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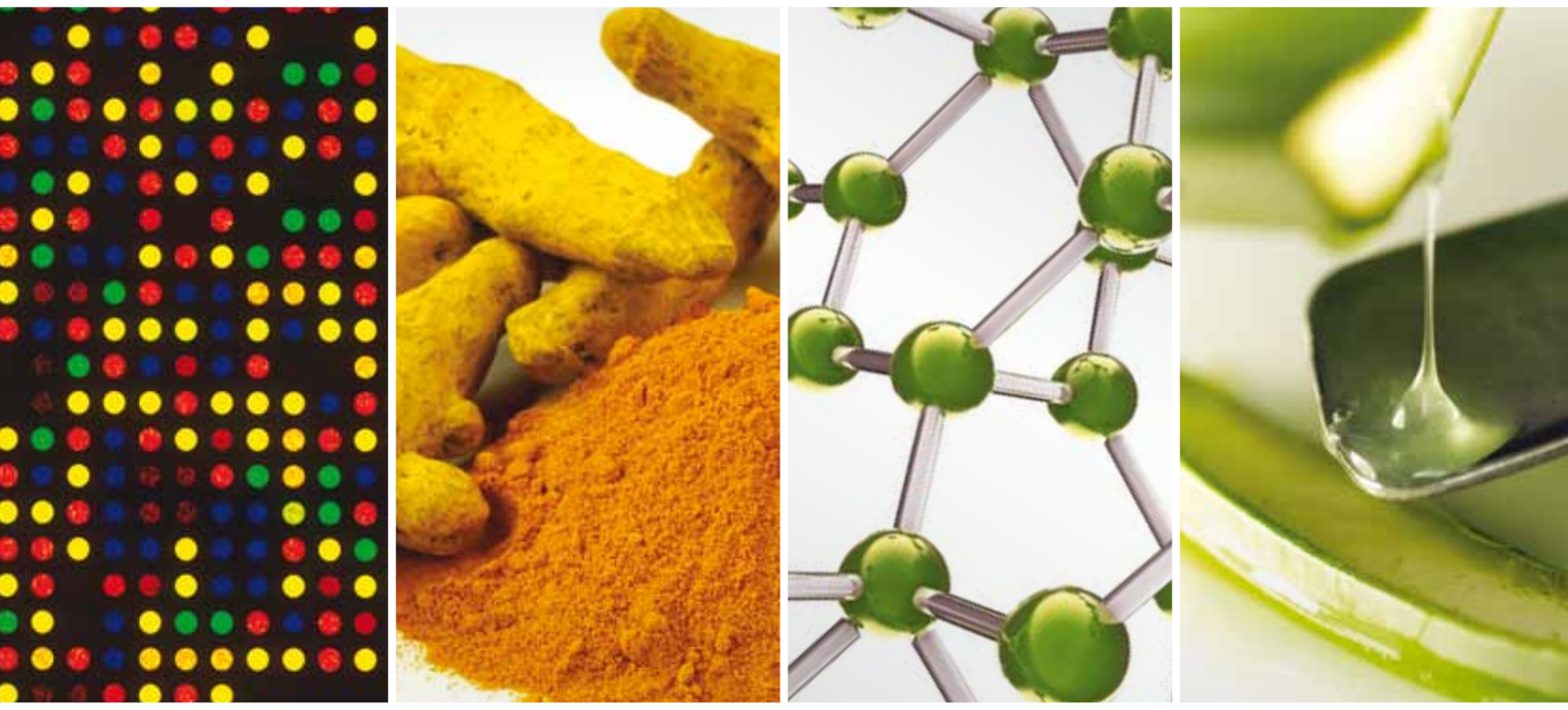
# Tropical Protozoan Diseases: Natural Product Drug Discovery and Development

Guest Editors: Liliana Muschietti, Roser Vila, Valdir Cechinel Filho, and William Setzer



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*Editorial*

# Tropical Protozoan Diseases: Natural Product Drug Discovery and Development

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Infections caused by protozoan parasites such as Chagas disease, human African trypanosomiasis, leishmaniasis, or malaria are responsible for considerable morbidity and mortality worldwide with devastating social and economic consequences (Table 1) [1]. Under normal circumstances (efficient epidemiological surveillance programs and sanitary education) the control of these diseases can be carried out effectively. Nevertheless, the implementation of an adequate health care system to palliate the necessities of the affected populations is hindered by the lack of financial and human resources, political instability in these countries, and often questionable government prioritization [2].

Currently, there is lack of effective, safe, and affordable therapies for the treatment of these diseases. The drugs used are far from ideal, and many of them were introduced decades ago; thus the development of new and more effective drugs with fewer side effects represents a crucial dare.

Over the last century, natural products have provided molecules with high structural diversity and "drug-like" properties from a physicochemical point of view. The reason resides in their chemical and steric complexity since they have well-defined three-dimensional structures, improved in terms of efficiency and selectivity for the molecular target [3, 4]. Natural product research has made a significant contribution to the chemotherapy of parasitic diseases such as quinine and artemisinin whose analogs are currently in use for the treatment of malaria.

This special issue brings significant works, done by leading scientists, and provides an overview on and an insight into recent advances that will contribute to the discovery of natural compounds with high potential against these protozoan parasites.

The readers will find, in nine papers, not only a wide range of topics including the identification of natural compounds with *in vitro* and *in vivo* activity against *Trypanosoma cruzi, Plasmodium falciparum,* or *Leishmania* spp. but also recent advances in the mechanism of action of bioactive compounds and the design of semisynthetic derivatives as new more effective chemotherapeutic agents with less toxicity.

Table 1: Protozoan infectious diseases.

| Parasites | Disease | Occurrence | Mortality | DALYs |
|---|---|---|---|---|
| *Plasmodium* spp. | Malaria | 219 million new cases* | 660,000 deaths* | 33,976,000 |
| *Leishmania* spp. | Leishmaniasis | 300,000 new cases of VL each year<br>1 million new cases of CL each year | Approx. 40,000 deaths due to VL | 1,974,000** |
| *Trypanosoma* spp. | Human African trypanosomiasis | 20,000 actual cases | | 1,673,000** |
| | Chagas disease | Approx. 8 million cases*** | 12,000 | 430,000** |

VL: visceral leishmaniasis, CL: cutaneous leishmaniasis, DALY's: Disability Adjusted Life Years.
*In 2010, according to the World Malaria Report 2012. **The Global Burden of Disease Report, WHO, 2004. ***Second WHO Report on Neglected Tropical Diseases 2013.

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*Liliana Muschietti*
*Roser Vila*
*Valdir Cechinel Filho*
*William Setzer*

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*Research Article*

# Antimalarial Activity of *Cocos nucifera* Husk Fibre: Further Studies

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In this study, the antimalarial and toxicity potentials of husk fibre extracts of five Nigerian varieties of *Cocos nucifera* were evaluated *in vitro*. The only active extract fraction, West African Tall (WAT) ethyl acetate extract fraction, was then evaluated for its phytochemical constituents, antimalarial and toxicity potentials at varying doses (31.25–500 mg/kg body weight) using various organ function indices. The results revealed that WAT ethyl acetate extract fraction (WATEAEF) contained alkaloids, tannins, and flavonoids and was active against *Plasmodium falciparum* W2 strain maintained in continuous culture, with a selectivity index of 30.3. The same extract fraction was active *in vivo* against *Plasmodium berghei* NK65, causing more than 50% reduction in parasitaemia on days 4 and 6 after inoculation at various doses administered. WATEAEF did not significantly alter ($P > 0.05$) function indices of the liver and cardiovascular system at all doses administered but significantly increased ($P < 0.05$) plasma creatinine concentration at 250 and 500 mg/Kg body weight compared to controls. The results of this study suggest that WATEAEF possesses antimalarial activity and may not adversely affect normal liver function nor predispose subjects to cardiovascular diseases but may impair normal kidney function at higher doses. Further studies are underway to isolate the active principles.

## 1. Introduction

Malaria is one of the most dreaded human parasitic diseases in the tropics and subtropics, especially in Africa where 81% of cases and 91% of deaths have been estimated to occur, with children under five years of age and pregnant women being most severely affected [1]. Nigeria accounts for a quarter of all malaria cases in Africa [2], mostly caused by *Plasmodium falciparum* [3], with an estimated 100 million malaria cases and over 300,000 deaths per year [4]. In addition to its direct health impact, malaria imposes a huge economic burden on afflicted individuals and nations, through high healthcare cost, missed days at work, and reduced economic output and productivity [5].

The continuous spread of *P. falciparum* resistance to antimalarial drugs poses a serious threat to malaria control programs. This, in addition to the high cost of the potent antimalarial drugs, has left the poor masses to be heavily reliant on traditional herbal medicines, which are often affordable and available [3]. Thus, the use of plant remedies has steadily increased worldwide in recent years, as well as the search for new phytochemicals that could be developed as

useful drugs for the treatment of malaria and other infectious diseases [6]. *Cocos nucifera* husk fibre and white flesh are used in folk medicine for the treatment of malaria [3, 7]. Our recent *in vitro* studies have authenticated the acclaimed antimalarial action of the husk fibre extract of *Cocos nucifera* [8]. The present study was, therefore, set out to evaluate the antimalarial and toxicity potentials of the husk fibre extracts of five Nigerian varieties of *Cocos nucifera in vitro* and the most active *in vivo*, with the aim of identifying the most potent variety for rational antimalarial drug design.

## 2. Materials and Methods

*2.1. Chemicals.* Absolute n-hexane, ethyl acetate, methanol, and ethanol were obtained from Sigma-Aldrich Laborchemikalien GmbH, Germany. Giemsa stain was obtained from Anosantec Laboratory, UK. Sodium chloride and glucose were both obtained from British Drug House Chemical limited, Poole, England. Sodium citrate was obtained from Merck, Darmstadt, Germany. Disodium hydrogen phosphate and potassium dihydrogen phosphate were obtained from Kermel Chemicals, China. Immersion oil was obtained from Panzonar laboratory Supplies, Button road, Canada. RPMI 1640 medium, sodium bicarbonate, L-glutamine, D-sorbitol, and HEPES were obtained from Sigma (St. Louis, MO, USA). Other reagents were of analytical grade and were prepared with injection water.

*2.2. Animals.* Sixty-five adult Swiss albino mice with an average weight of 18 ± 2 g were obtained from the Animal Breeding Unit of the Faculty of Pharmacy, Obafemi Awolowo University, Ile Ife, Osun State, Nigeria.

*2.3. Plant Materials.* Husk fibres of five varieties of *Cocos nucifera* dried at room temperature under shade were obtained from Nigeria Institute for Oil Palm Research (NIFOR), Badagry, Lagos State, Nigeria, in October 2010. They include West African Tall, Dwarf Red, Dwarf Yellow, Dwarf Green, and Hybrid varieties. They were botanically authenticated at the institute by Mr. Igbene Collins.

*2.4. Parasite Strains.* A chloroquine sensitive strain of *Plasmodium berghei* (NK-65) was obtained from the Department of Pharmacology, Obafemi Awolowo University, Ile-Ife, Nigeria. The W2 clone, CQ-resistant and mefloquine-sensitive strain of *P. falciparum* was originally received from the New York University Medical School and the *in vitro* tests were performed at Laboratório de Malária, Centro de Pesquisas René Rachou, FIOCRUZ, Belo Horizonte, MG, Brazil.

*2.5. Preparation of Extracts.* The extracts were prepared according to the method of Adebayo et al. [9]. The fresh samples were allowed to dry under shade at room temperature and then pulverized into powder. Four hundred and fifty grams (450 g) of the powder of each variety was successively extracted with 5000 mL each of n-hexane, ethyl acetate, and methanol for 72 h per solvent in a tightly stoppered glass container. After each solvent extraction, the content was filtered with Whatman filter paper no. 1. The filtrates were then concentrated under pressure using rotary evaporator at 40°C, thereby generating the crude extracts.

*2.6. Qualitative Phytochemical Screening.* Phytochemical screening was carried out for the ethyl acetate extract fraction using standard procedures described by Sofowora [10] to evaluate the presence of tannins, anthraquinones, alkaloids, terpenes, saponins, flavonoids, phenols, steroids, and cardiac glycoside.

*2.7. In Vitro Studies*

*2.7.1. [$^3$H]-Hypoxanthine Incorporation Assay.* The *in vitro* tests were performed with blood parasites of *P. falciparum* W2 clone, which are chloroquine resistant, kept in continuous culture at 37°C in human erythrocytes, using the candle jar method as described by Trager and Jensen [11]. The antiplasmodial effects of the extract fractions were measured through inhibition of parasite growth, by the [$^3$H]-hypoxanthine incorporation assay, as described by Desjardins et al. [12] and modified by Zalis et al. [13]. For the test, a stock solution of each extract fraction was diluted in complete culture medium without hypoxanthine (RPMI 1640 containing 10% human serum, 2% glutamine, and 75% $NaHCO_3$). Blood stage parasites in the ring form obtained in sorbitol-synchronized blood (180 $\mu$L/well) were cultured in 96-well culture plates at 1% parasitemia and 1% hematocrit and then incubated with the extracts. Controls without extracts or with chloroquine, used as the reference antimalarial drug, were run in parallel. After a 24 h incubation period, 20 $\mu$L of medium containing [$^3$H]-hypoxanthine (0.5 $\mu$Ci/well) was added to each well, followed by incubation for 18 h at 37°C. The plates were frozen and thawed, and the cells were harvested (Tomtec 96-Harvester; Tomtec Inc., Handem, CT, USA) on prewet glass-fiber filters (Wallac Ou, Turku, Finland), which were placed in sample bags (Wallac) and immersed in scintillation fluid (OptiPhase Super Mix, Wallac). Radioactive emission was counted in a 1450 Microbeta reader (Wallac).

The half-maximal inhibitory concentrations ($IC_{50}$) as compared to the drug-free controls were estimated by using curve-fitting software (Microcal Origin Software 8.0, Inc.). Each experiment was repeated at least two times. The microtitre plates used for the radioactive assay were kept in a designated place and are meant to be there for a minimum of eighty years.

*2.7.2. Histidine-Rich Protein 2 (HRP2) Assay.* This was performed according to the method of Noedl et al. [14]. The samples from the continuous cultures were washed and resuspended with RPMI 1640 (with 10% human serum) and uninfected erythrocytes to obtain 0.05% parasitemia and 1.5% hematocrit. For the test, a stock solution of each extract fraction was diluted in complete culture medium without hypoxanthine (RPMI 1640 containing 10% human serum, 2% glutamine, and 75% $NaHCO_3$). Serial twofold dilutions (seven concentrations and one drug-free control) of the drugs (20 $\mu$L/well) were dispensed into standard 96-well

microtitre plates (Costar 3599 plates), and 180 $\mu$L of CMM was added to each well. The plates were then incubated for 72 h at 37.5°C. They were subsequently frozen-thawed twice to obtain complete hemolysis. 100 $\mu$L each of the hemolyzed culture samples was transferred to the ELISA plates, which are precoated with monoclonal antibodies against *P. falciparum* HRP2 (capture antibody of the immunoglobulin M class; code CPF4), and the plates were incubated at room temperature for 1 h. Subsequently, the plates were washed four times with the washing solution provided with the test kit, and 100 $\mu$L of the diluted antibody conjugate (an indicator antibody of the immunoglobulin G1 isotype; code CPF6) was added to each well. After incubation for an additional 1 h, the plates were washed four times, and 100 $\mu$L of diluted (1 : 20) chromogen (tetramethylbenzidine) was added to each well. The plates were then incubated for another 15 min in the dark, and 50 $\mu$L of the stop solution was added. Spectrophotometric analysis was performed with an ELISA plate reader (SpectraMAX 340 Microplate spectrophotometer; Molecular Devices, Sunnyvale, CA, USA) at 450 nm.

*2.7.3. Cytotoxicity Test Using Cultures of Hepatoma Cell Line.* Hep G2 A16 hepatoma cells were kept at 37°C in RPMI supplemented with 5% fetal calf serum (complete medium), in a 5% $CO_2$ environment. Cells from confluent monolayers were trypsinized, washed, counted, resuspended in complete medium, distributed in 96-well microtiter plates ($4 \times 10^4$ cells/well), and then incubated for another 18 h at 37°C. The extract fractions prepared as stock solutions in DMSO were diluted in incomplete RPMI without fetal calf serum. After 24 h incubation at 37°C, 20 $\mu$L of MTT solution (5 mg/mL in RPMI 1640 without phenol red) was added to each well [15]. After 3 h incubation at 37°C, the supernatant was removed, and 100 $\mu$L of dimethyl sulphoxide was added to each well. The culture plates were read using a spectrophotometer with a 570 nm filter and a background of 630 nm. The minimum lethal dose (MLD) that killed 50% of the cells was determined as reported by do Ceu de Madureira et al. [16]; each assay was performed two times at least. Based on the values of cytotoxicity and antimalarial activity the selectivity index of activity, (SI) was calculated using the formula $SI = MLD_{50}/IC_{50}$.

### 2.8. In Vivo Studies

*2.8.1. Animal Handling.* The animals were housed in standard plastic cages and acclimatized for a period of two weeks. They were maintained under standard conditions (12 h light and 12 h dark cycle) and had access to chow (Bendel Feeds, Ewu, Edo State, Nigeria) and clean tap water *ad libitum*.

*2.8.2. 4-Day Suppressive Test in Animal Model.* The antimalarial tests were performed using *P. berghei* NK65 strain, maintained by serial weekly passages of infected blood in mice. Tests were performed as described by Peters [17] with some modifications [18]. Briefly, thirty-five mice inoculated by intraperitoneal route with $1 \times 10^5$ infected red blood cells were kept together for 2 to 16 h, divided randomly in seven groups of 5 mice per cage, and then treated daily by oral route for 3 consecutive days with various doses of the active extract fraction as follows.

Group A (control): administered appropriate volume of 5% DMSO.

Group B: administered 31.25 mg/Kg body weight of WAT ethyl acetate extract fraction.

Group C: administered 62.5 mg/Kg body weight of WAT ethyl acetate extract fraction.

Group D: administered 125 mg/Kg body weight of WAT ethyl acetate extract fraction.

Group E: administered 250 mg/Kg body weight of WAT ethyl acetate extract fraction.

Group F: administered 500 mg/Kg body weight of WAT ethyl acetate extract fraction.

Group G: administered 20 mg/Kg body weight of chloroquine

All solutions were freshly prepared before administration. The extract fraction was then dissolved in 5% DMSO and then diluted so that each mouse received 200 $\mu$L. At several days after parasite inoculation, blood smears were prepared from each mouse tail, methanol-fixed, stained with Giemsa, and then microscopically examined by counting parasitemia in up to 6000 erythrocytes. Inhibition of parasite growth in the drug-treated groups was calculated in relation to the nontreated control mice. The cumulative mortality of the animals was daily monitored up to day 30 after inoculation.

*2.8.3. Toxicity Test in Animal Model*

*Animal Grouping and Extract Administration*. Thirty adult Swiss mice were randomly divided into six groups of five mice each and daily administered 200 $\mu$L of the various doses of most active extract fraction (WAT ethyl acetate extract fraction) dissolved in 5% DMSO by the oral route for 7 days as follows.

Group A (control): administered 0.2 mL 5% DMSO.

Group B: administered 31.25 mg/Kg body weight of WAT ethyl acetate extract fraction.

Group C: administered 62.5 mg/Kg body weight of WAT ethyl acetate extract fraction.

Group D: administered 125 mg/Kg body weight of WAT ethyl acetate extract fraction.

Group E: administered 250 mg/Kg body weight of WAT ethyl acetate extract fraction.

Group F: administered 500 mg/Kg body weight of WAT ethyl acetate extract fraction.

*Sample Collection and Preparation*. At the end of the 7-day experimental period, the mice were sacrificed by slight diethyl ether anaesthesia, and venous blood was collected into EDTA bottle to prevent clotting. The EDTA blood sample was centrifuged at 3000 rpm for 5 min and the plasma pipetted out. This was stored frozen until needed for analysis.

Table 1: Antiplasmodial activities and cytotoxicities of fractions of *Cocos nucifera* husk fibre extracts.

| Extracts | $IC_{50}$ ($\mu$g/mL) *Plasmodium falciparum* W2 | | | Remark | $MLD_{50}$ ($\mu$g/mL)* | Selectivity index |
|---|---|---|---|---|---|---|
| | Hypoxanthine | HRP2 | Mean | | | |
| DG (H) | >50.00 | >50.00 | >50.00 | Inactive | >1000.00 ± 0.00 | — |
| DG (M) | — | >50.00 | >50.00 | Inactive | >1000.00 ± 0.00 | — |
| DR (EA) | >50.00 | >50.00 | >50 | Inactive | 452.50 ± 136.50 | — |
| DR (H) | 43.00 | >50.00 | >50.00 | Inactive | >1000.00 ± 0.00 | — |
| DR (M) | >50.00 | >50.00 | >50.00 | Inactive | >1000.00 ± 0.00 | — |
| DY (EA) | 9.80 | >50.00 | — | Inconclusive | >1000.00 ± 0.00 | — |
| DY (M) | >50.00 | >50.00 | >50.00 | Inactive | 635.70 ± 48.50 | — |
| DY (H) | >50.00 | >50.00 | >50.00 | Inactive | >1000.00 ± 0.00 | — |
| HB (EA) | >50.00 | >50.00 | >50.00 | Inactive | 332.70 ± 33.00 | — |
| HB (H) | >50.00 | >50.00 | >50.00 | Inactive | >1000.00 ± 0.00 | — |
| HB (M) | >50.00 | 26.70 | — | Inactive | >1000.00 ± 0.00 | — |
| WAT (EA) | 12.44 | 9.43 | 10.94 ± 2.00 | Active | 333.00 ± 25.50 | 30.30 |
| WAT (H) | 46.60 | 30.50 | 39.00 ± 8.00 | Inactive | 408.00 ± 42.40 | — |
| WAT (M) | 14.60 | >50.00 | — | Inconclusive | >1000.00 ± 0.00 | — |
| Chloroquine | 0.04 | 0.05 | 0.04 ± 0.02 | Active | 387.50 ± 47.50 | 9687.50 |

*Mean of 3 experiments ± SD. WAT: West African Tall variety; DG: Dwarf Green variety; DR: Dwarf Red variety; DY: Dwarf Yellow variety; HB: Hybrid variety; M: methanolic fraction; H: hexane fraction; EA: ethyl acetate fraction.

Table 2: Phytochemicals of ethyl acetate fraction of *Cocos nucifera* (West African Tall variety) husk fibre extract.

| Phytochemical | Status in extract fraction |
|---|---|
| Tannins | + |
| Saponins | ND |
| Alkaloids | + |
| Phlobatannins | ND |
| Glycosides | ND |
| Steroids | ND |
| Flavonoids | + |
| Phenols | ND |

+: present; ND: not detected.

*2.8.4. Biochemical Assays.* The protein content of the plasma was determined using the Biuret method as reported by Gornall et al. [19]. The plasma creatinine and urea concentrations were determined by the methods of Bartels and Bohmer [20] and Veniamin and Vakirtzi-Lemonias [21], respectively. Bilirubin concentration in the plasma was determined using the method described by Winsten and Cehely [22]. The procedure described by Doumas et al. [23] was used for the determination of plasma albumin concentration of the mice. The determination of plasma globulin concentration was done using the method described by Tietz [24] by subtracting the concentrations of liver and plasma albumin from the total liver and plasma protein concentrations, respectively. Alkaline phosphatase activity was determined by the method of Wright et al. [25]. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined by monitoring the concentrations of $\alpha$-keto acid hydrazones formed with 2, 4-dinitrophenyl hydrazine based on the procedure of Reitman and Frankel [26]. The method described by Wroblewski and La due [27], with slight modification, was used for the determination of lactate dehydrogenase activity. The method described by Bradley et al. [28], with slight modification, was used for the determination of glutamate dehydrogenase activity.

*2.9. Statistical Analysis.* Data were analyzed using Duncan multiple range test following one-way analysis of variance (ANOVA) using SPSS 16.0 computer software package (SPSS Inc., Chicago, IL, USA). Differences at $P < 0.05$ were considered significant.

## 3. Results

The *in vitro* evaluation of the extract fractions for antiplasmodial activities revealed that only the ethyl acetate fraction of the husk fibre extract of WAT was active against *Plasmodium falciparum* W2 strain maintained in continuous culture ($IC_{50}$ = 10.94 $\mu$g/mL), with a selectivity index of 30.3 (Table 1). Only extracts with $IC_{50}$ less than 25 $\mu$g/mL are generally considered active [18]. The phytochemicals present in the WAT ethyl acetate extract fraction were found to be alkaloids, tannins, and flavonoids (Table 2). The 4-day suppressive test revealed that the WAT ethyl acetate extract fraction, at the doses of 31.25, 62.5, and 125 mg/Kg body weight, caused 50.2%, 97.5%, and 98.6% reduction in parasitemia, respectively, on day 4 after inoculation and 56.6%, 73.4%, and 71.1% reduction in parasitemia, respectively, on day 6 after inoculation (Table 3). There was no significant change ($P > 0.05$) in plasma urea concentration caused by the extract fraction at all doses administered whereas the extract fraction significantly increased ($P < 0.05$) plasma creatinine concentration at the doses of 250 and 500 mg/Kg body weight compared to controls (Table 4). Administration

Table 3: Parasitaemia in *P. berghei* NK65-infected mice treated with ethyl acetate fraction of *Cocos nucifera* (West African Tall variety) husk fibre extract.

| Groups (dose in mg/kg b.w.) | Parasitaemia (% reduction) | | | |
|---|---|---|---|---|
| | 4° | 6° | 8° | 10° |
| Untreated control | 2.85 | 5.71 | 6.43 | 9.48 |
| 31.25 | 1.42 (50.2) | 2.48 (56.6) | 5.20 (19.1) | 7.80 (17.7) |
| 62.5 | 0.07 (97.5) | 1.52 (73.4) | 0.75 (88.3) | 3.58 (62.2) |
| 125 | 0.04 (98.6) | 1.65 (71.1) | 0.88 (86.3) | 2.49 (73.7) |
| 250 | 2.02 (29.1) | 2.00 (64.9) | 1.52 (76.4) | 1.60 (83.1) |
| 500 | 0.37 (87.0) | 0.68 (88.1) | 0.15 (97.7) | 1.21 (87.2) |
| Chloroquine (20) | 0.04 (98.6) | 1.23 (78.5) | 0.65 (89.95) | 0.23 (97.6) |

Values are means ± SD of 5 replicates. °Days after inoculation.

Table 4: Effects of ethyl acetate fraction extract of *Cocos nucifera* (West African Tall variety) husk fibre extract on plasma urea and creatinine concentrations in mouse.

| Groups (dose in mg/kg b.w.) | Urea (mmol/L) | Creatinine (mmol/L) |
|---|---|---|
| 5% DMSO (control) | $7.63 \pm 0.73^a$ | $75.62 \pm 5.67^a$ |
| 31.25 | $6.32 \pm 0.50^a$ | $88.70 \pm 4.29^a$ |
| 62.5 | $6.16 \pm 1.15^a$ | $83.98 \pm 1.52^a$ |
| 125 | $6.39 \pm 0.15^a$ | $87.62 \pm 1.29^a$ |
| 250 | $6.16 \pm 0.39^a$ | $115.77 \pm 6.52^b$ |
| 500 | $7.28 \pm 0.90^a$ | $115.03 \pm 2.92^b$ |

Values are expressed as mean ± SEM ($n = 5$). Values in the same column with different superscripts are significantly different ($P < 0.05$).

Table 5: Effect of ethyl acetate fraction of *Cocos nucifera* (West African Tall variety) husk fibre extract on total and conjugated bilirubin concentrations in mouse plasma.

| Groups (dose in mg/kg b.w.) | Total bilirubin ($\mu$mol/L) | Conjugated bilirubin ($\mu$mol/L) |
|---|---|---|
| 5% DMSO (control) | $333.86 \pm 97.53^a$ | $113.58 \pm 21.94^a$ |
| 31.25 | $471.13 \pm 119.26^a$ | $121.23 \pm 41.88^a$ |
| 62.5 | $568.98 \pm 126.14^a$ | $128.23 \pm 34.50^a$ |
| 125 | $371.99 \pm 65.24^a$ | $155.88 \pm 42.59^a$ |
| 250 | $467.15 \pm 85.70^a$ | $151.54 \pm 42.45^a$ |
| 500 | $370.50 \pm 139.87^a$ | $103.13 \pm 25.09^a$ |

Values are mean ± SEM of 5 replicates. Values in the same column with same letter superscripts are not significantly different ($P > 0.05$).

of WAT ethyl acetate extract fraction, at all doses investigated, did not significantly ($P > 0.05$) alter the plasma total and conjugated bilirubin concentrations (Table 5), as well as plasma albumin and globulin compared to controls (Table 6). All doses of the extract fraction administered caused no significant alteration ($P > 0.05$) in plasma total cholesterol concentration, HDL cholesterol concentration, and atherogenic index compared to controls (Table 7). However, the extract fraction significantly increased ($P < 0.05$) the plasma triglyceride concentration at the dose of 31.25 mg/kg body weight compared to control. Moreover, WAT ethyl acetate extract fraction, at all doses administered, did not significantly alter ($P > 0.05$) plasma alkaline phosphatase and alanine aminotransferase activities but significantly reduced ($P < 0.05$) plasma aspartate aminotransferase activity compared to controls (Table 8). However, plasma lactate dehydrogenase activity was increased significantly ($P < 0.05$) at the dose of 250 mg/kg body weight and glutamate dehydrogenase activity at the doses of 62.5 and 250 mg/Kg body weight compared to controls (Table 9).

## 4. Discussion

*Cocos nucifera* is abundant in Nigeria and other parts of West Africa [29]. The plant husk fibre has been reported to be used in the middle belt region of Nigeria as an antimalarial remedy, which has also been authenticated through *in vitro* studies [8]. The results of this study lend credence to our earlier report on the antiplasmodial activity of the widely grown tall variety of *Cocos nucifera* [8] and also established the *in vivo* antimalarial activity of the active extract fraction, WAT ethyl acetate extract fraction. Phytochemical screening of WAT ethyl acetate extract fraction in this study revealed the presence of flavonoids, tannins, and alkaloids (Table 2). This result is similar to the report of Silva et al. [30] on the phytochemicals present in the ethyl acetate extract of *Cocos nucifera* (Olho de Cravo variety) husk fibre. Moreover, tannins and polyphenolic compounds such as flavonoids, of which catechins are the most prominent, have been reported to be present in abundance in the coconut husk fibre and are responsible for its antibacterial, antiviral, antileishmanial, antinociceptive and, free radical scavenging activities [31, 32]. Flavonoids and tannins present, which are phenolic compounds, act primarily as antioxidants or free radical scavengers, and may alleviate the oxidative stress associated with malaria, which plays an important role in the development of anaemia in malaria [33].

The concentrations of total protein, bilirubin, and albumin in blood can be used to ascertain different types of liver damage [34]. Bilirubin is an important product of haemoglobin catabolism with biological and diagnostic values [35]. Absence of a significant ($P > 0.05$) change in both total and conjugated bilirubin suggests that the extract

Table 6: Effects of ethyl acetate fraction of *Cocos nucifera* (West African Tall variety) husk fibre extract on total protein, albumin, and globulin concentrations in mouse plasma.

| Groups (dose in mg/kg b.w.) | Total protein (mg/mL) | Albumin (g/L) | Globulin (g/L) |
|---|---|---|---|
| 5% DMSO (control) | $22.64 \pm 0.86^{b}$ | $27.86 \pm 1.59^{a}$ | $5.86 \pm 0.44^{ab}$ |
| 31.25 | $24.84 \pm 1.03^{b}$ | $30.09 \pm 1.57^{a}$ | $6.35 \pm 1.96^{ab}$ |
| 62.5 | $26.19 \pm 2.04^{b}$ | $25.25 \pm 1.95^{a}$ | $1.98 \pm 0.57^{a}$ |
| 125 | $21.76 \pm 1.05^{b}$ | $25.58 \pm 1.74^{a}$ | $5.66 \pm 1.12^{ab}$ |
| 250 | $16.46 \pm 2.31^{a}$ | $24.95 \pm 0.93^{a}$ | $8.50 \pm 3.02^{b}$ |
| 500 | $21.41 \pm 1.32^{b}$ | $27.61 \pm 1.01^{a}$ | $6.20 \pm 0.38^{ab}$ |

Values are mean ± SEM of 5 replicates. Values in the same column with different letter superscripts are significantly different ($P < 0.05$).

Table 7: Effects of ethyl acetate fraction of *Cocos nucifera* (West African Tall variety) husk fibre extract on plasma lipid profile.

| Groups (dose in mg/kg b.w.) | Chol (mmol/L) | HDL-chol (mmol/L) | TG (mmol/L) | Atherogenic index |
|---|---|---|---|---|
| 5% DMSO (control) | $2.11 \pm 0.36^{a}$ | $1.01 \pm 0.32^{a}$ | $0.91 \pm 0.15^{a}$ | $2.09 \pm 0.06^{a}$ |
| 31.25 | $1.96 \pm 1.43^{a}$ | $0.90 \pm 0.22^{a}$ | $1.50 \pm 0.41^{b}$ | $2.18 \pm 0.32^{a}$ |
| 62.5 | $1.48 \pm 0.89^{a}$ | $0.71 \pm 0.12^{a}$ | $1.18 \pm 0.18^{ab}$ | $2.11 \pm 0.22^{a}$ |
| 125 | $1.71 \pm 1.29^{a}$ | $0.82 \pm 0.32^{a}$ | $1.01 \pm 0.22^{a}$ | $2.10 \pm 0.36^{a}$ |
| 250 | $2.84 \pm 1.5^{a}$ | $1.25 \pm 0.33^{a}$ | $1.09 \pm 0.28^{a}$ | $2.62 \pm 0.41^{a}$ |
| 500 | $1.50 \pm 0.89^{a}$ | $0.85 \pm 0.27^{a}$ | $0.91 \pm 0.60^{a}$ | $1.15 \pm 0.04^{a}$ |

Values are means ± SEM, $n = 5$. Values in each column with different letter superscripts are significantly different ($P < 0.05$).

fraction, at the doses administered in this study, is not capable of causing haemolysis and impairment of the secretion of conjugated bilirubin into the bile duct in the liver [36].

Albumin is synthesized in the liver. Thus, the lack of alteration in plasma albumin concentration suggests that the synthetic function of the liver has not been compromised, at all doses of the extract fraction administered [37]. Globulins are heterogeneous complex mixture of protein molecules with diverse functions in the body [38]. Also, there was no significant change in the globulin concentrations in plasma caused by the extract fraction at the doses administered in this study. Thus, the roles of these plasma proteins in the transportation of nutrients, defense mechanism, coagulation processes, maintenance of blood osmotic pressure, and buffering capacity of the blood may not be affected by the extract fraction [38].

Serum urea and creatinine concentrations are used for the assessment of renal sufficiency. Higher than normal levels serum urea and creatinine are indicators of deficiency in renal function [39]. Moreover, increase in serum urea level may also be due to the increase in protein catabolism [40] while the decreased urea level may be attributed to impairment of the urea cycle leading to reduced production of urea [9]. The extract fraction, at all doses administered, showed no significant alteration in the plasma urea concentration, suggesting that the urea cycle and protein catabolism were not adversely affected by the extract fraction. However, the increase in plasma creatinine concentration at the doses of 250 and 500 mg/Kg body weight suggests that the extract fraction may impair renal function at higher doses.

In recent times, there has been an increase in the prevalence of coronary heart disease (CHD) and CHD-related deaths possibly due to the mismanagement of the risk factors that predispose to this disorder [41]. The major identified risk factors are elevated serum LDL-cholesterol concentration, reduced serum HDL-cholesterol concentration, and high blood pressure [42, 43]. Studies have shown that lowering levels of serum cholesterol decreases the incidence of coronary heart diseases [44]. The atherogenic index (total cholesterol/HDL-cholesterol) is a reliable and strong indicator of cardiovascular diseases. Myocardial infarction increases considerably when the ratio is higher than 5 [45, 46]. All the values for atherogenic index observed in this study were less than 3 and were not significantly different ($P > 0.05$) from control, suggesting that WAT ethyl acetate extract fraction may not predispose subjects to coronary heart disease, thereby not giving rise to further cardiovascular complications during malaria treatment.

The measurement of the activities of enzymes in tissues and body fluids plays a paramount and well-known role in disease investigation and diagnosis [47]. These enzymes, such as phosphatases, dehydrogenases, and transferases, get into the blood through leakage from disrupted cell membranes in damaged tissues [48, 49]. Alkaline phosphatase (ALP) has been reported to be a marker enzyme for plasma membrane and endoplasmic reticulum [50]. ALT activity in the plasma is a more specific indicator of liver damage affecting cell integrity [51]. ALP and ALT activities, at all doses of the extract fraction administered, were not significantly changed in the plasma, suggesting that the extract fraction may not interfere with plasma membrane integrity and other metabolic activities mediated by ALP in the liver [52]. Lack of change in plasma ALP activity also suggests that the extract fraction does to cause hepatobiliary obstruction [51]. The reduction in plasma AST activity, at all doses of the extract fraction administered in this study, suggests inhibition of the enzyme *in situ* rather than damage to the liver or heart. Generally, plasma lactate dehydrogenase activity was not affected

Table 8: Effects of ethyl acetate fraction of *Cocos nucifera* (West African Tall variety) husk fibre extract on mouse plasma alkaline phosphatase, aspartate, and alanine aminotransferases activities.

| Groups (dose in mg/kg b.w.) | ALP ($\mu$mol/mg prot./min) | ALT (mmol pyr/min/mg prot.) | AST ($\times 10^{-5}$ mmol pyr/min/mg prot.) |
|---|---|---|---|
| 5% DMSO (control) | $2.49 \pm 0.36^{a}$ | $81.20 \pm 17.05^{a}$ | $20.57 \pm 0.30^{e}$ |
| 31.25 | $3.25 \pm 0.18^{a}$ | $159.39 \pm 50.31^{a}$ | $4.47 \pm 0.48^{b}$ |
| 62.5 | $2.74 \pm 0.22^{a}$ | $122.83 \pm 35.09^{a}$ | $7.50 \pm 1.00^{c}$ |
| 125 | $2.79 \pm 0.17^{a}$ | $222.50 \pm 35.32^{a}$ | $7.93 \pm 0.24^{c}$ |
| 250 | $2.75 \pm 0.20^{a}$ | $151.83 \pm 22.59^{a}$ | $0.60 \pm 0.01^{a}$ |
| 500 | $2.77 \pm 0.39^{a}$ | $154.74 \pm 11.76^{a}$ | $15.00 \pm 1.00^{d}$ |

Values are mean ± SEM of 5 replicates. Values with different letter superscripts from the control are significantly different ($P < 0.05$).

Table 9: Effects of ethyl acetate fraction of *Cocos nucifera* (West African Tall variety) husk fibre extract on activities of mouse plasma lactate and glutamate dehydrogenases.

| Groups (dose in mg/kg b.w.) | Lactate dehydrogenase ($\mu$M/mg protein/min) | Glutamate dehydrogenase (U/mg protein) |
|---|---|---|
| 5% DMSO (control) | $0.11 \pm 0.01^{a}$ | $0.08 \pm 0.01^{a}$ |
| 31.25 | $0.13 \pm 0.01^{a}$ | $0.09 \pm 0.02^{a}$ |
| 62.5 | $0.12 \pm 0.01^{a}$ | $0.36 \pm 0.07^{b}$ |
| 125 | $0.14 \pm 0.01^{a}$ | $0.04 \pm 0.010^{a}$ |
| 250 | $0.19 \pm 0.03^{b}$ | $0.25 \pm 0.06^{b}$ |
| 500 | $0.13 \pm 0.01^{a}$ | $0.07 \pm 0.01^{a}$ |

Values are mean ± SEM of 5 replicates. Values with different letter superscripts from the control are significantly different ($P < 0.05$).

by the extract fraction, except at the dose of 250 mg/kg body weight, suggesting that the extract fraction not predispose subjects to myocardial infarction and haemolysis [51].

In conclusion, the results of this study suggest the husk fibre of the West African Tall variety of *Cocos nucifera* as a potential source for novel antimalarial drug, pinpointing its ethyl acetate extract fraction as being responsible for its antimalarial activity. This active extract fraction may not possess hepatotoxicity potential nor predispose subjects to cardiovascular diseases. However, it may impair normal kidney function at higher doses. Further studies on biofractionation are underway to isolate the active compound(s) from the active extract fraction, with the aim of getting rid of the nephrotoxicity of the extract fraction at higher doses and reducing the expensive cost of the treatment of the disease, especially amidst the poor populace of the country.

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*Research Article*

# Trypanocidal Activity of Thioamide-Substituted Imidazoquinolinone: Electrochemical Properties and Biological Effects

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Three thioamide-substituted imidazoquinolinone, which possess a heterocyclic center similar to tryptanthrin and are named **C1**, **C2**, and **C3**, were studied regarding (a) their *in vitro* anti-*Trypanosoma cruzi* activity, (b) their cytotoxicity and electrochemical behaviour, and (c) their effect on cell viability, redox state, and mitochondrial function. The assayed compounds showed a significant activity against the proliferative forms, but only **C1** showed activity on the trypomastigote form (for **C1**, $IC_{50\,epi} = 1.49\,\mu M$; $IC_{50\,amas} = 1.74\,\mu M$; and $IC_{50\,try} = 34.89\,\mu M$). The presence of an antioxidant compound such as ascorbic acid or dithiotreitol induced a threefold increase in the antiparasitic activity, whereas glutathione had a dual effect depending on its concentration. Our results indicate that these compounds, which exhibited low toxicity to the host cells, can be reduced inside the parasite by means of the pool of low molecular weight thiols, causing oxidative stress and parasite death by apoptosis. The antiparasitic activity of the compounds studied could be explained by a loss of the capacity of the antioxidant defense system of the parasite to keep its intracellular redox state. **C1** could be considered a good candidate for *in vivo* evaluation.

## 1. Introduction

Chagas' disease is endemic in Latin America, directly affecting around 20 million of inhabitants, but it is also to be taken in account that more than 200 million are at risk of infection [1]. *Trypanosoma cruzi* is the etiological agent of this illness, a hemoflagellate parasite whose life cycle involves the obligatory passage through both vertebrate and invertebrate hosts (hematophagous triatomine bugs). Currently, only two drugs were commercially available for its treatment: Benznidazole (Bnz) and Nifurtimox (Nfx); however, the latter has been discontinued. Both drugs can reduce serological titters in acute and early chronic infections by eliminating patent parasitemia. Nevertheless, these drugs are not active against all *T. cruzi* strains and are known to produce toxic effects on the host [2]. All of these facts highlight the urgent need for the development of new, cheap, safe, and more efficient compounds for treating Chagas' disease.

A variety of natural products are known to have antitrypanosomal activity. Tryptanthrin (indolo[2,1-b]quinazoline-6,12-dione) is a weak basic alkaloid isolated from medicinal plants, such as *Polygonum tinctorium*, *Isatis indigotica*, and *Strobilanthes cusia* [3]. This alkaloid has a broad spectrum of biological functions, including anti-inflammatory, antifungal,

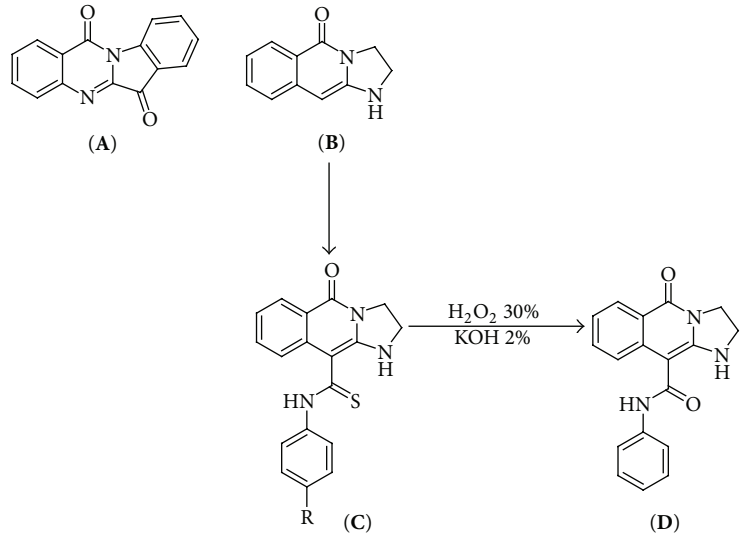


Figure 1: Tryptanthrin and structurally related compounds. (**A**) Tryptanthrin. (**B**) heterocycle precursor. (**C**) C-10 thioamide-substituted compounds, denominated **C1** (R: H), **C2** (R: Cl), and **C3** (R: $CH_3$). (**D**) C-10 amide-substituted compound.

antibacterial, and antitumor effects [4] and its references. In addition, tryptanthrin derivatives have been shown to possess activity against several protozoan pathogens. Specifically, these compounds have been reported to inhibit some strains of *Leishmania* spp. [5, 6], *Trypanosoma brucei* [7], *Plasmodium falciparum* [8], and *Toxoplasma gondii* [9]. Closely related to the tryptanthrin structure (Figure 1(**A**)), we have synthesized the 2,3-dihydroimidazo[1,2-b]isoquinolin-5(1-*H*)-one molecule, which is a heterocycle precursor (Figure 1(**B**)) to then obtain new N-1 or C-10 substituted imidazoquinolinones derivatives, similar to tryptanthrin [10, 11]. Importantly, when a computational analysis was run over, comparing tryptanthrin and the heterocycle precursor structures, which consist in the overlapped structures (HyperChem software, Hypercube. Inc), a significantly great similarity was found (root-mean-square deviation, RMSD $1.69 \times 10^{-3}$ Å; unpublished data). Recalling that thioamide derivatives substituted in C-10 (Figure 1(**C**)) exhibited the best antichagasic activity on *T. cruzi* epimastigotes [11], we have therefore selected the compounds of the **C** series (**C1**, **C2**, and **C3**, Figure 1), which showed superior activity to that of Nfx, to study their effect *in vitro* against the different stages of the parasite. **D** was used as a non-C-10 thioamide substituted imidazoquinolinone derivative (Figure 1(**D**)). The mechanism of action of the **C** compounds was also investigated. In case that **C** compounds were efficient antichagasic agents, it is worth to emphasize that they present the additional advantages over tryptanthrin and its derivatives of being obtained more cheaply, simply, and efficiently.

## 2. Materials and Methods

*2.1. Chemicals.* Bnz was kindly provided by Roche (Argentina). The compounds assayed (Figure 1) N-phenyl-5-oxo-1,2,3,5-tetrahydroimidazo[1,2-b]isoquinolin-10-carbothioamide (**C1**), N-(4-chlorophenyl)-5-oxo-1,2,3,5-tetrahydroimidazo[1,2-b]isoquinolin-10-carbothioamide (**C2**), N-(4-methylphenyl-5-oxo-1,2,3,5-tetrahydroimidazo[1,2-b]isoquinolin-10-carbothioamide (**C3**), and 5-oxo-N-phenyl-1,2,3,5-tetrahydroimidazo[1,2-b]isoquinolin-10-carboxamide (**D**) were synthesized according to Bollini et al. [11]. Standard solutions of these compounds were prepared in dimethyl sulphoxide (DMSO), and their final concentrations in the experiments never exceeded 0.5%.

*2.2. Parasites.* *Trypanosoma cruzi* epimastigotes (Tulahuen strain) were grown as previously described [12]. Bloodstream trypomastigotes were obtained from infected CF1 mice by cardiac puncture. Tulahuen strain expressing the $\beta$-galactosidase gene was kindly provided by Dr Buckner (University of Washington, USA) [13].

*2.3. Animals.* Inbred CF1 male mice were nursed at Facultad de Medicina, Universidad de Buenos Aires. Animals were handled in accordance with the guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC).

*2.4. In Vitro Assays for Anti-T. cruzi Activity.* To evaluate the growth inhibition of epimastigotes, $1.5 \times 10^6$ parasites/mL were cultivated with different concentrations of the compounds (0.50 to 15 $\mu$M) or Bnz (2.50 to 15 $\mu$M) for 4 days. Cells growth was assessed by counting the number of cells per mL of culture using a Neubauer chamber and was expressed as cellular density (CD). The percentage of inhibition (%$I$) was calculated as $\%I = \{1 - [(CD_{4t} - CD_0)/(CD_{4c} - CD_0)]\} \times 100$, where the different CDs represent the cellular density of $CD_{4t}$, treated parasites on day 4; $CD_0$, parasites on day 0; and $CD_{4c}$, untreated parasites (control) on day 4.

The trypanocidal effects of **C1**, **C2**, **C3**, **D** and Bnz were also tested on bloodstream trypomastigotes according to a standard WHO protocol slightly modified [14]. Briefly, mouse blood containing trypomastigotes was treated with different concentrations of each compound (0.30 to 350 $\mu$M) or Bnz

(0.38 to 38 $\mu$M). Plates were incubated for 24 h, and surviving parasites were counted in a Neubauer chamber as previously described [15]. Results were expressed as the percentage of lysed parasites (%$L$) relative to the number of parasites in the control: $\%L = [1 - (CD_t/(CD_c)] \times 100$, where $CD_t$ and $CD_c$ represent the cellular density of treated and untreated parasites, respectively.

For analysis of amastigotes, J774 cells were infected with bloodstream trypomastigotes expressing the $\beta$-galactosidase gene at a parasite: cell ratio of 10 : 1. After 24 h, cell cultures were washed and each compound (2 to 100 $\mu$M) was added in fresh RPMI medium without phenol red (to avoid interference with absorbance readings at 570 nm). After 7 days, the assay was developed as described [15]. The galactosidase activity was quantified using CPRG as substrate and measuring absorbance at 570 nm in a Microplate Reader (Bio-Rad Laboratories). Since the assayed compounds are coloured, blanks including uninfected cells with different doses of each compound were performed. The percentage of inhibition was calculated as $\%I = \{1-[(A_{it}-A_{nit})/(A_{ic}-A_{nic})]\}\times 100$, where $A$ represents the mean $A_{570}$ value recorded for $A_{it}$, treated infected cells; $A_{nit}$, treated noninfected cells; $A_{ic}$, untreated infected cells; and $A_{nic}$, untreated noninfected cells.

*2.5. Cytotoxicity Assay.* Vero cells were cultivated with different concentrations of each compound (12.5–100.0 $\mu$M) or Bnz (3.0–3000 $\mu$M). After 48 h of incubation, cells were washed and viability was measured by the MTT assay as previously described [15]. The selectivity index (SI) was calculated as the 50% cytotoxic concentration ($CC_{50}$) obtained with Vero cells divided by the 50% inhibitory concentration ($IC_{50}$) obtained with *T. cruzi*.

*2.6. Electrochemical Behaviour of Thioamide-Substituted Imidazoquinolinones.* Cyclic voltammograms for **C1**, **C2**, and **C3** dissolved in 1% DMSO were carried out using an EQMAT instrument with an EQSOFT Processor, at a sweep rate of 0.2 V/s under a nitrogen atmosphere at room temperature, employing lithium perchlorate as supporting electrolyte. A three-electrode cell was used equipped with a vitreous carbon as working electrode, a gold wire as auxiliary electrode, and a saturated calomel as reference electrode.

*2.7. Biochemical Assays to Characterize the Antitrypanosomal Action.* Epimastigotes of *T. cruzi* from a 4 days culture were incubated with **C1** (7.5–22.5 $\mu$M) during 5–36 hours. Cells were harvested, washed and then the following biochemical assays were carried out.

*2.7.1. Evaluation of Oxidative Stress*

*(a) Assay of Intracellular Oxidative Activity.* The intracellular oxidative activity was assessed by flow cytometry using the oxidant-sensitive fluorescent probe $H_2$DCFDA. As a positive control cells were treated with 0.1 mM $H_2O_2$. Stained cells were then analyzed by a FACSCalibur flow cytometer (Becton Dickinson) with an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Flow cytometry results were expressed by the ratio $Gm_t/Gm_c$, where $Gm_t$ and $Gm_c$ correspond to the geometric mean of histograms obtained for treated and untreated cells, respectively.

*(b) Determination of Antioxidant Enzymes Activity.* The activities of superoxide dismutase (SOD), ascorbate peroxidase (APx), and trypanothione reductase (TryR) were assayed as previously established [15].

Protein concentration was determined according to the method described by Lowry et al. [16]. These values were used to express the specific enzymatic activities as activity per mg of protein.

*(c) Determination of Total Thiol Groups.* The thiol groups content was determined employing the chromogenic compound 5,5′-dithiobis-2-nitrobenzoate (DTNB), as already described [12].

*2.7.2. Evaluation of Parasite Death.* Cell viability and phosphatidylserine (PS) exposure on the parasite surface were assessed by propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (FITC) staining, according to the manufacturer's instructions (Invitrogen). Epimastigotes exposed to 30% fresh human serum for 2 h at 28°C were used as positive control. Parasites were analysed by flow cytometry acquiring 20,000 events per sample.

*2.7.3. Evaluation of Mitochondrial Damage.* Mitochondrial membrane potential was assessed using two well-established assays: 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) staining and the cytochrome c release.

For the $DiOC_6$ assay, epimastigotes ($10^6$) were permeabilized for 20 min at room temperature with 0.01% saponin, washed, and incubated with 30 nM $DiOC_6$ for 30 min at 37°C. The positive control was done with 250 nM trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) as depolarizing agent. Stained cells were analyzed by flow cytometry with an excitation wavelength of 484 nm and an emission wavelength of 511 nm.

To evaluate the cytochrome c release, parasites ($3 \times 10^8$) were resuspended in PBS containing 200 $\mu$g/mL digitonin, incubated on ice for 15 min and then centrifuged at 9 000 ×g for 10 min at 4°C. Both, mitochondrial-rich fraction (pellet, resuspended in 50 $\mu$L of PBS) and cytosolic fraction (150 $\mu$L) were subjected to Western-blot analysis for cytochrome c. Protein extracts (10 $\mu$L and 30 $\mu$L of mitochondrial and cytosolic fractions, resp.) were resolved by 14% SDS/PAGE, transferred to nitrocellulose membranes, blocked with 3% skimmed milk in PBS, and then incubated with specific antibodies, according to the protocol of Piacenza et al. [17]. Relative intensities of bands were quantified by densitometry using Scion Image software (Scion). Results were expressed in arbitrary units.

*2.8. Statistical Analysis.* The results presented are representative of three to four separate experiments, performed in duplicates or triplicates. All data are expressed as means ± standard errors of the mean (SEM). To calculate the $IC_{50}$ values, the %$I$ or %$L$ values were plotted against the log of drug concentration ($\mu$M) and fitted with a straight line

Table 1: Values of $IC_{50}$ for the activity of the imidazoquinolinones on epi-, trypo-, and amastigotes forms of *Trypanosoma cruzi*.

| Compound | Epimastigotes | | Trypomastigotes | | Amastigotes | |
|---|---|---|---|---|---|---|
| | $IC_{50}$ ($\mu$M) | SI | $IC_{50}$ ($\mu$M) | SI | $IC_{50}$ ($\mu$M) | SI |
| **C1** | 1.49 ± 0.28 | >67.1 | 34.89 ± 1.20 | >2.9 | 1.74 ± 0.30 | >57.5 |
| **C2** | 5.57 ± 0.53 | >18.0 | 306 ± 15 | >0.3 | 3.63 ± 0.51 | >27.5 |
| **C3** | 1.50 ± 0.30 | >66.7 | 246 ± 18 | >0.4 | 1.50 ± 0.32 | >66.7 |
| **D** | >25 | ND | ≫300 | ND | 11.44 ± 1.21 | ND |
| Bnz | 5.49 ± 0.89 | 15.1 | 30.26 ± 2.85 | 2.7 | ND | ND |

$IC_{50}$ and SI values were calculated as indicated in Section 2. ND: not determined.

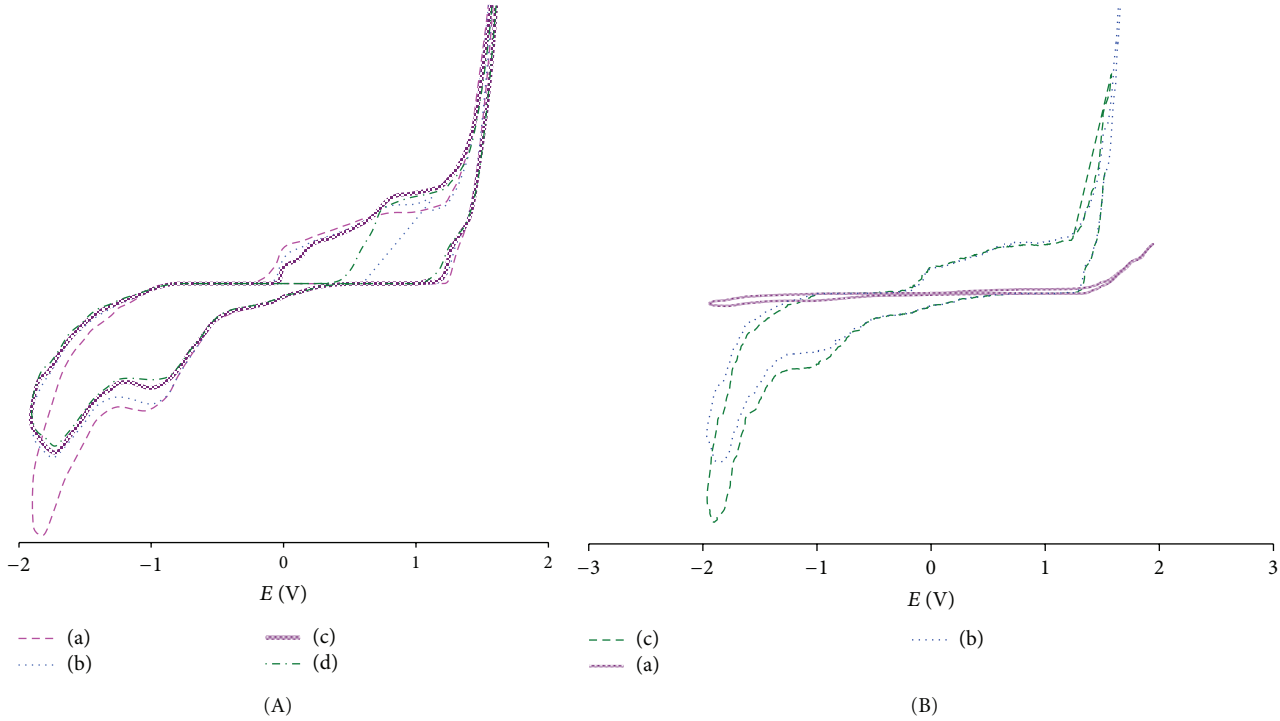


Figure 2: Cyclic voltammograms of **C1**. Profiles corresponding to: (A) different concentrations of drug in 1% DMSO: (a) 0.014 $\mu$M; (b) 0.14 $\mu$M; (c) 0.28 $\mu$M; and (d) 0.90 $\mu$M. (B) (a) **C1**; (b) GSH: **C1** (1 : 1); and (c) GSH: **C1** (2 : 1). Experimental conditions were as described in Section 2.

determined by a linear regression (Sigma Plot 10 software). The significance of differences was evaluated using Student' $t$ test, taking a $P < 0.05$ as significant. Flow cytometry data were analyzed employing the WinMDI 2.9 software.

## 3. Results

*3.1. In Vitro Antitrypanosomal Activity.* Results for the *in vitro* assays against the different stages of the parasite are shown in Table 1. Even though the three compounds of the series **C** were found to be active, with very similar $IC_{50}$ values for epi- and amastigotes, on trypomastigotes **C1** was 10 times more active than **C2** and **C3**. All the tested compounds showed the lowest *in vitro* trypanocidal activity when evaluated on the trypomastigote forms. Comparing the values obtained for **C** compounds and **D** (C-10 amide substituted analogue) it can be concluded that the thioamide group is essential for drugs to have a considerable anti-*T. cruzi* activity. Because of these findings, **C1** was selected as the most active compound of the **C** series, rendering the highest antiparasitic activity against the three stages of *T. cruzi*. Notably, although $IC_{50}$ of **C1** and Bnz were similar on trypomastigote stage, **C1** was found to be 3.5 times more active than Bnz on the epimastigote form (Table 1).

*3.2. Cytotoxicity Assay.* Unlike Bnz, which displayed a $CC_{50}$ of 82.79 ± 2.75 $\mu$M, none of the compounds evaluated were cytotoxic at any of the concentration assayed (12.5–100.0 $\mu$M). Since all the compounds displayed a $CC_{50}$ greater than 100.0 $\mu$M, then the highest SI was obtained for drugs that had the lowest $IC_{50}$ value (Table 1). **C1** was the compound presenting good SI values for the three stages.

*3.3. Electrochemical Behaviour.* Cyclic voltammetry is a methodology extensively used to determinate redox properties of molecules in solution. Figure 2(A) shows the cyclic

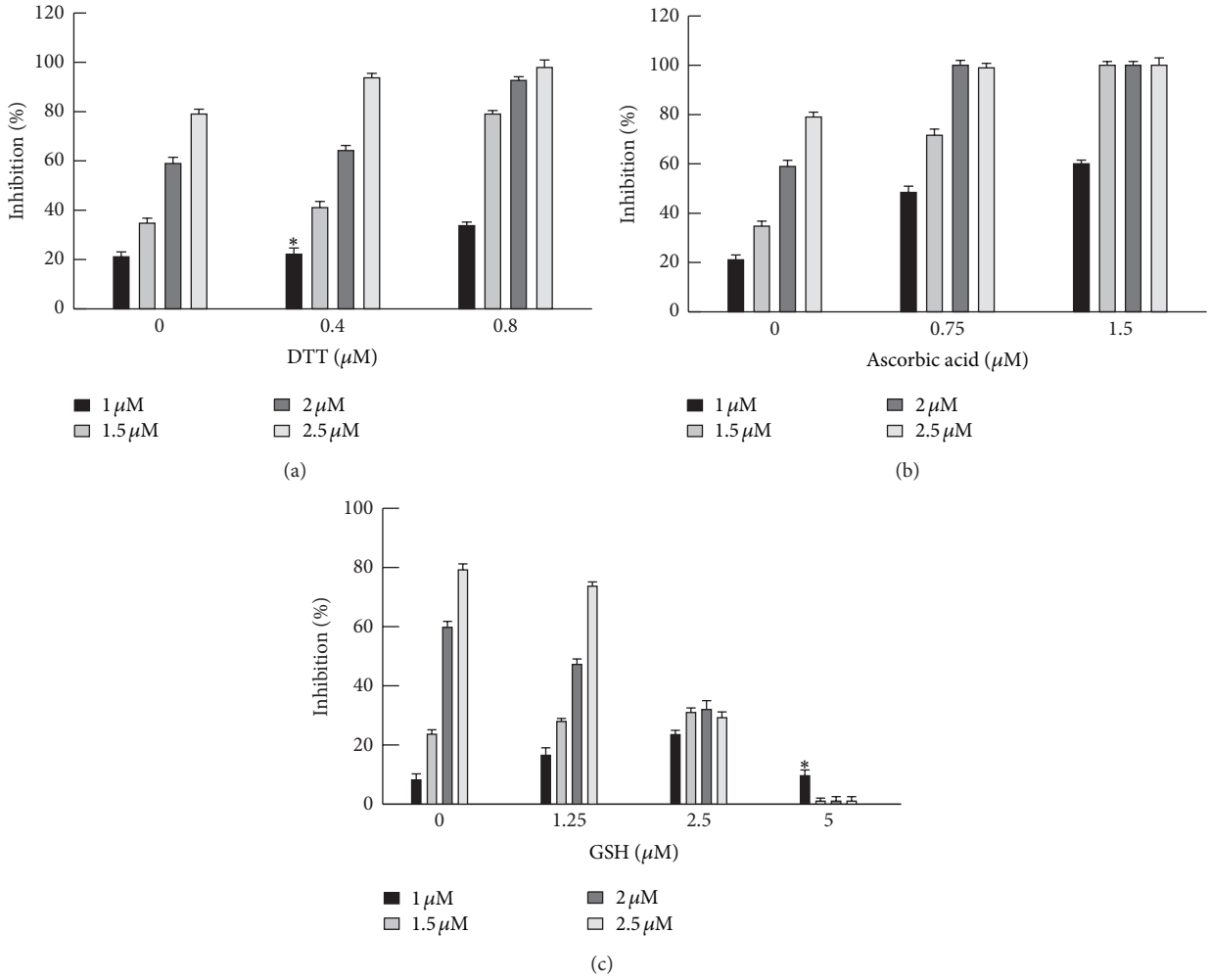


Figure 3: Effect of DTT, ascorbic acid and GSH on the anti-*T. cruzi* activity of **C1**. Experimental conditions were as described in Section 2. The concentrations of **C1** tested were from 1 up to 2.5 $\mu$M. The value 0% of inhibition corresponds to parasites cultured in the absence of both **C1** and antioxidant compound. *No significant differences ($P > 0.05$) were found when compared to the control (0 $\mu$M antioxidant compound) as assessed by Student' $t$ test.

voltammograms for increasing amounts of compound **C1** in 1% DMSO. A cathodic peak at −1.04 V and an anodic peak at 0.78 V, corresponding to irreversible reactions of reduction and oxidation, respectively, were observed. A very good linear correlation between the cathodic peak current ($i_{cp}$) and concentration of **C1** from 0.014 to 0.28 mM was observed (graphic not shown). For higher drug concentrations (0.90 mM) the saturation of the electrode was evident. A similar behaviour was obtained for the other two compounds of the **C** series (data not shown). Neither the cathodic nor the anodic peaks were measurable for the heterocycle precursor (Figure 1(**B**)) when subjected to the same potentials as **C1**.

The electrochemical properties of **C1** in the presence of a biologically relevant thiol, glutathione (GSH), were also studied. Figure 2(B) shows the typical cycle voltammograms of **C1** in the absence and in the presence of increasing amounts of GSH. When GSH was added at the ratio GSH : **C1** (1 : 1) it produced a significant increase in the current of both anodic and cathodic peaks with a concomitant displacement of the cathodic peak to low potentials. At the ratio GSH : **C1** (2 : 1) only the cathodic peak was increased, whereas the anodic peak was the same as that obtained with the 1 : 1 ratio. The GSH signals, at the studied concentrations, did not interfere with the signals of **C1**. The electrochemical profile obtained by adding GSH suggests that new electroactive entities (oxidation and reduction products) had been generated. The presence of a adduct **C1**-GSH was supported by the remarkable increase in the cathodic peak current with a concomitant displacement to lower potentials.

To obtain additional information regarding the mechanism of action of **C** compounds, the effect of antioxidant agents, such as dithiothreitol (DTT), ascorbic acid, and GSH on *in vitro* anti-*T. cruzi* activity of **C1** was evaluated (Figure 3). At the concentrations tested both DTT and ascorbic acid considerably enhanced the inhibitory action of the drug. Higher concentrations of these antioxidants were not tested because *in vitro* they showed significant antiparasitic activity *per se*. For GSH, a dual effect was observed; this

Table 2: Effect of treatment with **C1** on antioxidant enzymes activities.

| Time of treatment (hours) | Drug ($\mu$M) | SOD activity (%) | APx activity (%) | TryR activity (%) |
|---|---|---|---|---|
| 5 | 0 | 100.00 ± 3.60 | 100.00 ± 3.89 | 100.00 ± 4.56 |
| | 7.5 | 95.85 ± 2.50 | 111.26 ± 7.60 | 152.23 ± 6.38* |
| | 15.0 | 99.31 ± 4.30 | 98.31 ± 2.10 | 177.17 ± 15.56* |
| | 22.5 | 90.09 ± 2.30 | 105.20 ± 6.20 | 143.89 ± 4.80* |
| 12 | 0 | 100.00 ± 4.20 | 100.00 ± 1.50 | 100.00 ± 8.30 |
| | 7.5 | 99.75 ± 2.90 | 98.30 ± 2.30 | 188.60 ± 12.30* |
| | 15.0 | 103.92 ± 4.30 | 95.91 ± 4.10 | 251.62 ± 15.56* |
| | 22.5 | 88.48 ± 3.20* | 93.48 ± 6.20 | 190.55 ± 14.29* |
| 24 | 0 | 100.00 ± 4.50 | 100.00 ± 4.22 | 100.00 ± 6.30 |
| | 7.5 | 98.74 ± 7.30 | 108.35 ± 9.20 | 165.23 ± 9.80* |
| | 15.0 | 89.78 ± 3.50* | 105.30 ± 6.56 | 198.27 ± 9.30* |
| | 22.5 | 78.27 ± 4.20* | 96.25 ± 5.80 | 163.56 ± 7.60* |

Experimental conditions were as described in Section 2. For each time of treatment the activity value obtained in the absence of **C1** was considered as the control value (100%). *Significant differences ($P > 0.05$) were found when compared to the control as assessed by Student' $t$ test.

compound was able to either enhance or inhibit the effect of **C1**, depending on the concentration employed. GSH concentrations up to 2.5 $\mu$M potentiated the effect of low concentrations of **C1** (no greater than 1.5 $\mu$M), whereas the effect of **C1** concentrations above 1.5 $\mu$M was diminished. At a concentration of GSH of 5 $\mu$M the inhibitory effect of **C1** was completely abrogated.

### 3.4. Biochemical Assays to Characterize the Antitrypanosomal Action

*3.4.1. Evaluation of Oxidative Stress.* For this study, epimastigotes are cultured in the presence of high concentrations of **C1** (7.5, 15, and 22.5 $\mu$M) during short times of exposure (5, 12, and 24 h). These concentrations and times were selected because they were suitable for the parasite to manifest a response but without causing its death nor/or allowing it to revert such response. According to their electrochemical properties, we expected that **C1** would act as an electrophilic compound able to generate oxidative stress inside the parasite. The fluorescence of $H_2$DCFDA-loaded epimastigotes was not significantly modified by **C1** treatment. Thus, the ratio $Gm_t/Gm_c$ was around 1 independently on **C1** concentration and the time of exposure (data not shown). For the same treatment conditions, the activities of SOD and APx (Table 2) were not significantly different from those obtained with the control (only for SOD at longer times and high drug concentrations a slight decline in activity was observed), whereas TryR activity values showed a significant increase for all concentrations of **C1** and all exposure times tested (Table 2). Simultaneously, the level of low molecular mass thiols was found to remain constant for all **C1** concentrations tested, at exposition times of 5 and 12 h (data not shown). After 24 h of incubation a slight decrease (not more than 20%) was only observed for the highest concentration of the drug (22.5 $\mu$M). This behaviour, similar to that showed with SOD activity, would indicate that the deleterious action of 22.5 $\mu$M **C1** begins to be evident within 24 h of treatment. The constant level of thiols was not surprising due to the high activity of TryR.

*3.4.2. Evaluation of Cell Death and Mitochondrial Damage.* For these assays, parasites treated with **C1** at 22.5 $\mu$M for 8, 24, and 36 h were used. These experimental conditions were known to lead to parasite death. Annexin-V FITC/PI staining was used as a parameter to detect apoptotic cells. Results demonstrated that the number of apoptotic cells increased during the treatment with **C1** in a time-dependent manner (Figure 4(a)). The most significant differences in the levels of apoptotic cells were observed for early apoptotic cells for which values of 0.8%, 8.7%, and 51.0% were obtained for 0, 24, and 36 h of treatment, respectively. The number of late apoptotic cells also increased with time of treatment but in a less marked and significant manner, obtaining values of 2.8%, 4.9% and 7.7% for 0, 24, and 36 h of treatment, respectively. The number of viable nonapoptotic cells reached values of 84.8% and 40.4% for 24 and 36 h of incubation, respectively, versus 95.9% for control (0 h).

Simultaneously, the mitochondrial membrane depolarization which was evident after 24 h of treatment remained at similar levels up to 36 h (Figure 4(b)). Depolarized cells reached values of 77% and 84% of the evaluated cells after 24 and 36 h of treatment, respectively, versus 31% for the control (0 h). Given that the depolarization of the outer mitochondrial membrane causes the release of cytochrome c into the cytoplasm, then, the presence of cytochrome c in both mitochondrial and cytosolic fractions was assessed (Figure 4(c)). Cytochrome c was detected in the parasite cytosol after 24 h of treatment with **C1** increasing up to 2.5–3 times at 36 h. Densitometric analysis of the immunoblots showed that the total cytochrome c (mitochondrial + released into cytosol) in **C1**-treated parasites remained constant (the mitochondrial fraction of parasites nontreated was considered as control).

## 4. Discussion

In this work we have studied, on all stages of *Trypanosoma* cruzi, the trypanosomal activity of three totally synthetic C-10 thioamide substituted imidazoquinolinones, named **C1**, **C2**, and **C3** (Figure 1(**C**)). As illustrated in Table 1, the three compounds showed a considerable activity against epimastigotes

and amastigotes, whereas **C1** (selected as the best antichagasic compound) was the only one displaying an activity value similar to that obtained with Bnz for the infective form. The high $IC_{50}$ values observed for all tested imidazoquinolinones on trypomastigotes could be due to drug instability in presence of whole blood and/or to their association with serum components. Only tested on epimastigotes, the heterocycle precursor did not show antichagasic activity ($IC_{50}$ higher than 25 $\mu$M, data not shown). This result together with those obtained for compounds **C** on epimastigotes (Table 1) are consistent with slight differences due to the different methodology used, to the values previously reported by Bollini et al. [11]. The derivatives showed activities between 5 (for **C2**) to 25 (for **C1** and **C3**) times greater than the heterocyclic precursor. Regarding this, the heterocycle precursor and **C** series compounds show on *T. cruzi* a behaviour similar to tryptanthrin and its derivatives on *T. brucei* where the most potent derivative had a 50% effective concentration more than 25 times lower than that of tryptanthrin [11].

From the electrochemical study we would conclude that the reduction of **C** compounds (cathodic peak near −1.04 V) could take place *in vivo*. The absence of the correspondent anodic peak would indicate that this electronic transfer process is irreversible. The anodic scans showed a peak at 0.78 V that would correspond to an irreversible oxidation reaction. Considering that no redox reactivity was measurable for the heterocycle precursor (Figure 1(**B**)) when subjected to the same potentials as **C1**, it can be expected that the reduction of the thioamide group in C-10 could take place in the biological environment and represent a key event in the mechanism of action of these drugs. The reactions (1) and (2) shown below could justify the presence of the two peaks mentioned previously. Because we have found that the cathodic peak current is directly proportional to the **C1** concentrations (Randles-Sevcik equation) then, cyclic voltammetry can be used to quantify concentrations of compounds **C** between 0.014 to 0.28 mM.

On the other hand, the electrochemical profile obtained with GSH : **C1** (1 : 1) suggests that new reduction (cathodic peak) and oxidation (anodic peak) reactions have taken place. Reactions (3) and (4) would justify the cathodic and anodic peaks, respectively. The presence of the **C1**-GSH adduct was supported by both the increase in current and the shift of the cathodic peak to lower potentials. Reaction (4) reaches the saturation when the ratio GSH : **C1** is higher than 1 : 1,

$$\mathrm{RNH{-}R'C{=}S + 2H^+ + 2e^- \longrightarrow RNH{-}R'CH{-}SH} \quad (1)$$

$$\mathrm{2RNH{-}R'CH{-}SH \longrightarrow RNH{-}R'CH{-}S{-}S{-}HCR'{-}HNR + 2H^+ + 2e^-} \quad (2)$$

$$\mathrm{RNH{-}R'CH{-}SH + GSH \longrightarrow Adduct\left[RNH{-}R'CH{-}SH - (GSH)\right]} \quad (3)$$

$$\mathrm{RNH{-}R'CH{-}SH + GSH \longrightarrow RNH{-}R'CH{-}S{-}S{-}G + 2H^+ + 2e^-} \quad (4)$$

R: $-C_6H_5$ (for **C1**). R′: anion of precursor heterocycle (Figure 1(**B**)).

Considering that trypanosomatids possess high levels of low molecular mass thiols, we could expect that the reaction (2) was not physiologically significant. Then the probable mechanism of action of **C** compounds could be represented by the reactions (1), (3), and (4). It is important to remember that the only low molecular mass thiol of *T. cruzi* is not glutathione because this parasite has significant amounts of trypanothione ($N^1$, $N^8$ bis glutathionyl-spermidine), glutathionylspermidine, and ovothiol A [18]. Therefore, GSH would not be the only species to accomplish reactions (3) and (4) within the parasite. Independently of the interaction between the reduced compound and low molecular thiols, the possibility of an interaction with essential thiols from parasite's proteins (as enzymes) should be considered. The stimulatory effect of trypanosomal activity of **C1** observed by adding antioxidants such as DTT, ascorbic acid, or low concentrations of GSH (Figure 3) would support the participation of reaction (1) as part of the mechanism of action of this drug. Since the presence of high concentrations of GSH abolishes the antiparasitic effect of **C1**, we could postulate that reactions (3) and (4) would be involved in the metabolism of the drug inside the parasite. The latter reactions would block its effect.

According to these results, **C1** could act as an electrophilic compound, and therefore it could be able of producing oxidative stress inside the parasite. Using **C1** concentrations (7.5–22.5 $\mu$M) and exposure times (5–24 h) for which most of the cells remain viable (Figure 4(a)), the intracellular oxidative state, SOD and APx activities, and the levels of –SH groups remained unchanged. On the other hand, the only parameter that was significantly increased even for the lower concentration and shorter treatment time was the activity of TryR (Table 2). An oxidation and/or a decrease in the levels of thiols may have occurred (according to the reactions mentioned previously) as a consequence of the addition of **C1**, but the level of –SH groups was not altered, whereas it may be restored by the TryR.

Finally, we have found that treatment with **C1** (22.5 $\mu$M) at times up to 36 h produced time-dependent changes in the mitochondrial membrane potential, cytochrome c release from mitochondria into the cytoplasm, and the exposure of PS on the outer surface leaflet of the plasma membrane (Figure 4). These results would suggest that **C1** induces the parasite death by apoptosis.

## 5. Conclusions

Our findings led us to postulate that (1) to exert their effect, the thioamide-substituted imidazoquinolinones must undergo reduction inside the parasite, (2) the target of these drugs would be the pool of low molecular weight thiols, considering the principal redox buffer in the parasitic protozoa [19], and (3) the antiparasitic activity of these drugs may be associated with a loss of the antioxidant defense system capacity of the parasite to keep the intracellular redox state; however, as it has been reported for other drugs, the activity of C compounds could also be associated with a dysfunction

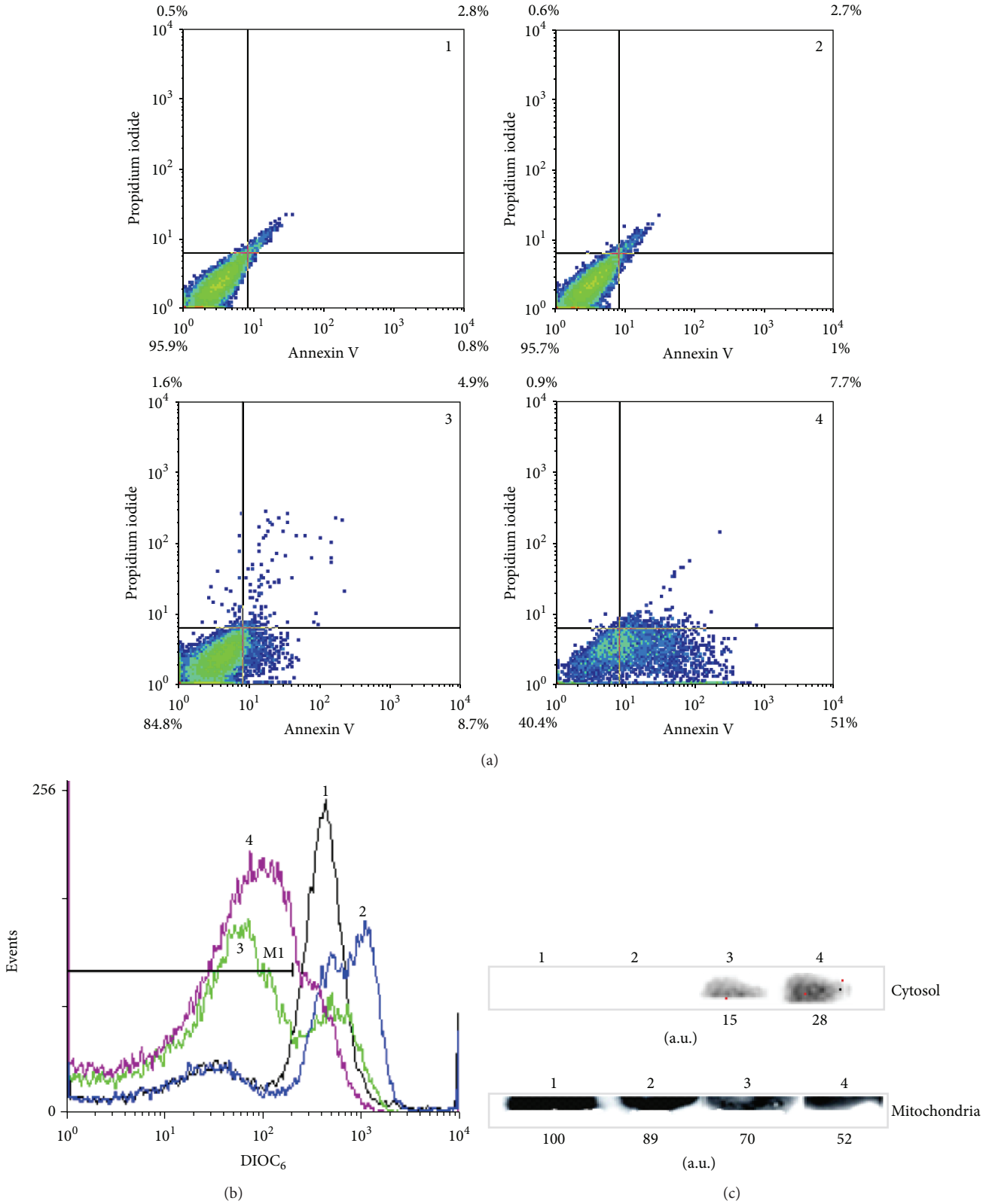


Figure 4: Effect of **C1** on cell death and state of the mitochondria evaluated by flow cytometry and Western blot. Epimastigotes of *T. cruzi* were treated with **C1** 22.5 $\mu$M during 8, 24, and 36 h. Parasites were (a) stained by Annexin V-FITC/PI, (b) stained by $DIOC_6$, or (c) incubated with digitonin, to obtain the cytosolic and mitochondrial fractions, which were subjected to Western-blot analysis for cytochrome c. The numbers correspond to 1: untreated cells and 2, 3, and 4: treated cells with **C1** during 8, 24, and 36 h, respectively. Methodology and data analysis were carried out as described in Section 2.

of the only mitochondria of these organisms (concerning this regard, additional studies are necessary to further sustained this proposed) mediated by the loss of mitochondrial membrane potential [20]. Both stress oxidative or mitochondrial damage finally would lead to cell death by apoptosis. These results provide supporting evidence to test *in vivo* the trypanocidal action of **C1** in an animal model of Chagas' disease. Considering the low income of the population suffering from Chagas' disease, it is important to take in account that the synthesis of imidazoquinolinones is highly efficient, simple, fast, and inexpensive; moreover, final products do show good stability. The previous reasons convert these compounds to an attractive therapeutic alternative more interesting than using tryptanthrin or its derivatives, to fight this parasitosis.

## Abbreviations

| | |
|---|---|
| Bnz: | Benznidazole |
| APx: | Ascorbate peroxidase |
| CPRG: | Chlorophenol Red-$\beta$-D-galactopyranoside |
| DCF: | Dichlorofluorescein |
| $H_2$DCFDA: | 2′,7′-Dichlorodihydrofluorescein diacetate |
| $DIOC_6$: | 3,3′-Dihexyloxacarbocyanine iodide |
| DTT: | Dithiothreitol |
| FCCP: | Trifluoromethoxy carbonyl cyanide phenyl hydrazone |
| FITC: | Fluorescein isothiocyanate |
| GSH: | Reduced glutathione |
| PI: | Propidium iodide |
| PS: | Phosphatidylserine |
| ROS: | Reactive oxygen species |
| SOD: | Superoxide dismutase |
| TCA: | Trichloroacetic acid |
| Try: | Trypanothione |
| TryR: | Trypanothione reductase. |

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contribution

Alejandra B. Ciccarelli and Fernanda M. Frank contributed equally to this work.

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*Research Article*

# The Effects of *N*-Butyl-1-(4-dimethylamino)phenyl-1,2,3,4-tetrahydro-$\beta$-carboline-3-carboxamide against *Leishmania amazonensis* Are Mediated by Mitochondrial Dysfunction

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Leishmaniasis is a disease that affects millions of people worldwide. The drugs that are available for the treatment of this infection exhibit high toxicity and various side effects. Several studies have focused on the development of new chemotherapeutic agents that are less toxic and more effective against trypanosomatids. We investigated the effects of *N*-butyl-1-(4-dimethylamino)phenyl-1,2,3,4-tetrahydro-$\beta$-carboline-3-carboxamide (**C4**) and its possible targets against *L. amazonensis*. The results showed morphological and ultrastructural alterations, depolarization of the mitochondrial membrane, the loss of cell membrane integrity, and an increase in the formation of mitochondrial superoxide anions in *L. amazonensis* treated with **C4**. Our results indicate that **C4** is a selective antileishmanial agent, and its effects appear to be mediated by mitochondrial dysfunction.

## 1. Introduction

Leishmaniasis is a disease caused by the protozoa of the genus *Leishmania*, which belongs to the order Kinetoplastida, family Trypanosomatidae. An estimated 12 million people are affected by this disease [1], with 2 million new cases worldwide [2] reported in 88 countries and four continents [3]. This disease has two clinical forms, visceral and cutaneous leishmaniasis.

The currently available first-line treatments are pentavalent antimonials [4]. These drugs have high toxicity and adverse side effects [5]. Amphotericin B and pentamidine are second-line therapies but are associated with long-term treatment, limited effectiveness, significant side effects, and toxicity [6–8]. Consequently, an urgent need exists to discover new drugs that are effective against leishmaniasis.

Several studies are being conducted to find new antileishmanial compounds. Carbolines comprise a class of compounds that have an alkaloid indole nucleus and hydrogenated six-member pyridine ring [9]. They are distributed throughout nature in many living beings, including vegetables, fungi, animals, and even human fluids [10]. Carbolines can be divided into three groups: fully aromatic, dihydrocarbolinic, and tetrahydro-$\beta$-carboline [11]. Interestingly, natural and synthetic $\beta$-carbolines are well known to possess several biological properties. For example, our group

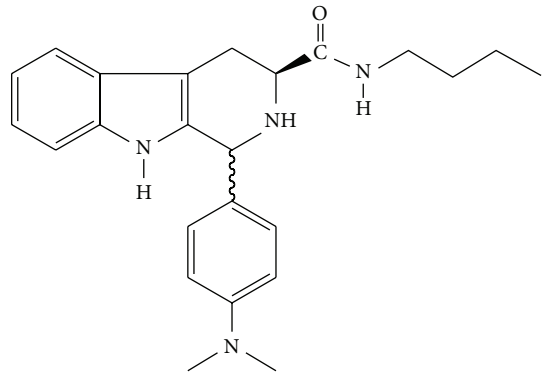


Figure 1: Chemical structure of *N*-butyl-1-(4-dimethylamino)phenyl-1,2,3,4-tetrahydro-$\beta$-carboline-3-carboxamide (**C4**).

recently reported the effective trypanocidal activity of the synthetic compound *N*-butyl-1-(4-dimethylamino)phenyl-1,2,3,4-tetrahydro-$\beta$-carboline-3-carboxamide (**C4**; Figure 1) against *Trypanosoma cruzi* [12, 13].

The mechanism of action of $\beta$-carboline compounds involves changes in DNA and inhibition of the respiratory chain in epimastigote forms of *T. cruzi* [10]. Although the mode of biological action of this compound in different cell types is not fully understood, previous studies have shown that the $\beta$-carboline ring in the molecule might be deposited in base pairs of DNA, thus contributing to its biological activity [14, 15] or inducing apoptosis [16].

Considering the low efficacy of drugs against leishmaniasis and previous studies of the effects of $\beta$-carboline compounds on *T. cruzi*, we investigated the potential effect of the synthetic compound *N*-butyl-1-(4-dimethylamino)phenyl-1,2,3,4-tetrahydro-$\beta$-carboline-3-carboxamide (**C4**) against *L. amazonensis* and its possible targets in this protozoan.

## 2. Materials and Methods

*2.1. Chemicals.* Antimycin A, carbonyl cyanide m-chlorophenylhydrazone (CCCP), digitonin, dimethylsulfoxide (DMSO), and rhodamine 123 (Rh123) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 were obtained from Invitrogen (Grand Island, NY, USA). 3,8-Phenanthridinediamine-5-(6-triphenylphosphoniumhexyl)-5,6-dihydro-6-phenyl (MitoSOX) and propidium iodide (PI) were obtained from Invitrogen (Eugene, OR, USA). All of the other reagents were of analytical grade.

*2.2. Synthesis of **C4** Compound.* **C4** was synthesized as previously described by Valdez et al. [12].

*2.3. Preparation of Drugs.* The **C4** compound was prepared in DMSO. All of the groups, including controls, were tested at final concentrations of less than 1% DMSO, a concentration that was found to not affect parasite or mammalian cells (data not shown).

*2.4. Parasites and Cell Culture. L. amazonensis* (strain WHOM/BR/75/JOSEFA) was originally isolated from a patient with diffuse cutaneous leishmaniasis by C.A. Cuba-Cuba (Universidade de Brasília, Brazil). Promastigotes were cultured in Warren's medium (brain heart infusion, hemin, and folic acid; pH 7.0) supplemented with 10% inactivated FBS at 25°C. Axenic amastigotes were cultured in Schneider's medium (Sigma, St. Louis, MO, USA; pH 4.6) supplemented with 20% FBS at 32°C.

Macrophages (J774G8) were maintained in tissue flasks with RPMI-1640 (pH 7.2), added with sodium bicarbonate and L-glutamine (As annex), and supplemented with 10% FBS at 37°C in a 5% $CO_2$ atmosphere.

*2.5. Antiproliferative Assay.* Promastigote and axenic amastigote forms in the logarithmic phase ($1 \times 10^6$ cells/mL) were cultured on a 24-well plate in Warren's and Schneider's media, respectively, supplemented with FBS in the presence or absence of 2.56, 12.8, 25.6, 128.0, and 256.0 $\mu$M of **C4**. The activity against promastigotes and axenic amastigotes was evaluated after 72 h of incubation. The results are expressed as a percentage, and the $IC_{50}$ (i.e., 50% inhibitory concentration) was determined after-incubation [17].

*2.6. Cytotoxicity Assay in Macrophage Cells.* Cytotoxicity was evaluated in J774G8 macrophage cells. A suspension of $5 \times 10^5$ cells/mL was cultured in RPMI-1640 medium supplemented with 10% FBS and added to each well in 96-well microplates. The plates were incubated at 37°C in a 5% $CO_2$-air mixture to obtain the confluent growth of the cells. After 24 h, the compound was added to each well at increasing concentrations (160.0, 320.0, 640.0, and 1,280.0 $\mu$M), and the plates were incubated for 48 h in a 5% $CO_2$-air mixture at 37°C. After treatment, the medium was removed and washed with phosphate-buffered saline (PBS), and 50 $\mu$L of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide formazan; 2 mg/mL) was added to each well for 4 h in a 5% $CO_2$-air mixture at 37°C. DMSO (50 $\mu$L) was then added, and the plates were homogenized. Absorbance was read in a 96-well plate reader (BIO-TEK Power Wave XS spectrophotometer) at 492 nm. The percentage of viable cells was calculated relative to controls, consisting of cells cultured in medium alone, according to $CC_{50}$ values (i.e., 50% cytotoxicity concentration). The $CC_{50}$ was determined by logarithm regression analysis.

*2.7. Scanning Electron Microscopy.* The promastigote forms of the parasite in the logarithmic phase ($1 \times 10^6$ cells/mL) in the presence or absence of 16.0 and 103.0 $\mu$M of **C4** for 48 h were washed with 0.01 M PBS and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h at room temperature. The parasites were adhered on poly-L-lysine-coated coverslips, dehydrated in different concentrations of ethanol, critical-point dried with $CO_2$, sputter coated with gold, and observed in a Shimadzu SS-550 scanning electron microscope [18].

*2.8. Transmission Electron Microscopy.* The promastigote forms of the parasite in the logarithmic-phase ($1 \times 10^6$ cells/mL) in the presence or absence of 16.0 and 103.0 $\mu$M of **C4** for 48 h were harvested by centrifugation and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The cells were then post-fixed in a solution that contained 1% osmium tetroxide and 0.8% potassium ferrocyanide at room temperature for 60 min, dehydrated in different concentrations of acetone, and embedded in Epon resin. Thin sections were stained with uranyl acetate and lead citrate and examined in a JM 1400 JEOL transmission electron microscope [19].

*2.9. Mitochondrial Membrane Potential and Cell Membrane Integrity Assay.* The promastigote forms of the parasite in the logarithmic phase ($5 \times 10^6$ cells/mL) in the presence or absence of 16.0 and 103.0 $\mu$M of **C4** for 24 h were harvested and washed with PBS. The parasites were then washed and incubated at 37°C with Rh 123 (5 $\mu$g/mL for 15 min) to evaluate mitochondrial membrane potential ($\Delta\Psi$m) and PI (0.2 $\mu$g/mL for 10 min) to verify possible alterations in cell membrane integrity. CCCP (100 $\mu$M) and digitonin (40 $\mu$M) were used as positive controls for mitochondria membrane potential alterations and cell membrane alterations, respectively. The material was kept on ice until analysis. The mean fluorescence intensity of the cells was analyzed by flow cytometry using FACSCalibur and CellQuest software. A total of 10,000 events were acquired in the region that was previously established as the one that corresponded to the parasites [20].

*2.10. Fluorimetric Detection of Mitochondrial-Derived $O_2^{\bullet-}$.* Promastigote forms of the parasite in the logarithmic phase ($2 \times 10^7$ cells/mL) were harvested and washed with Krebs-Henseleit (KH) solution buffer that contained 15 mM $NaHCO_3$, 5 mM KCl, 120 mM NaCl, 0.7 mM $Na_2HPO_4$, and 1.5 mM $NaH_2PO_4$ (pH 7.3). The cells were loaded with 5 $\mu$M MitoSOX reagent. The parasites were incubated for 10 min at room temperature (25°C) and protected from light. After incubation with MitoSOX reagent, the parasites were washed twice with KH buffer and treated or not with 16.0, 103.0, and 205.0 $\mu$M of **C4**. Antimycin A (10 $\mu$M), a stimulus that is known to induce superoxide anion ($O_2^{\bullet-}$) production by mitochondria, was used as a positive control. MitoSOX detection was performed using black 96-well plates for 3 h. Fluorescence was measured using a fluorescence microplate reader (Victor X3; PerkinElmer) at 194 $\lambda$ex = 510 nm and $\lambda$em = 580 nm [21].

Table 1: Effect of **C4** against promastigote and axenic amastigote forms of *Leishmania amazonensis*, cytotoxicity in macrophages cells, and selectivity index.

| Cells | **C4** ($\mu$M) $IC_{50}$ [a] | **C4** ($\mu$M) $IC_{90}$ [b] | SI[c] |
|---|---|---|---|
| Promastigotes | 16.0 ± 2.28 | 103.0 ± 20.17 | 45.1 |
| Axenic amastigotes | 16.3 ± 2.38 | 118.0 ± 45 | 44.2 |
| Macrophages ($CC_{50}$)[d] | 722.0 ± 45 | — | — |

[a]Concentration that inhibited 50% of growth.
[b]Concentration that inhibited 90% of growth.
[c]Selectivity index (SI; $CC_{50}$ macrophages/$IC_{50}$).
[d]50% cytotoxicity concentration in macrophages.

*2.11. Statistical Analysis.* The data shown in the graphs are expressed as the mean ± standard error of at least three independent experiments. The data were analyzed using analysis of variance (ANOVA). Significant differences among means were identified using Tukey post hoc test. Values of $P \leq 0.05$ were considered statistically significant. The statistical analyses were performed using Statistica software.

## 3. Results

*3.1. Antileishmanial Activity.* The treatment of the parasites with **C4** dose dependently inhibited the growth of the promastigote and axenic amastigote forms of *L. amazonensis*. The inhibition percentages of the parasites and concentrations that corresponded to 50% ($IC_{50}$) and 90% ($IC_{90}$) of growth inhibition of the promastigotes were calculated by plotting the concentration *versus* percentage growth inhibition using linear regression after directly counting free-living parasites in a Neubauer chamber. The $IC_{50}$ and $IC_{90}$ in promastigotes were 16.0 ± 2.28 $\mu$M and 103.0 ± 20.17 $\mu$M, respectively. In axenic amastigotes, the $IC_{50}$ and $IC_{90}$ were 16.3 ± 2.38 $\mu$M and 118.0 ± 45 $\mu$M, respectively (Table 1).

*3.2. Cytotoxicity Assay.* The cytotoxic effect (50% cytotoxic concentration [$CC_{50}$]) of **C4** in J774G8 macrophages after 48 h of treatment was 722.0 ± 45 $\mu$M (Table 1). The toxicity in macrophages was compared with activity against the promastigote and axenic amastigote forms, yielding the selectivity index (SI). **C4** was more selective against the promastigote and axenic amastigote forms than against mammalian cells, with SIs of 45.1 and 44.2, respectively (Table 1).

*3.3. Scanning and Transmission Electron Microscopy.* Morphological alterations in promastigotes treated with **C4** were observed using scanning electron microscopy. The control showed normal characteristics of the parasite, such as an elongated body and free flagellum (Figure 2(a)). Parasites treated with the $IC_{50}$ of **C4** exhibited distortions in the cell body (Figures 2(b) and 2(c)), and the group treated with the $IC_{90}$ showed rounding of the cell body (Figure 2(d)).

Ultrastructural changes in promastigotes treated with **C4** at the $IC_{50}$ and $IC_{90}$ are illustrated in Figure 2, showing significant alterations. The mitochondria showed intense swelling (Figures 2(f) and 2(g)), the presence of concentric membrane structures inside the organelle (Figure 2(f)), and alterations

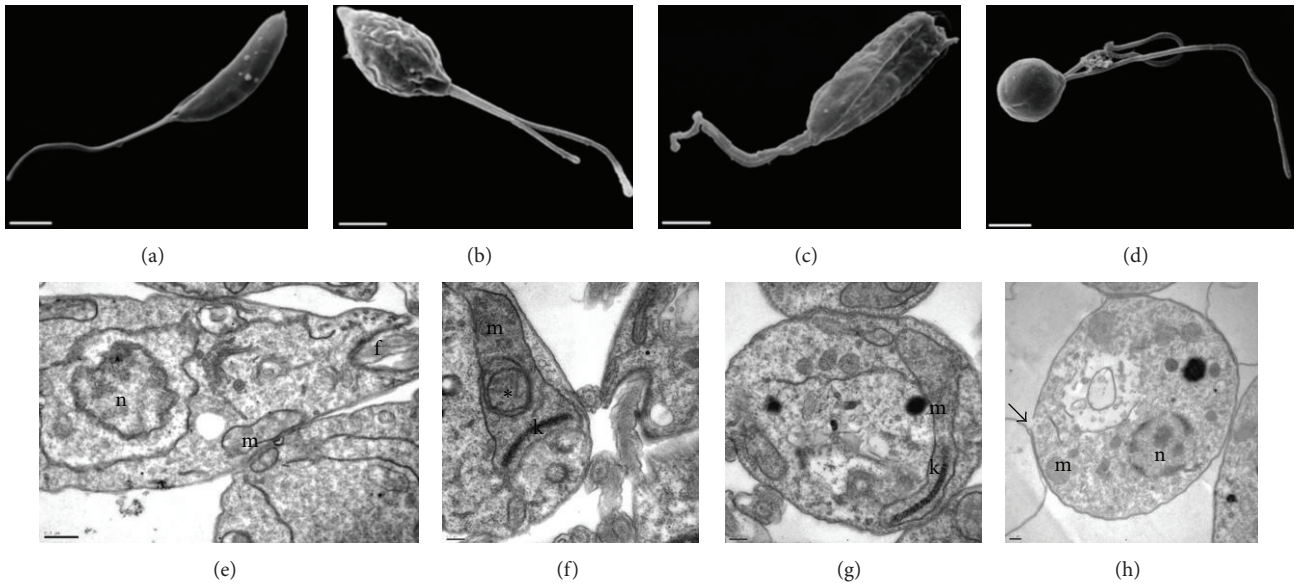


Figure 2: Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of *Leishmania amazonensis* promastigotes after 48 h of treatment with **C4**. (a) SEM image of an untreated promastigote, showing typical elongated morphology. (b) and (c) SEM images of promastigotes after treatment with the $IC_{50}$ (16.0 $\mu$M), showing distortion of the cell body. (d) SEM image of promastigote after treatment with the $IC_{90}$ (103.0 $\mu$M), showing rounding of the cell body. Scale bar: 2 $\mu$m. (e) TEM image of untreated promastigote, showing normal mitochondria (m), nucleus (n), and flagellum (f). (f) and (g) TEM images of promastigotes treated with **C4** at 16.0 $\mu$M, showing mitochondrial swelling and the presence of concentric membranes inside mitochondria (black asterisk). (h) Promastigotes treated with **C4** at 103.0 $\mu$M, showing alteration of the plasma membrane (black arrow). Scale bar: 0.5 $\mu$m in (e) and 0.2 $\mu$m in (f)–(h).

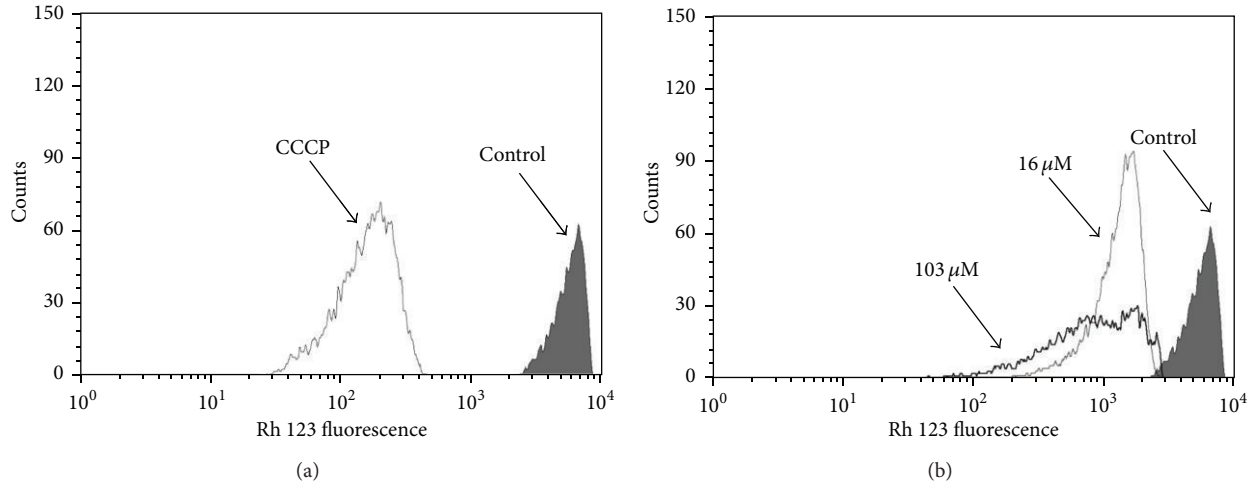


Figure 3: Flow cytometry analysis of promastigotes of *Leishmania amazonensis* treated with **C4** for 24 h and stained with Rh 123. (a) Promastigotes treated with 100 $\mu$M CCCP (positive control). (b) Promastigotes treated with 16.0 and 103.0 $\mu$M of **C4**. The control group (i.e., untreated parasites) is also shown.

of the plasma membrane (Figure 2(h)). These ultrastructural changes were not observed in untreated parasites (Figure 2(e)).

*3.4. Mitochondrial Membrane Potential.* The effect of **C4** on mitochondrial membrane potential ($\Delta\Psi$m) in promastigote forms was assessed by flow cytometry using Rh 123, a fluorescent marker that indicates mitochondrial membrane potential. The $IC_{50}$ and $IC_{90}$ induced 73.3% and 82.5% decreases in total Rh 123 fluorescence intensity, respectively, compared with the control group, indicating depolarization of mitochondrial membrane potential (Figure 3(b)). Promastigotes treated with CCCP showed a 94.8% decrease in membrane potential (Figure 3(a)).

*3.5. Plasma Membrane Integrity.* Cell membrane integrity in promastigotes treated with **C4** was assessed by flow cytometry using PI, which diffuses across disrupted plasma

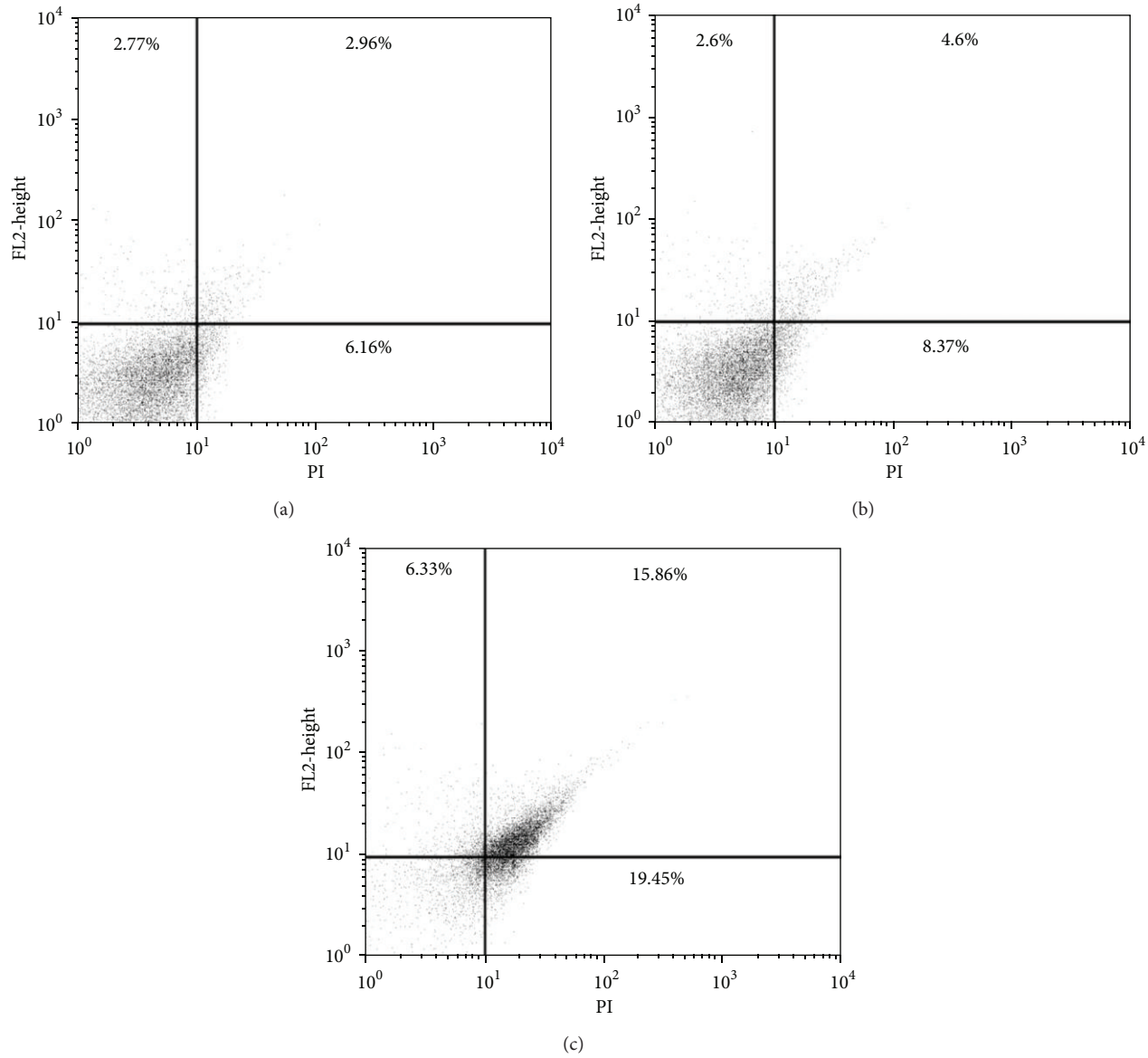


Figure 4: Flow cytometry analysis of promastigotes of *Leishmania amazonensis* treated with **C4** for 24 h and stained with propidium iodide (PI). (a) Control group (i.e., untreated cells). (b) Promastigotes treated with 16.0 $\mu$M of **C4**. (c) Promastigotes treated with 103.0 $\mu$M of **C4**. The percentages of PI-stained positive cells are shown in the upper right and left quadrants.

membranes of cells and binds to nucleic acids. **C4** at the $IC_{90}$ increased total PI fluorescence intensity by 22.2% (Figure 4(c)), compared with the control group (5.7%; Figure 4(a)), indicating the alteration of cell membrane integrity. However, **C4** at the $IC_{50}$ did not induce any membrane alterations (Figure 4(b)). The positive control (i.e., digitonin) also showed an increase in fluorescence (data not shown).

*3.6. Detection of Mitochondria-Derived $O_2^{\bullet-}$.* The production of $O_2^{\bullet-}$ was evaluated in promastigotes treated with **C4** using MitoSOX reagent, which measures the accumulation of mitochondrial superoxide. Figure 5 shows that **C4** increased the production of mitochondrial $O_2^{\bullet-}$ at all concentrations and times tested compared with the control group. However, **C4** induced a significant increase in $O_2^{\bullet-}$ production only after 2 and 3 h of treatment at the higher concentration (205 $\mu$M). The positive control (i.e., antimycin A) also showed an increase in fluorescence (data not shown).

## 4. Discussion

Several natural and synthetic compounds have been studied for the treatment of leishmaniasis [22–29]. Nevertheless, the treatment of this infection remains a problem because of the high toxicity and adverse side effects. $\beta$-Carbolines have been previously reported to have various biological properties, including antioxidant, antimicrobial [30], antiparasitic [12, 13], antitumoral [31], and antiviral effects [32]. These compounds also exhibited activity against protozoa, such as *T. cruzi*, especially against bloodstream trypomastigotes.

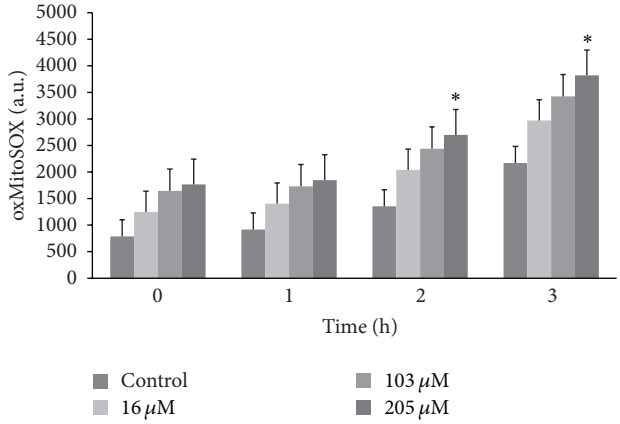


Figure 5: Mitochondrial $O_2^{\bullet-}$ production in promastigote forms of *Leishmania amazonensis* treated with **C4** for up to 3 h. Mitochondrial $O_2^{\bullet-}$ production was evaluated using the fluorescent probe MitoSOX. At the indicated times, promastigotes were used to measure oxidized MitoSOX (oxMitoSOX). The results are expressed in arbitrary units (mean ± SE of at least three independent experiments). $^*P \leq 0.05$, significant difference compared with the control group (i.e., untreated cells; two-way analysis of variance followed by Tukey post hoc test).

Our previous studies demonstrated the effective and selective action of **C4** against *T. cruzi* [12, 13]. The present study evaluated the antileishmanial activity of **C4** against *L. amazonensis* and its possible targets in this parasite.

**C4** compound inhibited the growth of the promastigote forms of the parasite and caused morphological and ultrastructural alterations, especially in mitochondria. The Rh 123 assay confirmed the mitochondrial action of **C4**, reflected by a decrease in Rh 123 fluorescence intensity. The mitochondria of trypanosomatids are attractive chemotherapeutic targets because they have structural and functional characteristics that are distinct from mammalian mitochondria [33]. In fact, increasing reports have described compounds that target parasite mitochondrial destabilization and disorganization [24, 26].

Mitochondrial alterations may be a consequence of many potentially harmful effects induced by both exogenous and endogenous toxic compounds. We currently consider **C4** as an exogenous toxic compound. However, **C4** may also induce the production of endogenous toxic compounds, including reactive oxygen species (ROS), that may be responsible for mitochondrial dysfunction and induce oxidative damage in lipids and proteins, the main macromolecules of biological membranes. This indirect effect of **C4** was demonstrated in the MitoSOX and PI assays in the present study, similar to the effects of compound reported by Desoti et al. [34].

In conclusion, our data indicate that **C4** is a leishmanicidal compound that is able to induce parasite disorders that are mainly mediated by mitochondrial dysfunction. Further *in vitro* and *in vivo* studies are necessary to increase our understanding of the mode of action of this compound and determine whether it can be exploited alone or in combination with other drugs for the treatment of antileishmaniasis.

## Conflict of Interests

There is no conflict of interests declared by the authors.

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*Research Article*

# Evaluation of Antileishmanial Activity of Selected Brazilian Plants and Identification of the Active Principles

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This study evaluated extracts, fractions, and isolated compounds from some selected Brazilian medicinal plants against strains of promastigotes of *Leishmania amazonensis* and *L. brasiliensis in vitro*. The cell viability was determined, comparing the results with reference standards. The dichloromethane fractions of the roots, stems, and leaves of *Allamanda schottii* showed $IC_{50}$ values between 14.0 and 2.0 $\mu$g/mL. Plumericin was the main active compound, with $IC_{50}$ of 0.3 and 0.04 $\mu$g/mL against the two species of *Leishmania* analyzed. The hexane extract of *Eugenia umbelliflora* fruits showed $IC_{50}$ of 14.3 and 5.7 $\mu$g/mL against *L. amazonensis* and *L. brasiliensis*, respectively. The methanolic extracts of the seeds of *Garcinia achachairu* and guttiferone A presented $IC_{50}$ values of 35.9 and 10.4 $\mu$g/mL, against *L. amazonensis*, respectively. The ethanolic extracts of the stem barks of *Rapanea ferruginea* and the isolated compound, myrsinoic acid B, presented activity against *L. brasiliensis* with $IC_{50}$ of 24.1 and 6.1 $\mu$g/mL. Chloroform fraction of *Solanum sisymbriifolium* exhibited $IC_{50}$ of 33.8 and 20.5 $\mu$g/mL, and cilistol A was the main active principle, with $IC_{50}$ of 6.6 and 3.1 $\mu$g/mL against *L. amazonensis* and *L. brasiliensis*, respectively. It is concluded that the analyzed plants are promising as new and effective antiparasitic agents.

## 1. Introduction

*Leishmania* are protozoan parasites responsible for a spectrum of diseases known as leishmaniasis. There are two main forms of leishmaniasis: cutaneous, characterized by skin sores; and visceral, which affects the internal organs (e.g., the spleen, liver, and bone marrow). This disease is classified as a neglected disease and is one of major public health problem, causing significant morbidity and mortality in various countries. Leishmaniasis is considered by the World Health Organization as one of six major infectious diseases, with a high detection rate and ability to produce deformities [1–3].

Currently, the treatment of this disease is based on a limited number of chemotherapeutic agents, which represent high toxicity and cost. The search for new medicinal agents has become extremely important. The higher plants are a very rich source of new and selective substances, with therapeutic potential against these ailments. There are approximately 250.000 plant species worldwide, of which only a fraction has so far been studied. However, much research is needed to identify plants as sources of drugs or their phytoconstituents. The World Health Organization also advocates the use of traditional medicine for the treatment of these tropical diseases [4–6].

Our research group participated in an Iberoamerican program (RIBIOFAR/CYTED/CNPq) to search for Brazilian plants with therapeutic potential to treat several diseases, including the so-called neglected diseases. Some species have been previously analyzed with respect to their chemical composition and biological properties. Now we are interested in exploring the potential of the crude plant extracts, semi-purified fractions, and chemically defined molecules, in terms of their activity against leishmaniasis. Table 1 gives a list of the plants analyzed, that were selected based on factors related to the discovery of new drugs from the biodiversity, the abundance of these plants, and previous pharmacological studies conducted at our laboratories. The selected plants, *Allamanda schottii*, *Rapanea ferruginea*, *Eugenia umbelliflora*, *Garcinia achachairu*, and *Solanum sisymbriifolium*, have exhibited various biological properties, including anti-proliferative antinociceptive, anti-inflammatory, antimicrobial, and gastroprotective effects [7–15].

## 2. Material and Methods

*2.1. Plant Material.* Different parts (leaves, stems, and roots) of *Allamanda schottii* were collected in the city of Blumenau, in December of 2006. The fruits of *Eugenia umbelliflora* Berg were collected in June 2008, in the town of Itapema. *Rapanea ferruginea,* synonymously *Myrsine coriacea* (Ruiz and Pavon) Mez, was collected in Blumenau in July 2007. *Garcinia achachairu* was collected in Camboriú, in March 2007. Aerial parts of *Solanum sisymbriifolium* Lam. were collected in Itapema, in January 2006. All the species were identified or authenticated by Professor Oscar Benigno Iza (Universidade do Vale do Itajaí), and vouchers were deposited at the Barbosa Rodrigues Herbarium (HBR, Itajaí), under numbers HBR 52525 (*A. schottii*), VC-Filho 50 (*E. umbelliflora*), HBR 52715 (*R. ferruginea*), HBR 52637 (*M. coriacea*), and VC-Filho 037 (*S. sisymbriifolium*).

*2.2. Preparation of Extracts and Fractions.* The dried vegetal material (100 g) of each part (roots, stems, and leaves) of *A. schottii* was macerated with 95% ethanol at room temperature, for seven days. Solvent removal was carried out under reduced pressure at temperatures below 45°C, until the desired concentrations were achieved, in order to obtain the ethanolic extracts. Part of each extract was dissolved separately in methanol : water (90 : 10) and successively partitioned with hexane, dichloromethane, and ethyl acetate to obtain the respective fractions, after the removal of solvents. The samples were stored under refrigeration and protected from light until analysis.

The fruits of *E. umbelliflora* were dried under air circulation (37°C) for 2 days and powdered using a knife grinder. The dried fruits (570 g) were extracted twice by maceration with *n*-hexane for five days. Next, solvent was removed under vacuum to yield a dry residue of 2.62% hexane extract. The fruits were then reextracted using dichloromethane and methanol, following the methods described above. The separate extracts were submitted to concentration to obtain dichloromethane and methanolic extract 1 with yields of 1.16% and 4.47%, respectively. The methanolic extract 2 was obtained by extracting the dried fruits with methanol at room temperature for seven days.

The dried material 100 g of each part (leaves, stem barks and fruits) of *R. ferruginea* was macerated with 95% ethanol at room temperature, for seven days. Solvent removal was carried out under reduced pressure at temperatures below 45°C, until the desired concentrations were achieved, in order to obtain ethanolic extracts with yields of 16%, 15%, and 13% from leaves, stem, and fruits, respectively. The chloroform extract was obtained by the stem barks and is described in Hess et al. [9].

The air-dried and powdered seeds, leaves and branches (250 g each) of *G. achachairu* were separately extracted at room temperature with methanol for seven days. The macerated material was filtered and concentrated under reduced pressure, yielding 9.01 g (3.6%), 15.0 g (6%), and 12.0 g (4.8%) of crude methanolic extract, respectively.

The powdered leaves, and stem (680.0 g) of *S. sisymbriifolium* were extracted with MeOH for ten days. The concentrated methanolic extract was diluted with a water : methanol mixture (9 : 1, 300 mL) and extracted with hexane (11.5 g; 1.70%), chloroform (11.2 g; 1.64%), and ethyl acetate (1.18 g; 0.17%).

*2.3. Isolation and Identification of Components.*

*A. schottii*. The ethanolic extract of *A. schottii* stems (15.1 g) was chromatographed in a silica-gel column with a hexane: ethylacetate: ethanol gradient. The fraction eluted with *n*-hexane : ethyl acetate 7 : 3 yielded plumericin (15.2 mg). Elution with a hexane : ethyl acetate ratio of 6.5 : 3.5 yielded ursolic acid (62.4 mg). The fraction eluted with ethyl acetate : ethanol 8 : 2 yielded plumieride (39.4 mg) as described previously by Schmidt et al. [7] and Malheiros et al. [29].

*R. ferruginea*. The $CHCl_3$ extract of *R. ferruginea* (63.4 g) was subjected to column chromatography packed with silica gel 60–230 mesh and eluted with hexane gradually enriched in ethyl acetate. The fraction eluted with 40% ethyl acetate in hexane provides impure myrsinoic acid B. This fraction (8.5 g) was chromatographed in a silica-gel column with hexane : ethyl acetate to provide pure myrsinoic acid B (4.3 g) as described previously by Baccarin et al. [30].

*E. umbelliflora*. The complete procedure used to obtain the compound isolated from *E. umbelliflora* fruits, the meroterpenoid Eugenial A, was described previously by Faqueti et al. [11].

*G. achachairu*. The methanolic extract of the seeds (5.0 g) was chromatographed on a silica-gel column (0.063–0.20 mm, 84.0 g, 2.5 × 50 cm, Merck) and eluted with a gradient of $CHCl_3$–MeOH (100 -> 0) yielding guttiferone A as described previously by Dal Molin et al. [13].

*S. sisymbriifolium*. The chloroform fraction was purified by CC on silica gel, yielding Cilistol A (60.0 mg) and cilistadiol

Table 1: Traditional uses of the selected Brazilian medicinal plants and previous pharmacological studies.

| Species | Popular use | Biological studies | Reference |
|---|---|---|---|
| *A. schotii* | Latex: used as scabicide and in louse control<br>The infusion of flowers is purgative and antihelminthic<br>The stems are used against hepatic tumors | antiproliferative effect leukemic cells | [7, 16–20] |
| *E. umbelliflora* | Fruits and leaves used to treat various ailments such as infections, inflammation, and diabetes | Antimicrobial, gastroprotective, antifungal | [10, 21–23] |
| *G. achachairu* | Rheumatism, inflammation, pain, and gastric disorders | Low genotoxicity, gastroprotective, and antinociceptive activity | [13–15, 24] |
| *R. ferruginea* | The leaves or the bark prepared as a tea is indicated as a diuretic, to combat diseases of the urinary tract and also is a good cleanser. It is used too for itching, rashes, hives, eczema, rheumatism, and diseases of the liver | Anti-inflammatory and analgesic activity | [9, 12, 25] |
| *S. sisymbriifolium* | Diarrhea, respiratory and urinary infections | Hypotensive and molluscicidal activity | [26–28] |

(25.0 mg) as described previously by Niero et al. [8]. The molecular structures of isolated compounds are presented in Figure 1.

### 2.4. Leishmanicidal Activity

*2.4.1. Promastigotes of Leishmania amazonensis Clone 1.* AML (MHOM/BR/76/LTB-012) and *Leishmania braziliensis* (M2904 C192 RJA) obtained from *in vitro* cultures of IIFB (20 $\mu$L) were fixed with glutaraldehyde (5%, 180 $\mu$L) and counted in a Neubauer chamber. The population was adapted to $3 \times 10^6$ parasites/mL with Schneider medium (pH = 6.8) and Fetal Bovine Serum (10%), distributed (100 $\mu$L/well) in 96-microwell plates. Solutions of the samples, at different concentrations, were added (100 $\mu$L). DMSO (1%) and Amphotericin B (0.5 $\mu$g/mL) were used as parasite growth control. Each test was performed in triplicate and the plates were incubated for 72 hours at 26°C.

To each well of the plate was added (50 $\mu$L/well) a solution of XTT (1 mg/mL) in phosphate buffer (pH 7.0, 37°C) with PMS (Sigma-Aldrich, 0.06 mg/mL), which was incubated for 4 h at 26°C. The plates were read on a computer Stat Fax (Model 2100 Series-Plate Reader) at 450 nm. The $IC_{50}$ values of 50% inhibitory concentration of parasites were calculated using Microsoft Excel 2000 [31].

*2.5. Statistical Analysis of Data.* The samples were analyzed in replicates with $n = 4$, using two methods of viability assessment (XTT and Neutral Red). The data are mean ± stand error, and statistical differences were determined by the one-way ANOVA followed by Tukey-Kiover ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

## 3. Results and Discussion

In this work we tested extracts, fractions, and compounds of five Brazilian medicinal plants to determine their *in vitro* antiparasitic effect against promastigotes of *L. amazonensis* and *L. brasiliensis*. For the initial screening, extracts with $IC_{50}$ less than 100 $\mu$g/mL were considered active. The results of the minimal inhibitory concentrations are shown in Tables 2 and 3.

The ethanolic extracts from different parts of *A. schottii* (roots, stems, and leaves) exhibited pronounced leishmanicidal activity (Table 2). The best results were obtained from the root extracts with $IC_{50}$ of 43.8 $\mu$g/mL for *L. amazonensis* and 8.5 $\mu$g/mL for *L. brasiliensis*. The extracts were submitted to partition with hexane, dichloromethane, and ethyl acetate. The activity was concentrated in the dichloromethane fraction for all extracts with $IC_{50}$ of 2.1 to 13.4 $\mu$g/mL. The best activity was observed in the dichloromethane fraction of the roots with $IC_{50}$ of 2.1 $\mu$g/mL for *L. amazonensis* and 8.8 $\mu$g/mL for *L. brasiliensis*, suggesting that the iridoid plumericin and the triterpene ursolic acid are the main active principles. These compounds were isolated from the ethanolic extract of the stems and detected in all the dichloromethane fractions by thin layer chromatography. Plumericin presented $IC_{50}$ of 0.3 $\mu$g/mL (0.98 $\mu$M) for *L. amazonensis* and 0.04 $\mu$g/mL (0.13 $\mu$M) for *L. brasiliensis*. Ursolic acid was active against the two species evaluated with $IC_{50}$ of 66.1 $\mu$g/mL and 8.3 $\mu$g/mL for *L. amazonensis* and *L. brasiliensis*, respectively. Amphotericin B, used as positive control, exhibited activity with $IC_{50}$ of 0.6 $\mu$g/mL and 0.7 $\mu$g/mL, respectively, for the two species evaluated (Table 3). Another evaluated iridoid was plumieride, a glycoside iridoid, which was detected in all the ethyl acetate fractions, presenting $IC_{50}$ of 21.3 $\mu$g/mL for *L. brasiliensis*. This compound may be responsible for the activity observed in ethyl acetate fractions of different parts of this plant.

The Apocynaceae family is as a rich source of species with antileishmanicidal activity and for some species, plumericin is the main active compound. This compound, previously isolated from *Himatanthus sucuuba*, presented $IC_{50}$ values of 0.9 and 1.0 $\mu$M against promastigote and amastigote forms of *L. amazonensis* [32] and $IC_{50}$ of 3.17 ± 0.12 and 1.41 ± 0.03 $\mu$M against promastigotes and amastigotes of *L. donovani*, respectively [33].

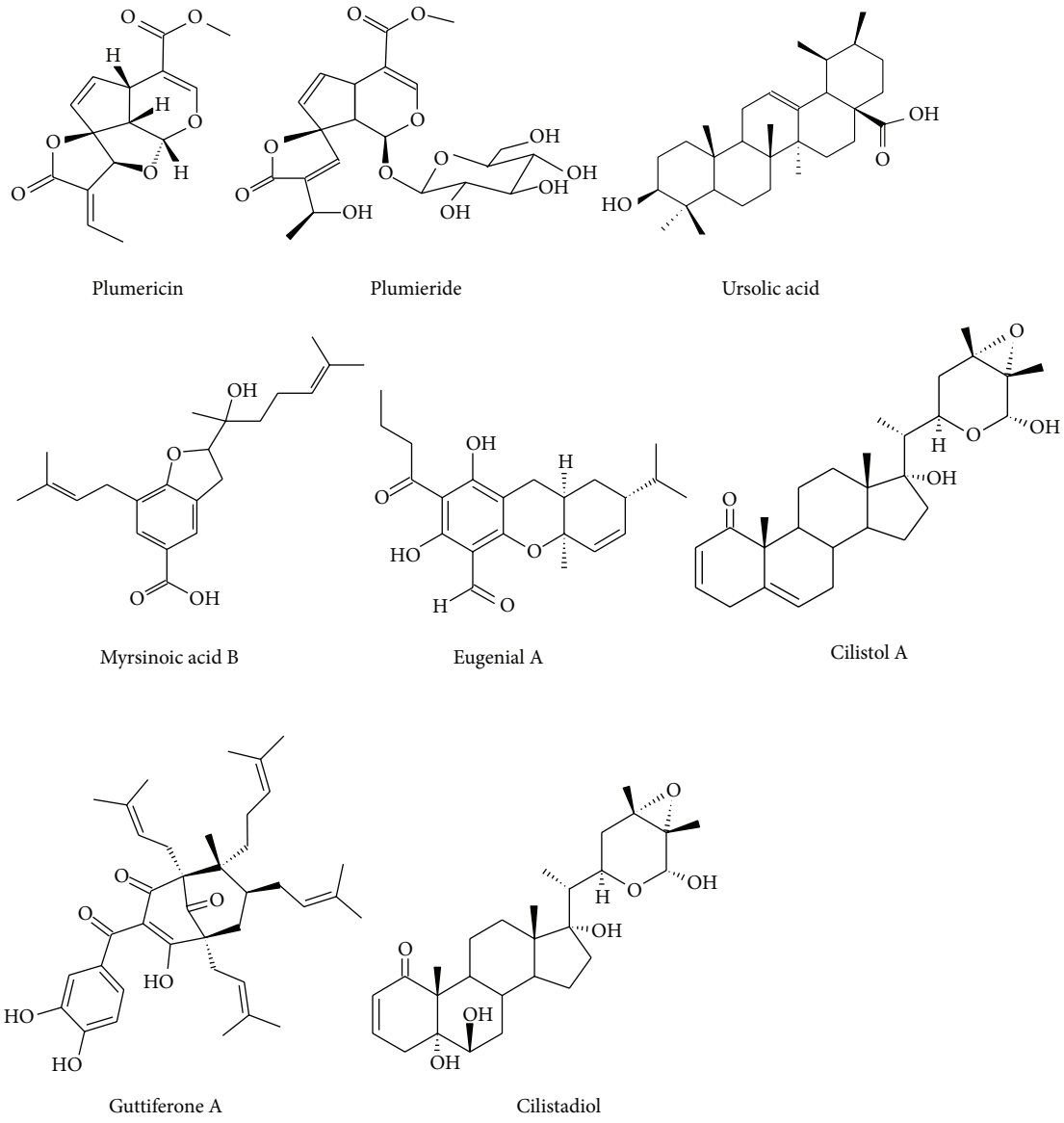


Figure 1: Molecular structure of the isolated compounds from analyzed plants.

On the other hand, ursolic acid was isolated as an active compound from extracts of *Baccharis dracunculifolia* (Asteraceae) against *Leishmania donovani* with $IC_{50}$ of 3.7 $\mu$g/mL [34]. Our results, together with those reported in the literature, suggest that the compounds found in *Allamanda genus* are promising antileishmanial agents.

The antiparasitic investigation against promastigotes of *L. amazonensis* and *L. brasiliensis* with respect to *E. umbelliflora* fruits indicated that the hexane extract was the most active with $IC_{50}$ of 14.3 ± 0.86 and 5.7 ± 0.92 $\mu$g/mL, respectively. When the fruits were directly submitted to methanol extraction, activity was observed with $IC_{50}$ of 12.5 ± 0.5 and 7.8 ± 0.4 $\mu$g/mL against *L. amazonensis* and *L. brasiliensis*, respectively. Despite reports in the ethnobotanical literature on the medicinal uses of *Eugenia* species to treat some diseases, there have been few scientific studies that validate their antileishmanial activity. Previous studies with *Eugenia* genus have suggested that terpenic compounds found in the essential oil may have potential anti-leishmanial activity [35].

Continuing our screening program in the search for bioactive molecules from Brazilian plants, we have investigated *G. achachairu* for its leishmanicidal activity. Thus, the methanolic extract, some fractions, and isolated compounds were evaluated. As can be seen in Table 2, the methanolic extract of the seeds, leaves, and branches obtained from *G. achachairu* exhibited $IC_{50}$ values of 35.9 and 100 $\mu$g/mL in promastigote forms of *L. amazonensis* and 28.2, 100, and 81.6 $\mu$g/mL against promastigote of *L. brasiliensis*, respectively. The most bioactive extract (from the seeds) was chromatographed on a silica-gel column yielding guttiferone A as the main constituent. This compound, after evaluation in promastigote forms of *L. amazonensis* and *L. brasiliensis*

Table 2: *In vitro* leishmanicidal activity of extracts on promastigotes of *Leishmania amazonensis* and *L. brasiliensis*.

| Species | Sample | *L. amazonensis* $IC_{50}$ ($\mu$g/mL)[a] | *L. brasiliensis* $IC_{50}$ ($\mu$g/mL)[a] |
|---|---|---|---|
| *A. schottii* | Ethanolic extract roots | 43.8 ± 1.13 | 8.5 ± 0.47 |
| | Hexane fraction | 49.4 ± 2.27 | 48.6 ± 1.23 |
| | Dichloromethane fraction | 2.1 ± 0.25 | 8.8 ± 0.37 |
| | Ethyl acetate fraction | 85.6 ± 3.57 | 67.8 ± 0.14 |
| | Ethanolic extract stems | >100 | 39.0 ± 0.56 |
| | Hexane fraction | >100 | 17.8 ± 1.83 |
| | Dichloromethane fraction | 13.6 ± 3.80 | 8.2 ± 0.07 |
| | Ethyl acetate fraction | >100 | 47.9 ± 3.60 |
| | Ethanolic extract leaves | 63.8 ± 1.10 | 65.7 ± 6.71 |
| | Hexane fraction | >100 | 36.7 ± 6.80 |
| | Dichloromethane fraction | 13.4 ± 1.27 | 8.9 ± 0.63 |
| | Ethyl acetate fraction | >100 | 32.9 ± 6.10 |
| *E. umbelliflora* | Hexane extract fruits | 14.3 ± 0.86 | 5.7 ± 0.92 |
| | Dichloromethane extract fruits | 37.0 ± 1.02 | 20.7 ± 0.05 |
| | Ethyl acetate extract fruits | 27.2 ± 4.80 | 37.6 ± 0.44 |
| | Methanolic extract fruits 1 | >100 | >100 |
| | Methanolic extract fruits 2 | 12.5 ± 0.50 | 7.8 ± 0.40 |
| *G. achachairu* | Methanolic extract seeds | 35.9 ± 0.52 | 28.2 ± 0.35 |
| | Methanolic extract leaves | >100 | >100 |
| | Methanolic extract branches | >100 | 81.6 ± 0.25 |
| *R. feruginea* | Ethanolic extract fruits | >100 | >100 |
| | Ethanolic extract leaves | >100 | >100 |
| | Ethanolic extract stem barks | 66.4 ± 2.68 | 24.9 ± 1.79 |
| *S. sisymbriifolium* | Hexane fraction | >100 | 74.3 ± 0.22 |
| | Chloroform fraction | 33.8 ± 0.81 | 20.5 ± 0.76 |
| | Ethyl acetate fraction | >100 | >100 |
| Positive control | Catetanol | 21.1 ± 4.27 | 21.4 ± 5.53 |
| | Amphotericin B | 0.6 ± 0.36 | 0.7 ± 0.36 |

[a]Data are expressed as mean ± standard deviation of three determinations.

Table 3: *In vitro* leishmanicidal activity of isolated compounds on promastigotes of *Leishmania amazonensis* and *L. brasiliensis*.

| Species | Isolated compounds | *L. amazonensis* $IC_{50}$ ($\mu$g/mL)[a] | *L. brasiliensis* $IC_{50}$ ($\mu$g/mL)[a] |
|---|---|---|---|
| *A. schottii* | Plumericin | 0.3 ± 0.07 | 0.04 ± 0.007 |
| | Plumieride | >100 | 21.3 ± 2.80 |
| | Ursolic acid | 66.1 ± 1.22 | 8.3 ± 0.84 |
| *R. ferruginea* | Myrsinoic acid B | 24.1 ± 0.52 | 6.1 ± 0.24 |
| *E. umbelliflora* | Eugenial A | >100 | 53.8 ± 1.71 |
| *G. achachairu* | Guttiferone A | 10.4 ± 0.50 | 18.4 ± 0.20 |
| *S. sisymbriifolium* | Cilistol A | 6.6 ± 0.22 | 3.1 ± 0.25 |
| | Cilistadiol | >100 | 59.8 ± 0.32 |
| Positive control | Catetanol | 21.1 ± 4.27 | 21.4 ± 5.53 |
| | Amphotericin B | 0.6 ± 0.36 | 0.7 ± 0.36 |

[a]Data are expressed as mean ± standard deviation of three determinations.

(Table 3), showed a significant activity with $IC_{50}$ values of 10.4 and 18.4 $\mu$g/mL.

The ethanolic extracts of fruits, leaves, and stem bark of *R. ferruginea* were evaluated against promastigotes of *L. amazonensis* and *L. brasiliensis*. Only the extracts of the stem bark presented activity, with $IC_{50}$ of 66.4 $\mu$g/mL for *L. amazonensis* and 24.9 $\mu$g/mL for *L. brasiliensis*. In this extract, the main compound was a prenylated benzoic acid derivative known as myrsinoic acid B, with $IC_{50}$ of 24.1 $\mu$g/mL for *L. amazonensis* and 6.1 $\mu$g/mL for *L. brasiliensis*. These extracts were previously evaluated by high performance liquid chromatography, and myrsinoic acid B was confirmed

as the main compound in the stem bark extract. In leaves and fruits, it is present in lower concentrations [30]. It is interesting to note that species from the *Rapanea* genus are not known for their leishmanicidal activity, our studies being the first to suggest its importance as a possible source of antileishmanicidal agents.

According to the literature data, some biological activities of natural products, such as antimicrobial and trypanocidal products, are associated with their prenylated compounds, which may be increased by increasing the number of prenyl residues attached by an increase in lipophilicity [36, 37]. Thus, considering that guttiferone A and myrsinoic acid B are prenylated compounds, the activity observed for these compounds could be explained.

Regarding *S. sisymbriifolium*, we have observed an important antileishmanial activity. As shown in Table 2, the chloroform fraction exhibited $IC_{50}$ values of 33.8 $\mu$g/mL against promastigote forms of *L. amazonensis*. On the other hand, the hexane and ethyl acetate fractions exhibited $IC_{50}$ values of 100 $\mu$g/mL. In relation to the promastigote forms of *L. brasiliensis*, both hexane and chloroform fractions presented $IC_{50}$ values of 74.3 and 20.50 $\mu$g/mL, respectively. However, the ethyl acetate fractions exhibited an $IC_{50}$ value of 100 $\mu$g/mL, suggesting that the less polar compounds are responsible for the observed activity. This can be confirmed by the activity of two compounds isolated from the chloroform fractions and identified as cilistol A and cilistadiol (Figure 1). These compounds exhibited $IC_{50}$ values of 6.60 and more than 100 $\mu$g/mL against the promastigote forms of *L. amazonensis* and $IC_{50}$ values of 3.1 and 59.8 $\mu$g/mL for the promastigote forms of *L. brasiliensis*, respectively (Table 3).

## 4. Conclusions

Our results, together with those reported in the literature, strongly suggest that *Allamanda schottii*, *Rapanea ferruginea*, *Eugenia umbelliflora*, *Garcinia achachairu*, and *Solanum sisymbriifolium* and, in particular, the compounds plumericin, plumieride, ursolic acid, guttiferone A, cilistol A, and cilistadiol could be promising for the treatment of leishmaniasis caused by protozoans, demanding a search for new chemotherapeutic agents. However, further studies (*in vitro* and *in vivo*) need to be carried out, in order to understand the mechanisms of action and to evaluate the toxicity, searching for a clinical use for these bioactive compounds.

## Conflict of Interests

The authors declare that they have no conflict of interests and no financial connection to any commercial entity mentioned in the paper.

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*Research Article*

# Trypanocidal Activity of *Smallanthus sonchifolius*: Identification of Active Sesquiterpene Lactones by Bioassay-Guided Fractionation

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In order to find novel plant-derived biologically active compounds against *Trypanosoma cruzi*, we isolated, from the organic extract of *Smallanthus sonchifolius*, the sesquiterpene lactones enhydrin (**1**), uvedalin (**2**), and polymatin B (**3**) by bioassay-guided fractionation technique. These compounds showed a significant trypanocidal activity against the epimastigote forms of the parasite with $IC_{50}$ values of 0.84 $\mu$M (**1**), 1.09 $\mu$M (**2**), and 4.90 $\mu$M (**3**). After a 24 h treatment with 10 $\mu$g/mL of enhydrin or uvedalin, parasites were not able to recover their replication rate. Compounds **1** and **2** showed $IC_{50}$ values of 33.4 $\mu$M and 25.0 $\mu$M against *T. cruzi* trypomastigotes, while polymatin B was not active. When the three compounds were tested against the intracellular forms of *T. cruzi*, they were able to inhibit the amastigote replication with $IC_{50}$ of 5.17 $\mu$M, 3.34 $\mu$M, and 9.02 $\mu$M for **1**, **2**, and **3**, respectively. The cytotoxicity of the compounds was evaluated in Vero cells obtaining $CC_{50}$ values of 46.5 $\mu$M (**1**), 46.8 $\mu$M (**2**), and 147.3 $\mu$M (**3**) and the selectivity index calculated. According to these results, enhydrin and uvedalin might have potentials as agents against Chagas disease and could serve as lead molecules to develop new drugs.

## 1. Introduction

Chagas disease, also called American trypanosomiasis is an endemic disease that remains as a major public health problem in Latin America. It is estimated that approximately 10 million people are infected and 100 million are at risk worldwide, mainly due to population migrations. The disease is caused by a kinetoplastid protozoan parasite, *Trypanosoma cruzi*, which is primarily transmitted by blood-sucking insects widely known in endemic countries as "kissing bugs." The acute clinical stage of the disease (in which 5% of children die) is characterized by fever, generalized lymphadenopathy, and hepatosplenomegaly. The chronic stage often involves mainly cardiac and/or digestive disturbances being a leading cause of infectious cardiomyopathy worldwide [1].

Current treatments are based on the nitro-derivatives benznidazole and nifurtimox, two drugs developed more than four decades ago. They are employed in acute and early chronic cases. However, these drugs have an unsatisfactory cure rate in the chronic disease, are frequently not well tolerated, and cause toxic side effects [2]. Hence, improved treatment options are needed for all stages of *T. cruzi* infection.

Medicinal plants produce a variety of chemical compounds and are still a major source of innovative therapeutic agents for various diseases, directly in their native form, or more often after optimization by structural modifications or by the synthesis of analogs with improved pharmacological properties [3]. Many reports concerning the antiprotozoal activity of natural compounds from plant origin have been reported [4–9].

*Smallanthus sonchifolius* (Asteraceae), a herbaceous perennial plant native to South America, is locally known as "yacon," "llacuma," and "jiquima" or "poire de terre" and "yacon strawberry" in Europe. The history of yacon goes far back beyond the Incas. This plant originates from the Andean region, whence it has spread to New Zealand, Japan, and Brazil. It is grown throughout the Andes, from Colombia to Northwestern Argentina [10, 11], and its cultivation and consumption have expanded in recent decades to several Asian and European countries [12]. Yacon has been identified as a traditional food in the Andean region, and it is also used as an offering during religious festivities [11]. Besides from its use as food, yacon is also acknowledged as a medicinal plant. Antidiabetic properties have been attributed to yacon leaves, which are dried and used in the preparation of tea [12]. Previous biological works have demonstrated anti-inflammatory, antifungal, and antibacterial activities [13, 14]. Different chemical compounds such as phenolic and ent-kaurenoic acids, related diterpenoid substances, acetophenone phytoalexins, and sesquiterpene lactones (STLs) were identified in yacon leaves [15, 16].

We have already shown that STLs, mainly occurring in the Asteraceae family, represent a class of compounds with potential as trypanocidal leads [17–19]. Given the increasing interest that yacon has raised in recent years due to its health promoting properties, its potential as a medicinal plant, and its content in STLs, we have selected *S. sonchifolius* in the search for trypanocidal molecules against *T. cruzi*.

## 2. Materials and Methods

*2.1. Plant Material.* Leaves of *Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson (Asteraceae), clone LIEY 97-2, were collected in May 2010 from experimental crops located at Centro Universitario "Horco Molle," Universidad Nacional de Tucumán (26°47′ S, 65°19′ W, 547 m a.s.l.). A voucher specimen was deposited at the Herbarium of Instituto Miguel Lillo, S. M. de Tucumán, Argentina (LIL 607176).

*2.2. Extraction of Plant Material.* Approximately 350 g of air-dried and ground leaves were extracted by soaking in dichloromethane (6.8 L) at room temperature for 30 min. The procedure was repeated, and the filtrates combined. Evaporation of the solvent under reduced pressure provided 9.29 g of organic extract (OE; 2.65%).

*2.3. Bioassay-Guided Fractionation of OE and Isolation of Compounds.* The OE (9.0 g) was separated by column chromatography (CC) on silica gel 60 (Merck, 0.063–0.2 mm/70–230 mesh; 67 g) affording 10 fractions ($F_{1A}$–$F_{5B}$). Each fraction was eluted with 250 mL of the following eluents: *n*-hexane 100% ($F_{1A}$-$F_{1B}$), *n*-hexane : ethyl acetate 1 : 1 ($F_{2A}$-$F_{2B}$.), ethyl acetate 100% ($F_{3A}$-$F_{3B}$), ethyl acetate : methanol 1 : 1 ($F_{4A}$-$F_{4B}$), and methanol 100% ($F_{5A}$-$F_{5B}$). Eluates were monitored by thin-layer chromatography (TLC) using silica gel 60 $F_{254}$ (Merck) using hexane : ethyl acetate 1 : 1 as mobile phase. Visualization of compounds was done by a solution of anisaldehyde/sulfuric acid followed by heating.

Fractions $F_{2B}$ and $F_{3A}$ were separately chromatographed over silica gel CC (10 g) and eluted isocratically with *n*-hexane : ethyl acetate (1 : 1) as mobile phase yielding 20 subfractions of $F_{2B}$ ($F_{2B1}$–$F_{2B20}$) and 22 subfractions from $F_{3A}$ ($F_{3A1}$–$F_{3A22}$), of 10 mL each. According to their TLC profile, fractions $F_{2B5}$–$F_{2B7}$; $F_{2B8}$–$F_{2B12}$; $F_{3A6}$–$F_{3A8}$ and $F_{3A9}$–$F_{3A13}$ were pooled and subjected to preparative TLC on silica gel 60 employing *n*-hexane : ethyl acetate 1 : 1 as mobile phase to afford compounds **1**, **2**, and **3**.

These compounds were recrystallized from ethanol at 96°C and identified by spectroscopic techniques: $^1$H- and $^{13}$C-nuclear magnetic resonance (NMR), gas chromatography coupled to mass spectrometry (GC/MS), and infrared spectroscopy (IR).

*2.4. Identification of Compounds **1**, **2**, and **3**.* The structure elucidation of the isolated compounds was performed by proton nuclear magnetic resonance ($^1$H NMR) and carbon NMR ($^{13}$C NMR) (Bruker 300 MHz-Karlsruhe, Germany). Compounds were dissolved in deuterated chloroform ($Cl_3CD$), and tetramethylsilane (TMS) was used as internal standard (Sigma). FTIR spectra were recorded on a Nicolet 380 spectrometer using KBr pellets. The material was dried and placed in a desiccator at 20°C prior to pellet preparation. Gas chromatography/mass spectroscopy spectra were recorded on a Agilent 5973 network mass selective detector (MS), Agilent 6890 Series GC system (GC).

*2.5. Parasites, Cell Lines, and Media. T. cruzi* epimastigotes (RA strain) were grown in liver infusion tryptose medium (LIT) supplemented with 10% fetal bovine serum (Natocor). Cultures were routinely maintained by weekly passages at 28°C. *T. cruzi* trypomastigotes were routinely maintained by infecting 21-day-old male CF1 mice. *T. cruzi* amastigotes were obtained by infecting J774 cells with bloodstream trypomastigotes.

*2.6. In Vitro Trypanocidal Activity against T. cruzi Epimastigotes and Trypomastigotes. S. sonchifolius* OE and fractions $F_{1A}$–$F_{5B}$ were tested for trypanocidal activity against *T. cruzi* epimastigotes, at final concentrations of 10 $\mu$g/mL and 100 $\mu$g/mL, by a [$^3$H] thymidine uptake assay as previously described [17]. Compounds **1**, **2**, and **3** were tested at final concentrations ranging from 0.01 $\mu$g/mL to 50 $\mu$g/mL. Stock solutions of the samples were prepared in ethanol : water (1 : 1). Epimastigotes in exponential growth phase were adjusted to $1.5 \times 10^6$ parasites/mL and seeded on 96-well plates in the presence of the different concentrations of OE, fractions, or compounds. Parasites were cultured in triplicate for 72 h. Control parasites were cultured in absence or presence of benznidazole (20 $\mu$M; Roche-Rio de Janeiro,

Brazil). Percentage inhibition was calculated as 100 − {[(cpm of treated parasites)/(cpm of untreated parasites)] × 100}. The compound concentration at which the parasite growth was inhibited by 50% (inhibitory concentration 50, $IC_{50}$) was determined after 72 h.

To determine whether the parasites could recover after treatment with the pure compounds, *T. cruzi* epimastigotes were incubated with compounds **1–3** (0.1, 1, and 10 $\mu$g/mL) for 24 h. Parasites were then centrifuged at 3000 rpm for 10 min, resuspended in fresh medium, and incubated for 2 additional days, counting the number of parasite in a Neubauer chamber on a daily basis.

The trypanocidal effect of compounds **1**, **2**, and **3** was also tested on bloodstream trypomastigotes as previously described [17]. Briefly, mouse blood containing trypomastigotes was adjusted to a concentration of $1.5 \times 10^6$ parasite/mL and seeded (150 $\mu$L/well) by duplicate into a 96-well microplate, in the presence of each compound (1 to 50 $\mu$g/mL, final concentration). Plates were incubated for 24 h, and the remaining live parasites were counted in a haemocytometer.

*2.7. In Vitro Trypanocidal Activity against T. cruzi Amastigotes.* To evaluate the effect of compounds **1–3** on intracellular forms of *T. cruzi*, 96-well plates were seeded with J774 murine macrophages at $5 \times 10^3$ per well in 100 $\mu$L of culture medium and incubated for 2 h at 37°C in a 5% $CO_2$ atmosphere. Cells were infected with transfected blood trypomastigotes expressing $\beta$-galactosidase at a parasite : cell ratio of 10 : 1. After 2 h of coculture, plates were washed twice with PBS to remove free parasites and compounds **1–3** were added at 0.1–50 $\mu$g/mL per well in 150 $\mu$L of fresh complete RPMI medium without phenol red (Gibco, Rockville, MD). Controls included infected nontreated cells (100% infection control) and uninfected cells (0% infection control). The assay was developed 48 h later by addition of chlorophenol red-$\beta$-d-galactopyranoside (100 $\mu$M CPRG) and 1% Nonidet P40. Plates were incubated for 4–6 h at 37°C. Wells with galactosidase activity turned the media from yellow to red, and this reaction was quantified at 590 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA). Percentage inhibition was calculated as 100 − {[(absorbance of treated infected cells)/(absorbance of untreated infected cells)] × 100} and the $IC_{50}$ value calculated.

*2.8. Cytotoxicity Assay.* The cytotoxic effect of compounds **1**, **2**, and **3** on Vero cells was evaluated by using the MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) colorimetric assay. Cells ($5 \times 10^4$ cells/well) were seeded at a final volume of 150 $\mu$L in a flat-bottom 96-well microplate and cultured at 37°C in a 5% $CO_2$ atmosphere in the absence or presence of increasing concentrations of the compounds (1–50 $\mu$g/mL). After 24 h, MTT was added at a final concentration of 1.5 mg/mL and plates were incubated for 2 h at 37°C. The purple formazan crystals were completely dissolved by adding 150 $\mu$L of ethanol, and the absorbance was detected at 570 nm in a microplate reader. Results were calculated as the ratio between optical density in the presence and absence of the compound multiplied by 100. The 50% cytotoxic concentration ($CC_{50}$) was calculated for each compound. All experiments were made in duplicate.

The selectivity index (SI) of each compound was calculated as the $CC_{50}$ obtained with Vero cells divided by the $IC_{50}$ obtained against *T. cruzi* amastigotes.

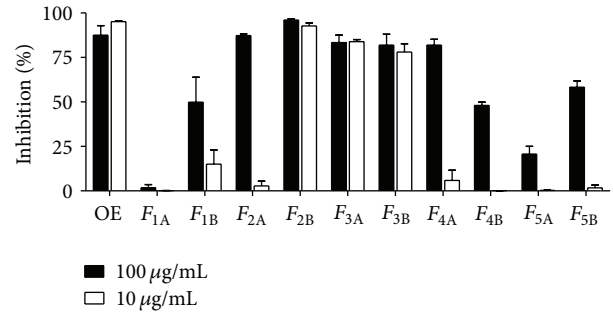


Figure 1: Growth inhibition of *T. cruzi* epimastigotes by *S. sonchifolius* OE and fractions $F_{1A}$ to $F_{5B}$. Epimastigotes were cultured in triplicate in the presence of 10 or 100 $\mu$g/mL of OE or each fraction. Cultures were done in 96-well plates with $1.5 \times 10^6$ parasites/mL during 72 h with the addition of [$^3$H] thymidine for the last 16 h. Bars represent means ± SEM.

*2.9. Statistical Analysis.* All values were presented as mean ± SEM The GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA) was employed to carry out calculations. To calculate the $IC_{50}$ values, the percentages of inhibition were plotted against the drug concentration and fitted with a straight line determined by a linear regression (Sigma Plot 10 software). Results presented are representative of three to four independent experiments.

## 3. Results

*3.1. In Vitro Trypanocidal Activity of S. sonchifolius OE.* The trypanocidal activity of the organic extract of *S. sonchifolius* (OE) was evaluated *in vitro* against *T. cruzi* epimastigotes by a [$^3$H] thymidine uptake assay. The extract was found to be active showing a growth inhibition of 87.6% ± 5.3 and 95.1% ± 0.5 at 100 and 10 $\mu$g/mL, respectively.

The fractionation of the OE by CC yielded 10 fractions ($F_{1A}$–$F_{5B}$), the effects of which were tested against epimastigote forms of *T. cruzi* as described previously (Figure 1). The results showed that at the lowest concentration tested (10 $\mu$g/mL) fractions $F_{2B}$, $F_{3A}$, and $F_{3B}$ showed the highest trypanocidal activity with percentages of growth inhibition of 92.7 ± 1.7%, 83.8 ± 1.1%, and 82.7 ± 1.0%, respectively.

*3.2. Bioassay-Guided Fractionation of S. sonchifolius OE.* The bioassay-guided fractionation of fractions $F_{2B}$ and $F_{3A}$ led to the isolation of compounds **1**, **2**, and **3** that were purified by a combination of column chromatography, preparative TLC, and precipitation techniques. Compound **1** (35 mg; 0.39%), compound **2** (6.4 mg; 0.07%), and compound **3** (2.4 mg; 0.03%) were identified by comparing their spectral data with published values [20–22] as the STLs *enhydrin*

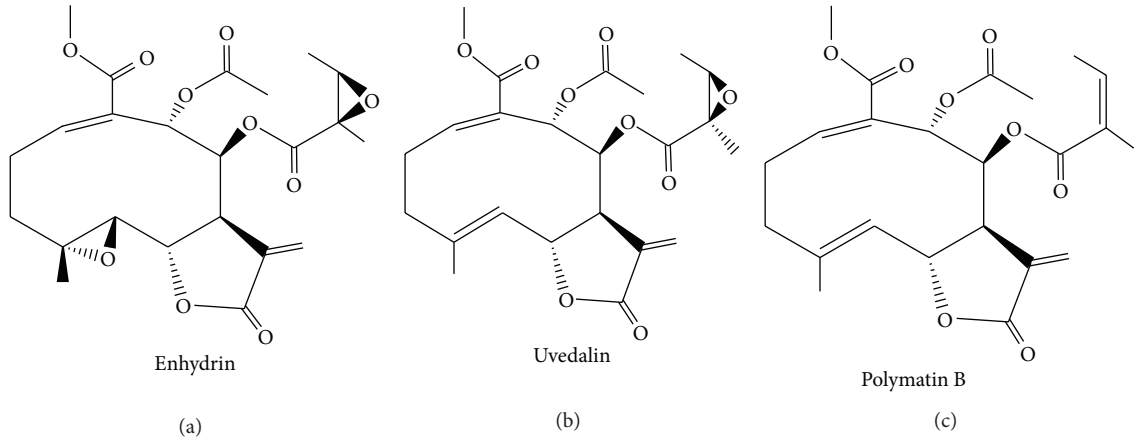


Figure 2: Chemical structures of the sesquiterpene lactones enhydrin, uvedalin, and polymatin B.

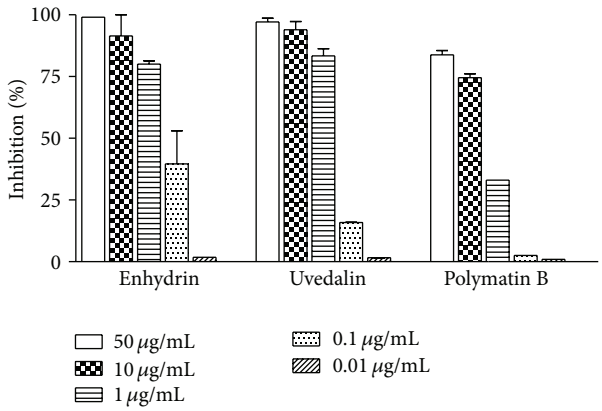


Figure 3: Inhibition of *T. cruzi* epimastigotes growth by enhydrin, uvedalin, and polymatin B. Parasites were adjusted at $1.5 \times 10^6$/mL and incubated in triplicate in the presence of 0.01 to 50 $\mu$g/mL of each compound. Parasites were cultured for 72 h, with the addition of [$^3$H] thymidine for the last 16 h.

(4$\alpha$,5$\beta$-epoxy-8$\beta$-(2′S,3′S)-epoxyangeloyloxy-9$\alpha$-acetyloxy-1(10)E,11(13)-germacradien-12,6$\alpha$-olide-14-oic-acid methyl ester), *uvedalin* (8$\beta$-(2′S,3′S)-epoxyangeloyloxy-9$\alpha$-acetyloxy-1(10)E,4E,11(13)-germacratrien-12,6$\alpha$-olide-14-oic acid methyl ester), and *polymatin B* (8$\beta$-angeloyloxi-9$\alpha$-acetyloxi-1(10)E,4E,11(13)-germacratrien-12,6$\alpha$-olide-14-oic acid methyl ester) (Figure 2). Purity of the compounds (>95%) was confirmed by gas chromatography (GC).

*3.3. In Vitro Trypanocidal Activity against T. cruzi Epimastigotes and Trypomastigotes.* The $IC_{50}$ values of the pure compounds were calculated on different *T. cruzi* evolutive stages. Figure 3 shows the effect of the pure compounds on the growth of epimastigotes of *T. cruzi*. The $IC_{50}$ values for enhydrin, uvedalin, and polymatin B were 0.39 $\mu$g/mL (0.84 $\mu$M), 0.49 $\mu$g/mL (1.09 $\mu$M), and 2.12 $\mu$g/mL (4.90 $\mu$M), respectively, after 72 h of incubation.

Moreover, after the 24 h treatment with 10 $\mu$g/mL enhydrin or uvedalin, a drastic reduction in the amount of parasites could be observed. Besides, two days after removal of the compounds, no recuperation of the epimastigotes was observed suggesting that 24 h treatment at high doses is sufficient to kill the parasites (Figure 4). When 0.1 $\mu$g/mL of polymatin B was removed at 24 h after treatment, epimastigotes recovered their replication rates at values similar to the control parasites.

The effect of the compounds against the infective form of *T. cruzi* is shown in Figure 5. When bloodstream trypomastigotes were incubated with the pure STLs, we observed that enhydrin and uvedalin were active with $IC_{50}$ values of 15.5 $\mu$g/mL (33.4 $\mu$M) and 11.2 $\mu$g/mL (25.0 $\mu$M), respectively. By contrast, polymatin B showed no activity against this parasite form.

*3.4. In Vitro Trypanocidal Activity against T. cruzi Amastigotes.* In order to evaluate the ability of the pure compounds to inhibit the intracellular amastigote forms of *T. cruzi*, J774 macrophages were infected with transfected blood trypomastigotes expressing $\beta$-galactosidase. Forty-eight hours after the addition of the pure compounds, the percentage of inhibition was determined. All the tested STLs were able to inhibit amastigotes replication with $IC_{50}$ values of 2.4, 1.5, and 3.9 $\mu$g/mL (5.17, 3.34, and 9.02 $\mu$M) for enhydrin, uvedalin, and polymatin B, respectively (Figure 6).

*3.5. Cytotoxicity Activity in Vero Cells.* *In vitro* cytotoxicity of the STLs on Vero cells was analyzed using the MTT assay. Results are shown in Figure 7. When cell suspensions were treated with enhydrin, uvedalin, and polymatin B, the $CC_{50}$ were 21.6, 21.0, and 63.7 $\mu$g/mL (46.5, 46.8, and 147.3 $\mu$M), respectively. The SI was used to compare the toxicity for mammalian cells and the activity against the parasites. The SI for the intracellular form of the parasites was 9, 14, and 16.3 for enhydrin, uvedalin, and polymatin B, respectively.

## 4. Discussion

In this work, the trypanocidal activity of the species *Smallanthus sonchifolius* has been evaluated by *in vitro* assays. The dichloromethane extract (OE) of this plant induced a

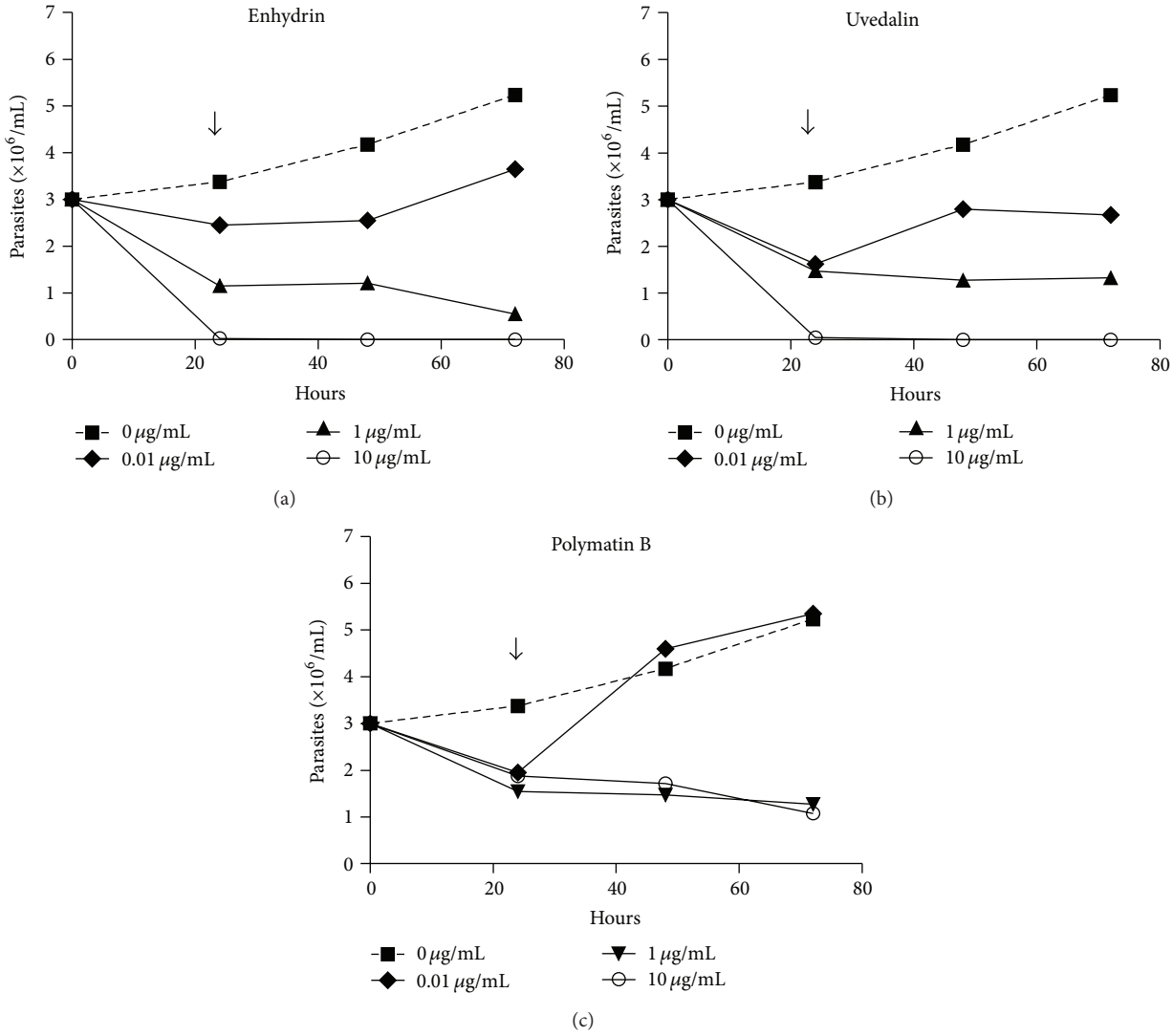


Figure 4: Residual effect of enhydrin, uvedalin, and polymatin B on the growth of *T. cruzi* epimastigotes. Parasites were incubated in the absence or presence of 0–10 $\mu$g/mL of the compounds for 24 h. The culture medium was replaced by a fresh one (arrow) without the compound, and parasites were allowed to grow for 2 days. Parasites were counted in a Neubauer chamber. Symbols represent the mean ± SEM.

significant growth inhibition (95.1%) when tested against *T. cruzi* epimastigotes at a concentration of 10 $\mu$g/mL. This result prompted us to carry out a bioassay-guided fractionation of the OE by chromatographic techniques. Among the tested fractions, $F_{2B}$, $F_{3A}$, and $F_{3B}$ presented the highest *in vitro* inhibitory activity, against epimastigotes, with percentages of growth inhibition higher than 80% at the lower concentration tested. Further purification of $F_{2B}$ and $F_{3A}$, by a series of chromatographic separations, led to the isolation of three structurally related germacranolide STLs of the melampolide type, which were identified as enhydrin, uvedalin, and polymatin B.

STLs are naturally occurring plant terpenoids with over 5000 known structures and which are mainly present in members of the Asteraceae family [23]. They exhibit a variety of skeletal arrangements and are the largest and most diverse category of natural products with an $\alpha$-methylene-$\gamma$-lactone motif [3]. STLs have been related to a broad spectrum of biological activities ranging from anticancer, antiviral, antibacterial, antifungal, and antiprotozoal. Many are described as the active constituents of medicinal plants used in traditional medicine for the treatment of inflammatory diseases [24]. The vast majority of STLs are considered quite "drug-like" molecules with respect to their physicochemical properties [25].

Enhydrin, uvedalin, and polymatin B were firstly evaluated for their trypanocidal activity on *T. cruzi* epimastigotes showing a marked activity with $IC_{50}$ values of 0.84 $\mu$M, 1.09 $\mu$M, and 4.90 $\mu$M, respectively. These values indicated that an increase in the trypanocidal activity was achieved along the purification process.

Besides, epimastigotes treated for 24 h with 10 $\mu$g/mL of enhydrin or uvedalin were not able to recover their initial replication rates suggesting that the treatment at high doses was sufficient to kill the parasites. Parasites treated with either

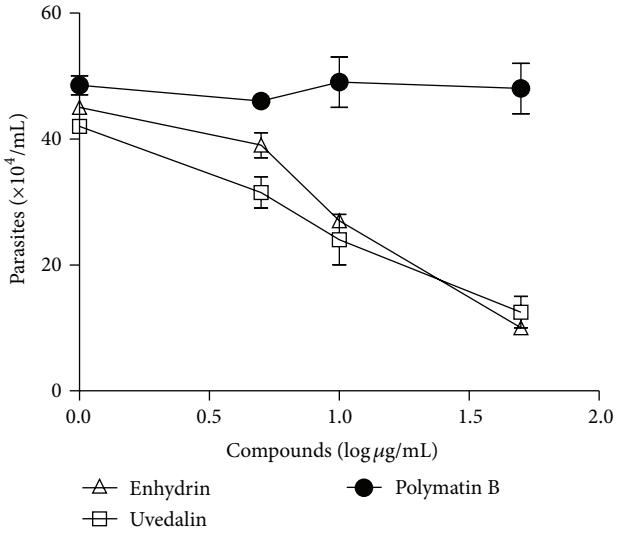


Figure 5: Effect of enhydrin, uvedalin, and polymatin B on *T. cruzi* trypomastigotes. Bloodstream trypomastigotes were cultured in duplicate in the presence of 1 to 50 $\mu$g/mL of the compounds. The assay was performed employing $1.5 \times 10^6$ parasites/mL over 24 h. Remaining live parasites were counted in a Neubauer chamber. Symbols represent the mean ± SEM.

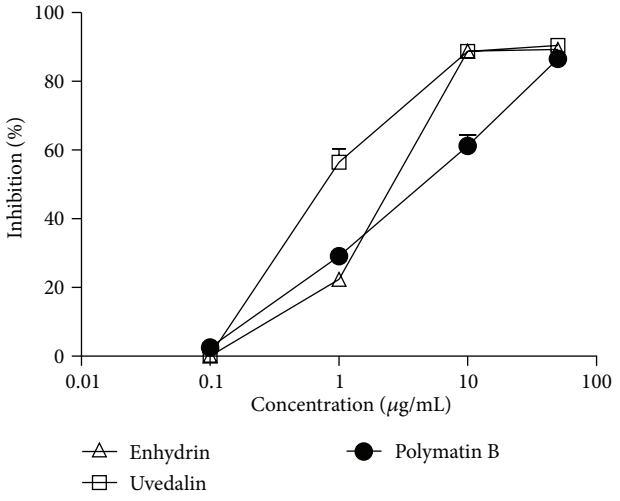


Figure 6: Effect of enhydrin, uvedalin, and polymatin B on *T. cruzi* amastigotes. J774 phagocytic cells were infected with transfected trypomastigotes expressing $\beta$-galactosidase (10 : 1 parasite : cell ratio). After removing free parasites, STLs were added at concentrations ranging from 0.1 to 50 $\mu$g/mL. Three days after infection, nonidet P40 and chlorophenol red-$\beta$-d-galactopyranoside (CPRG) were added and the galactosidase activity was determined at 590 nm. Values represent the mean ± SEM.

enhydrin or uvedalin, at 1 $\mu$g/mL, or with polymatin B, at 10 $\mu$g/mL, presented a reduction in the replication rate that was close to 50% at 24 h after treatment. On the other hand, recovery was evident when 0.1 $\mu$g/mL of the compounds was employed.

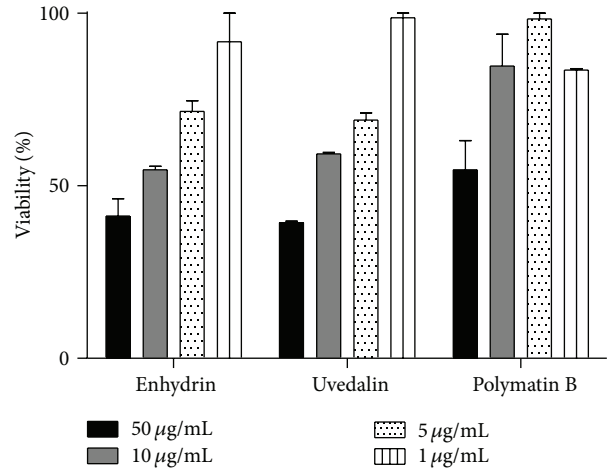


Figure 7: Cytotoxicity of enhydrin, uvedalin, and polymatin B on Vero cells. Cultures were kept for 24 h in the presence of different concentrations (1 to 50 $\mu$g/mL) of the STLs. Cell viability was determined by the MTT method and was expressed as the ratio between viable cells in the presence and absence of the compound multiplied by 100. Bars represent the mean ± SEM of three experiments carried out in duplicate.

When the isolated compounds were evaluated against *T. cruzi* trypomastigotes, the results ($IC_{50}$ = 33.4 $\mu$M for enhydrin and $IC_{50}$ = 25.0 $\mu$M for uvedalin) showed that this parasite stage is less sensitive than epimastigotes to the compounds. During the initial acute phase of infection, the nonreplicative bloodstream trypomastigotes invade different mammalian cell types, where they transform into replicative intracellular amastigotes and multiply within the host's cells cytoplasm. Interestingly, when enhydrin, uvedalin, and polymatin B were tested against the amastigote forms, they were able to inhibit replication. The three STLs were active with $IC_{50}$ values of 5.17 $\mu$M, 3.34 $\mu$M, and 9.02 $\mu$M, respectively. The ability of compounds to inhibit the intracellular growth of *T. cruzi* amastigotes is a more rigorous and relevant test of anti-*T. cruzi* activity, as it is applied to a stage which is the predominant form in mammals and because the killing assay requires that the drug cross the host cell membrane [26]. It is well known that the treatment with benznidazole is especially useful for patients in the acute phase, when trypomastigotes may be easily found in blood, while its effectiveness during the asymptomatic or chronic stage, is still controversial. The fact that these compounds proved to be active against amastigotes is of particular interest, since the DNDi organization prioritizes the development of drugs that are useful during the indeterminate and chronic phases of the infection, where parasites remain intracellular [1].

The therapeutic potential and the lack of cytotoxic effects on mammalian cells are important criteria to be considered when novel compounds with activity against *T. cruzi* are investigated. Compounds containing an *exo*methylene moiety conjugated with a carbonyl group can react as Michael-type acceptors with the thiol groups of macromolecules such as enzymes [3]. The presence of this reactive group in the isolated STLs could explain the trypanocidal effects and

the cytotoxicity of these compounds. When analyzing the chemical structure of the STLs isolated in this work, it could be observed that, unlike enhydrin and uvedalin, polymatin B does not present an epoxide as substituent. Biological activity of $\alpha$-methylene-$\gamma$-lactones may vary according to their different number of alkylating structure elements such as conjugated cyclopentones, conjugated side-chain esters, and epoxides [25]. These features may be related to the increased activity shown by enhydrin and uvedalin compared to the nonepoxide substituted polymatin B. Moreover, when comparing the toxicity of the lactones in Vero cells with the activity against *T. cruzi* amastigotes, we could observe that enhydrin, uvedalin, and polymatin B had some selective toxicity against the parasites (SI of 9, 14, and 16.3, resp.).

The three STLs had been previously isolated from *S. sonchifolius* [11, 13, 15]. Enhydrin and uvedalin have shown cytotoxic activity in cervical cancer cells [27]. For enhydrin hypoglycemic, antibacterial, anti-inflammatory, and antihyperalgesic activities have also been reported [11, 28, 29], while this is the first report of the anti *T. cruzi* activity of these compounds.

## 5. Conclusion

Three STLs with promising antitrypanocidal properties were isolated from the leaves of *S. sonchifolius* by a bioassay-guided fractionation method. The compounds, identified as enhydrin, uvedalin, and polymatin B, efficiently inhibited both the epimastigote and the replicative intracellular amastigotes, being more selective for the parasites than for mammalian cells. According to the results obtained, enhydrin and uvedalin may be considered promising antitrypanosomal lead molecules. However, the potential of these compounds still have to be improved by obtaining derivatives that might be used as therapeutic agents against the parasite.

Further studies will involve evaluation of the underlying mechanisms as well as *in vivo* studies of enhydrin and uvedalin.

## Conflict of Interests

The authors declare that they have no conflict of interests.

## Authors' Contribution

F. M. Frank, J. Ulloa, S. I. Cazorla, C. Catalán, and L. V. Muschietti contributed equally to this paper.

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*Research Article*

# Natural Sesquiterpene Lactones Induce Oxidative Stress in *Leishmania mexicana*

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Leishmaniasis is a worldwide parasitic disease, caused by monoflagellate parasites of the genus *Leishmania*. In the search for more effective agents against these parasites, the identification of molecular targets has been attempted to ensure the efficiency of drugs and to avoid collateral damages on the host's cells. In this work, we have investigated some of the mechanisms of action of a group of natural sesquiterpene lactones that are effective against *Leishmania mexicana mexicana* promastigotes. We first observed that the antiproliferative effect of mexicanin I (Mxc), dehydroleucodine (DhL), psilostachyin (Psi), and, at lesser extent, psilostachyin C (Psi C) is blocked by 1.5 mM reduced glutathione. The reducing agent was also able to reverse the early effect of the compounds, suggesting that lactones may react with intracellular sulfhydryl groups. Moreover, we have shown that all the sesquiterpene lactones, except Psi C, significantly decreased the endogenous concentration of glutathione within the parasite. Consistent with these findings, the active sesquiterpene lactones increased between 2.7 and 5.4 times the generation of ROS by parasites. These results indicate that the induction of oxidative stress is at least one of the mechanisms of action of DhL, Mxc, and Psi on parasites while Psi C would act by another mechanism.

## 1. Introduction

Leishmaniasis is a parasitic disease caused by flagellated parasites of the genus *Leishmania* and transmitted by phlebotomine sandflies. These parasites exhibit a heteroxenous life cycle, alternating between intracellular amastigotes in the mammalian cells and flagellate promastigotes in the vector.

Leishmaniasis affects about 12 million people worldwide and, according to the World Health Organization (WHO), 2 million of new cases occur annually and 350 million people are considered at risk of contracting leishmaniasis [1]. The clinical forms of the disease depend on the species of *Leishmania* involved and include local infections of the skin, subcutaneous tissue, and regional lymphatic nodes (cutaneous leishmaniasis); metastatic infections of the oronasal mucosa (mucocutaneous leishmaniasis); and disseminated infection involving visceral organs (visceral leishmaniasis) [2].

Leishmaniasis is distributed worldwide with foci of infection in Central and South America, Southern Europe, North and East Africa, the Middle East and India [3]. In Argentina, this parasitosis affects the northern region of the country with an incidence that has increased over the last two decades [4].

Current drugs used to treat leishmaniasis include pentavalent antimonials, pentamidine, and amphotericin B, which induce serious toxic effects on patients. Parasite resistance to these drugs has also been described. New formulations, such as liposomal amphotericin B and other drugs (miltefosine, paromomycin), have serious drawbacks such as parenteral route of administration, duration of the

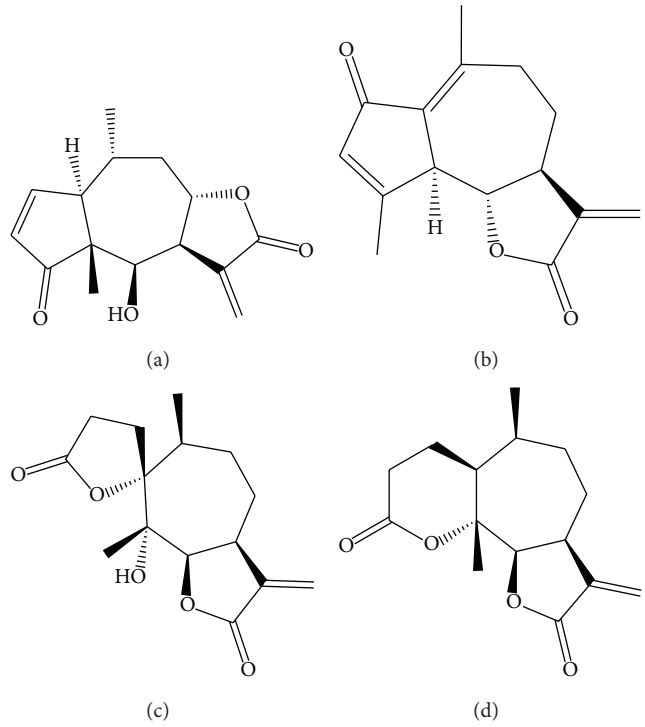


Figure 1: Chemical structures of the sesquiterpene lactone: mexicanin I (a), dehydroleucodine (b), psilostachyin (c), and psilostachyin C (d).

treatment, teratogenic effects, toxicity, and cost of treatment, which limit their use in endemic areas [5]. Therefore, there is an urgent need for novel candidates to treat this parasitic disease.

Sesquiterpene lactones, a group of natural compounds characteristic of the Asteraceae family, have been pointed out as good candidates for antiprotozoal therapy since many of them are active against trypanosomatids [6–8]. Moreover, we have previously described the trypanocidal and leishmanicidal activity of natural sesquiterpene lactones isolated from Argentinean Asteraceae species [9–16].

One of the most important aspects in antiprotozoal drug discovery is to determine the mechanism of action of the potential candidates and to identify the possible molecular targets upon which these compounds act. Among other mechanisms, it is presumed that sesquiterpene lactones could exert their leishmanicidal activity by the generation of an oxidative environment within the parasite [17, 18]. The particular defense mechanism against oxidative stress in trypanosomatids makes parasites susceptible to these kinds of compounds.

In this sense, the aim of the present work was to evaluate the possible effect of four bioactive sesquiterpene lactones: dehydroleucodine (DhL); mexicanin I (Mxc). psilostachyin (Psi), and psilostachyin C (Psi C) on the defense mechanism of *Leishmania mexicana mexicana* against oxidative stress.

## 2. Materials and Methods

*2.1. Compounds.* Mexicanin I (Mxc) was isolated from the aerial parts of *Gaillardia megapotamica* and dehydroleucodine (DhL) was isolated from *Artemisia douglasiana* as previously described [19]. Psilostachyin (Psi) and psilostachyin C (PsiC) have been isolated from *Ambrosia tenuifolia* and *A. scabra*, respectively [11, 13].

*2.2. Parasites.* Axenic cultures of *Leishmania mexicana mexicana* promastigotes were grown in Diamond's liquid medium (0.106 M NaCl, 29 mM $KH_2PO_4$, 23 mM $K_2HPO_4$, 12.5 g/L tryptone, 12.5 g/L tryptose, and 12.5 g/L yeast extract, adjusted to pH 7.2) supplemented with 75 $\mu$M hemine, 75 IU/mL penicillin, 75 $\mu$g/mL streptomycin, and 20% fetal bovine serum at 25°C.

*2.3. Treatments.* *Leishmania mexicana mexicana* promastigotes ($2 \times 10^6$ parasites) were incubated with 0.5 $\mu$g/mL of Mxc, Psi, or Psi C or 2.5 $\mu$g/mL of DhL, at 25°C, either in the presence or in the absence of 1.5 mM glutathione (GSH). The concentrations used for each compound were those corresponding to each $IC_{50}$, as previously determined (data not shown). Aliquots of the parasites were collected every 24 h and counted in a Neubauer hemocytometer [16]. In other experiments, parasites were preincubated with the compounds for 30 min and the reducing agent was then added. Alternatively, the lactones were withdrawn after incubation for 1 h and before adding GSH. Controls were carried out in the presence of DMSO (less than 0.05%) which was used to dissolve the compounds.

*2.4. Measurement of ROS.* The fluorescent probe, $H_2$DCFDA, was used to measure the intracellular generation of ROS, according to Duranteau et al. [20]. Briefly, parasites ($1 \times 10^6$ cells) were previously treated with the lactones (10 $\mu$g/mL

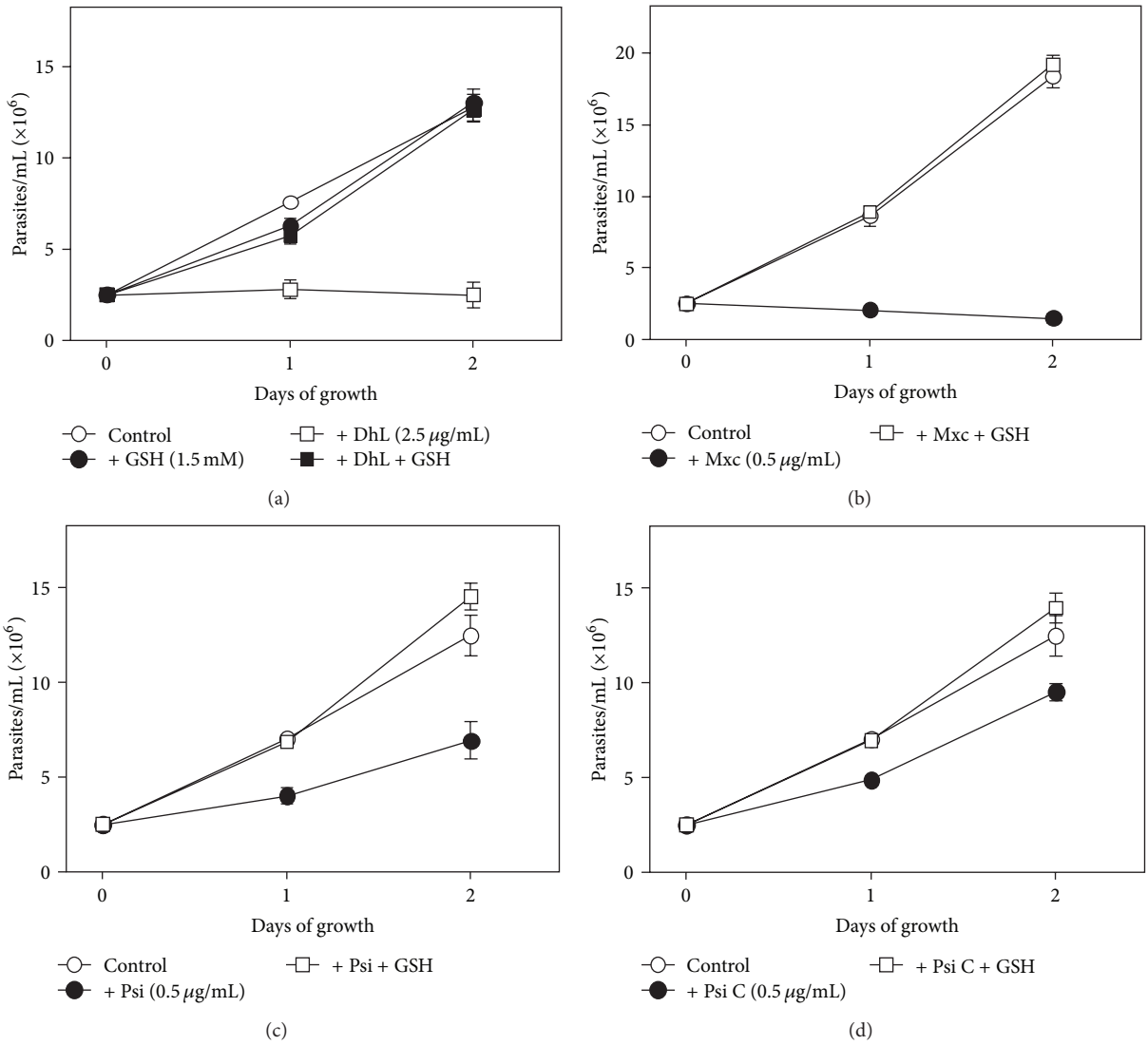


Figure 2: The effect of sesquiterpene lactones on the growth of *L. mexicana mexicana* is blocked by adding of GSH. Parasites were incubated with the lactones; dehydroleucodine (DhL) (a), mexicanin I (Mxc) (b), psilostachyin (Psi) (c), or psilostachyin C (Psi C) (d) in the presence or in the absence of 1.5 mM glutathione (GSH), as indicated in the figure. Parasite counts were done daily. Glutathione alone did not affect the parasite growth (a).

of each sesquiterpene lactone for 3 h) and then incubated with 10 $\mu$M of the probe for 1 h at room temperature in the dark. The fluorescence intensity of $H_2$DCFDA was measured at 507 nm excitation and 538 nm emission wavelengths. To validate the assay, generation of ROS by 4 mM $H_2O_2$ was used as a positive control.

*2.5. Measurement of Reduced Glutathione.* Endogenous GSH was measured in parasite lysates by using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), according to Beutler et al. [21]. Briefly, parasites ($1 \times 10^7$ cells/mL) were previously incubated with the lactones (10 $\mu$g/mL) for 3 h at 25°C then pelleted, lysed with 200 $\mu$L lysis solution (10% EDTA, 0.5% Triton X-100 in bidistilled water) during 30 min, and centrifuged at 12,000 ×g. Supernatants were mixed with 300 $\mu$L of solution P (0.2 M $HPO_3$, 5 mM EDTA, and 5.1 M NaCl) and centrifuged again at 12,000 ×g. Supernatants were mixed with 800 $\mu$L of 0.3 M $Na_2HPO_4$, and 200 $\mu$L of DTNB (in 1% sodium citrate). Absorbances were then measured in a spectrophotometer at 412 nm, and the concentration of GSH was derived from a standard curve.

*2.6. Statistical Analysis.* Results are presented as mean ± SD. The level of statistical significance was determined by using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test.

## 3. Results and Discussion

We had previously reported the antileishmanial activity of Mxc, DhL, Psi, and Psi C (Figure 1) [9–16]. The common

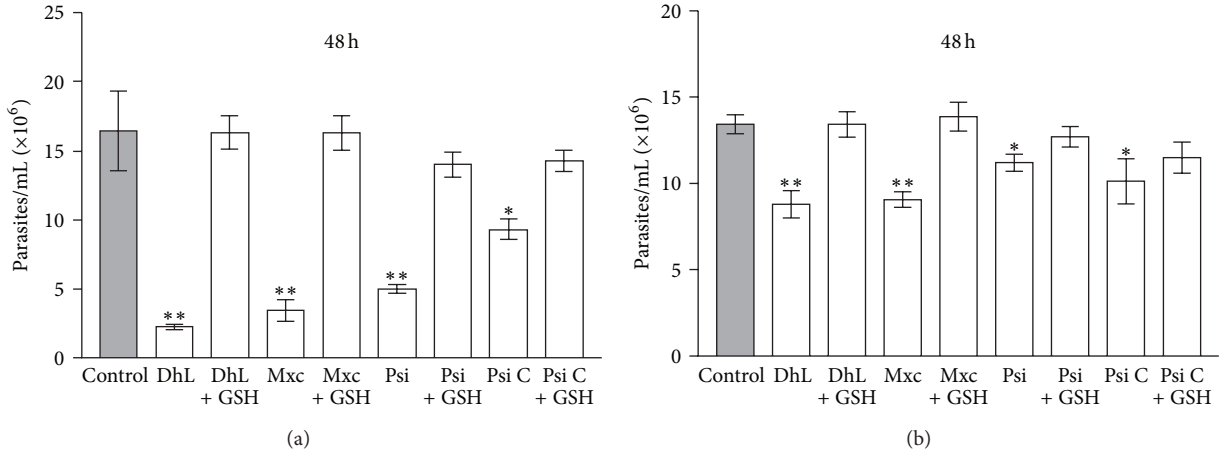


Figure 3: The effect of glutathione (GSH) on the number of parasites preincubated with 0.5 $\mu$g/mL mexicanin I (Mxc), psilostachyin (Psi) or psilostachyin C (Psi C) or with 2.5 $\mu$g/mL of dehydroleucodine (DhL), for 30 min (a) or preincubated 1 h with the lactones and followed by withdrawal of the compounds before adding the reducing agent (b). Bars represent the means of parasite concentration ± SD from three independent experiments. (∗∗) and (∗) indicate significant differences with the control ($P < 0.01$ and $P < 0.05$ resp.).

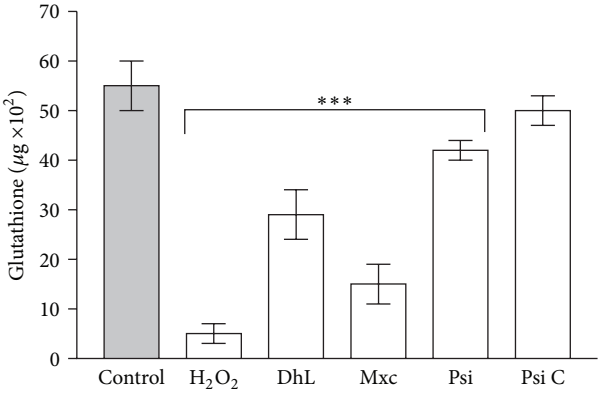


Figure 4: Concentration of endogenous glutathione in the parasites after treatment with 10 $\mu$g/mL dehydroleucodine (DhL), mexicanin I (Mxc), psilostachyin (Psi), or psilostachyin C (Psi C), as described in materials and methods. (∗ ∗ ∗): significant differences with the control ($P < 0.02$). $H_2O_2$ (5 mM) was used as positive control.

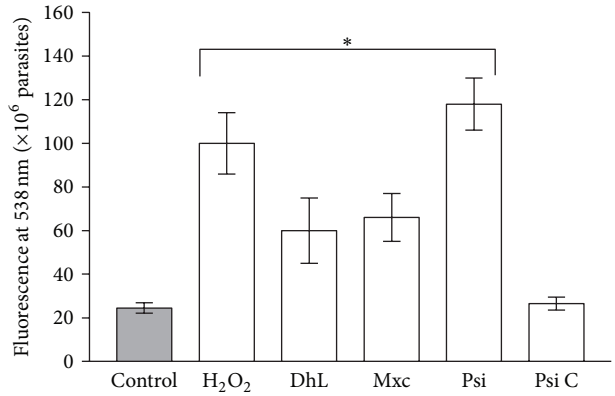


Figure 5: Generation of ROS by the parasites after treatment with 10 $\mu$g/mL of mexicanin I (Mxc), dehydroleucodine (DhL), psilostachyin (Psi), and psilostachyin C (Psi C). Values are expressed as units of fluorescence emitted by the probe at 538 nm. Bars represent the means of fluorescence ± SD from three independent experiments. (∗): significant differences with the control ($P < 0.05$).

functional group $\alpha$-methylene-$\gamma$-lactone present in the sesquiterpene lactones is believed to be responsible for their antiprotozoal activity. However, the presence of other alkylating groups such as $\alpha,\beta$-unsaturated cyclopentenones and other factors, such as lipophilicity, molecular geometry, and chemical environment, may also influence their bioactivity [22].

In this work we have corroborated the antiproliferative effect of the four lactones on *L. mexicana mexicana* promastigotes and we have demonstrated that this effect was blocked by 1.5 mM GSH (Figure 2). As these lactones are nonpolar molecules they could easily pass through the parasite's plasmalemma. The blocking effect of GSH might be due to the transformation of the compounds into derivatives unable to traverse the plasmalemma. However, it is more likely that the compounds interfere with the intracellular concentration of GSH, as the antiproliferative effect of lactones can be reversed by GSH when the reducing agent is added 30 min after incubation with the compounds or 1 h after incubation followed by withdrawal of the lactones (Figure 3). In addition, it was observed that DhL, Mxc, and Psi, but not Psi C, reduced the concentration of endogenous GSH (Figure 4).

On the other hand, treatment with DhL, Mxc, or Psi, but not Psi C, induced a significant increase of ROS in *L. mexicana* promastigotes (Figure 5).

The generation of free radicals in *Leishmania* by the sesquiterpene lactones would be deleterious for trypanosomatids, as the regulation of oxidative stress is crucial for parasite survival. It is known that sesquiterpene lactones

react with sulfhydryl groups by the Michael-type addition and therefore could act by inhibiting the activity of enzymes that are vital against oxidative stress (e.g., trypanothione reductase) [17]. This situation could lead to an increase in the level of reactive oxygen species and to parasite damage via the generation of an oxidative burst by a deregulation of the redox balance within the parasite [23]. However, a direct interaction of the compounds with GSH or trypanothione should not be ruled out.

The decrease in the concentration of glutathione within the parasites induced by the sesquiterpene lactones Mxc, DhL, and Psi would lead to an enhancement in the production of reactive oxygen species. These results are in accordance with ROS production and the *in vitro* leishmanicidal activity, with psilostachyin C being the less active compound against *L. mexicana*. Given that sesquiterpene lactones can also induce GSH depletion and ROS generation in certain mammalian cells (e.g., tumor cells) [24], these compounds should be improved before use as therapeutic agents against *Leishmania*.

One vital step in the process of drug development is the identification of the molecular target/s of such drugs. Taking into consideration the data obtained, we can suggest that Mxc, DhL, and Psi were able to affect the defense mechanism against oxidative stress in *L. mexicana*. This mechanism could be related to inhibition of key enzymes that maintain redox balance in the parasite.

This study must be complemented by further investigations on amastigotes forms of *Leishmania* and on *in vivo* models of leishmaniasis.

## Authors' Contribution

Patricia Barrera and Valeria P. Sülsen contributed equally to this work.

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*Research Article*

# Synergistic Effect of Lupenone and Caryophyllene Oxide against *Trypanosoma cruzi*

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The *in vitro* trypanocidal activity of a 1 : 4 mixture of lupenone and caryophyllene oxide confirmed a synergistic effect of the terpenoids against epimastigotes forms of *T. cruzi* ($IC_{50}$ = 10.4 $\mu$g/mL, FIC = 0.46). In addition, testing of the terpenoid mixture for its capacity to reduce the number of amastigote nests in cardiac tissue and skeletal muscle of infected mice showed a reduction of more than 80% at a dose level of 20.8 mg·kg$^{-1}$·day$^{-1}$.

## 1. Introduction

Chagas disease is a chronic parasitosis caused by the flagellate protozoan *Trypanosoma cruzi,* which is transmitted by an insect vector of the *Reduviidae* family causing cardiac injury leading to death [1]. The disease represents an important public health problem in Latin America, with an estimated 10 million people infected and 25 million people under risk of infection [2]. At present, there is no satisfactory chemotherapy for the disease also known as American trypanosomiasis; the drugs currently used, which include nifurtimox, benznidazole, and allopurinol, are toxic, have severe side effects, and are effective mainly in the acute phase, while their activity in the chronic phase of the disease is low and controversial [3–5]. Because of this, the WHO has emphasized the need to develop new and better trypanocidal drugs with none or limited side effects [6].

A strategy for the development of new and more efficient pharmaceuticals is to evaluate the synergism between two or more products as part of a treatment of combined therapy. Often, the therapeutic activity of a combination of drugs is greater than the activity of each product when administrated separately; additionally, synergism can improve the efficiency of the treatment, broaden its spectrum of action, limit the development of resistant strains, and reduce its duration and toxicity [7, 8]. These arguments, supported by the recent recommendation by the WHO that oral artemisinin-based monotherapies are withdrawn from the market and replaced with artemisinin-based combination therapies for the treatment of malaria [9], emphasize the importance of considering combined therapies as an alternative for the treatment of protozoan diseases.

We have recently carried out a study of native plants of the Yucatán Peninsula and reported the presence of trypanocidal activity in the leaf extract of *Serjania yucatanensis* [10]. The bioassay-guided purification of the bioactive crude extract resulted in the identification of a 1 : 1 mixture of terpenoids, lupenone (a triterpene) and caryophyllene oxide (an oxygenated sesquiterpene), as that responsible for the originally detected trypanocidal activity; the mixture also proved to

inhibit the egress of trypomastigotes from infected Vero cells without being cytotoxic [11]. We wish to report herein on the synergism of a 1 : 4 mixture of lupenone and caryophyllene oxide when tested *in vitro* for trypanocidal activity and *in vivo* when tested against the amastigote form of the parasite during the chronic phase of the infection.

## 2. Materials and Methods

*2.1. Lupenone and Caryophyllene Oxide.* Commercial caryophyllene oxide (Sigma-Aldrich) and lupenone obtained from the oxidation of commercial lupeol (Sigma-Aldrich) were used in all tests. Mixtures were prepared by combining the two terpenoids in different proportions (1 : 0, 1 : 4, 2 : 3, 1 : 1, 3 : 2, 4 : 1, and 0 : 1; w/w).

*2.2. Parasites and Their Growth Conditions.* Epimastigotes forms of the Tulahuen strain and blood trypomastigote forms of the H4 strain (isolated from a patient with Chagas disease in Yucatán, Mexico) of *T. cruzi* were used in this study [12]. Epimastigotes were obtained from liver infusion tryptose medium supplemented with 10% fetal bovine serum [13] and blood trypomastigotes were obtained by successive infections of BALB/c mice.

*2.3. Evaluation of the In Vitro Trypanocidal Activity.* The trypanocidal activity was assayed on epimastigotes of *T. cruzi* (Tulahuen strain). Experiments were carried out using 96-well microplates containing $1 \times 10^5$ epimastigotes/mL. The different proportions of mixture of lupenone and caryophyllene oxide (1 : 0, 1 : 4, 2 : 3, 1 : 1, 3 : 2, 4 : 1, and 0 : 1) as well as the crude extract of *S. yucatanensis* and the low-polarity (hexane) fraction obtained from the crude extract [10, 11] were dissolved in dimethylformamide (DMF; final solvent concentration not greater than 1%) and were evaluated at 100, 50, 25, and 12.5 $\mu$g/mL. For each experiment there were controls of parasites growing in the presence and absence of DMF. The different mixtures and their corresponding concentrations were added to the wells, and the plates were incubated at 28°C for 72 h. All assays were performed in duplicate. The activity was evaluated using the XTT colorimetric method, which is based on the reduction of the sodium salt of 2,3-bis(2metoxi-4-nitro-5-sulfophenyl-2-h-tetrazolium-5-carboxanilide) by mitochondrial dehydrogenases to produce formazan crystals [14]; 50 $\mu$L of a solution of XTT/PMS (1 mg/mL XTT)/(0.001 mg/mL PMS) were added to each well, and the plates were incubated for an additional 4 h. The plates were read in an ELISA plate spectrophotometer at 450 nm. The activity is expressed as $IC_{50}$ ($\mu$g/mL). Amphotericin B was used as positive control because this polyene antibiotic has been used as a reference drug for the *in vitro* testing of crude extracts and purified natural products on *Trypanosoma* cultures [15, 16].

*2.4. Evaluation of Synergism.* Fractional inhibitory concentrations (FIC) were calculated as previously described [17]. We have FIC = FEa + FEb, where FEa = $IC_{50}$ a + b/$IC_{50}$ a and FEb = $IC_{50}$ a + b /$IC_{50}$ b. Values of FIC < 1 indicate synergism, values = 1 indicate additive effect, and values >1 indicate antagonism.

*2.5. In Vivo Assay against Amastigotes of T. Cruzi.* Eight-week old BALB/c mice and trypomastigotes of *T. cruzi* H4 strain were used to assay for antitrypanosomal activity. Animals were maintained on a light-dark cycle and had access to food and water *ad libitum* during the entire assay.

Thirty BALB/c mice weighing approximately 23 g were randomly divided into five groups ($n = 6$ each). The animals were infected with 100 trypomastigotes through intraperitoneal injection; inoculation conditions were selected based to the reported in previous studies in mice infected with *T. cruzi* in chronic phase [18, 19]. Mice were divided into five groups: negative control (CN): infected animals treated with PBS; positive control (CP): infected animals treated with allopurinol (8.5 $\mu$g/g); hexane fraction (FHex): animals treated with the hexane fraction from the leaf crude extract of *S. yucatanensis* (41.6 mg·kg$^{-1}$·day$^{-1}$); dose 1 (D1): infected mice treated with a 20.8 mg·kg$^{-1}$·day$^{-1}$ 1 : 4 mixture of lupenone and caryophyllene oxide; dose 2 (D2): infected mice treated with a 41.6 mg·kg$^{-1}$·day$^{-1}$ 1 : 4 mixture of lupenone and caryophyllene oxide. All treatments were administered resuspended in phosphate buffer saline (PBS, NaCl 13.7 mM, KCl 2.7 mM, $Na_2HPO_4$ 4.3 mM y $KH_2PO_4$ 1.4 mM pH 7.4). Administration started after 45 days post infection, during the chronic phase; the mice in the experimental groups received each treatment orally (adjusted to 50 $\mu$L per animal), every 24 hours, for 15 days.

*2.6. Histopathology Study.* Samples of cardiac tissue and skeletal muscle from groups of treated and control mice were collected and fixed in 10% formaldehyde for further processing. Paraffin embedded tissue sections were stained with hematoxylin-eosin and examined under a light microscope (40x). The number ofamastigotenests was quantified in 100 fields for each heart tissue and skeletal muscle sample. The experiments were carried out under the approval of the Bioethics Committee of Centro de Investigaciones Regionales "Dr. Hideyo Noguchi," in Mérida, Yucatán, México.

*2.7. Statistical Analyses.* The statistical analyses were performed using Prism program 5.0 software. Data are presented as mean values ± S.D. Statistical analyses: one-way ANOVA and post hoc Tukey's test were used to compare different experimental groups ($P < 0.05$).

## 3. Results and Discussion

*In vitro* testing of the leaf crude extract of *S. yucatanensis*, the low-polarity (hexane) fraction from the crude extract, and the different proportions of lupenone and caryophyllene oxide (1 : 0, 1 : 4, 2 : 3, 1 : 1, 3 : 2, 4 : 1, and 0 : 1) against epimastigotes of *T. cruzi* (Tulahuen strain) showed that the crude extract of *S. yucatanensis* and the hexane fraction had a similar activity ($IC_{50}$ = 74.5 and 61.5 $\mu$g/mL, resp.) than that previously reported against *T. cruzi* (Y strain) [11]. Similarly, the trypanocidal activity observed for the 1 : 1 mixture of lupenone

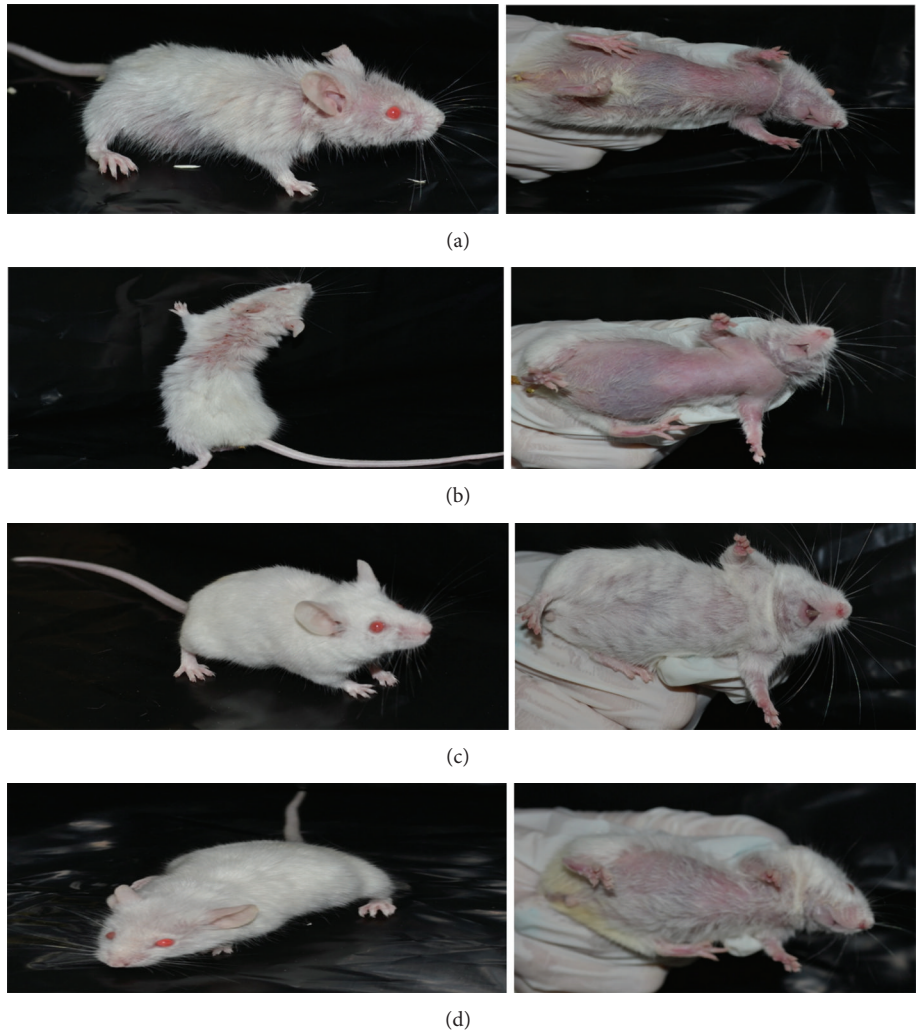


Figure 1: Mice belonging to the different groups: (a) negative control (CN): infected animals treated with PBS; (b) positive control (CP): infected animals treated with allopurinol (8.5 $\mu$g/g); (c) dose 1 (D1): infected mice treated with a 20.8 mg·kg$^{-1}$·day$^{-1}$ 1 : 4 mixture of lupenone and caryophyllene oxide; (d) dose 2 (D2): infected mice treated with a 41.6 mg·kg$^{-1}$·day$^{-1}$ 1 : 4 mixture of lupenone and caryophyllene oxide.

and caryophyllene oxide ($IC_{50}$ = 80.3 $\mu$g/mL; Table 1) is similar to that reported for the original mixture of terpenoids obtained in a approximate ratio of 1 : 1 from the leaf extract of *S. yucatanensis* ($IC_{50}$ = 80.3 $\mu$g/mL) [11]. However, the highest activity ($IC_{50}$ = 10.4 $\mu$g/mL) was observed for the 1 : 4 mixture of lupenone and caryophyllene oxide; the synergistic effect of this mixture of terpenoids against epimastigotes was confirmed by a potentiation FIC value of <1 (FIC = 0.46) (Table 1). It is interest to point out that the rest of the terpenoid mixtures proportions showed FIC values >1 indicating an antagonistic effect and suggesting that the mixture of terpenoids in a 1 : 4 proportion is necessary for the full expression of trypanocidal activity and that higher proportions of lupenone result in lower activity. The fact that lupenone does not show significant antiprotozoal activity, and that the activity of caryophyllene oxide is only moderate, is in agreement with reports in the literature describing a fraction containing lupenone as not showing trypanocidal activity against trypomastigotes of *T. cruzi* [20] and caryophyllene oxide with only a moderate inhibitory activity against cruzipain of *T. cruzi* [21]. However, lupenone has been reported to have anticarcinogenic activity in mouse melanoma [22] and to inhibit the protein tyrosine phosphatase 1B, an attractive target for the development of new drugs for type 2 diabetes and obesity [23]; the biological activities reported for caryophyllene oxide include antifungal [24], anti platelet aggregation [25], and not being cytotoxic to Vero and THP-1 cells [26].

Having the synergism of lupenone and caryophyllene oxide confirmed and taking into account that, to date, there are no reports on their combined trypanocidal activity, the 1 : 4 mixture was evaluated *in vivo* at two different doses, 20.8 mg·kg$^{-1}$·day$^{-1}$ and 41.6 mg·kg$^{-1}$·day$^{-1}$, against amastigotes, the parasite form present in the mammalian host and the one responsible for maintaining the infection. The first, most evident results observed were that mice treated with both doses of the 1 : 4 mixture of lupenone and caryophyllene oxide did not show the physical deterioration observed in mice

Table 1: *In vitro* trypanocidal activity ($IC_{50}$ values in $\mu$g/mL) of mixtures of lupenone and caryophyllene oxide.

| Sample | *Trypanosoma cruzi* Tulahuen strain | FIC |
|---|---|---|
| SYH | 74.5 $\mu$g/mL | — |
| FHex | 61.5 $\mu$g/mL | — |
| Lupenone + caryophyllene oxide 1 : 0 | 85.0 $\mu$g/mL | — |
| Lupenone + caryophyllene oxide 4 : 1 | >100 $\mu$g/mL | 5.62 |
| Lupenone + caryophyllene oxide 3 : 2 | 80.0 $\mu$g/mL | 3.59 |
| Lupenone + caryophyllene oxide 1 : 1 | 86.5 $\mu$g/mL | 3.88 |
| Lupenone + caryophyllene oxide **1 : 4** | **10.4** $\mu$g/mL | **0.46** |
| Lupenone + caryophyllene oxide 2 : 3 | 39.8 $\mu$g/mL | 1.79 |
| Lupenone + caryophyllene oxide 0 : 1 | 30.1 $\mu$g/mL | — |
| Anfotericina B | 0.7 $\mu$g/mL | — |

FIC: fractional inhibitory concentrations; SYH: leaf extract of *Serjania yucatanensis*; FHex: hexane fraction.

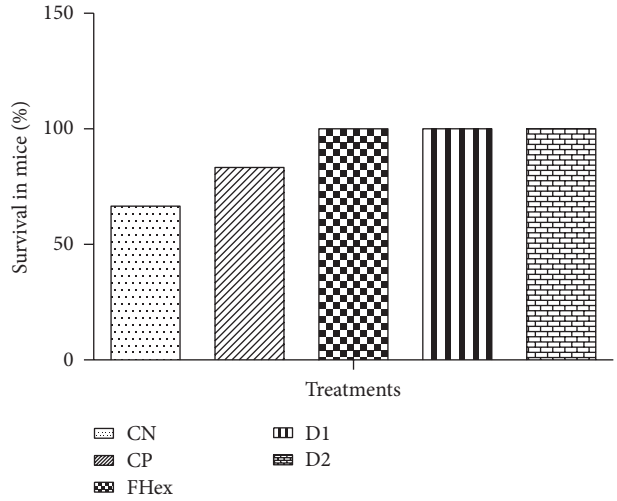


Figure 2: Survival rates of the five groups of *T. cruzi*-infected mice during the *in vivo* treatment: CN: negative control, infected animals treated with PBS; CP: positive control, infected animals treated with allopurinol (8.5 $\mu$g/g); FHex: hexane fraction, animals treated with the hexane fraction from the leaf crude extract of *S. yucatanensis* (41.6 mg·kg$^{-1}$·day$^{-1}$); D1: dose 1, infected mice treated with a 20.8 mg·kg$^{-1}$·day$^{-1}$ 1 : 4 mixture of lupenone and caryophyllene oxide; D2: dose 2, infected mice treated with a 41.6 mg·kg$^{-1}$·day$^{-1}$ 1 : 4 mixture of lupenone and caryophyllene oxide.

belonging to the negative control group (i.e., infected animals only treated with PBS; Figures 1(a)–1(d)), which presented the clinical signs commonly observed in BALB/c mice infected with *T. cruzi* [27], namely, adinamia and alopecia in neck and chest. Additionally, the group treated with both doses of the 1 : 4 mixture of lupenone and caryophyllene oxide showed a better survival rate (100% of survival after 60 days of infection, Figure 2) than that observed in the positive control (83% of survival, Figure 2), which proved to be only slightly higher than that of the negative control (66% of survival; Figure 2).

Finally, testing of the hexane fraction (41.6 mg·kg$^{-1}$·day$^{-1}$) and both doses of the 1 : 4 mixture of terpenoids for their capacity to reduce the number of amastigote nests in infected mice showed that both doses of the 1 : 4 mixture of lupenone and caryophyllene oxide reduced the presence of amastigote nests in cardiac tissue by more than 80% ($P < 0.05$) when compared to untreated mice (Figure 3). The 1 : 4 mixture of terpenoids also showed an important reduction in the number of amastigotes nests in skeletal muscle, with the lowest dose (20.8 mg·kg$^{-1}$·day$^{-1}$) showing a reduction of more than 98% ($P < 0.05$) when compared to the negative control (Figure 4). The activity of the 1 : 4 terpenoid mixture against amastigotes of *T. cruzi* is particularly important because most drugs presently used for the treatment of Chagas disease are effective mainly in the acute phase of the disease but not in the chronic phase (3–5).

It is interesting to point out that the hexane fraction, with *in vitro* activity against epimastigotes, showed no antitrypanosomal activity *in vivo* against amastigote of *T. cruzi* in both heart tissue and skeletal muscle. Additionally,

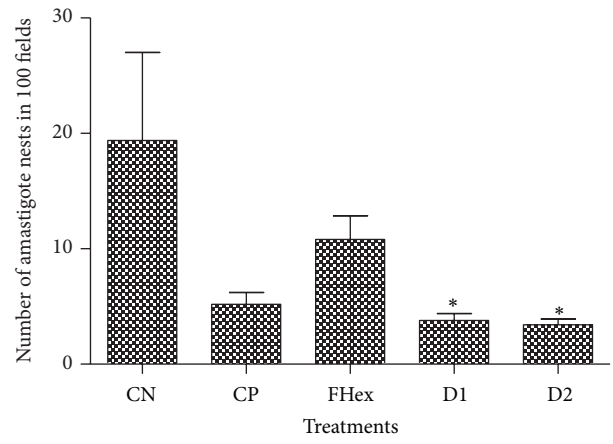


Figure 3: *In vivo* antitrypanosomal activity of the different treatments, determined by the number of amastigote nests observed in cardiac tissue from infected mice. CN: negative control, infected animals treated with PBS; CP: positive control, infected animals treated with allopurinol (8.5 $\mu$g/g); FHex: hexane fraction, animals treated with the hexane fraction from the leaf crude extract of *S. yucatanensis* (41.6 mg·kg$^{-1}$·day$^{-1}$); D1: dose 1, infected mice treated with a 20.8 mg·kg$^{-1}$·day$^{-1}$ 1 : 4 mixture of lupenone and caryophyllene oxide; D2: dose 2, infected mice treated with a 41.6 mg·kg$^{-1}$·day$^{-1}$ 1 : 4 mixture of lupenone and caryophyllene oxide. Statistical analysis was performed using one-way ANOVA and post hoc Tukey's test: $^{*}P < 0.05$ compared with negative control.

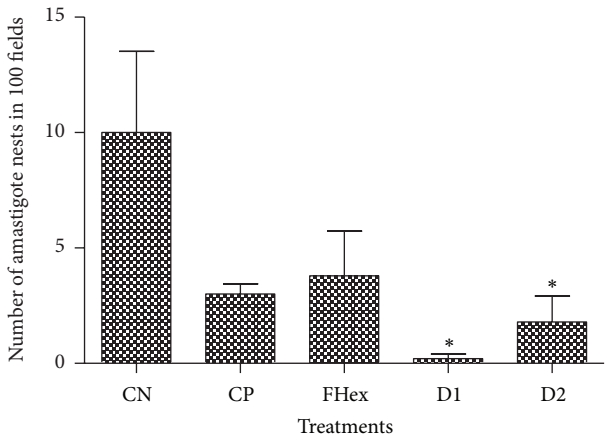


Figure 4: *In vivo* antitrypanosomal activity of the different treatments, determined by the number of amastigote nests observed in skeletal muscle of infected mice. CN: negative control, infected animals treated with PBS; CP: positive control, infected animals treated with allopurinol (8.5 $\mu$g/g); FHex: hexane fraction, animals treated with the hexane fraction from the leaf crude extract of *S. yucatanensis* (41.6 mg·kg$^{-1}$·day$^{-1}$); D1: dose 1, infected mice treated with a 20.8 mg·kg$^{-1}$·day$^{-1}$ 1 : 4 mixture of lupenone and caryophyllene oxide; D2: dose 2, infected mice treated with a 41.6 mg·kg$^{-1}$·day$^{-1}$ 1 : 4 mixture of lupenone and caryophyllene oxide. Statistical analysis was performed using one-way ANOVA and post hoc Tukey's test: $^{*}P < 0.05$ compared with negative control.

although mice treated with the reference drug allopurinol showed a decrease in the number of amastigote nests in cardiac tissue and skeletal muscle, the value was not significantly different to that observed in untreated mice (Figures 3 and 4); furthermore, the mortality and physical deterioration observed in the animals treated with allopurinol might be due to the reported toxicity of the reference drug when used in the chronic phase of the disease [4, 5].

To date there are few reports on the use of synergism as a combined therapy against *T. cruzi*; these include the synergistic effect observed between amiodarone and posaconazole [28] and between aspirin and nifurtimox; the latter reported to be a consequence of the capacity of aspirin to increase the antiparasitic activity of macrophages [29]. A synergistic effect between parthenolide, a terpenoid isolated from *Tanacetum vulgare*, and benznidazole has also been confirmed [30], and the combination of benznidazole and ketoconazole is reported to act synergistically to inhibit the parasite in the acute phase of Chagas disease in mice infected with CL and Y strains of *T. cruzi* [31]. Taking into account that, to date, there is no adequate treatment for Chagas disease and that the number of studies on the use of synergism as a combined treatment strategy against *T. cruzi* and in the chronic phase of the disease is still limited, the synergistic effect shown by the 1 : 4 mixture of lupenone and caryophyllene oxide against *T. cruzi in vivo* represents an important option for the future use of two commercially available natural products to treat this parasitosis.

## 4. Conclusions

This is the first report on the trypanocidal activity of a mixture of lupenone and caryophyllene oxide against *T. cruzi in vitro* and *in vivo*. Our results showed that the 1 : 4 mixture of lupenone and caryophyllene oxide is active in the chronic phase of the disease, reducing significantly the number of amastigote nests in both cardiac tissue and skeletal muscle. Future studies will include the evaluation of the trypanocidal activity of the terpenoid mixture at lower concentrations and its administration to infected mice for longer periods of time, together with a better understanding of its mode of action.

## Conflict of Interests

No conflict of interests exists in the results being presented in this paper.

## Acknowledgments

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*Research Article*

# Eupomatenoid-5 Isolated from Leaves of *Piper regnellii* Induces Apoptosis in *Leishmania amazonensis*

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*Leishmania* spp. are protozoa responsible for leishmaniasis, a neglected disease that kills up to 50,000 people every year. Current therapies mainly rely on antimonial drugs that are inadequate because of their poor efficacy and safety and increased drug resistance. An urgent need exists to find new and more affordable drugs. Our previous study demonstrated the antileishmanial activity of eupomatenoid-5, a neolignan obtained from leaves of *Piper regnellii* var. *pallescens*. The aim of the present study was to clarify the mode of action of eupomatenoid-5 against *L. amazonensis*. We used biochemical and morphological techniques and demonstrated that eupomatenoid-5 induced cell death in *L. amazonensis* promastigotes, sharing some phenotypic features observed in metazoan apoptosis, including increased reactive oxygen species production, hypopolarization of mitochondrial potential, phosphatidylserine exposure, decreased cell volume, and G0/G1 phase cell cycle arrest.

## 1. Introduction

Leishmaniasis remains a significant neglected tropical disease that puts 350 million people in 88 countries on four continents at risk for infection, with approximately 50,000 deaths per year [1]. Approximately 21 species have been found to cause three different clinical manifestations of leishmaniasis: cutaneous, in which the lesions are confined to the site of inoculation by the sandfly, and mucocutaneous, which affects mucosal tissues, and visceral leishmaniasis affecting the inner organs [2].

Current chemotherapy for leishmaniasis is still based on the use of pentavalent antimonials as first-line drugs and pentamidine, amphotericin B (free or liposomal forms), paromomycin, and miltefosine as second-line drugs. Although these drugs are usually effective, they have limitations, such as toxicity in the host and long-term treatment [3, 4]. These drawbacks reveal the urgent need to develop new therapeutic agents for the treatment of leishmaniasis.

Natural compounds, known to be valuable sources of new medicinal agents, have been exhaustively evaluated against *Leishmania* [5–7]. One example is eupomatenoid-5, a neolignan obtained from the leaves of *Piper regnellii* var. *pallescens,* representing a subclass with various biological activities, such as antifungal, antibacterial, insecticidal, and trypanocidal effects [8–12]. Our previous study reported the antileishmanial effect of eupomatenoid-5 [13]. Considering such antileishmanial activity, the aim of the present study was to better characterize the biochemical alterations induced by this compound against promastigote forms of *L. amazonensis* and elucidate the mechanism of action of eupomatenoid-5 involved in the cell death of this protozoan parasite.

## 2. Materials and Methods

*2.1. Chemicals.* Actinomycin D, antimycin A, bovine serum albumin, carbonyl cyanide m-chlorophenylhydrazone

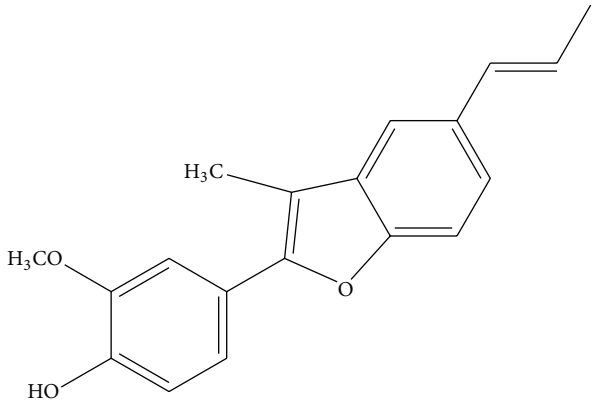


Figure 1: Structure of eupomatenoid-5 isolated from the leaves of *Piper regnellii* var. *pallescens*.

(CCCP), digitonin, dimethylsulfoxide (DMSO), rhodamine 123 (Rh123), 2′,7′-dichlorofluorescin diacetate ($H_2$DCFDA), and 5-5′-dithio*bis*(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Invitrogen (Grand Island, NY, USA). Annexin-V FITC, 3,8-phenanthridine diamine-5-(6-triphenylphosphonium hexyl)-5,6-dihydro-6-phenyl (MitoSOX) and propidium iodide (PI) were obtained from Invitrogen (Eugene, OR, USA). The protein assay kit was obtained from Bio-Rad (Hercules, CA, USA). All of the other reagents were of analytical grade.

*2.2. Isolation of Eupomatenoid-5 from Leaves of Piper regnellii Var. pallescens.* Eupomatenoid-5 (Figure 1) was isolated from the leaves of *P. regnellii*, which was collected in the Professor Irenice Silva Garden of Medicinal Plants on the campus of the State University of Maringa (UEM) in Parana, Brazil. A voucher specimen (no. HUM 8392) was deposited at the UEM Herbarium. Briefly, the dry plant material was extracted by exhaustive maceration at room temperature in the dark in an ethanol : water ratio of 90 : 10. Fractionation was performed from the ethyl acetate extract to obtain the hexane fraction, and a dihydrobenzofuran neolignan, eupomatenoid-5, was isolated from this fraction as described previously [9]. The compound was purified using absorption-chromatographic methods and identified by analyzing the ultraviolet, infrared, $^1$H nuclear magnetic resonance (NMR), $^{13}$C NMR, distortionless enhancement polarization transfer (DEPT), correlated spectroscopy (COSY), heteronuclear correlation (HETCOR), nuclear overhauser effect spectroscopy (NOESY), heteronuclear multiple bond correlation (HMBC), and gas chromatography/mass spectrometry (GC/MS) spectra. The data were compared with the literature [14].

Stock solutions of eupomatenoid-5 were prepared aseptically in DMSO and diluted in culture medium so that the DMSO concentration did not exceed 1% in the experiments. The concentrations of eupomatenoid-5 used in the assays were 30.0, 85.0, and 170.0 $\mu$M, representing the $IC_{50}$, $IC_{90}$, and twofold $IC_{90}$, respectively [13].

*2.3. Parasites.* *Leishmania amazonensis* promastigotes (MHOM/BR/Josefa) were maintained at 25°C in Warren's medium (brain-heart infusion plus hemin and folic acid; pH 7.2) supplemented with 10% heat-inactivated FBS.

*2.4. Determination of Mitochondrial Transmembrane Potential ($\Delta\Psi m$).* Promastigotes ($1 \times 10^7$ cells/mL) were treated or untreated with 30.0, 85.0, and 170.0 $\mu$M eupomatenoid-5 for 24 h at 37°C, harvested, and washed with phosphate-buffered saline (PBS). The parasites were incubated with 1 mL (5 mg/mL in ethanol) of Rh123, a fluorescent probe that accumulates in mitochondria, for 15 min, resuspended in 0.5 mL PBS, and incubated for an additional 30 min. The assay was conducted according to the manufacturer's instructions. The parasites were analyzed using a BD FACSCalibur flow cytometer and CellQuest Pro software (Becton Dickinson and Company, USA, 1997). A total of 10,000 events were acquired in the region that corresponded to the parasites. CCCP at 100 $\mu$M was used as a positive control [15].

*2.5. Measurement of Reactive Oxygen Species.* Promastigotes ($1 \times 10^7$ cells/mL) were treated or untreated with 30.0, 85.0, and 170.0 $\mu$M for 3 and 24 h, centrifuged, washed, and resuspended in PBS (pH 7.4). Afterward, these parasites were loaded with 10 $\mu$M of a permeant probe, $H_2$DCFDA, in the dark for 45 min [16]. Reactive oxygen species (ROS) were measured as an increase in fluorescence caused by the conversion of nonfluorescent dye to highly florescent 20,70-dichlorofluorescein, with an excitation wavelength of 488 nm and emission wavelength of 530 nm in a fluorescence microplate reader (Victor X3, PerkinElmer, Finland).

*2.6. Fluorimetric Detection of Mitochondrial-Derived $O_2^{\bullet-}$.* Promastigotes ($2 \times 10^7$ cells/mL) were loaded with 5 $\mu$M of a fluorescent $O_2^{\bullet-}$-sensitive, mitochondrial-targeted probe, MitoSOX, for 10 min at 25°C and then washed with Krebs-Henseleit (KH) buffer (pH 7.3) that contained 15 mM $NaHCO_3$, 5 mM KCl, 120 mM NaCl, 0.7 mM $Na_2HPO_4$, and 1.5 mM $NaH_2PO_4$ [17]. After the parasites were treated or untreated with 30.0, 85.0, and 170.0 $\mu$M eupomatenoid-5, the fluorescence intensity was detected after 1, 2, 3, and 4 h of treatment using a fluorescence microplate reader (Victor X3, PerkinElmer, Finland), with an excitation wavelength of 510 nm and emission wavelength of 580 nm. Oxidized MitoSOX (oxMitoSOX) becomes highly fluorescent upon binding to nucleic acids. Cells were exposed to 10 $\mu$M antimycin A, a stimulus known to induce mitochondrial $O_2^{\bullet-}$ production.

*2.7. Estimation of Decrease in Reduced Thiol Level.* Thiol levels were determined using DTNB. Promastigotes ($1 \times 10^7$ cells/mL) were treated or untreated with 30.0, 85.0, and 170.0 $\mu$M eupomatenoid-5 for 24, 48, and 72 h at 25°C. Afterward, the parasites were centrifuged, dissolved in 10 mM Tris-HCl buffer (pH 2.5), and sonicated. Acidic pH was used during sonication to prevent oxidation of the free thiol groups. Cellular debris was removed by centrifugation, and 100 $\mu$L of the supernatant and 100 $\mu$L of 500 mM phosphate

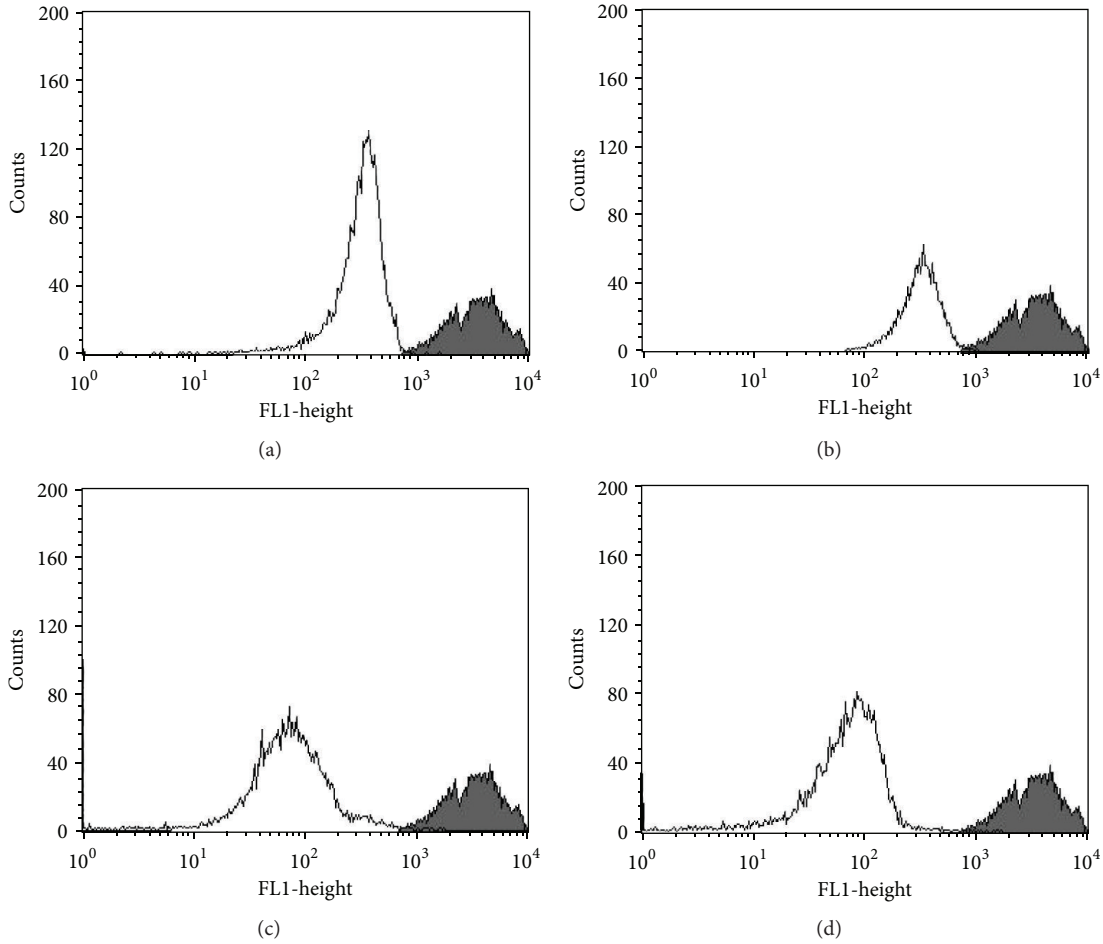


Figure 2: Mitochondrial membrane potential assay in promastigote forms of *L. amazonensis* treated with eupomatenoid-5 at (b) 30.0 $\mu$M, (c) 85.0 $\mu$M, and (d) 170.0 $\mu$M for 24 h and stained with Rh123, which accumulates in mitochondria. (a) Positive control (CCCP). The gray area corresponds to the control group (i.e., untreated parasites). Typical histograms of at least three independent experiments are shown.

buffer (pH 7.5) were taken in each microtiter well, followed by the addition of 20 $\mu$L of 1 mM DTNB to each well. Absorbance was measured at 412 nm [16].

*2.8. Phosphatidylserine Exposure.* Promastigotes ($1 \times 10^7$ cells/mL) were treated or untreated with 30.0, 85.0, and 170.0 $\mu$M eupomatenoid-5 for 24 h at 25°C. Afterward, the parasites were washed and resuspended in 100 $\mu$L of binding buffer (140 mM NaCl, 5 mM $CaCl_2$, and 10 mM HEPES-Na, pH 7.4), followed by the addition of 5 $\mu$L of a calcium-dependent phospholipid binding protein, annexin-V FITC, for 15 min at room temperature. Binding buffer (400 $\mu$L) and 50 $\mu$L PI were then added. Antimycin A (125.0 $\mu$M) was used as a positive control. Data acquisition and analysis were performed using a BD FACSCalibur flow cytometer equipped with CellQuest software. A total of 10,000 events were acquired in the region that was previously established as the one that corresponded to the parasites. Cells that were stained with annexin-V (PI-positive or -negative) were considered apoptotic, and cells that were only PI-positive were considered necrotic [18].

*2.9. Determination of Cell Volume of Parasites.* Promastigotes ($1 \times 10^7$ cells/mL) were treated or untreated with 30.0, 85.0, and 170.0 $\mu$M eupomatenoid-5 for 24 h at 25°C, harvested, and washed with PBS. Subsequently, the parasites were analyzed using a BD FACSCalibur flow cytometer and CellQuest Pro software. Histograms were generated, and FSC-H represented the cell volume. A total of 10,000 events were acquired in the region that corresponded to the parasites. Actinomycin D (20.0 mM) was used as a positive control [19].

*2.10. Determination of Cellular Membrane Integrity.* Promastigotes ($1 \times 10^7$ cells/mL) were treated or untreated with 30.0, 85.0, and 170.0 $\mu$M eupomatenoid-5 for 24 h at 32°C, harvested, and washed with PBS. The parasites were incubated with 50 mL of 2 mg/mL PI for 5 min according to the instructions provided by the manufacturer. Immediately thereafter, the parasites were analyzed using a BD FACSCalibur flow cytometer equipped with CellQuest software. A total of 10,000 events were acquired in the region that corresponded to the parasites. Digitonin (40.0 $\mu$M) was used as a positive control [19].

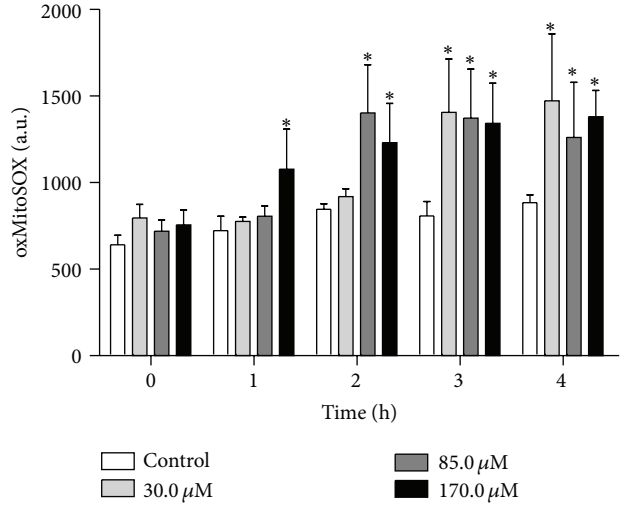


Figure 3: Mitochondrial $O_2^{\bullet-}$ production in promastigote forms of *L. amazonensis* treated with eupomatenoid-5 at 30.0, 85.0, and 170.0 $\mu$M for up to 4 h using the fluorescence probe MitoSOX. At the indicated times, parasites were used to fluorometrically measure oxidized MitoSOX (oxMitoSOX). The data are expressed as the mean fluorescence (in arbitrary units) ± SD of at least three independent experiments. $^*P \leq 0.05$, significant difference compared with the control group (i.e., untreated parasites).

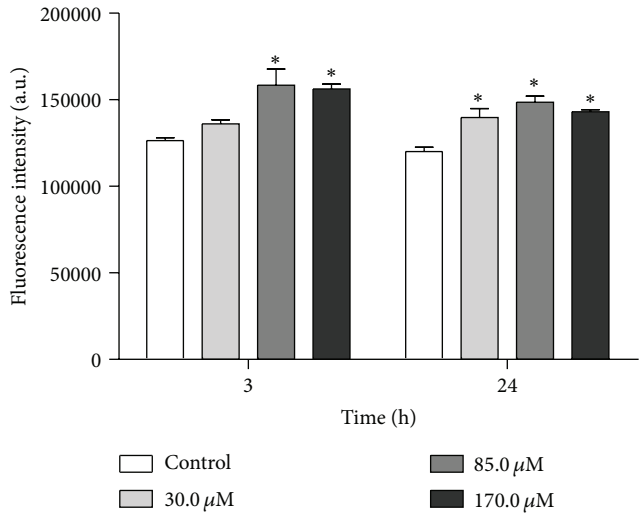


Figure 4: Total ROS production in promastigote forms of *L. amazonensis* treated with eupomatenoid-5 at 30.0, 85.0, and 170.0 $\mu$M for 3 and 24 h using the fluorescence probe $H_2$DCFDA. The data are expressed as the mean fluorescence (in arbitrary units) ± SD of at least three independent experiments. $^*P \leq 0.05$, significant difference compared with the control group (i.e., untreated parasites).

*2.11. Scanning Electron Microscopy.* Promastigotes ($1 \times 10^6$ cells/mL) were treated or untreated with 30.0 and 85.0 $\mu$M eupomatenoid-5 for 48 h at 25°C and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1–3 h. Subsequently, the parasites were adhered on poly-L-lysine-coated coverslips and dehydrated in increasing concentrations of ethanol. The samples were critical point dried in $CO_2$, coated with gold, and observed in a Shimadzu SS-550 (Japan) scanning electron microscope [20].

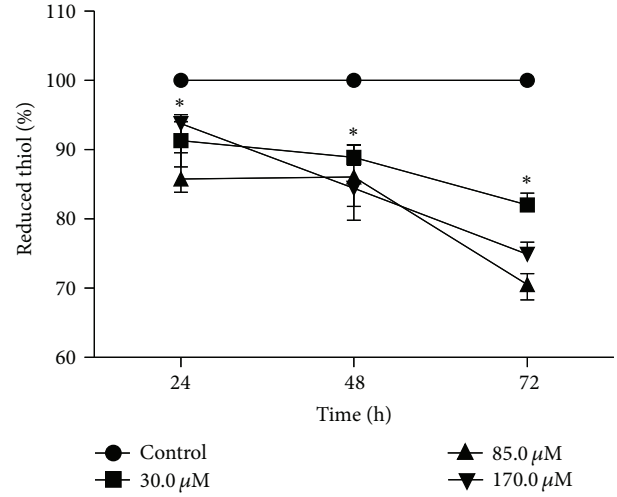


Figure 5: Thiol levels in promastigote forms of *L. amazonensis* treated with eupomatenoid-5 at 30.0, 85.0, and 170.0 $\mu$M for 24, 48, and 72 h using DTNB. The data are expressed as the mean ± SD of at least three independent experiments. $^*P \leq 0.05$, significant difference compared with the control group (i.e., untreated parasites).

*2.12. Cell Cycle.* Promastigotes ($1 \times 10^7$ cells/mL) were treated or untreated with 30.0, 85.0, and 170.0 $\mu$M eupomatenoid-5 for 24 h at 25°C. After incubation, the parasites were centrifuged and washed twice in PBS (pH 7.4). The resultant pellet was resuspended in 500 $\mu$L of a cold methanol/PBS (70% v/v) mixture and maintained at 4°C for 1 h. Afterward, the pellet was centrifuged, resuspended in PBS with 10 $\mu$g/mL PI and 20 $\mu$g/mL of DNase-free RNase (200 mg), and incubated for 45 min at 37°C. Data were acquired using a BD FACSCalibur flow cytometer and analyzed using CellQuest Pro software [21].

*2.13. Statistical Analysis.* The data shown in the graphs are expressed as the means ± standard deviation (SD) of the mean of at least three independent experiments. The data were analyzed using one- and two-way analysis of variance (ANOVA). Significant differences among means were identified using the Tukey *post hoc* test. Values of $P \leq 0.05$ were considered statistically significant. Statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, CA, USA, 2007).

## 3. Results

*3.1. Mitochondrial Membrane Potential.* Our previous study used transmission electron microscopy and found that eupomatenoid-5 caused damage and significant changes in promastigote mitochondria [13]. Based on this, we decided to evaluate the $\Delta\Psi$m in eupomatenoid-5-treated parasites using flow cytometry and Rh123, a fluorescent marker that indicates mitochondrial membrane potential. The histograms showed

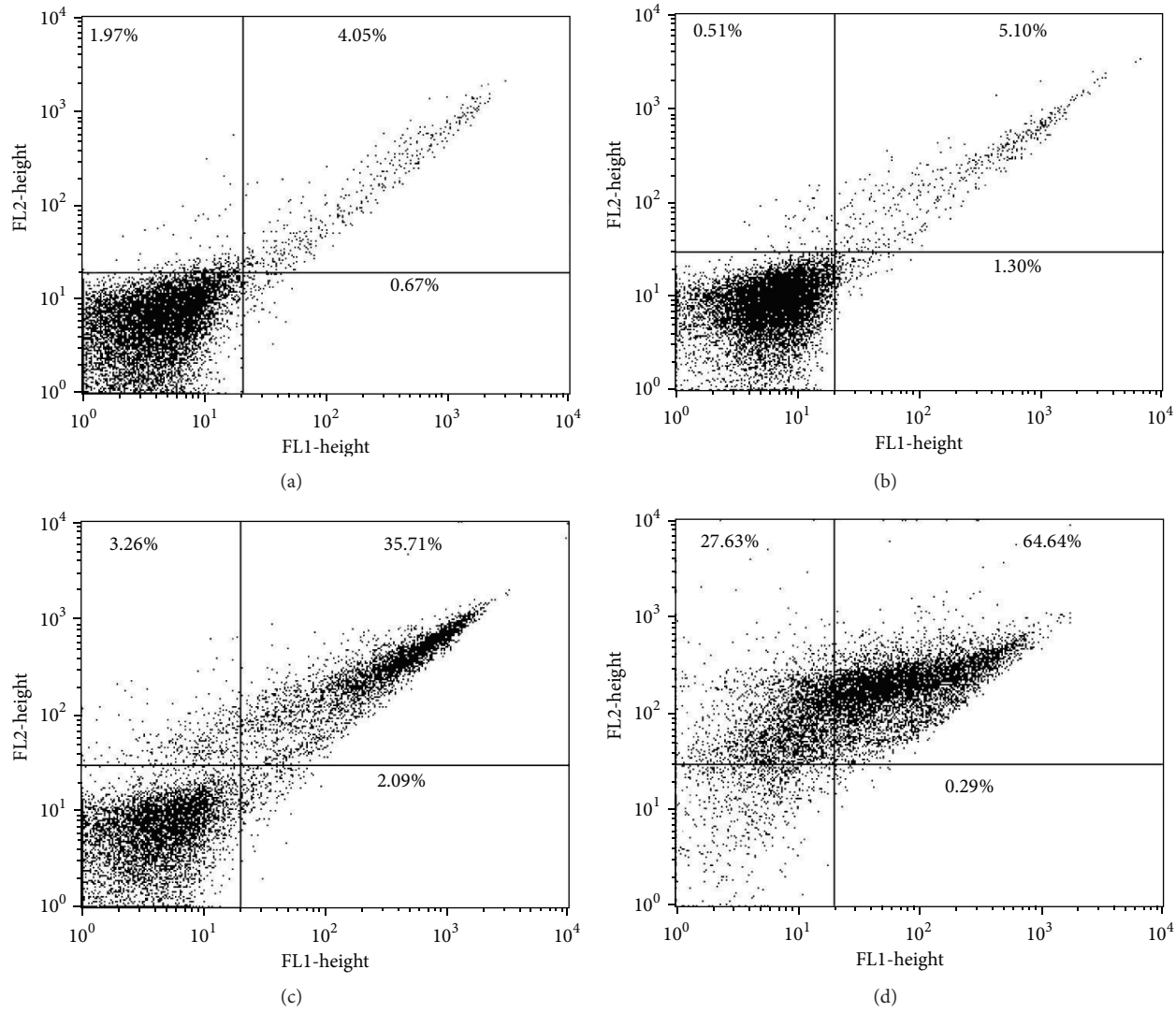


Figure 6: Phosphatidylserine exposure in promastigote forms of *L. amazonensis* treated with eupomatenoid-5 at (b) 30.0 $\mu$M, (c) 85.0 $\mu$M, and (d) 170.0 $\mu$M for 24 h using annexin-V FITC and PI. (a) corresponds to the control group (i.e., untreated parasites). The results are presented in a dot plot together with the negative control. Typical histograms of at least three independent experiments are shown.

that eupomatenoid-5 decreased total Rh123 fluorescence intensity at all of the concentrations tested compared with the control group, indicating mitochondrial depolarization (Figures 2(a)–2(c)). This loss of $\Delta\Psi$m was higher at the $IC_{90}$ (95.9%) than that at the $IC_{50}$ (76.8%), and at the twofold $IC_{90}$, the loss of $\Delta\Psi$m remained the same as the $IC_{90}$ (94.9%). The positive control, CCCP, induced a decrease of 62.5% in mitochondrial membrane potential.

*3.2. Mitochondrial-Derived $O_2^{\bullet-}$ Production.* Based on our $\Delta\Psi$m results, we evaluated $O_2^{\bullet-}$ production in eupomatenoid-5-treated parasites. Mitochondrial-derived $O_2^{\bullet-}$ production was evaluated using MitoSOX reagent, which measures the mitochondrial accumulation of superoxide based on its hydrophobic nature and positively charged triphenylphosphonium moiety [20]. Figure 3 shows that eupomatenoid-5 significantly increased the production of mitochondrial $O_2^{\bullet-}$ at most of the concentrations and times tested compared with the control group. After 1 and 2 h of treatment, eupomatenoid-5 increased $O_2^{\bullet-}$ production by approximately 40% only at higher concentrations. In contrast, after 3 and 4 h of treatment, eupomatenoid-5 increased $O_2^{\bullet-}$ production by more than 50% at all of the concentrations tested. The positive control, antimycin A, induced a two-fold increase in mitochondrial $O_2^{\bullet-}$ production.

*3.3. Reactive Oxygen Species Level.* Based on the MitoSOX data, we evaluated the effects of total ROS production in eupomatenoid-5-treated parasites using a fluorescent probe, $H_2$DCFDA. This probe primarily detects $H_2O_2$ and hydroxyl radicals and fluoresces after forming dichlorofluorescein [22]. Our results showed that eupomatenoid-5 increased total ROS production at all of the concentrations and times tested compared with the control group (Figure 4). However, a

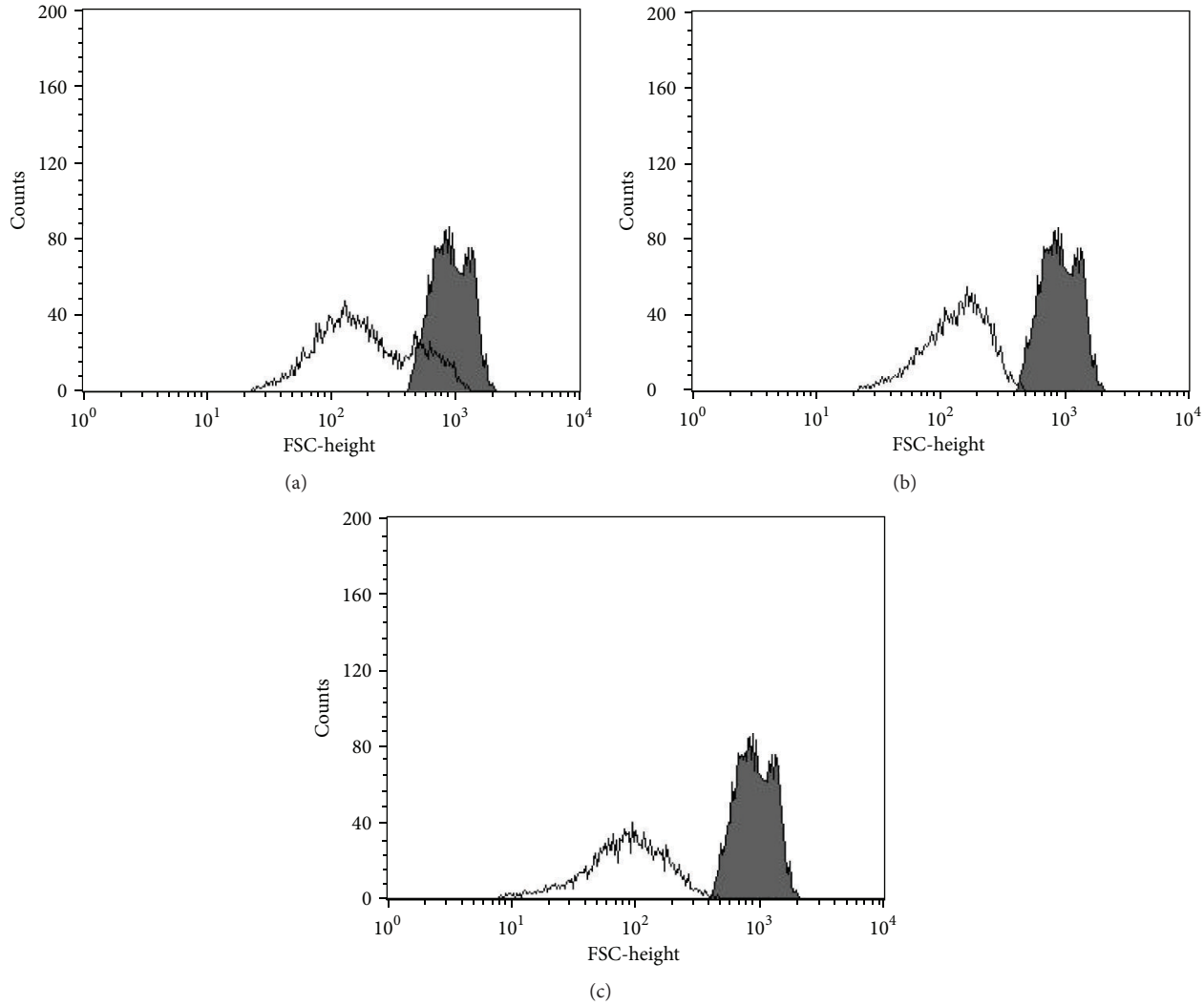


Figure 7: Cell volume in promastigote forms of *L. amazonensis* treated with eupomatenoid-5 at (a) 30.0 $\mu$M, (b) 85.0 $\mu$M, and (c) 170.0 $\mu$M. FSC-H was considered a function of cell size. The gray area corresponds to the control group (i.e., untreated parasites). Typical histograms of at least three independent experiments are shown.

significant increase in total ROS production of approximately 25% was observed after 3 h of treatment at 85.0 and 170.0 $\mu$M. After 24 h treatment, total ROS production increased by 16.3% even at the lower concentration.

*3.4. Reduced Thiol Levels.* Our data suggest that eupomatenoid-5 induces oxidative imbalance, attributable to enhanced ROS production. However, oxidative imbalance conditions depend on both increased oxidant species and decreased antioxidant effectiveness [23]. Therefore our next step was to assess the effect of eupomatenoid-5 on the level of reduced thiols, which might be decreased, for example, by a reduction in trypanothione reductase (TR) activity. Eupomatenoid-5 dose-dependently decreased total reduced thiol levels at all of the concentrations and times tested compared with the control group (Figure 5). This decrease might also be considered time-dependent, with reductions of thiol levels of approximately 10, 15, and 20% after 24, 48, and 72 h treatment, respectively.

*3.5. Phosphatidylserine Exposure.* To determine whether the mechanism of cell death triggered by eupomatenoid-5 involves apoptosis, we evaluated the externalization of phosphatidylserine, an apoptotic marker that is present in the outer leaflet of plasmalemma [24] in promastigotes treated with eupomatenoid-5 for 24 h and double stained with FITC-conjugated annexin-V and PI. As shown in Figure 6, eupomatenoid-5 increased annexin-V fluorescence intensity more than 30% at higher concentrations (85.0 and 170.0 $\mu$M) compared with the control group, indicating phosphatidylserine exposure (Figures 6(c) and 6(d)).

*3.6. Cell Volume.* In addition to biochemical alterations, apoptosis also induces morphological alterations. Based on this, we evaluated cell shrinkage, a hallmark of apoptotic

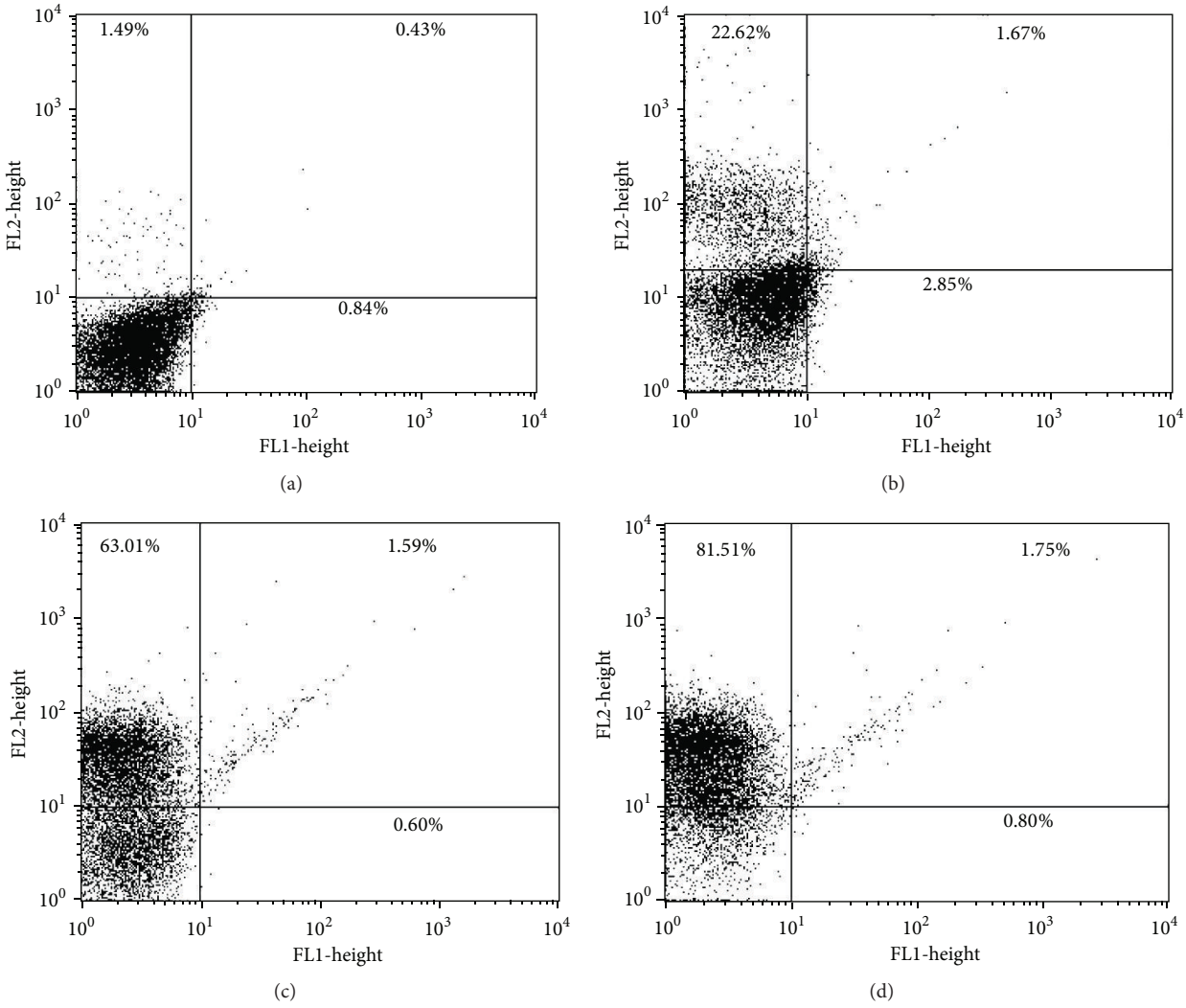


Figure 8: Cell membrane integrity assay in promastigote forms of *L. amazonensis* treated with eupomatenoid-5 at (b) 30.0 $\mu$M, (c) 85.0 $\mu$M, and (d) 170.0 $\mu$M for 24 h and stained with PI. The numbers show the percentage of PI-positive parasites in (a) and (b). (a) corresponds to the control group (i.e., untreated parasites). Typical histograms of at least three independent experiments are shown.

death, in eupomatenoid-5-treated parasites [25]. As shown in Figure 7, a dose-dependent decrease in cell volume (30.4, 63.1, and 84.3%, resp.) was observed at all of the concentrations tested compared with the control group.

*3.7. Cell Membrane Integrity.* To determine whether the mechanism of cell death triggered by eupomatenoid-5 also involves the necrotic death pathway, we evaluated plasma membrane integrity in eupomatenoid-5-treated promastigotes stained with PI, which diffuse across permeable membranes and bind to nucleic acids. As shown in Figure 8, eupomatenoid-5 at 30.0, 85.0, and 170.0 $\mu$M dose-dependently increased PI-stained parasites from 1.9% (untreated promastigotes; Figure 8(a), upper quadrants) to 24.3%, 64.6%, and 86.3%, respectively (Figures 8(b)–8(d), upper quadrants), indicating permeabilization of the plasma membrane. The positive control, digitonin, showed a 47.7% increase in the gated percentage of PI-stained cells.

*3.8. Scanning Electron Microscopy.* To confirm that eupomatenoid-5 induced morphological alterations in promastigotes, we further evaluated morphological alterations using scanning electron microscopy. Photomicrographs revealed that untreated protozoa had typical characteristics, with an elongated shape and terminal flagellum. In contrast, eupomatenoid-5 dose-dependently altered the size and shape of the treated parasites, including a reduction and rounding of the cellular body (Figure 9).

*3.9. Cell Cycle.* To evaluate the ratio of pseudohypodiploid cells, flow cytometry after cell permeabilization and PI labeling were used. In a given cell, the amount of bound dye correlates with the DNA content, and thus DNA fragmentation

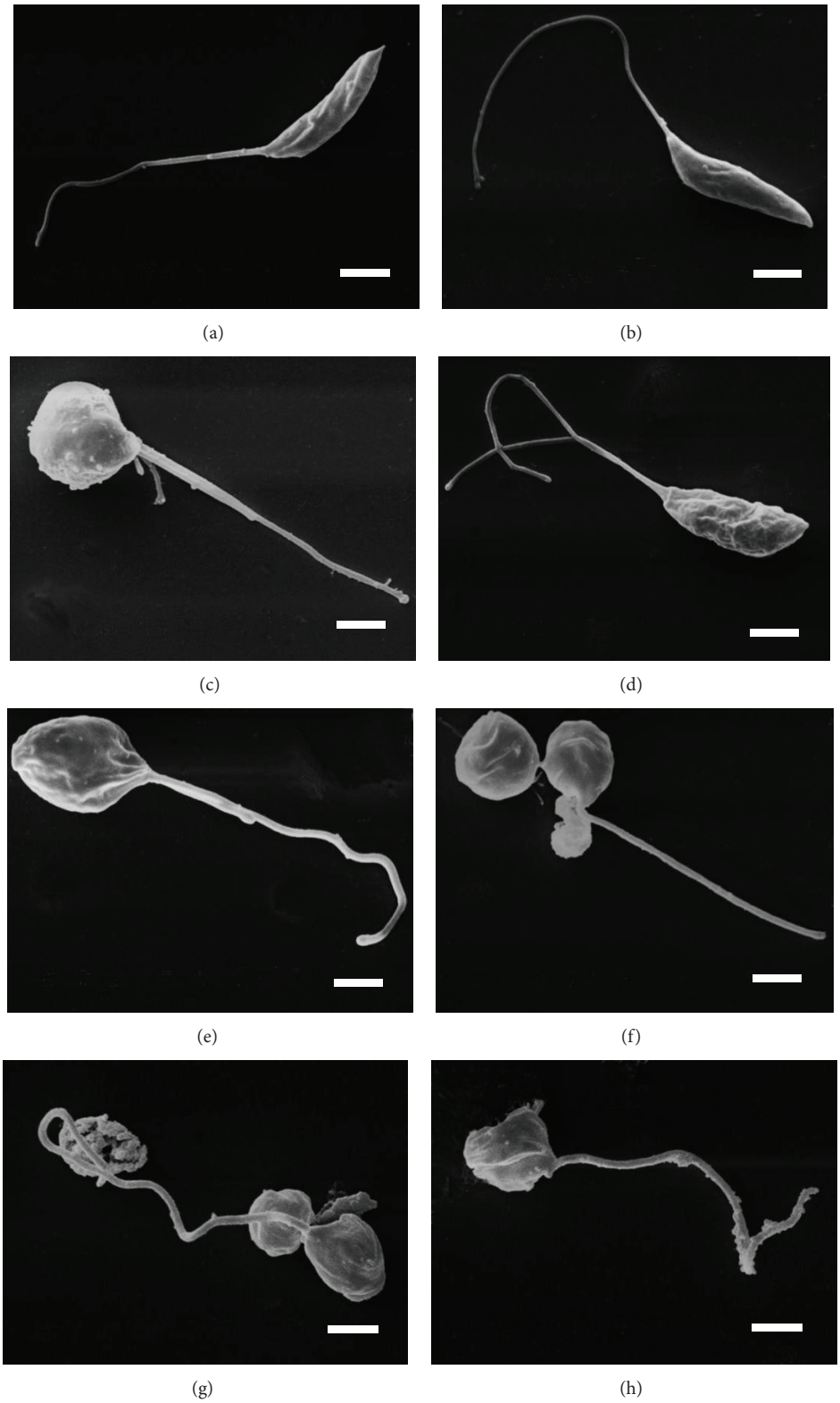


Figure 9: Scanning electron microscopy images of promastigote forms of *L. amazonensis* incubated in the ((a) and (b)) absence or presence of eupomatenoid-5 at ((c)–(e)) 30.0 $\mu$M and ((f)–(h)) 85.0 $\mu$M for 48 h. Scale bar = 2 $\mu$m.

Table 1: Effect of eupomatenoid-5 on the cell cycle of *L. amazonensis* promastigotes ($1 \times 10^7$ cells/mL) that were treated with 30.0, 85.0, and 170.0 $\mu$M eupomatenoid-5 for 24 h and processed for cell cycle analysis as described in Section 2. The data are expressed as percentages.

| Group | Sub-G0/G1 (M1) | G0/G1 (M2) | S and G2/M (M3) |
|---|---|---|---|
| Control | 4.16 | 57.31 | 37.16 |
| 30.0 $\mu$M | 16.28 | 55.29 | 21.20 |
| 85.0 $\mu$M | 28.11* | 47.03 | 16.96 |
| 170.0 $\mu$M | 28.41* | 45.44 | 15.84 |

in apoptotic cells is reflected by fluorescence intensity that is lower than that of G0/G1 cells (i.e., a sub-G0/G1 peak) [26]. The incubation of promastigotes with eupomatenoid-5 for 3 and 24 h resulted in a 16% increase in the proportion of cells in the sub-G0/G1 phase at the lower concentration (30.0 $\mu$M) and a 28% increase at the higher concentrations (85.0 and 170.0 $\mu$M) compared with the control group (Table 1). In contrast, an increase in the number of cells in the sub-G0/G1 phase led to a decrease in the number of cells in the G2/M phase compared with untreated cells.

## 4. Discussion

Despite recent advances, the treatment of leishmaniasis continues to be unsatisfactory. Pentavalent antimonials remain the first-line treatment for this infection in most endemic areas, despite their limitations, such as high toxicity and increased drug resistance [27, 28]. Thus, an urgent need exists to develop new drugs and therapeutic strategies. We have been extensively exploring plant resources to find effective antileishmanial agents. Our previous studies indicated that eupomatenoid-5 contained in the crude extracts and chloroform fractions of *P. regnellii* leaves are responsible for the antileishmanial activity of this plant [13]. Furthermore, eupomatenoid-5 showed more selective against protozoan than macrophage cells [13]. Additionally, transmission electron microscopy revealed many ultrastructural alterations, especially in the mitochondria of treated parasites, indicating damage and significant changes in this organelle [13]. The present study sought to further elucidate the mechanism of action of eupomatenoid-5 in the cell death of this protozoan.

We initially focused on investigating mitochondrial alterations and their consequences, especially with regard to parasite death. We found that parasites treated with eupomatenoid-5 exhibited a decrease in $\Delta\Psi$m and increase in mitochondrial ROS production. *Leishmania* is known to have a single mitochondrion, and so the maintenance of mitochondrial transmembrane potential is essential for parasite survival [29]. In fact, a number of studies have been published describing compounds that target at Trypanosomatids mitochondria [16, 30]. Eupomatenoid-5 also induced a time-dependent decrease in reduced thiol levels of treated parasites. The trypanothione system is unique in trypanosomatid parasites and plays an important role in the homeostasis of parasite redox metabolism [31, 32]. In this system, trypanothione is reduced to a dithiol T(SH)2 by trypanothione/trypanothione reductase (TR). The inhibition of TR decreases total reduced thiol [16]. Our data suggest that eupomatenoid-5 induces oxidative imbalance in promastigote forms through two pathways, increasing ROS production by disrupted mitochondria and decreasing hydroperoxide detoxification by reducing TR activity.

Additionally, eupomatenoid-5 induced the externalization of phosphatidylserine and a reduction of parasite volume, indicated by flow cytometry and scanning electron microscopy. Altogether, our data showed that eupomatenoid-5-treated parasites exhibited apoptotic-like events. An increase in the generation of ROS in the cytosol, an established event in most apoptotic cells, might direct the cell toward this death pathway [33]. Interestingly, programmed cell death in protists appears to share some morphological features with apoptosis in multicellular organisms, including cell shrinkage, the loss of mitochondrial membrane potential, and the externalization of phosphatidylserine [34, 35]. Following phosphatidylserine flip, apoptotic cells lose their plasma membrane integrity. This signal was also induced by eupomatenoid-5 and revealed by the addition of PI, a cellimpermeable nuclear dye [36]. Interestingly, similar results were found in parasitic forms of *Trypanosoma cruzi* after treatment with eupomatenoid-5 [19].

Finally, we demonstrated that eupomatenoid-5 induced G0/G1 phase cell cycle arrest using flow cytometry and PI labeling, in which the amount of bound dye correlated with the DNA content, and thus DNA fragmentation in apoptotic cells is translated into fluorescence intensity that was lower than that of G0/G1 cells (i.e., a sub-G0/G1 peak) [26, 37].

## 5. Conclusion

In parasites, apoptosis appears to be the predominant form of cell death [38], which has been observed in kinetoplastids in response to chemotherapeutic agents, such as amphotericin B and plant extracts, such as Aloe Vera leaf exudates [39, 40]. We used biochemical and morphological techniques and demonstrated that eupomatenoid-5 induced cell death, sharing some phenotypic features observed in metazoan apoptosis, including increased ROS, hypopolarization of mitochondrial potential, phosphatidylserine exposure, a reduction of cell volume, and G0/G1 phase cell cycle arrest. The study of the major pathways involved in *Leishmania* apoptosis-like death will provide insights into the future design of newer chemotherapeutic strategies.

## Conflict of Interests

The authors have no conflict of interests.

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*Research Article*

# *Eugenia uniflora* L. Essential Oil as a Potential Anti-*Leishmania* Agent: Effects on *Leishmania amazonensis* and Possible Mechanisms of Action

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*Eugenia uniflora* L. is a member of the Myrtaceae family and is commonly known as Brazilian cherry tree. In this study, we evaluated the chemical composition of *Eugenia uniflora* L. essential oil (EuEO) by using gas chromatography-mass spectrometry (GC-MS) and assessed its anti-*Leishmania* activity. We also explored the potential mechanisms of action and cytotoxicity of EuEO. Thirty-two compounds were identified, which constituted 92.65% of the total oil composition. The most abundant components were sesquiterpenes (91.92%), with curzerene (47.3%), $\gamma$-elemene (14.25%), and *trans*-$\beta$-elemenone (10.4%) being the major constituents. The bioactivity shown by EuEO against promastigotes ($IC_{50}$, 3.04 $\mu g \cdot mL^{-1}$) and amastigotes ($IC_{50}$, 1.92 $\mu g \cdot mL^{-1}$) suggested significant anti-*Leishmania* activity. In the cytotoxicity determination, EuEO was 20 times more toxic to amastigotes than to macrophages. Hemolytic activity was 63.22% at the highest concentration tested (400 $\mu g \cdot mL^{-1}$); however, there appeared to be no toxicity at 50 $\mu g \cdot mL^{-1}$. While the data show that EuEO activity is not mediated by nitric oxide production, they do suggest that macrophage activation may be involved in EuEO anti-*Leishmania* activity, as evidenced by increases in both the phagocytic capacity and the lysosomal activity. More studies are needed to determine *in vivo* activity as well as additional mechanisms of the anti-*Leishmania* activity.

## 1. Introduction

Protozoan parasites of the genus *Leishmania* are responsible for a spectrum of diseases collectively known as leishmaniasis, which affects the skin, mucous membranes, and internal organs. Over 12 million cases of leishmaniasis have been reported worldwide, with 1 to 2 million new cases being reported annually [1, 2]. Increase in the incidence of leishmaniasis is associated with urban development, deforestation, environmental changes, and increased migration to areas where the disease is endemic [3]. Despite its epidemiological importance, treatment is still performed with chemotherapeutic drugs that are delivered parenterally, require medical supervision, and have many side effects [4, 5].

The need to identify new anti-*Leishmania* compounds that are more effective and less toxic than conventional drugs has motivated research of substances derived from plant species. In this context, essential oils containing a group of secondary metabolites consisting mainly of monoterpenes, sesquiterpenes, and phenylpropanoids have demonstrated proven anti-*Leishmania* activity *in vivo* and *in vitro* on promastigote and/or amastigote forms of *Leishmania*. Included

among these are *Croton cajucara* [6], *Ocimum gratissimum* [7], *Copaifera cearensis* [8], *Chenopodium ambrosioides* [9], *Cymbopogon citratus* [10, 11], and *Lippia sidoides* [12]. These studies have shown that essential oils can be a promising source of new drugs with anti-*Leishmania* activity.

*Eugenia uniflora* L., commonly known as "pitangueira" or Brazilian cherry tree, is a species belonging to the family Myrtaceae, which is native to South America and common in regions with tropical and subtropical climate [13]. In Brazil, it is used in the treatment of digestive disorders [14] and is thought to have anti-inflammatory and antirheumatic activities [15]. It is an aromatic species and its essential oil has pharmacological properties that are well characterized in the literature as antioxidant and antimicrobial [16]. *E. uniflora* has known antihypertensive [17], antitumor [18], and antinociceptive properties [19], and it shows good performance against microorganisms, demonstrating antiviral, antifungal [20], anti-*Trichomonas gallinae* [21], and anti-*Trypanosoma cruzi* properties [22].

Considering the potential pharmacological benefits of *E. uniflora* and the increasing interest in the discovery of essential oils with anti-*Leishmania* activity, the aim of this study was to investigate the anti-*Leishmania* activity of leaf essential oil from this species, the chemical composition of the oil, its cytotoxicity, and possible mechanisms of action.

## 2. Materials and Methods

*2.1. Chemicals.* Dimethyl sulfoxide (DMSO: 99%), anhydrous sodium sulfate, glacial acetic acid, ethanol, formaldehyde, sodium chloride, calcium acetate, zymosan, and neutral red were purchased from Merck Chemical Company (Germany). The *n*-alkane ($C_8$–$C_{20}$) homologous series, Schneider's medium, RPMI 1640 medium, fetal bovine serum (FBS), MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide), Griess reagent (1% sulfanilamide in $H_3PO_4$ 10% (v/v) in Milli-Q water), and the antibiotics penicillin and streptomycin were purchased from Sigma Chemical (St. Louis, MO, USA). The antibiotic amphotericin B (90%) was purchased from Cristália (São Paulo, SP, Brazil).

*2.2. Plant Material.* *E. uniflora* leaves were collected (January 2010) from a mature tree in the flowering stage in São Luís ($2^\circ 30' 45.3'' S$ and $44^\circ 18' 1.1'' W$) in the state of Maranhão in northeast Brazil. The samples were collected from a cultivated plant and its fruits have red color. A voucher specimen (no. 0998/SLS017213) was deposited at the Herbarium "Ático Seabra" of the Federal University of Maranhão, and plant identification was confirmed by botanists of the Herbarium "Murça Pires" from Museu Paraense Emílio Goeldi, Belém, PA, Brazil.

*2.3. Extraction of the Essential Oil.* The plant material was air-dried for 7 days, cut into small pieces, and subjected to hydrodistillation using a Clevenger-type apparatus (300 g, 3 h) to obtain a sesquiterpene-rich essential oil. Once collected, the essential oil was dried over anhydrous sodium sulfate, filtered, and weighed, and then the oil yield was calculated in terms of % (w/w). Its percentage content was estimated based on the plant dry weight by calculating the water content by using a moisture analyzer prior to distillation. EuEO was then stored in a dark flask and refrigerated (at +5°C) until use.

*2.4. Gas Chromatography-Mass Spectrometry Analysis of the Essential Oil.* EuEO was analyzed using a THERMO DSQ II GC-MS instrument (Thermo Fisher Scientific, Austin, TX, USA), under the following conditions: DB-5 ms (30 m × 0.25 mm i.d.; 0.25 $\mu$m film thickness) fused silica capillary column, with the following temperature program: 60°C, subsequently increased by 3°C/min up to 240°C; injector temperature: 250°C; carrier gas: helium (high purity), adjusted to a linear velocity of 32 cm/s (measured at 100°C). The injection type was splitless. The oil sample was diluted 1 : 100 in hexane solution, and the volume injected was 0.1 $\mu$L. The split flow was adjusted to yield a 2 : 1000 ratio, and the septum sweep was constant at 10 mL/min. All mass spectra were acquired in electron impact (EI) mode with an ionization voltage of 70 eV over a mass scan range of 35–450 amu. The temperature of the ion source and connection parts was 200°C.

*2.5. Identification and Quantification of Constituents.* The quantitative data regarding the volatile constituents were obtained by peak-area normalization using a FOCUS GC/FID operated under conditions similar to those used in the GC-MS assay, except for the carrier gas, which was nitrogen. The retention index was calculated for all the volatile constituents by using an *n*-alkane ($C_8$–$C_{20}$) homologous series. When possible, individual components were identified by coinjection with authentic standards. Otherwise, the peak assignment was performed by comparison of both mass spectrum and GC retention data by using authentic compounds previously analyzed and stored in our private library, as well as with the aid of commercial libraries containing retention indices and mass spectra of volatile compounds commonly found in essential oils [23, 24]. Percentage (relative) of the identified compounds was computed from the GC peak area.

*2.6. Parasites and Mice.* *Leishmania* (*Leishmania*) *amazonensis* (IFLA/BR/67/PH8) was used for the determination of the anti-*Leishmania* activity. Parasites were grown in supplemented Schneider's medium (10% heat-inactivated fetal bovine serum (FBS), 100 U·mL$^{-1}$ penicillin, and 100 $\mu$g/mL streptomycin at 26°C). Murine macrophages were collected from the peritoneal cavities of male and female BALB/c mice (4-5 weeks old) from Medicinal Plants Research Center (NPPM/CCS/UFPI), located at Teresina, PI, Brazil. The macrophages were maintained at a controlled temperature (24 ± 1°C) and light conditions (12 h light/dark cycle). All protocols were approved by the Animal Research Ethics Committee (CEEAPI no. 001/2012).

*2.7. Anti-Leishmania Activity Assay.* Promastigotes in the logarithmic growth phase were seeded in 96-well cell culture plates at $1 \times 10^6$ *Leishmania* per well. Then, essential oil was added to the wells in serial dilutions of 400, 200, 100, 50, 25, 12.5, 6.25, and 3.12 $\mu$g·mL$^{-1}$. The plate was kept at 26°C in a biological oxygen demand (BOD) incubator, and

*Leishmania* was observed and counted by using a Neubauer hemocytometer after 24, 48, and 72 h to monitor growth and viability [25]. Assays were performed in triplicate and were repeated 3 times on different days.

*2.8. Cytotoxicity Determination.* Cytotoxicity of EuEO was assessed using the MTT test. In a 96-well plate, 100 $\mu$L of supplemented RPMI 1640 medium and about $1 \times 10^5$ macrophages were added per well. They were then incubated at 37°C in 5% of $CO_2$ for 2 h to allow cell adhesion. After this time, 2 washes with supplemented RPMI 1640 medium were performed to remove cells that did not adhere. Subsequently, EuEO was added, in triplicate, after being previously diluted in supplemented RPMI 1640 medium to a final volume of 100 $\mu$L for each well at the tested concentrations (100, 50, 25, 12.5, 6.25, and 3.12 $\mu g \cdot mL^{-1}$). Cells were then incubated for 48 h. At the end of the incubation, 10 $\mu$L of MTT diluted in PBS was added at a final concentration of 5 $mg \cdot mL^{-1}$ (10% of volume, i.e., 10 $\mu$L for each 100 $\mu$L well) and was incubated for an additional 4 h at 37°C in 5% $CO_2$. The supernatant was then discarded, and 100 $\mu$L of DMSO was added to all wells. The plate was then stirred for about 30 min at room temperature to complete formazan dissolution. Finally, spectrophotometric reading was conducted at 550 nm in an ELISA plate reader [26].

*2.9. Hemolytic Activity.* The hemolytic activity was investigated by incubating 20 $\mu$L of serially diluted essential oil in phosphate-buffered saline (PBS; 400, 200, 100, 50, 25, 12.5, 6.25, and 3.12 $\mu g \cdot mL^{-1}$) with 80 $\mu$L of a suspension of 5% red blood cells (human $O^+$) for 1 h at 37°C in assay tubes. The reaction was slowed by adding 200 $\mu$L of PBS, and then the suspension was centrifuged at 1000 g for 10 min. Cell lysis was then measured spectrophotometrically (540 nm). The absence of hemolysis (blank control) or total hemolysis (positive control) was determined by replacing the essential oil solution with an equal volume of PBS or Milli-Q sterile water, respectively. The results were determined by the percentage of hemolysis compared to the positive control (100% hemolysis), and the experiments were performed in triplicate [27].

*2.10. Treatment of Infected Macrophages.* Macrophages were collected in 24-well culture plates at a concentration of $2 \times 10^5$ cells/500 $\mu$L in RPMI 1640, containing sterile 13 mm round coverslips. They were then incubated at 37°C in 5% of $CO_2$ for 2 h to allow cell adhesion. After this time, the medium was replaced with 500 $\mu$L of supplemented RPMI 1640 and incubated for 4 h. The medium was then aspirated, and a new medium containing promastigotes (in the stationary phase) at a ratio of 10 promastigotes to 1 macrophage was added to each well. After 4 h of incubation in 5% $CO_2$ at 37°C, the medium was aspirated to remove free promastigotes, and the test samples were added at nontoxic concentrations to the macrophages (3.12, 1.56, and 0.78 $\mu g \cdot mL^{-1}$). This preparation was then incubated for 48 h, after which the coverslips were removed, fixed in methanol, and stained with Giemsa. For each coverslip, 100 cells were evaluated and both the number of infected macrophages and the amount of parasites per macrophage were counted [26].

*2.11. Lysosomal Activity.* Measurement of the lysosomal activity was carried out according to the method of Grando et al. [28]. Peritoneal macrophages were plated and incubated with EuEO in serial dilutions of 100, 50, 25, 12.5, 6.25, and 3.12 $\mu g \cdot mL^{-1}$. After 4 h of incubation at 37°C in 5% $CO_2$, 10 $\mu$L of neutral red solution was added and incubated for 30 min. Once this time had elapsed, the supernatant was discarded, the wells were washed with 0.9% saline at 37°C, and 100 mL of extraction solution was added (glacial acetic acid 1% v/v and ethanol 50% v/v dissolved in bidistilled water) to solubilize the neutral red inside the lysosomal secretion vesicles. After 30 min on a Kline shaker (model AK 0506), the plate was read at 550 nm by using an ELISA plate reader.

*2.12. Phagocytosis Test.* Peritoneal macrophages were plated and incubated with EuEO in serial dilutions of 100, 50, 25, 12.5, 6.25, and 3.12 $\mu g \cdot mL^{-1}$. After 48 h of incubation at 37°C in 5% $CO_2$, 10 $\mu$L of zymosan color solution was added and incubated for 30 min at 37°C. After this, 100 mL of Baker's fixative (formaldehyde 4% v/v, sodium chloride 2% w/v, and calcium acetate 1% w/v in distilled water) was added to stop the phagocytosis process. Thirty minutes later, the plate was washed with 0.9% saline in order to remove zymosan that was not phagocytosed by macrophages. The supernatant was removed and added to 100 mL of extraction solution. After solubilization in a Kline shaker, the absorbances were measured at 550 nm by using an ELISA plate reader [28].

*2.13. Nitric Oxide (NO) Production.* In 96-well plates, $2 \times 10^5$ macrophages were added per well and incubated at 37°C in 5% $CO_2$ for 4 h to allow cell adhesion. A new medium containing promastigotes (in the stationary phase) at a ratio of 10 promastigotes per macrophage was added to half of the wells. The essential oil was added after being previously diluted in a culture medium containing different concentrations (100, 50, 25, 12.5, 6.25, and 3.12 $\mu g \cdot mL^{-1}$) in the absence or presence of *L. amazonensis*, and was then incubated again at 37°C in 5% $CO_2$ for 24 h. After this period, the cell culture supernatant was collected and transferred to another plate for measurement of nitrite. A standard curve was prepared with 150 $\mu$M sodium nitrite in Milli-Q water at varying concentrations of 1, 5, 10, 25, 50, 75, 100, and 150 $\mu$M diluted in a culture medium. At the time of dosing, 100 $\mu$L of either the samples or the solutions prepared for obtaining the standard curve was mixed with an equal volume of Griess reagent. The analysis was performed using an ELISA plate reader at 550 nm [28].

*2.14. Statistical Analysis.* All assays were performed in triplicate and in 3 independent experiments. The half-maximal inhibitory concentration ($IC_{50}$) values and 50% cytotoxicity concentration ($CC_{50}$) values, with 95% confidence intervals, were calculated using a probit regression model and Student's *t*-test. Analysis of variance (ANOVA) followed by a Bonferroni test were performed, taking a $P$ value of $<0.05$ as the minimum level required for statistical significance.

## 3. Results

*3.1. Analysis of the Essential Oil.* The yield of EuEO was 0.3%. Thirty-two components were identified in this oil by GC-MS, constituting 92.65% of the total mixture. EuEO was shown to be rich in oxygenated sesquiterpenes (62.55%) and sesquiterpene hydrocarbons (29.37%). Curzerene was the major constituent (47.3%), followed by $\gamma$-elemene (14.25%) and *trans*-$\beta$-elemenone (10.4%). The chemical constituents are listed in Table 1.

*3.2. Anti-Leishmania Activity Assay.* The inhibitory profile of EuEO on *L. amazonensis* promastigotes showed a significant concentration-dependent decrease ($P < 0.05$) in parasite viability, with 100% inhibition of promastigote growth at concentrations of 400, 200, and 100 $\mu g \cdot mL^{-1}$ (Figure 1). The $IC_{50}$ was 1.75 $\mu g \cdot mL^{-1}$ at 72 h of exposure (Table 2). In cultures treated with EuEO, we used an optical microscope to observe morphological changes in the promastigotes, such as cells with rounded or completely spherical shapes, as well as cellular debris, in contrast to the spindle forms present in the control (data not shown). Amphotericin B was used as a positive control and was tested at a concentration of 2 $\mu g \cdot mL^{-1}$. The highest inhibitory effect of amphotericin B was observed after 24 h.

*3.3. Cytotoxicity and Hemolysis Assay.* The cytotoxicity of EuEO for murine peritoneal macrophages and erythrocytes is shown in Figures 2 and 3, respectively, and in Table 2. The EO from *E. uniflora* significantly altered ($P < 0.05$) macrophage viability at a concentration of 6.25 $\mu g \cdot mL^{-1}$ and was able to reduce 50% of the macrophage viability ($CC_{50}$) at 45.3 $\mu g \cdot mL^{-1}$. The cytotoxicity of EO from *E. uniflora* for human blood type $O^+$ erythrocytes was 63.22% at the highest concentration tested (400 $\mu g \cdot mL^{-1}$); however, it appeared to be nontoxic at concentrations less than 50 $\mu g \cdot mL^{-1}$ (Figure 3).

*3.4. Treatment of Infected Macrophages.* Figures 4 and 5 show the results of EuEO treatment of macrophages infected with *L. amazonensis*. The values obtained revealed a significant and concentration-dependent reduction in macrophage infection at 48 h with all the 3 EuEO concentrations studied. The reduction of infection was 27%, 44.67%, and 50.67% at 0.78, 1.56, and 3.12 $\mu g \cdot mL^{-1}$, respectively. EuEO also decreased the number of amastigotes per infected cell in a concentration-dependent manner (Figure 5). The $IC_{50}$ at 48 h of exposure to EuEO was 1.92 $\mu g \cdot mL^{-1}$ (Table 2). These values and the $CC_{50}$ values for macrophages were used to calculate the selectivity index that is useful for assessing the safety level of EuEO in mammalian cells (Table 2). This index represents the relationship between $CC_{50}$ and $IC_{50}$ for amastigotes.

*3.5. Lysosomal Activity and Phagocytosis Assay.* Lysosomal activity was assessed based on the retention of neutral red in macrophage lysosomes and was determined colorimetrically. The lysosomal activity of macrophages treated with EuEO showed significant reduction at concentrations of 100 and 50 $\mu g \cdot mL^{-1}$ (Figure 6(a)) due to cytotoxicity ($CC_{50}$ 45.3 $\mu g \cdot mL^{-1}$). However, we observed a statistically significant increase in lysosomal activity at concentrations from 12.5 to 3.12 $\mu g \cdot mL^{-1}$ compared to control. Another parameter reflecting macrophage activation was the phagocytosis of stained zymosan when the cells were exposed to an external stimulus. The phagocytic ability of macrophages incubated with EuEO is shown in Figure 6(b). There was a significant increase in phagocytosis of zymosan particles in the wells

Table 1: Chemical composition and retention indices of the constituents of *Eugenia uniflora* essential oil.

| Constituents[a] | RRI | | % A[d] |
|---|---|---|---|
| | Cal.[b] | Lit.[c] | |
| Myrcene | 989 | 988 | 0.19 |
| Limonene | 1026 | 1029 | 0.18 |
| Linalool | 1095 | 1095 | 0.16 |
| (*Z*)-3-Hexenyl butyrate | 1186 | 1184 | 0.10 |
| Cuminaldehyde | 1240 | 1238 | 0.10 |
| $\delta$-Elemene | 1335 | 1338 | 1.32 |
| $\alpha$-Cubebene | 1349 | 1345 | 0.10 |
| $\beta$-Elemene | 1390 | 1389 | 5.51 |
| Sativene | 1394 | 1390 | 0.06 |
| (*E*)-Caryophyllene | 1417 | 1417 | 4.33 |
| *trans*-$\alpha$-Bergamotene | 1437 | 1432 | 0.05 |
| **$\gamma$-Elemene** | **1434** | **1434** | **14.25** |
| Aromadendrene | 1440 | 1439 | 0.07 |
| $\alpha$-Humulene | 1455 | 1452 | 0.21 |
| Alloaromadendrene | 1462 | 1458 | 0.11 |
| $\beta$-Chamigrene | 1475 | 1476 | 0.38 |
| Germacrene D | 1482 | 1484 | 1.19 |
| $\beta$-Selinene | 1487 | 1489 | 0.70 |
| **Curzerene** | **1498** | **1499** | **47.3** |
| $\gamma$-Cadinene | 1513 | 1513 | 0.12 |
| $\delta$-Cadinene | 1523 | 1522 | 0.28 |
| Selina-3,7-(11)-diene | 1546 | 1545 | 0.24 |
| Germacrene B | 1558 | 1559 | 0.45 |
| Spathulenol | 1578 | 1577 | 0.17 |
| Caryophyllene oxide | 1581 | 1582 | 0.13 |
| Globulol | 1585 | 1590 | 0.25 |
| Viridiflorol | 1590 | 1592 | 0.08 |
| ***trans*-$\beta$-Elemenone** | **1597** | **1601** | **10.4** |
| Atractilona | 1655 | 1657 | 2.38 |
| Germacrone | 1694 | 1693 | 1.51 |
| Eudesm-7(11)-en-4-ol | 1700 | 1700 | 0.33 |
| Monoterpene hydrocarbons | | 0.37 | |
| Oxygenated monoterpenes | | 0.36 | |
| Sesquiterpene hydrocarbons | | 29.37 | |
| Oxygenated sesquiterpenes | | 62.55 | |
| Total | | 92.65 | |

[a]Compounds listed in order of elution on the DB-5ms column. [b]Relative retention indices (RRIs) experimentally determined against *n*-alkanes by using the DB-5ms column. [c]RRIs reported in the literature [23, 24]. [d]Content expressed as percentages obtained by integration of the GC peak area.

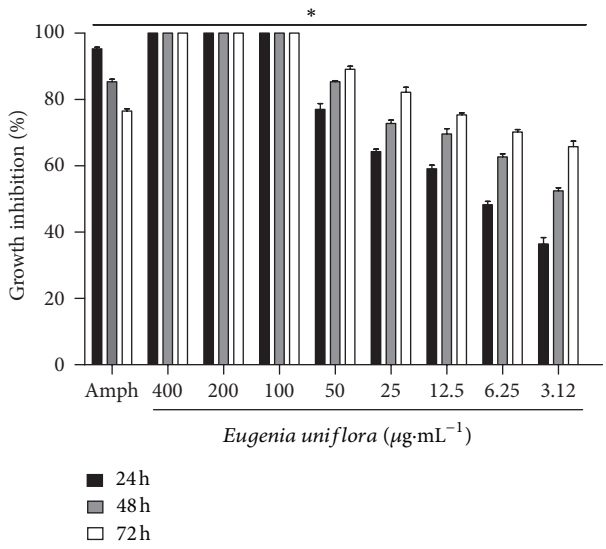


Figure 1: Effect of *Eugenia uniflora* essential oil (400, 200, 100, 50, 25, 12.5, 6.25, and 3.12 $\mu g \cdot mL^{-1}$) or amphotericin B (Amph) (2 $\mu g \cdot mL^{-1}$) on *Leishmania amazonensis* promastigotes. Cultures of log-phase promastigotes ($1 \times 10^6$) were incubated at 26°C for 24, 48, and 72 h in different essential oil concentrations. Data represent the mean percentage of growth inhibition ± standard error of 3 experiments carried out in triplicate. $^*P < 0.05$.

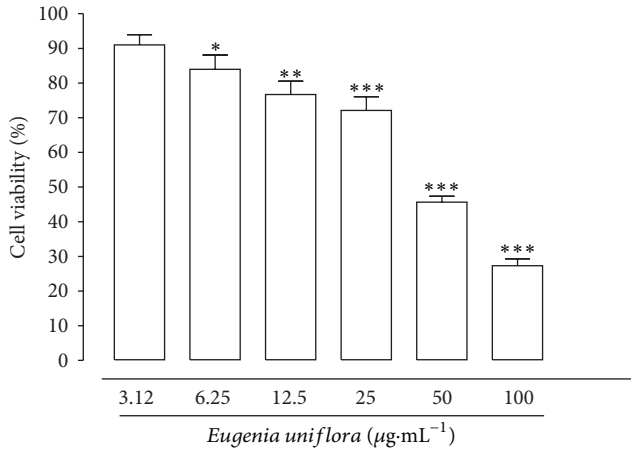


Figure 2: Cytotoxicity of *Eugenia uniflora* essential oil on the viability of murine peritoneal macrophages. Peritoneal macrophages were seeded at $1 \times 10^5$/well in 96-well microplates and incubated for 48 h in the presence of *E. uniflora* L. essential oil at concentrations of 100, 50, 25, 12.5, 6.25, and 3.12 $\mu g \cdot mL^{-1}$. Viability was determined with 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), and the optical density was determined at 540 nm. Data represent the mean parasite density ± standard error of 3 experiments carried out in triplicate. $^*P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

treated with EuEO at concentrations ranging from 25 to 3.12 $\mu g \cdot mL^{-1}$.

*3.6. NO Production.* NO production was determined indirectly by measuring nitrite produced by macrophages treated with EuEO and stimulated (or not stimulated) by *L. amazonensis* promastigotes. Macrophages treated with EuEO, but not stimulated by *Leishmania*, showed significant reduction in NO production at concentrations of 100 and 50 $\mu g \cdot mL^{-1}$ (Figure 7(a)), while macrophages stimulated by the parasite showed increased NO at a concentration of 3.12 $\mu g \cdot mL^{-1}$, albeit without statistical significance ($P > 0.05$) (Figure 7(b)).

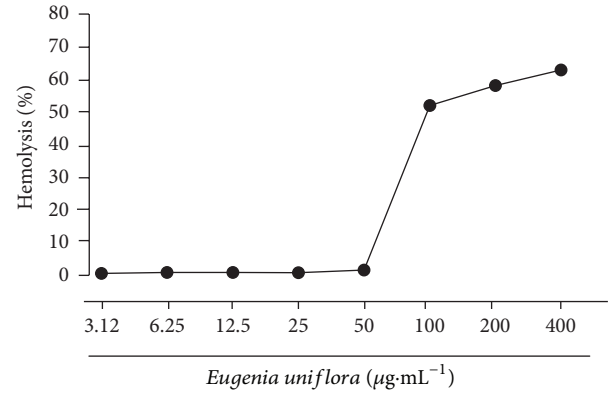


Figure 3: Hemolytic activity of *Eugenia uniflora* essential oil in a 4% suspension of human $O^+$ red blood cells after 1 h of incubation.

## 4. Discussion

The need to identify new compounds with anti-*Leishmania* properties that are more effective and less toxic than conventional drugs has motivated research on natural products isolated from plant species. These substances predominantly consist of alkaloids, terpenes, flavonoids, benzopyrans, phenolics, and sesquiterpene lactones and have been identified in plant species with documented anti-*Leishmania* activity [29, 30]. The chemical composition of EuEO obtained in this study revealed sesquiterpenes to be the dominant chemical class, corroborating the findings of many previous studies [16, 31–33]. Despite the prevalence of sesquiterpenes, the species has different chemotypes, with different major constituents being observed in different studies. The specimen analyzed in this study had curzerene as the major constituent, which again corroborated previous reported findings [32, 34]. According to Adio [35], germacrone is a heat-sensitive compound that may degrade to *trans*-$\beta$-elemenone during GC analysis. This may be the explanation of the presence of *trans*-$\beta$-elemenone among the major compounds of EuEO. Some germacrone products have shown pharmacological activity, as antitumor [35].

According to Costa et al. [20] the composition of the essential oil from *E. uniflora* leaves might be influenced by fruit colors. The plants with red fruit colors have curzerene as the major component as in our studied plant. In Maranhão, where the samples were collected, the majority of plants of *E. uniflora* have red fruit color [32]. Selina-1,3,7(11)-trien-8-one [36], germacrene B [16, 33], and $\alpha$- and $\beta$-selinene [37] have been found to be the major constituents of other *E. uniflora* chemotypes. Genetic variability, geographical and environmental conditions, and the method of environmental management directly influence the yield and composition of

Table 2: Anti-*Leishmania* and cytotoxic effects of *Eugenia uniflora* essential oil.

| | Macrophage | Promastigote | | | | Amastigote | |
|---|---|---|---|---|---|---|---|
| | $CC_{50}$ ($\mu g \cdot mL^{-1}$) | $IC_{50}$ ($\mu g \cdot mL^{-1}$) | | | SI | $IC_{50}$ ($\mu g \cdot mL^{-1}$) | SI |
| | 48 h | 24 h | 48 h | 72 h | | 48 h | |
| EuEO | 45.3 ± 2.45 | 6.96 ± 1.02 | 3.04 ± 0.75 | 1.75 ± 0.53 | 14.9[a] | 1.92 ± 0.8 | 23.59[b] |

[a]$SI_{pro}$ (selectivity index) = $CC_{50}$ macrophages/$IC_{50}$ promastigote forms (48 h).

[b]$SI_{ama}$ (selectivity index) = $CC_{50}$ macrophages/$IC_{50}$ amastigote forms.

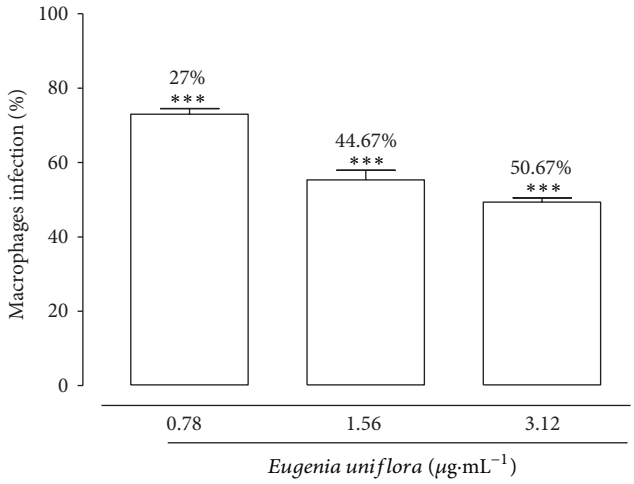


Figure 4: Effect of *Eugenia uniflora* essential oil on macrophage infection after 48 h of exposure. Peritoneal macrophage cells were infected with promastigotes of *Leishmania amazonensis* and then treated with 3.12, 1.56, or 0.78 $\mu g \cdot mL^{-1}$ of *Eugenia uniflora* essential oil. Data represent the mean parasite density ± standard error of 3 experiments carried out in triplicate. $^{***}P < 0.001$.

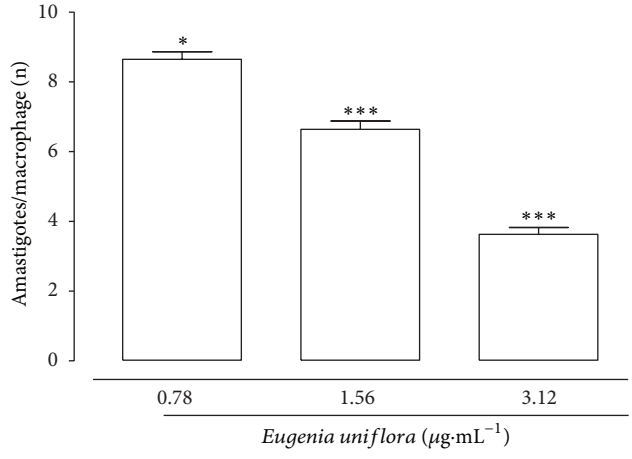


Figure 5: Effect of *Eugenia uniflora* essential oil on the survival of *Leishmania amazonensis* amastigotes internalized in macrophages. *Leishmania amazonensis*-infected mouse peritoneal macrophages were treated with 3.12, 1.56, or 0.78 $\mu g \cdot mL^{-1}$ of *Eugenia uniflora* essential oil. After 48 h of incubation, amastigote survival was assessed. Data represent the mean parasite density ± standard error of 3 experiments carried out in triplicate. $^{***}P < 0.001$.

essential oils [38]. It is the first report of essential oil analyzed from *E. uniflora* collected in São Luís, MA, Brazil and this could be an excellent source of natural product with interest for pharmaceutical industry.

The present study was conducted to evaluate the anti-*Leishmania* activity of EuEO on promastigotes and amastigotes of *L. amazonensis*, its potential mechanisms of macrophage activation, and its cytotoxicity in mammalian cells. The EuEO showed significant concentration-dependent activity against promastigotes of *L. amazonensis*, with an $IC_{50}$ of 1.75 $\mu g \cdot mL^{-1}$. Previous studies have suggested that these classes of natural products may have potential anti-*Leishmania* activity. $\beta$-Caryophyllene (a sesquiterpene hydrocarbon) and nerolidol (an oxygenated sesquiterpene) are examples of terpenic substances with well-characterized anti-*Leishmania* activity, possibly associated with inhibition of cellular isoprenoid biosynthesis [8, 39].

It has been shown that the lipophilic components of essential oils may affect layers of polysaccharides, fatty acids, and phospholipids in plasma membranes of promastigotes of *Leishmania* spp. This then leads to cell lysis and release of macromolecules [40]. In the cytoplasm, these substances can disrupt the specific metabolic pathways of lipids and proteins or stimulate depolarization of mitochondrial membranes, which can lead to cell necrosis or apoptosis [41, 42].

Amphotericin B is a drug of choice for the treatment of different forms of leishmaniasis. Because of this it was used as a positive control. We observed a significant inhibitory effect on promastigote growth after 24 h of exposure to amphotericin B, but leading to an increase in viable promastigote forms at 48 and 72 h. Similar results were observed in previous studies [43]. Some *Leishmania* strains have shown mechanisms of resistance to amphotericin B. This may has happened with the strain used in this study. The reproduction of resistant parasites may have led to a higher cell viability after 48 and 72 h [44, 45].

Experimental models using amastigotes internalized into macrophages can produce results that more closely approximate those obtained using animal models, since these forms are responsible for the clinical manifestations of leishmaniasis [46]. Amastigotes can be found in parasitophorous vacuoles of infected macrophages; therefore, a therapeutic drug will only be effective against *Leishmania* spp. if it can cross the host cell membrane and act on the amastigotes inside the vacuole. Thus, assays of activity against intracellular amastigotes are the most effective way to relate the *in vitro* activity of a possible substance with its *in vivo* effectiveness [47]. The inhibition of amastigote growth was even greater at

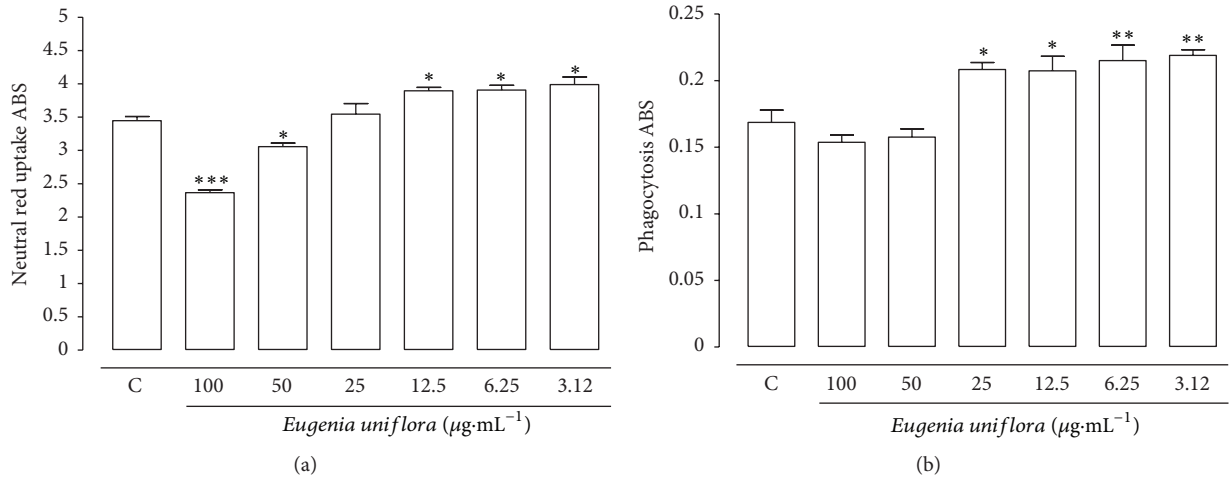


Figure 6: The influence of *Eugenia uniflora* essential oil on the lysosomal activity (a) and phagocytic activity (b) of peritoneal macrophages at concentrations of 100, 50, 25, 12.5, 6.25, and 3.12 $\mu g \cdot mL^{-1}$. Data represent the mean density ± standard error of 3 experiments carried out in triplicate. $^{*}P < 0.05$ and $^{***}P < 0.001$. C: control. ABS: absorbance.

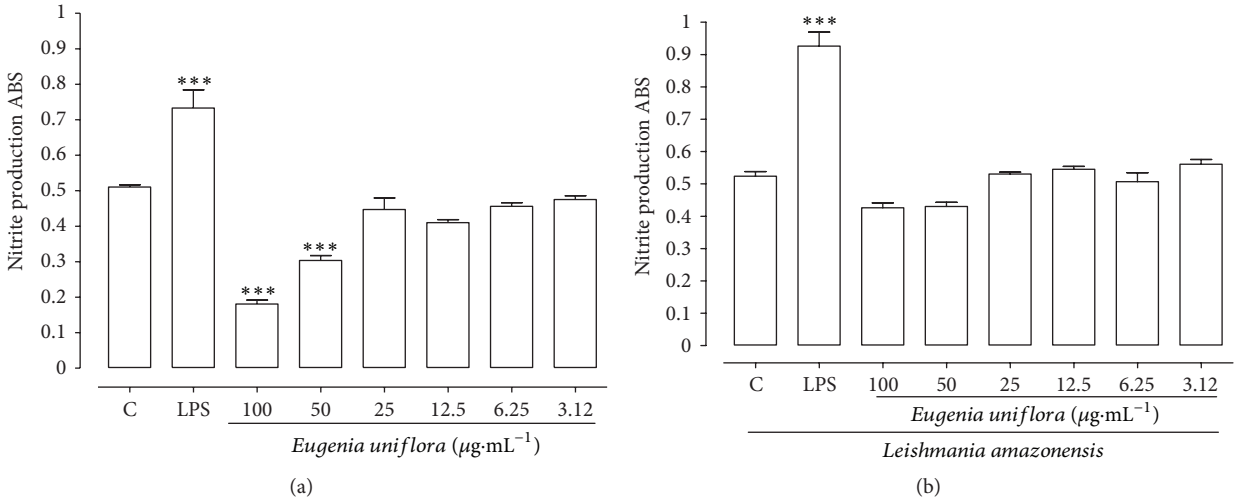


Figure 7: Production of nitric oxide (NO). Murine macrophages ($2 \times 10^5$) were treated with *Eugenia uniflora* essential oil (100, 50, 25, 12.5, 6.25, and 3.12 $\mu g \cdot mL^{-1}$) in the absence (a) or presence of *Leishmania amazonensis* (b) over 24 h. Data represent the mean density ± standard error of 3 experiments carried out in triplicate. $^{***}P < 0.001$. C: control. ABS: absorbance.

48 h after exposure to oil, with an $IC_{50}$ of 1.92 $\mu$g/mL, whereas the $IC_{50}$ for promastigotes was 3.04 $\mu g \cdot mL^{-1}$ for the same duration of exposure. Previous studies demonstrated greater activity against amastigotes versus promastigotes of essential oils of *Artemisia absinthium* and *Satureja punctata* [48].

Mechanisms of anti-*Leishmania* action on *Leishmania* spp. amastigotes have also been described for essential oils and their terpene compounds. Monoterpenes such as linalool, isolated from leaves of *Croton cajucara* (Euphorbiaceae), significantly increased NO production in macrophages infected with *L. amazonensis* and acts directly on the parasite, as evidenced by mitochondrial swelling and changes in the kinetoplast and the organization of nuclear chromatin [6]. In this study, the production of NO was determined by measuring nitrite. The results from macrophages treated with EuEO revealed that EuEO does not induce NO production, which demonstrates that its promising anti-*Leishmania* activity does not occur by this route.

Natural products that are able to activate macrophages, and are thus biological response modifiers, have been extensively studied [49, 50]. In this context, the EuEO demonstrated potential in the activation of macrophages by increasing phagocytic capacity and lysosomal activity, immunomodulatory activities possibly involved in its anti-*Leishmania* activity. Phagocytosis and the lysosomal system are critical to macrophage function, because of their roles in internalization, degradation, and eventually presentation of peptides derived from antigens required for host defense. In addition, antigen presentation occurs through phagocytosis and endosomal/lysosomal targeting systems [51–53].

Due to the need for anti-*Leishmania* substances that are more selective for the parasite and less toxic to host cells and since EuEO showed low $IC_{50}$ values, it was important to investigate the cytotoxic activity of EuEO against mammalian cells. EuEO showed significant toxicity in macrophages, with a $CC_{50}$ of 45.3 $\mu g \cdot mL^{-1}$, but when compared with its $IC_{50}$ value in amastigotes, this indicates a secure selectivity index above 20. The literature recommends that for amastigotes internalized in macrophages, this index must be demonstrated as near to or greater than 20 [54]. The investigation of the toxic action of new substances with anti-*Leishmania* activity on macrophages is important, since they are the main cells of the vertebrate host parasitized by *Leishmania* spp. [55, 56]. Furthermore, various other cytotoxic assays can be conducted using animal and human cells to determine the safety of a test substance, leading to a future treatment *in vivo*. Tests performed with erythrocytes enable assessment of a drug's potential to cause injury to the plasma membrane of a cell, either by forming pores or by total rupture, leading to cellular damage or changes in membrane permeability [57]. This is a model used for the preliminary study of the protective and toxic effects of substances, being a possible indicator of this type of damage to cells *in vivo* [58–60]. The results obtained in this test with EuEO revealed low toxicity to erythrocytes, since EuEO showed 0% hemolysis at concentrations of 50 $\mu g \cdot mL^{-1}$ and lower.

## 5. Conclusions

In conclusion, the data in this study show that the essential oil of leaves from *E. uniflora* (EuEO), characterized by sesquiterpenes, has anti-*Leishmania* activity in both stages of *L. amazonensis*. This study also demonstrated that EuEO activity is not mediated by NO production. On the other hand, activation of macrophages may be involved in the mechanisms of EuEO anti-*Leishmania* activity, as evidenced by increases in both phagocytic capacity and the lysosomal compartment. Further investigation is needed to determine additional mechanisms involved in the anti-*Leishmania* activity of this plant species, as well as *in vivo* studies in models of leishmaniasis.

## Conflict of Interests

The authors declare no conflict of interests.

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