaluated its biological effects (Campbell et al., 1997; Cardoso et al., 2011; Gazim et al., 2010, Gazim et al., 2011, Gazim et al., 2013; Martins, 2008; van Puyvelde et al., 1988; Van Puyvelde et al., 1987; van Puyvelde et al., 1986). *Commiphora myrrh* (Nees) Engl. is real myrrh and has emerged as a good source of traditional medicine for the treatment of inflammation, obesity, arthritis, microbial infections, wounds, and pain in Africa, Arabia, and India. The medicinal functions and usage of real myrrh are recorded in ancient literature (Shen et al., 2012). The microbicidal and anti-inflammatory activity of *T. riparia* has scarcely been documented in the literature (Campbell et al., 1997; Gazim et al., 2010, Gazim et al., 2013; Okem et al., 2012; York et al., 2011, York et al., 2012). The diterpene 6,7-dehydroroyleanone isolated from *T. riparia* (TrROY) may have some biological effects such as antioxidant and antitermitic activity (Gazim et al., 2013; Kusumoto et al., 2009). The leishmaniostatic activity of TrEO against *L. (L.) amazonensis* were recently reported (Demarchi et al., 2013; Thomazella et al., 2013), but this potential effects of TrEO and then isolated compounds on *Leishmania* have not yet been investigated. Therefore, to determine their potential as an alternative therapy for leishmaniasis, we studied TrEO and TrROY effects on *L. (L.) amazonensis* promastigote and amastigote forms, cytotoxicity on human erythrocytes and murine macrophages, nitric production and inducible nitric oxide synthase (iNOS) mRNA expression.

2. Materials and methods

2.1. Plant material

T. riparia leaves were collected monthly between September 2006 and August 2007 in Umuarama, Paraná, Brazil (23°46′22″S and 53°16′73″W, 391 m). The plant was identified by Professor Ezilda Jacomasi of the Departamento de Farmácia of Universidade Paranaense (UNIPAR, Umuarama, Paraná, Brazil). A voucher specimen of plant was deposited in the UNIPAR Herbarium (code number 2502). The mean values for the maximum and minimum temperatures, precipitation, and relative humidity from September 2006 to August 2007 were reported by Gazim et al. (Gazim et al., 2010).

2.2. Essential oil extraction of T. riparia

The fresh leaves of *T. riparia* were used to extract the essential oil, which was obtained by hydrodistillation for 3 h using a Clevenger-type apparatus. The distilled oils were collected and dried over anhydrous sodium sulfate and stored in a freezer. The oil was analyzed by gas chromatography-mass spectrometry (GC–MS) using an Agilent 5973N GC–MS System that was operated in electron ionization mode and equipped with a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm, Agilent, PA, USA) that was used to inject 1 μl of a solution sample (Gazim et al., 2010; Omolo et al., 2004). The initial temperature of the column was 80 °C. The column was gradually heated to 260 °C°C at a rate of 4 °C/min. The injector (splitless, 0.5 min) and transfer line temperatures were held at 260 °C and 280 °C, respectively. Helium (1.0 mL/min) was used as a carrier gas. The same temperature program was used for gas chromatography with a flame ionization detector (GC-FID). The identification of the compounds was based on comparisons of their retention time that were obtained using various n-alkanes (C7 – C25). Their electron impact mass spectra were compared with the Wiley library spectra and literature (Gazim et al., 2010).

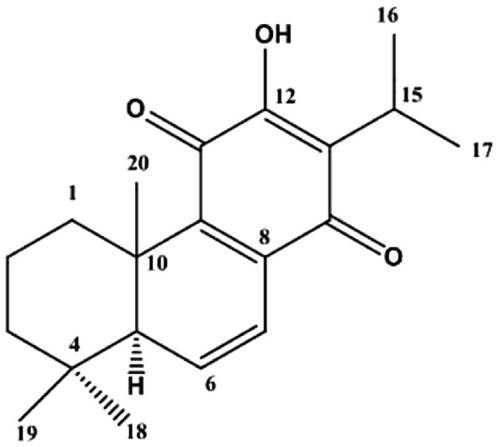

Fig. 1. Structural formula of compound 6,7-dehydroroyleanone derived from *T. riparia* (TrROY).

2.3. Isolating 6,7-dehydroroyleanone of T. riparia (TrROY)

Briefly, the TrEO (2 g) from leaves was subjected to column chromatography over a silica gel support and eluted with pentane, pentane-dichloromethane (9:1; 8:2; 7:3 and 1:1), dichloro methane-pentane (3:7), dichloromethane, dichloromethane-methanol (9:1; 7:3 and 1:1), and methanol, resulting in 29 fractions (Gazim et al., 2013). Fraction 17 (11.7 mg) pentane-dichloromethane (8:2) were identified by ^{1}H, ^{13}C, DEPT, HSQC, HMBC and NOESY according to Gazim et al. (2013) and by comparison with literature data. Fraction 17 orange crystalline powder gave an {M−H] − at *m*/*z* 313 was identified as TrROY (Fig. 1). Spectral data corresponded with data previously described by Kusumoto (Kusumoto et al., 2009).

2.4. Cytotoxicity assays

2.4.1. Hemolysis assay

Erythrocyte toxicity was determined as described by Valdez et al. (Valdez et al., 2009). Briefly, a 6% suspension of fresh defibrinated human blood was prepared in sterile 1% glucose saline solution. 6,7-dehydroroyleanone was dissolved in 5% dimethylsulfoxide (DMSO; obtained from Sigma-Aldrich, St. Louis, MO, USA). DMSO concentration promoted hemolysis from 6% v/v, and at 5% did not cytotoxicity. The compound was then serially distributed into culture plates at concentrations of 5.0−0.1 μg/mL for TrEO, 50−0.1 μg/mL for TrROY and 500−1 μg/mL for amphotericin B (AmB, Anforicin, Cristalia Laboratory, São Paulo, SP, Brazil). The compounds were incubated with erythrocytes in suspension at 37 °C. After 2 h, the samples were centrifuged at 300 × *g* for 3 min. Absorbance of the supernatant was determined at a wavelength 550 nm to estimate hemolysis. A solution of 4% Triton X-100 (Sigma−Aldrich, St. Louis, MO, USA) was used as the positive control, and the cell suspension was used as the negative control. The hemolysis was tested in duplicate. The results are expressed as a percentage of hemolysis based on the Hamolysis equation ($\% = 100 - [(Ap-As)/(Ap-Ac) \times 100]$; where *Ap*, *As* and *Ac* are the absorbance of the positive control, test sample and negative control, respectively).

2.4.2. Macrophages cytotoxicity

Peritoneal macrophages were obtained from BALB/c mice in accordance with the Ethics Committee on the Use of Experimental Animals of the Universidade Estadual de Maringá, Paraná State, Brazil (warrant no.133/2012). The peritoneal cavity was washed with 8 mL sterile RPMI 1640 medium. The cell suspension was adjusted to 1×10^6 macrophages/mL. Next, 100 μl was plated in 96-well culture plates (TPP, Switzerland). The plates were incubated for 2 h at 37 °C, 5% CO_2, and non-adherent cells were removed by sterile phosphate buffered saline (PBS) washing. TrROY was diluted in DMSO and RPMI 1640 medium from 100 to 0.1 μg/mL, and TrEO from 3 μg/mL to 30 ng/mL. The DMSO did not exceed 0.005% v/v, and it had not macrophage cytotoxicity effect. Non-treated cultures were used as viability control. The plates were maintained at 37 °C in a humid atmosphere containing 5% CO_2 during 24 h. The results were revealed using colorimetric cell viability XTT (2,3-bis[2-methyloxy-4- nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxa nilide) (Sigma Chemical Co; St. Louis, MO) (El-On et al., 2009; Williams et al., 2003). A XTT solution (500 μg/mL) was activated with 50 μg/mL phenazine methosulfate (PMS, Sigma Co Chemical; St. Louis, USA), and it was added over the cell monolayer to each well. After 3−5 h incubated at 37 °C, with 5% CO_2 and protected from light, the result was measured at a wavelength 450/650 nm using a spectrophotometer plate reader (ASYS Expert Plus, ASYS Hitech GMBH, Austria). The cytotoxicity concentration (CC_{50}) was defined as the dose of the compound that reduced 50% of the survival of macrophages comparing with untreated macrophages (viability control) (Cardoso et al., 2011). This test was done in duplicate.

2.5. Promastigote assay

2.5.1. Parasite strain and culture

L. (L.) amazonensis (MHOM/BR/1977/LTB0016) infection was induced by inoculating the left footpad of BALB/c mice with 1×10^7 parasites. Animals between 30 and 40 days of age were infected and after 30 days, they were sacrificed with 160 mg/kg ketamine (Agner União, Embu-Guaçu, SP, Brazil) and 50 mg/kg xylazine (Coopazine®, Invervet Schering-Plough, Cotia, SP, Brazil). The lymph node fragments were incubated in 199 culture medium supplemented with 10% fetal bovine serum, 1% human urine, 2 mM L-glutamine, and antibiotics (100 UI/mL penicillin and 0.1 mg/mL streptomycin). The cultures were incubated at 25 °C, and parasites were maintained by weekly transfers in 25 cm^2 culture flasks supplemented with 199 culture medium (Demarchi et al., 2012).

2.5.2. XTT tetrazolium method

Promastigote viability was evaluated using the XTT method. Promastigotes (4×10^6 parasites/100 μl/well) from a logarithmic growth phase culture were seeded into flat-bottomed 96-well plastic tissue culture microplates in triplicate. TrEO, TrROY, and AmB were dissolved in 1.6% v/v DMSO in the first well and diluted in series from 50 μg/mL to 1 ng/mL in a 96-well culture plate. After 24, 48, and 72 h incubation at 25 °C, 0.2 mg/mL XTT and 200 μM phenazine methosulfate (PMS, Sigma Co Chemical; St. Louis, USA) were added to each well and incubated for 3−5 h at 37 °C. The result was measured at a wavelength 450/650 nm using a spectrophotometer plate reader (ASYS Expert Plus, ASYS Hitech GMBH, Austria). The lethal dose (LD_{50}) was defined as the dose of the essential oil that reduced the survival of *Leishmania* parasites by 50% compared with untreated parasites (El-On et al., 2009; Williams et al., 2003). The therapeutic selectivity index ($TSI = CC_{50}/LD_{50}$) was calculated by ratio of toxicity to macrophage vs. toxicity to the parasites after 24 h incubation. The test was done in triplicate.

2.5.3. Promastigote growth inhibition

Promastigotes were cultured in Schneider's insect medium (Sigma−Aldrich, St. Louis, MO, USA), pH 7.2, supplemented with 10% FCS (v/v) and 2 mM L-glutamine until they reached the logarithmic growth phase. TrEO and TrROY were diluted in DMSO from 50 μg/mL to 1 ng/mL on a cell culture plate with 96 wells (TPP® test plate, Switzerland). The concentrations of DMSO not exceed 1.6% v/v DMSO in the first well, and it had no effect on the parasites. Next, 100 μl of the suspension containing 4×10^6 parasites/mL was distributed in each well of the culture plate. After 24 h at 25 °C, an aliquot of each well was added to a solution containing 10% eosin and 2% formalin, and the parasites were counted in a Neubauer chamber. All tests were performed in triplicate. The inhibitory concentration that caused a 50% decrease in survival (IC_{50}) of promastigotes was calculated. Values were compared to promastigotes cultures not treated. The results were evaluated by linear regression of the inhibition percentage (Demarchi et al., 2012). The selectivity index ($SI = CC_{50}/IC_{50}$) was calculated by ratio of toxicity concentration to macrophage vs. inhibitory concentration to the parasite growth after 24 h incubation.

2.5.4. Ultrastructural alterations

L. (L.) amazonensis promastigotes were grown in 199 medium during 3 days in 25 cm^2 culture vials at 25 °C. The LD_{50}

concentration of TrEO and 50% inhibitory concentration (IC_{50} = 30 ng/mL) (Thomazella et al., 2013) was added to the cultures and incubated at 25 °C for 24 h. After incubation, the IC_{50} was calculated according to the number of remaining parasites in a Neubauer chamber compared with control cultures without drugs and diluents (DMSO). The results were evaluated by linear regression of the inhibition percentage. An untreated culture was used as a negative control, and the ultrastructural changes were observed by transmission electron microscopy. For this, the samples were centrifuged at 12,000 *g*-force per minute for 10 min and fixed in Karnovsky solution 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 at 4 °C (Karnovsky, 1965). The material was fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. Block staining was performed with 2% uranyl acetate for 2 h, and the blocks were dehydrated in a graded series of alcohol and acetone. Embedding was performed in Epon 812 resin (Luft, 1961). Sections were obtained using an Sorval Porter Blum MT-2 ultramicrotome and concentrated in 2% uranyl acetate (Watson, 1958) and nitrate/acetate lead (Reynolds, 1963). The JEOL 1200EX II transmission electron microscopy was used for ultrastructural evaluation. The assays were conducted in duplicate.

2.6. Amastigote assay

2.6.1. Leishmania infection on macrophages to obtain amastigote forms

Peritoneal macrophages were obtained from BALB/c mice peritoneal cavity as already reported above. The cell suspension was adjusted to 1×10^6 macrophages/mL. Next, 500 μL were distributed on 13 mm-diameter sterile glass coverslips (Glastecnica, São Paulo, SP, Brazil), and placed in 24-well culture plates (TPP, Switzerland). The plates were incubated for 2 h at 37 °C. The non-adherent cells were removed by sterile PBS washing. Macrophages were infected with promastigote forms in the proportion of six parasites for each macrophage, and the plates were incubated for 4 h at 37 °C in an atmosphere of 5% CO_2.

2.6.2. Infection index

TrEO and TrROY were diluted in DMSO and RPMI 1640 medium. The final concentration of DMSO did not exceed 0.05%, and no cytotoxicity effect on the macrophages was observed. TrEO and TrROY were added to cultures at concentrations from 300 μg/mL to 30 ng/mL, and 100 to 0.1 μg/mL, respectively. After 24 h at 37 °C and 5% CO_2, the cells on coverslips were fixed in 95% ethanol and dyed with eosin and hematoxylin. At least 200 cells were counted in an optical microscope. To infection index was determined from the percentage of infected macrophages multiplied by the mean number of parasites per macrophage. The supernatant was stored at - 30 °C to nitrite assay.

2.7. Inducible nitric oxide synthase (iNOS) expression and nitrit assay

2.7.1. Cells and treatment

Peritoneal macrophages were obtained from BALB/c mice. Briefly, the peritoneal cavity was washed with 8 mL sterile RPMI 1640 culture medium. The cell suspension was adjusted to 1×10^6 macrophages/mL, 1 mL of which was added to each well of a 12-well culture plate. The plates were incubated for 1 h at 37 °C, and non-adherent cells were removed by three sterile washes in PBS. The adherent cells were incubated in RPMI 1640 culture medium. After a few minutes, macrophages were subjected to the following conditions: (1) stimulated with 5 μg/mL lipopolysaccharide (LPS, Sigma–Aldrich, Brazil), (2) untreated and uninfected (negative control), (3) infected by the promastigote form of *L. (L.) amazonensis* at a proportion of six parasites for each macrophage, (4) treated with 30 ng/mL TrEO, (5) infected with the promastigote form of *L. (L.) amazonensis* for 4 h and treated with 30 ng/mL TrEO, (6) treated with 100–0.1 μg/mL TrROY, (7) infected with the promastigote form of *L. (L.) amazonensis* for 4 h and treated with 100–1 μg/mL The final concentration of DMSO did not exceed 0.01%, and no cytotoxic effect on the macrophages was observed. The plates were maintained at 37 °C in a humid atmosphere that contained 5% CO_2 for 3, 6, and 24 h. The supernatant was also removed and stored at −30 °C for the nitrite assay. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was added to adhered cells for RNA extraction.

2.7.2. Nitrite assay

Nitric oxide was quantified by determining the accumulation of nitrite (NO^-_2) in the supernatants after 24, 48, and 72 h using the standard Griess assay (Green et al., 1982). Readings were performed in a spectrophotometer at a wavelength 550/620 nm (ASYS Expert Plus, ASYS Hitech GMBH, Austria). The experiments were performed in duplicate and on different days. The results are expressed as nitrite concentration (μM).

2.7.3. mRNA analysis by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA from the samples that were stored in Trizol reagent was extracted according to the manufacturer. cDNA was synthesized using 1 μg of total RNA as a template in a reverse transcription reaction (Superscript III reverse transcriptase, Invitrogen, Carlsbad, CA, USA). RNA quantification (ng/μl) and the determination of purity were performed using NANODROP 2000 UV–Vis Spectrophotometer (Thermo Fisher Scientific Inc, USA), considering an optimal purity level ≥ 1.8. PCR primers were: iNOS (Genbank accession no. NM_010927.3, forward 5′-CTGCAGCACTTGGATCAGGAACTG-3′; reverse 5′-GGGAGTAGCCTGTGTGCACCTGGAA-3′, 311 bp); Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) mRNA of *Mus musculus* was used as intern control (Genbank accession no. XM_001476707.3, forward 5′-CCACCATGGAGAAGGCTGGGGCTC-3′; reverse 5′-AGTGATGGCATGGACTGTGGTCAT-3′, 239 bp). The primers were chosen according to the BLAST tool (available in the Genbank database) and previous publications (Byrne et al., 2011; Chen et al., 2004). The PCR conditions were the following: 95 °C (5 min), 30 cycles of 95 °C (20 s) and 55 °C (25 s), followed by a standard denaturation curve. iNOS mRNA expression was evaluated by comparing the presence and absence of the expression between experimental conditions and incubation times. Semi-quantitative RT-PCR was conducted by quantifying the densitometry of the bands using ImageJ software (National Institutes of Health, USA). Each gene was normalized to GAPDH as a housekeeping gene (internal control) (Lee et al., 2011). The amplified DNA fragments were separated by 2% agarose gel electrophoresis and revealed with ethidium bromide in a transilluminator.

2.8. Statistical analysis

The values are presented as mean ± standard error of mean (SEM). Differences among means and values were tested for statistical significance using one-way analysis of variance (ANOVA) and the Tukey test. Analyses were performed using Statistic 7 software. Values of $p < 0.05$ were considered statistically significant.

3. Results and discussion

The essential oil and 6,7-dehydroroyleanone derived from *T. riparia* induced *Leishmania* promastigote forms death (Fig. 2). After 24, 48, and 72 h incubation, the LD_{50} of *T. riparia* essential oil was

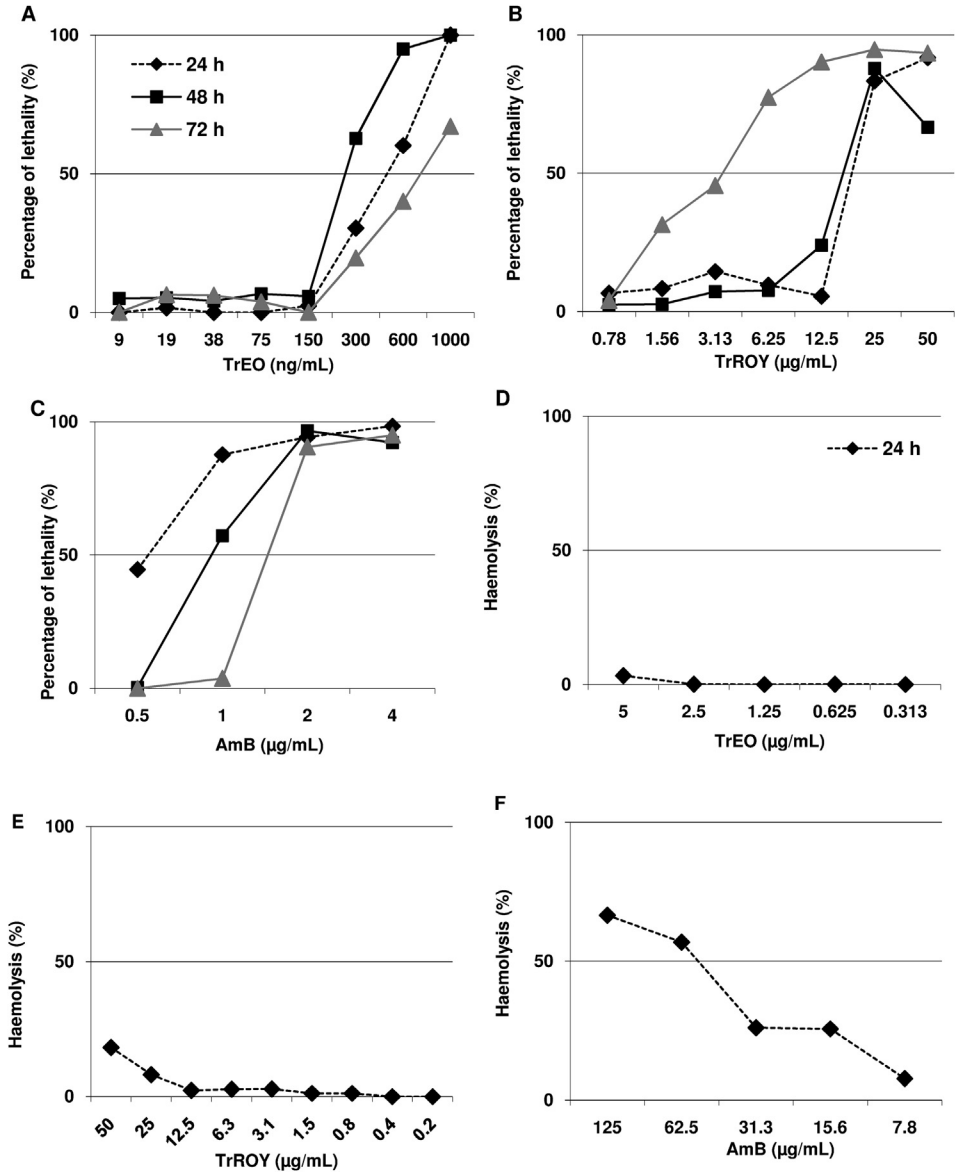

Fig. 2. Antileishmanial activity and hamolysis percentage of TrEO and TrROY. *Leishmania* promastigotes were incubated under the following conditions for 24, 48, and 72 h: (A) TrEO. (B) TrROY. (C) AmB (leishmaniasis reference drug). The lethality percentage was obtained using the XTT method. (D) Hamolysis percentage of TrEO (5.0–0.3 µg/mL) in erythrocytes. (E) Haemolysis percentage in erythrocytes treated with TrROY (50.0–0.2 µg/mL). (F) Haemolysis percentage of AmB (500–1 µg/mL) in erythrocytes. Each experiment was performed independently and in triplicate.

0.5, 0.3, and 0.8 µg/mL, respectively (Fig. 2A). The LD_{50} of TrROY was 16.9, 14.9, and 3 µg/mL (Fig. 2B), respectively. LD_{50} AmB was less than 1.5 µg/mL in all times (Fig. 2C). No difference was found between LD_{50} of TrEO and AmB ($p > 0.05$). TrROY LD_{50} was higher than AmB and TrEO ($p < 0.001$). Thus, TrEO had better microbicidal activity TrROY.

Essential oils are natural products that contain a complex mixture of pharmacological compounds. These compounds are most often terpenoids, which are hydrocarbons. The chemical composition of TrEO was analyzed by GC–MS in Gazim et al. (2010) showing the chemical identification and concentration (%) of compounds isolated from TrEO. The chemical composition revealed a complex terpenoids mixture such as diterpenes, sesquiterpenes and monoterpenes (Gazim et al., 2010) with anti-inflammatory activity and microbicidal effects (Campbell et al., 1997; Gazim et al., 2010, Gazim et al., 2013; Okem et al., 2012; York et al., 2011, York et al., 2012). Antileishmanial activity of TrEO and isolated substances from this plant has been poorly described (Torquilho et al., 1999; Thomazella et al., 2013). Thus, the substances with anti-*Leishmania* activity until remain unknown. We showed that a diterpene TrROY from TrEO is one of the substances with antileishmanial activity. Other substances isolated from TrEO should be evaluated against *Leishmania*, and also interactions between isolated compounds could be performed to identify the substances with anti-*Leishmania* activity. The several biological effects of essential oils can be explained by the interactions between different bioactive components may lead to synergistic, additive, or antagonistic effects.

The use of compound isolated from essential oils may not have the same effect promoted by essential oil requiring a greater amount of isolated to achieve the desired action (Bassole and Juliani, 2012). These reasons may explain the highest concentration of the isolated compound that was necessary to induce *Leishmania* death compared with TrEO. Checkerboard, graphical

Table 1
Antileishmanial and macrophage cytotoxicity effects of 6,7-dehydroroyleanone and the essential oil derived from *Tetradenia riparia*.

Treatment	Promastigote IC_{50}/24 h (μg/mL)	Promastigote LD_{50}/24 h (μg/mL)	Murine macrophage cytotoxicity CC_{50}/24 h (μg/mL)	Selectivity index (SI)	Therapeutic selectivity index (TSI)
6,7-dehydroroyleanone	2.45	16.9	0.53	0.22	0.03
T. riparia essential oil	0.03	0.5	0.17	5.67	0.34

IC_{50} = inhibitory concentration that reduces 50% parasite growth; LD_{50} = 50% lethal dose; CC_{50} = 50% cytotoxicity concentration; SI = CC_{50}/IC_{50}; TSI = CC_{50}/LD_{50}.

and Time-kill methods are widely used procedures to determine these interactions (Bassole and Juliani, 2012), but we have not yet assessed the interactions between essential oil components.

Terpenoids have antiparasitic effects on different *Leishmania* species (Ogungbe and Setzer, 2013). Some diterpenoid compounds that are isolated from plants have been reported to have leishmanicidal activity, particularly against *L. amazonensis* (Dos Santos et al., 2011; Jullian et al., 2005; Santos et al., 2013), but the effects of the diterpene TrROY on this species had not yet been demonstrated. Some studies showed that diterpenoids favor docking to glycerol-3-phosphate dehydrogenase (GPDH) of *L. mexicana* (Ogungbe and Setzer, 2013), which is also involved in the glycosomal and cytosolic glycolytic pathways of *Leishmania* (Zhang et al., 2013). One of these pathways involves NADH that is produced by GPDH, which can be evaluated using the XTT assay (Ramirez-Macias et al., 2012). This method has been used to study the

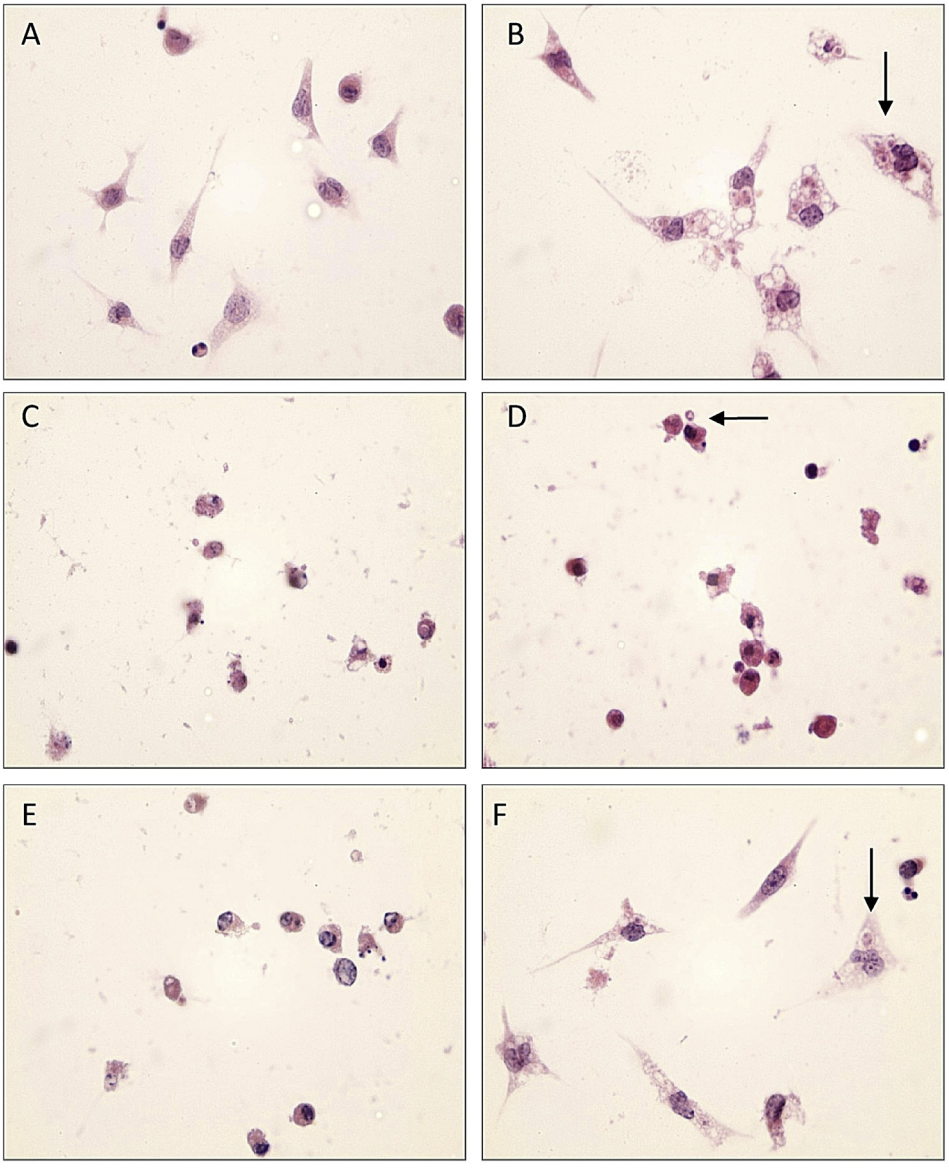

Fig. 3. Peritoneal macrophages of BALB/c mice infected and treated with TrEO and TrROY. (A) non-infected and non-treated macrophages (negative control). (B) macrophages infected with *L. (L.) amazonensis* (6 parasites/cell). (C) infected-macrophages treated with 100 μg/mL of TrROY. (D) infected-macrophages treated with 0.1 μg/mL of TrROY. (E) infected-macrophages treated with 3 μg/mL of TrEO. (F) infected-macrophages treated with 30 ng/mL of TrEO. The cells were stained with hematoxylin and eosin (100 × objective). Arrow indicates amastigote intracellular forms. All conditions were tested in duplicate and analyzed after 24 h of the incubation at 37 °C with 5% CO_2.

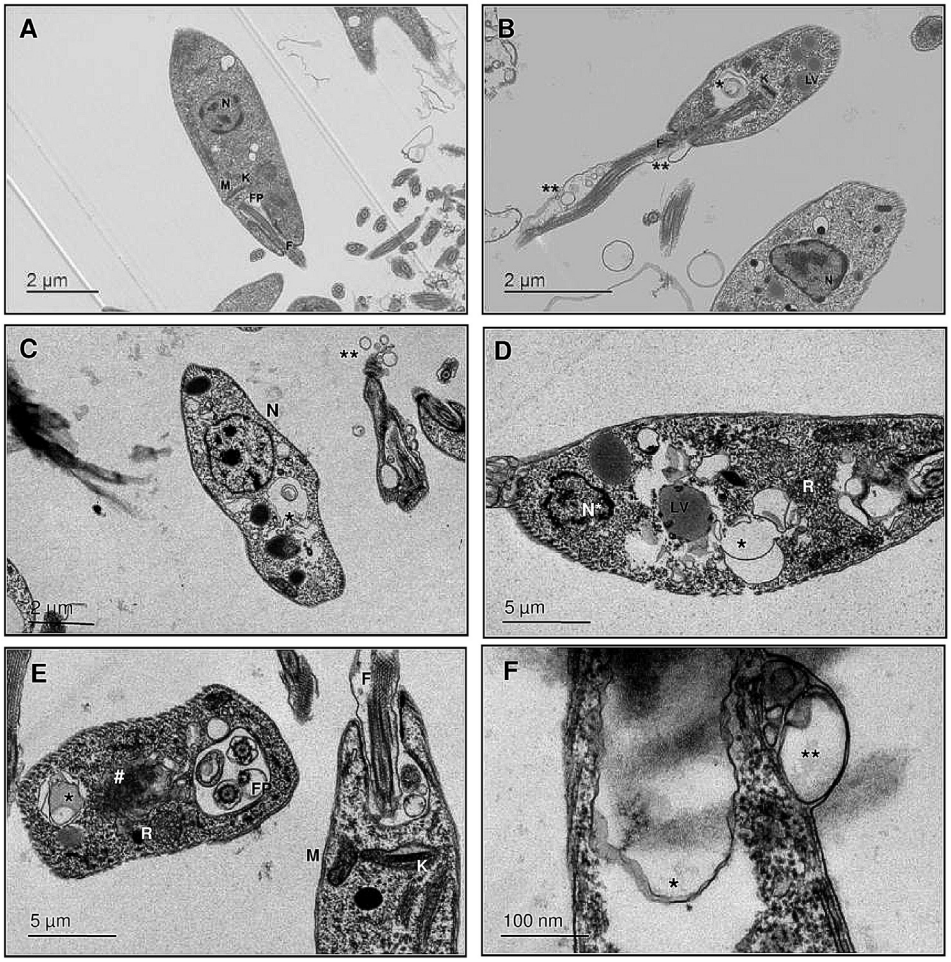

Fig. 4. Transmission electron microscopy of *L. (L.) amazonensis* treated with TrEO for 24 h. (A) *Leishmania* promastigotes. (B–F) Promastigotes treated with TrEO (30 ng/mL). N, nucleus; N*, abnormal chromatin condensation nuclear alterations; K, kinetoplast; M, mitochondria; FP, flagellar pocket; F, flagellum; V, vacuoles; LV, lipid vesicles; R, myelin-like figure appears in close association with the flagellar pocket membrane; *membranous profiles; **blebbing; #mitochondrial swelling.

mitochondrial metabolism and respiratory toxicity of drugs (El-On et al., 2009; Williams et al., 2003). We showed that the essential oil and TrROY derived from *T. riparia* probably promote *Leishmania* death through mitochondrial metabolism pathways.

TrEO at 5 µg/mL promoted 3.2% hamolysis in human erythrocytes (Fig. 2). TrROY exhibited low toxicity in human erythrocytes at 50 µg/mL (<18% hemolysis) comparing with reference drug (AmB, 50 µg/mL, > 50% hemolysis) (Fig. 2). Gazim et al. (2014) showed that TrEO and TrROY were not cytotoxic to human melanoma, human nervous system and human colon cells line. Although TrROY was not toxicity on human cells, we observed a cytotoxicity of this compound on murine macrophage (Table 1). TrROY CC50 was 0.53 µg/mL, and the selectivity index (SI) and therapeutic SI (TSI) were 0.22 and 0.03, respectively. TrEO CC_{50} was 0.17 µg/mL) resulting in 5.67 for SI and 0.34 for TSI. The TSI value obtained with TrEO was ~11 folds higher concentration compared to observe with TrROY (Table 1). The cytotoxicity effects on murine macrophages can also be observed in Fig. 3.

TrEO at 30 ng/mL (IC_{50}) and 0.5 µg/mL (LD_{50}) inhibited 62% and 80% of *Leishmania* promastigote growth after 24 h, respectively. Both concentrations modified the morphology of *L. (L.) amazonensis* promastigote viewed by transmission electronic microscopy. The ultrastructural changes observed with the IC_{50} included intense cytoplasm vacuolization, membranous profiles inside the organelle, lipid vesicles, and membrane blebbing that suggested autophagy, thickening of the kinetoplast, chromatin condensation, and nuclear fragmentation (Fig. 4).

Nuclear fragmentation suggests apoptosis, and a vesicle that engulfs a part of the cytoplasm indicates autophagic cell death. The presence of multivesicular bodies and several vacuoles that have membrane profiles and cellular debris is related to the presence of secondary lysosomes and organelles that are likely involved in the degradation of damaged structures (Rodrigues and de Souza, 2008; Santos et al., 2013). They may also correspond to the secretion of abnormal lipids that accumulate as consequence of drug treatment and alterations in the physical properties of lipids and membranes, leading to the accumulation of concentric membranes, and myelin-like structures. The lipid composition can induce irreversible changes in the structure and function of several organelles, leading to the appearance of autophagosomes to remove and recycle abnormal membrane structures and suggesting an intracellular remodeling process (Granthon et al., 2007; Lira et al., 2001). TrEO LD_{50} promoted the complete destruction of parasite membranes and other structures. These results indicate the high potential of this essential oil to induce *L. (L.) amazonensis* death.

Both TrEO and TrROY decreased the infection index of murine macrophages infected with *Leishmania* amastigote significantly. While the infection index 177 was obtained in macrophages infected and non-treated (positive control), TrEO index was 80 at 3 µg/mL, 73 (300 ng/mL) and 62 (30 ng/mL) reducing the infection

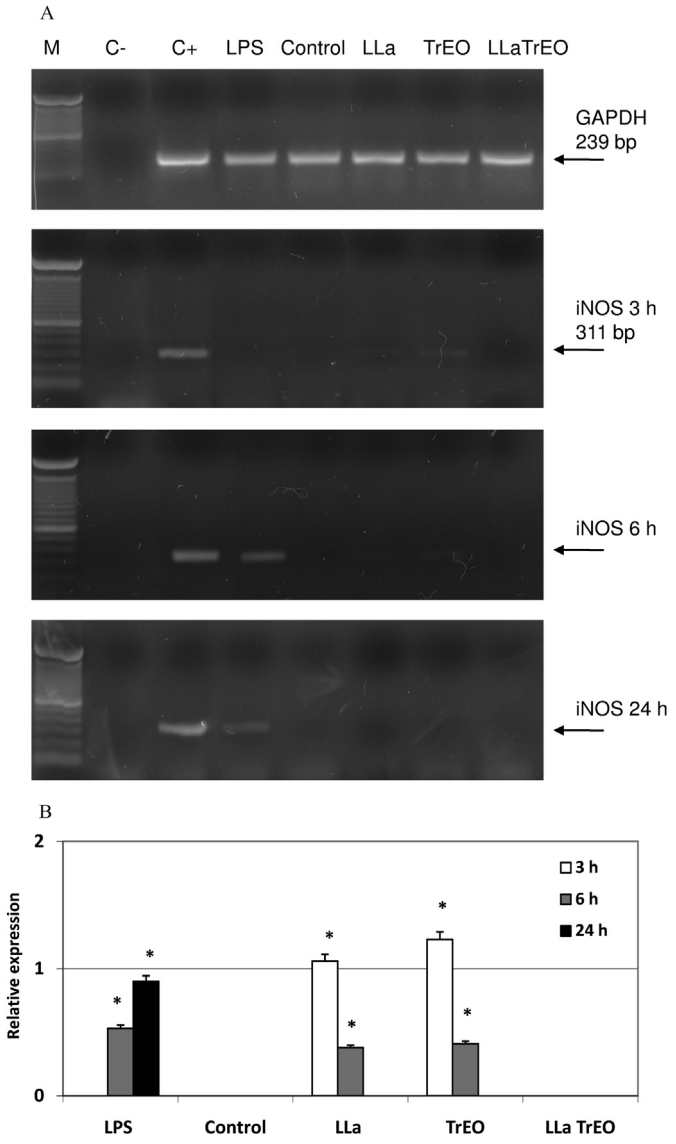

Fig. 5. iNOS mRNA expression using semi-quantitative RT-PCR. (A) DNA fragments were separated in 2% agarose gel electrophoresis and revealed with ethidium bromide in a transilluminator. (B) expression levels of iNOS mRNA were normalized to GAPDH as a housekeeping gene (internal control). M, molecular marker; C-, intern negative control (H_2O). C+, DNA obtained from *L. (L.) amazonensis* promastigotes cultured in 199 medium during 3 days. LPS, lipopolysaccharide-stimulated macrophages; Control, untreated and uninfected macrophages (negative control); LLa, macrophages infected with *L. (L.) amazonensis*; TrEO, macrophages treated with 30 ng/mL of TrEO; LLa TrEO, macrophages infected with *L. (L.) amazonensis* and treated with 30 ng/mL of TrEO. All of the conditions were analyzed with 3, 6, and 24 h incubation at 37 °C. $^{*}p < 0.05$.

in 55, 59 and 65%, respectively. TrROY infection index was 122 to 0.1 μg/mL, 96 (1 μg/mL), 93 (10 μg/mL) and 105 (100 μg/mL) decreasing in 31, 46, 48 and 41%, respectively. Thus, TrEO at 30 ng/mL showed the most appropriate results because it has not presented toxicity into the cells and promoted promastigote and amastigote forms death.

TrEO showed the outcome and shows promise in leishmaniasis treatment. Recent research on essential oils has shown a successful approach to obtain new antileishmanial alternatives (Monzote et al., 2007). Studies have showed the promising results of the essential oils from *Chenopodium ambrosioide* in BALB/c mice *Leishmania*-infected (Monzote et al., 2009, Monzote et al., 2010, Monzote et al., 2014, Monzote et al., 2007). The use of natural products could be an alternative to cutaneous leishmaniasis therapy, but there are few studies showing the effectiveness of essential oils. Thus, TrEO and TrROY could be explored to develop a new alternative treatment for leishmaniasis. We suggest that *in vivo* studies should be performed to evaluate the therapeutic efficacy of essential oil and its compounds from *T. riparia* in cutaneous leishmaniasis.

Some medicinal plants have anti-inflammatory effects and can act on immune response, and it can be demonstrated by reductions in iNOS expression and nitric oxide synthesis (Choudhari et al., 2013; Jeong et al., 2013). The *Leishmania* genus survives in macrophages, and cellular death is induced by reactive oxygen species, especially nitric oxide, which depends on iNOS expression (Amoo et al., 2012; Oliveira et al., 2014). In our study, iNOS mRNA was expressed by infected-macrophages after 3 and 6 h (Fig. 5A), and the iNOS mRNA levels were higher in treated-macrophages than in infected-macrophages, but infected-macrophage treated with TrEO did not expressed iNOS (Fig. 5B). After 24 h, iNOS mRNA was observed only in LPS-stimulated macrophages. The nitrite production was <0.1 μM under all of the experimental conditions at 3 and 6 h. After 24 h, the nitrite level was 5 μM to LPS-stimulated macrophages, 1.8 μM to treated-macrophages, 1.7 μM to infected and treated-macrophages, while it was 0.8 μM to *Leishmania*-infected cells ($p < 0.001$) comparing with 1.5 μM to negative control (only macrophage). Thus, the infection reduced nitrite levels but did not inhibit iNOS mRNA expression in macrophages. These results is in concordance with studies that show the infection caused by *L. amazonensis* can decrease the production of nitric oxide as an escape mechanism of the immune host (Amoo et al., 2012; Oliveira et al., 2014). TrROY also did not change the iNOS mRNA expression and nitrite levels (1.8 μM) comparing with control.

This difference between iNOS mRNA expression and nitrite production shows that the parasite affected post-transcriptional nitric oxide synthesis events, it may result in parasite survival and escape from the immune response. Calegari et al. (Calegari-Silva et al., 2009) reported that *L. amazonensis* has developed an adaptive strategy to escape from host defenses by activating the host transcriptional response, particularly nuclear factor κB, which in turn down regulates the expression of iNOS mRNA and favors the infection. In addition, *L. amazonensis* produces nitric oxide, which may lead to iNOS downregulation by macrophages (Balestieri et al., 2002; Genestra et al., 2006). TrEO increased iNOS mRNA expression, but it did not change nitrite production as well its isolate suggesting that TrEO did not act on iNOS and nitrite production during *Leishmania* infection, but TrROY and TrEO can modulate other immune mechanisms until not investigated.

Previous studies have shown the anti-inflammatory effects of *T. riparia* extract on prostaglandins, cyclooxygenase 1 (COX 1), and cyclooxygenase 2 (COX 2), but its effect on iNOS has been unknown (Ndhlala et al., 2011; Okem et al., 2012). In leishmaniasis, this increase in iNOS may influence the nitric oxide production and promote elimination of the parasite. TrEO did not affect this mechanism but still acted against *Leishmania*. Other mechanisms of the immune response are involved in *Leishmania* death such as a balance of the cytokines of host immune response that could solve the curse of infection (Amoo et al., 2012; Oliveira et al., 2014). The main immunopathogenesis competencies of *L. amazonensis* are to carry the anergic diffuse cutaneous leishmaniasis at the T-cell hyposensitivity pole and with a higher Th2-type immune response. This shift and imbalance in immunity leads to persistence of the disease and treatment failure (Silveira et al., 2009). New drugs with antileishmanial and immunomodulation activity may be promising to treatment leishmaniasis, and TrEO can be one of this, and the immunomodulatory effects of TrEO have been conducted by us.

Despite the African and other population have used this plant

for a variety diseases, the biological effects still remained to be investigated. We showed in vitro that the TrEO and TrROY have an ability to induce the *Leishmania* death by mitochondrial metabolism pathways, and induction of the secretion of abnormal lipids that accumulate as a consequence of drug treatment, and alterations in the physical properties of lipids and membranes. Although the isolated showed a high cytotoxicity in murine macrophages, the plant and the essential oil were not cytotoxicity against human cells. The cytotoxicity of TrROY must be considered before therapeutic application in leishmaniases. Other compound isolated from *T. riparia* may be investigated to the development of future leishmanicidal agents. For this, *in vivo* studies and other cytotoxicity assays must be conducted to ensure the safe use of this plant and its compounds to infections or inflammatory diseases. The isolation of antileishmanial compounds from the essential oil of *T. riparia* supports the traditional medicinal use of this plant as a treatment for parasitological infections as leishmaniasis.

Conflicts of interest

The authors declare that they have no conflicting interests.

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