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ORIGINAL ARTICLE

Evaluation of the hydroalcoholic extract of *Clarisia racemosa* as an antiparasitic agent: an in vitro approach

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Abstract

Clarisia racemosa Ruiz & Pav is a neotropical species found in humid forests from southern Mexico to southern Brazil. There are few studies related to the ethnopharmacological use of *C. racemosa*. Our objective was to evaluate the hydroalcoholic extract of *C. racemosa* as a potential antiparasitic agent. For this, we performed in vitro assays against strains of *Leishmania amazonensis*, *Trypanosoma cruzi*, *Plasmodium falciparum,* and *Schistosoma mansoni*. At the same time, immunomodulatory activity tests were carried out. The results demonstrated that the extract was able to stimulate and activate immune cells. In preliminary antiparasitic tests, structural modifications were observed in the promastigote form of *L. amazonensis* and in adult worms of *S. mansoni.* The extract was able to inhibit the growth of trypomastigote form of *T. cruzi* and finally showed low antiparasitic activity against strains of *P. falciparum*. It is pioneering work and these results demonstrate that *C. racemosa* extract is a promising alternative and contributes to the arsenal of possible forms of treatment to combat parasites.

Keywords Antiparasitic agent · Natural products · Amazon forest · *Clarisia racemosa*

Introduction

Neglected diseases are those caused by infectious agents or parasites and are considered endemic in low-income populations with limited access to health services (Biswas and Mandal 2023). These are mainly distributed among the poorest populations in Africa, Asia, and America (Biswas and Mandal 2023; Renslo and McKerrow 2006). Among the various diseases, we will highlight here only leishmaniasis (caused by parasites of the genus *Leishmania*), Chagas disease (*T. cruzi*), malaria (parasites of the genus *Plasmodium*), and schistosomiasis (*S. mansoni*) (Ferreira et al. 2022). These diseases remain one of the leading causes of morbidity and mortality worldwide and represent an important medical need that remains unmet (Renslo and McKerrow 2006).

Epidemiological data from the Pan American Health Organization show that, from 2001 to 2021, a total of 1105,545 cases of leishmaniasis were reported, corresponding to an average of 52,645 cases per year (PAHO 2023). According to data published by the World Health Organization (WHO), it is estimated that there are approximately 6–7 million people infected with Chagas disease worldwide, with 10,000 deaths each year (WHO 2023a). With regard to malaria in 2021, there were an average of 619,000 deaths from malaria worldwide (WHO 2023b). In relation to schistosomiasis, it was estimated that 251.4 million people would need preventive treatment for the disease in 2021 (WHO 2023c).

Even in the face of these alarming data, investments in research, drug production and control of these diseases are still low (Mengarda et al. 2023). In addition to this scenario,

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it is still necessary to take into account that many of these parasites have become resistant to commercially used drugs. Therefore, there is a growing interest in discovering new forms of treatment (synthetic or natural products) that may, in the near future, be used for the treatment of these diseases (Renslo and McKerrow 2006).

Plants are important sources for obtaining bioactive compounds. Studies have shown that natural products have shown promising results in combating various parasites (Igoli et al. 2022; El-Seedi et al. 2022; Cruz-Filho et al. 2023).

The Amazon rainforest is one of the main targets for discovering new drug candidates. This fact is related to the vast biodiversity. Among the various species of plants, *Clarisia racemosa*, a native tree, has stood out for being used as a renewable raw material as a wood product. However, few studies have shown for the use of this plant for pharmacological purposes. Albuquerque Nerys et al. (2022) published the results of chemical characterization and different biological activities (antiproliferative, antimicrobial, antioxidant, antiglycant, photoprotective, toxicity in vitro and in vivo) showing that the hydroalcoholic extract of *Clarisia racemosa* has therapeutic potential.

In this context, we aimed to evaluate the hydroalcoholic extract of *C. racemosa* as a potential antiparasitic agent. For this, we performed in vitro assays against strains of *L. amazonensis*, *T. cruzi*, *P. falciparum,* and *S. mansoni*. At the same time, tests were performed on immune cells to evaluate the immunomodulatory profile promoted by the extract. This work aims to contribute to the arsenal of possible forms of treatment to combat parasites.

Materials and methods

Reagents

Acetone (Merk, CAS 67-64-1), chloroquine (Merk, CAS: 50-63-5), EPON (Merk, CAS 90-72-2), fetal bovine serum (Thermo Fisher Scientific), gentamicin (Novafarma), glutaraldehyde (Merk, CAS 111-30-8), green fluorescent protein (BioLinker), Griess reagent (Merk), lead citrate (Chem Xinglu Chemical, CAS 512-26-5), MTT (Merk, CAS: 298-93-1), osmium tetroxide (Merk, CAS 20816-12-0), poly-l-lysine (Merk, CAS 25988-63-0), RPMI 1640 culture medium (Thermo Fisher Scientific), Schneider® culture medium (Sigma-Aldrich), sodium cacodylate (Merk, CAS 6131-99-3), sodium chloride calcium (Merk, CAS 10043-52-4), SYBR green I (Invitrogen, Thermo Fisher Scientific) and uranyl acetate (Metaquimica, Brazil) were used.

Plant: *Clarisia racemosa*

This study was carried out with *Clarisia racemosa* (The tree was 15 years old) trunks supplied by Mil Madeiras Preciosas, a subsidiary of the Swiss group Precious Woods (http://preciouswoods.com.br/). The plant was collected in the district of Itacoatiara Manaus, Amazonas Brazil, at the following location 03°08′31″ and 58°26′33″W longitude and latitude. The species was registered in SisGen (National System of Genetic Heritage and Associated Traditional Knowledge) n° AAF588D.

Obtaining the hydroalcoholic extract

Obtaining, chemical characterization of the evaluated extract of the in vitro and in vivo toxicity of the hydroalcoholic extract was carried out at the Laboratory of Chemistry and Therapeutic Innovation of the Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil and published by Albuquerque Nerys et al. (2022). Briefly, the extract was obtained by exhaustive maceration (using 100 g of *C racemosa* ground to 0.177 mm) with 70% ethanol for 7 days at room temperature. Then, the solvent was removed by rotary evaporation (Biovera, model IKA RV3) and lyophilized under vacuum pressure of 0.024 mBar and temperature of − 40 °C in a lyophilizer (model L-101, brand Liotop) for 24 h, then frozen at − 20 °C. The extract yield was 14.5%. The phytochemical characterization of the extract was found in the supplementary material. Contents of phenolics, flavonoids, flavonols, and tannic acid were determined by UV/Vis spectroscopy (Table S1). Chromatogram (HPLC) obtained for the hydroalcoholic extract was obtained from the stem of *C. racemosa* (Fig. S1). Main compounds identified for the hydroalcoholic extract were obtained from the stem of *C. racemosa* (Table S2).

In vitro immunomodulatory activity

In vitro immunomodulation assays were performed using a primary culture of splenocytes from mice treated with *C. racemosa* extract dissolved in distilled water at concentrations ranging from 3.9 to 1000 µg/mL. All tests were performed according to the methodology proposed by Cruz-Filho et al. (2019) and Araújo et al. (2022). This study was approved by the Committee on Ethics in the Use of Animals of the Instituto Aggeu Magalhães/Fundação Oswaldo Cruz, protocol number 164/2020.

Briefly, 45-day-old female Balb/c mice (Total $n = 10$) from the LIKA vivarium were anesthetized with 10 mg/kg of xylazine and 115 mg/kg of ketamine and aseptically euthanized by cervical dislocation, the spleens were removed.

Then, a homogenate was prepared with the spleens and the splenocytes were isolated with FicollPaque TM. Cells were obtained by centrifugation at 2500 × g at room temperature for 25 min.

The cells were isolated and the concentration obtained was 10^6 cells/mL. Then the cells were treated with different concentrations of extract for 24 h in a CO_2 oven at 30 °C. Cytotoxicity assays were performed using annexin V and propidium iodide (BD Biosciences, San Diego, CA). To evaluate cell proliferation, carboxyfluorescein succinimidyl ester (CFSE) was used. Cytokine concentrations were determined with the supernatant of treated cultures using a Th1/Th2/Th17 kit (Becton Dickinson Biosciences, USA) for simultaneous detection of IL-2, IL-4, IL-6, IL-10, IL- 17, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ).

Furthermore, the concentration of nitric oxide (NO) produced by the Griess colorimetric method was determined.

Finally, cytosolic and mitochondrial reactive oxygen species (ROS) levels, transmembrane potential and cytosolic Ca^{2+} concentration were determined. These assays were performed by flow cytometry using dihydroethidium (DHE) (Merck), MitoSox Red (Thermo Fischer Fisher-USA), MitoStatus (BD Biosciences-USA) and fluo-3AM (Thermo Fisher Scientific-USA), respectively. All experiments were performed in triplicate.

In vitro leishmanicidal activity

The experiments were carried out according to Araújo et al. (2022) and Cruz-Filho et al. (2023). Promastigotes of *L. amazonensis* (strain WHOM/00LTB0016) were maintained in Schneider medium (Sigma) supplemented with 1% streptomycin and 20% fetal bovine serum, respectively, grown in an incubator at 26 °C (Schneider's Drosophila Medium favors the growth of promastigote forms). The promastigotes forms were used in exponential phase of growth in all phases of the experiment (72 h). For the leishmanicidal activity assay, promastigotes were counted and diluted in complete Schneider medium (Sigma/Merck) at 2.3×10^6 cells/mL. The parasites were incubated at 26 °C in the presence of different extract concentrations (3.9–1000 µg/mL), diluted in distilled water, for 72 h. Parasites incubated only with culture medium and with Amphotericin B were used as negative and positive controls, respectively. Cell growth was evaluated and the IC_{50}/72h was determined by regression analysis. Each assay was performed in triplicate. The selectivity index (SI) was determined as the ratio between CC_{50} (concentration capable of inhibiting cell growth by 50%) and IC_{50} (concentration capable of inhibiting parasite growth by 50%).

The ultrastructural evaluation was performed by Araújo et al. (2022) and Cruz-Filho et al. (2023). The tests were carried out with the IC_{50} value promoted by the extract against the promastigote form to evaluate possible damage promoted in the parasites treated with the extract. The parasites were adhered to coverslips previously coated with poly-L-lysine (Sigma®). Coverslips were treated for 1 h with one containing 1% osmium tetroxide (OsO_4) in sodium cacodylate buffer. Then, the cells were dehydrated in an increasing series of ethanol and subjected to critical point drying in the Critical Point Dryer HCP-2 (Hitachi, Tokyo, Japan), covered with 20 nm gold in the JFC-1100 metallizer (Jeol, Tokyo, Japan). Japan) and visualized in a JEOL T-200 scanning electron microscope (Jeol, Tokyo, Japan). For visualization in transmission electronic microscopy, the fixed parasites were washed and post-fixed for 1 h in a solution containing 1% osmium tetroxide (OsO_4), 0.8% potassium ferricyanide, 5 mM $CaCl_2$ in cacodylate buffer of sodium. The parasites were dehydrated in increasing concentrations of acetone and included in EPON (Sigma® Aldrich, St. Louis, USA). Finally, ultrathin Sections (70 nm) were obtained with a Leica EMUC6 ultramicrotome (Leica, Wetzlar, Germany). These sections were stained with uranyl acetate and lead citrate and analyzed using a TecNai G2 Spirit TEM transmission microscope (FEI, Hillsboro, USA). The experiments were performed in triplicate.

Evaluation of in vitro trypanocidal activity of the extract against *T. cruzi* trypomastigotes

The in vitro tests with trypomastigotes forms of *T. cruzi* were performed according to the protocols established by Cruz-Filho et al. (2023). Briefly, *T. cruzi* parasites (Tulahuen strain) expressing the *Escherichia coli* β-galactosidase gene were cultured on a monolayer of J774 macrophages. Cultivations were carried out in RPMI 1640 medium (pH 7.4) phenol red plus 10% fetal bovine serum and glutamine (2 mM). Macrophages were seeded in 96-well tissue culture microplates at a concentration of 1.0×10^3 per well in a volume of 80 µL and incubated for 24 h. Then, trypomastigotes were added at a concentration of 1.0×10^4 per well in a volume of 20 µL. After 48 h, the medium was discarded and replaced by 180 µL of medium and the extract, diluted in distilled water, at different concentrations (3.9–1000 µg/mL). After 7 days of incubation, the reagents Chlorophenol red-*β*-*D*-galactopyranoside (CPRG) (final concentration 100 µM) and Nonidet P-40 (final concentration 0.1%) were added to the plates, followed by incubation for 24 h at 37 °C. Absorbance was determined at 570 nm in an automatic microplate reader. Benznidazole was used as a positive control. The results were expressed as percentage of growth inhibition for 50%

of the parasites (IC_{50}). Experiments were performed in triplicate.

Anti-*Plasmodium falciparum* activity in vitro

For this study, two strains of *P. falciparum* adapted to continuous culture were used: a strain sensitive to all drugs (3D7) and a strain resistant to chloroquine (Dd2). Both strains were obtained from the Malaria Research and Reference Reagent Resource Center (MR4). The tests were performed according to the methodology proposed by Araújo et al. (2022) and Cruz-Filho et al. (2023). Briefly, a desynchronized culture with 0.6% hematocrit and 0.5% parasitemia was incubated in a 96-well flat bottom plate with concentrations ranging from 0.17 to 10,000 ng/mL of extract for 72 h (37 °C and 5% CO_2).

Cytotoxicity assays of the extract against the chloroquine-sensitive strain were evaluated by flow cytometry (Beckman Coulter, Cytoflex) with a 96-well plate reader, using Fl-1 (green fluorescent protein [GFP]; excitation wavelength, 488 nm). On average, 20,000–40,000 erythrocytes were counted for each well. As for the resistant strain, it was previously stained with 0.5 × SYBR green I (Invitrogen, Thermo Fisher Scientific) for 30 min in the absence of light at 37 °C, washed once with PBS and then analyzed by cytometry under the same conditions. of the susceptible strain 3D7-GFP. The standards used were: chloroquine (for sensitive strain) and dihydroartemisinin (DHA) (resistant strain) at the same extract concentrations. Experiments were performed in triplicate.

In vitro schistosomicidal activity

The study was approved by the UFPE Animal Ethics Committee (n° 0060/2019 CEUA/UFPE). Initially, 20 female mice (Swiss Webster, 28 ± 2 g, 30 days old) obtained from the LIKA/UFPE vivarium were divided into two groups. The first group was infected with 3000 and the second with 120 cercariae of *S. mansoni* (strain BH, Belo Horizonte—MG—Brazil) for 45 days, this procedure was performed according to Lima-Aires et al. (2014). The lineage of *S. mansoni* belongs to the mollusc of the Federal University of Pernambuco and is maintained by successive passages in snails of the species *Biomphalaria glabrata* and mice (Mus musculus/Swiss Webster). Adult worms were recovered by perfusion, with sterile saline NaCl at 0.9% w/v, from the hepatic portal system and mesenteric vessels of mice on the 45th day of infection (Smithers and Terry 1965). After perfusion, the worms were transferred to a petri dish containing supplemented RPMI 1640 medium (20 mM HEPES, 100 µg/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum) and washed three times with this culture medium.

According to the methodology described by Ramirez et al. (2007), couples of adult *S. mansoni* worms were distributed in 24-well plates and incubated at 37 °C in a humid atmosphere containing 5% CO_2. Each well received 2 couples of adult worms. After 2 h of adaptation, *C. racemosa* hydroalcoholic extract was added at concentrations of 200, 100, and 75 µg/mL. In the first stage of the tests, the worms were incubated and evaluated every 3, 6, 12, and 24 h with the extract. In the second stage, the worms were incubated with the extract, evaluated every 24 h for 5 consecutive days and monitored with an inverted microscope for motor activity, oviposition and mortality rate (Duval and Dewitt 1967). Adult worm couples were incubated only in RPMI 1640 supplemented with 1% DMSO and 10 µM PZQ to form negative and positive control groups, respectively. They were performed in triplicate.

During the observation of schistosomicidal activity, the groups that showed 100% mortality and the highest concentrations were retained and fixed for SEM along with the treatment controls. Then, washed in 0.1 M sodium cacodylate buffer, pH 7.2 for subsequent fixation in 0.1 M sodium cacodylate buffer, 2.5% glutaraldehyde, and 4% paraformaldehyde. Post-fixation was performed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 90 min. Then, three washes were performed in 0.1 M cacodylate buffer for subsequent dehydration, using an increasing series of ethanol in percentages ranging from 30 to 100% for 10 min each step. After dehydration, the critical point was performed. Then, the material was metallized for visualization and analysis in the JEOL JSM-5600 LV scanning electron microscope.

Statistical analysis

Results were expressed as mean ± standard deviation. Differences between groups were determined by analysis of variance (ANOVA) followed by Tukey's test. Statistical analysis was performed using GraphPad Prism® 9.4.3 Software. The minimum significance level for rejecting the null hypothesis was set at 5% ($p < 0.05$).

Results and discussion

In vitro immunomodulatory activity assays

The cytotoxicity assays promoted by the hydroalcoholic extract against splenic cells were performed in a flow cytometer using fluorescent annexin V (apoptosis) and propidium iodide (late necrosis and apoptosis) as markers (Rieger et al. 2011). These results are shown in Fig. 1.

The cell viability results using annexin V/propidium iodide (Fig. 1) showed that the extract promoted low toxicity against splenocyte cells, presenting viability results

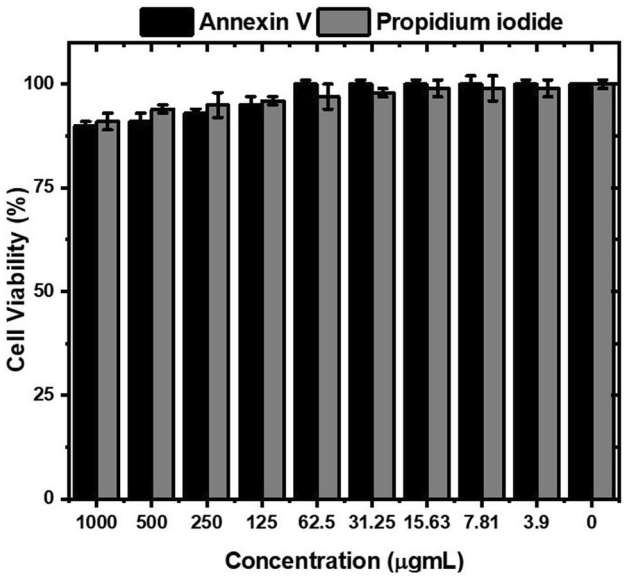


Fig. 1 Viability results promoted by hydroalcoholic extract of *C. racemosa* against splenocyte cells from mice evaluated by labeling with annexin V and propidium iodide. Horizontal bars represent the average of two independent experiments performed in triplicate

greater than 90% for the two cell death assays, in all evaluated concentrations.

The literature presents different hydroalcoholic extracts that present low cytotoxicity against immune cells. Among the works we can mention: Sousa et al. (2021) evaluating different extracts of the leaves of *Passiflora edulis* F. flavicarpa, against mouse lymphocytes, in concentrations ranging from 3 to 50 µg/mL, found that ethyl acetate extract did not induce significant cell death and ethanolic and hexanic extracts induced cytotoxicity at 50 µg/mL. Similar results were obtained by Silva et al. (2021) evaluating hexane, ethyl acetate and ethanol extracts from leaves of *Caesalpinia pulcherrima* against splenocytes from Balb/c mice. The authors found that the extracts promoted low cytotoxicity at concentrations lower than 25 µg/mL. Mashhadi et al. (2021) evaluated the ethanolic extracts of *Origanum vulgare L.* and *Origanum Majorana L.* and found low toxicity at concentrations ranging from 0.01 to 10 mg/mL against mouse splenocytes. These findings confirm the low toxicity of the extract against splenic cells.

In parallel with this study, assessment of the proliferative profile was performed with CFSE, a membrane-permeable fluorescent agent binding cytoplasmic amines (Rieger et al. 2011; Christina et al. 2022). The cell proliferation results showed that the extract was able to promote cell proliferation at all concentrations evaluated, in a non-significant way. Other hydroalcoholic extracts were also able to promote cellular proliferation of immune system cells. Barusrux et al. (2018), evaluating the effect of jujube extract on the proliferation of peripheral blood mononuclear cells, found cell proliferation at concentrations ranging from 50 to 1000 µg/mL. Kurnia Hartati et al. (2017), evaluating Indonesian black rice extract (*Oryza sativa* L. indica), found an increase in lymphocyte proliferation at concentrations ranging from 50 to 200 µg/mL.

In addition to the viability and proliferation profiles, the production of cytokines and nitric oxide was also investigated in the supernatant of cells treated with the extract at a concentration of 15.6 µg/mL (Table 1). This concentration was chosen because it is low, non-toxic, and close to that used in other studies, being sufficient to induce activity (Silva et al. 2021; Sousa et al. 2021; Araujo et al. 2022).

The results presented in Table 1 showed that the extract was able to provide an increase in the production of most anti-inflammatory cytokines, that is, of the Th2 profile, such as the cytokines IL-4, IL-10, and IL-6, the latter being pleiotropic. The other cytokines were produced at baseline values (similar to the control) and, therefore, did not change the immune status of the cultures. In addition, an increase in nitric oxide (toxic to organisms) was observed without promoting cell death. This fact indicates that the evaluated splenic cells are activated and that the extract may have anti-inflammatory properties. The mechanism of immunomodulatory activity promoted by different extracts is still not well understood (Parbat et al. 2021). This fact is related to the complex nature of the extracts. However, it is known that extracts that have a large number of phenolic constituents are easily recognized by the receptors found on immune cells and this binding can promote different responses (Ding et al. 2018; Mileo et al. 2019).

The literature presents different results related to cytokines produced by immune cells. Sousa et al. (2021) evaluating extracts obtained from the leaves of *Passiflora edulis* F. flavicarpa found that the ethanolic extract did not induce the production of cytokines. Silva et al. (2021) evaluating different extracts obtained from the leaves of *Caesalpinia pulcherrima* found that the ethanolic extract only induced the production of IL-6. Daltro et al. (2021) verified that the ethanolic extract of *Physalis angulata* reduced, in a concentration-dependent manner, the levels of IL-2, IL-6 and IFN-γ by splenocytes. In contrast, it induced a significant increase in IL-4 levels and did not modulate IL-10 levels.

In addition to cytokines, cytosolic and mitochondrial levels of reactive oxygen species (ROS), calcium release and changes in membrane potential were certain for different extract concentrations. The results were shown in Table 2.

The results shown in Table 2 showed that the extract was able to promote an increase in cytosolic and mitochondrial ROS production, in cytosolic calcium levels and in mitochondrial membrane potential. The response to oxidative stress, without inducing cell death, is an important step when

Table 1 Concentration of cytokines and nitric oxide produced by mouse splenocytes treated with different concentrations of hydroalcoholic extract (3.9–1000 µg/mL) for 24 h

Cytokines (pg/mL)	Control	3.9 µg/mL	7.81 µg/mL	15.63 µg/mL	31.25 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL
IL-2	7.34 ± 0.3	7.34 ± 0.1	7.35 ± 0.1	7.36 ± 0.01	7.34 ± 0.1	7.34 ± 0.02	7.34 ± 0.09	7.35 ± 0.0	77.35 ± 0.9	7.35 ± 0.8
IL-4	6.39 ± 0.1	9.73 ± 0.0	9.74 ± 0.01	9.73 ± 0.02	9.80 ± 0.0	9.82 ± 0.01	9.90 ± 0.4	9.95 ± 0.03	9.99 ± 0.05	10.01 ± 0.06
IL-6	10.57 ± 1.0	11.15 ± 0.8	11.17 ± 0.2	11.20 ± 0.01	11.31 ± 0.01	11.35 ± 0.1	11.40 ± 0.2	11.50 ± 0.02	11.60 ± 0.5	11.67 ± 0.9
IL-10	4.39 ± 0.8	6.56 ± 0.1	6.57 ± 0.01	6.84 ± 0.1	6.85 ± 0.01	6.85 ± 0.3	6.91 ± 0.9	6.97 ± 0.5	7.01 ± 0.1	7.6 ± 0.1
IL-17	5.38 ± 0.1	5.38 ± 0.3	5.39 ± 0.1	5.39 ± 0.3	5.39 ± 0.02	5.38 ± 0.2	5.40 ± 0.0	5.38 ± 0.1	5.39 ± 0.6	5.39 ± 0.4
TNF-α	6.89 ± 0.3	6.90 ± 0.1	6.93 ± 0.02	6.92 ± 0.01	6.91 ± 0.3	6.92 ± 0.3	6.92 ± 0.01	6.92 ± 0.03	6.92 ± 0.3	6.92 ± 0.9
IFN-γ	6.35 ± 0.2	6.36 ± 0.1	6.35 ± 0.01	6.35 ± 0.02	6.35 ± 0.01	6.35 ± 0.04	6.35 ± 0.04	6.35 ± 0.09	6.35 ± 0.01	6.35 ± 0.08
Nitric oxide (NO)	Control	3.9 µg/mL	7.81 µg/mL	15.63 µg/mL	31.25 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL
Concentration (µg/mL)	0.09 ± 0.0	0.18 ± 0.01	0.19 ± 0.0	0.20 ± 0.02	0.21 ± 0.01	0.25 ± 0.03	0.30 ± 0.0	0.31 ± 0.02	0.34 ± 0.0	0.42 ± 0.02

Mean ± standard deviation

it comes to the differentiation and activation of immune cells (Cruz-Filho et al. 2019). The results showed that the extract is a promising immunomodulatory agent under the conditions evaluated here.

In vitro leishmanicidal activity

For this in vitro leishmanicidal activity study, the metacyclic promastigote form of *L. amazonensis* (obtained in 72 h of growth/Fig. S3 of the supplementary material) was used as an experimental model. The metacyclic forms (infective) show great affinity for cells of the phagocytic system (Van Assche et al. 2011). These forms adhere to these cells (macrophages for example) via receptors and are captured by phagocytosis, remaining in phagosomes that fuse with lysosomes, finally forming phagolysosomes (Moradin and Descoteaux 2012).

Once inside the macrophages, promastigotes undergo biochemical and metabolic changes, transforming into amastigotes, which are the intracellular forms of the parasite (Van Assche et al. 2011; Moradin and Descoteaux 2012). Therefore, the evaluation of the toxicity of extracts against the promastigote form is a preliminary evaluation that provides fast and promising results (Gutiérrez-Rebolledo et al. 2017).

Through the cell viability curve at different concentrations (Fig. S4 of the supplementary material), it was possible to determine the IC_{50} (95.7 ± 0.2 µg/mL) of the extract against the promastigote form. This is a very promising result, since the extract against macrographic cells was able to provide a CC_{50} of 160.5 µg/mL (result obtained by Albuquerque Nerys et al. (2022)). The extract was more toxic against the parasite when compared to macrophage cells.

The prerequisite for using an extract as a leishmanicidal agent is to present lower toxicity against parasitic cells when compared to normal cells. According to the adapted arbitrary scale proposed by Gouveia et al. (2022) and Mariano et al. (2022) where $IC_{50} < 50$ µg/mL is considered active; $50 < IC_{50} < 100$ µg/mL moderately active and $IC_{50} > 100$ µg/mL inactive. The extract can be classified as moderately active. Through these results it was possible to determine the selectivity index (SI) being 1.7.

The results show that the extract showed greater toxicity against the promastigote form when compared to macrophages. These results when compared to the standard drug amphotericin B are lower IC_{50} of 0.15 ± 0.01 µg/mL against promastigotes, CC_{50} against J774 macrophages of 12.59 ± 0.01 µg/mL and selectivity index of 83.9, respectively. However, when compared to other extracts, it presents good results against the promastigote form of *L. amazonensis*. Among the different works we can mention those carried out by Pereira et al. (2020) evaluating the cytotoxicity of an ethanolic extract of *Croton blanchetianus* obtained an IC_{50} of 73.6 µg/mL. Pereira et al. (2010) obtained an IC_{50}

Table 2 Results of cytosolic and mitochondrial levels of reactive oxygen species (ROS), calcium release and mitochondrial membrane potential (ΔΨ), respectively

Concentration (μg/mL)	Cytosolic ROS (fluorescence)	Mitochondrial ROS (fluorescence)	Membrane potential (fluorescence)	Calcium ion concentration (nM)
0	25.0 ± 0.3	13.4 ± 0.1	20.1 ± 0.02	35.9 ± 0.12
3.9	30.0 ± 0.1	14.9 ± 0.2	20.9 ± 0.01	40.1 ± 0.25
7.81	37.0 ± 0.01	16.1 ± 0.01	30.5 ± 0.03	40.6 ± 0.32
15.63	44.0 ± 0.03	17.9 ± 0.09	32.9 ± 0.5	41.8 ± 0.13
31.25	49.0 ± 0.4	18.2 ± 0.03	33.7 ± 0.09	42.3 ± 0.33
62.5	50.0 ± 0.1	19.6 ± 0.09	34.3 ± 0.06	42.7 ± 0.43
125	53.0 ± 0.02	19.8 ± 0.08	35.6 ± 0.02	42.9 ± 0.91
250	58.0 ± 0.01	19.9 ± 0.4	37.6 ± 0.08	43.2 ± 0.76
500	61.0 ± 0.09	20.1 ± 0.9	38.1 ± 0.04	43.7 ± 0.16
1000	62.0 ± 1.0	21.2 ± 0.8	39.2 ± 0.1	43.9 ± 0.15

Mean ± standard deviation

of 22.93 μg/mL for the ethanolic extract of *Garcinia brasiliensis* Mart. Fruits. Santos et al. (2022) obtained an IC_{50} of 23.82 μg/mL for the ethanolic extract of *Capsicum chinense* unripe fruit (var. bode pepper). Mariano et al. (2022) evaluating the leishmanicidal effect of hydroalcoholic extracts from different parts of *Handroanthus impetiginosus* (Ipê-Roxo) obtained IC_{50} ranging from 2.24 to 49.12 μg/mL for 24 h of treatment and 4.69 to 47.27 μg/mL for 48 h.

Finally, to evaluate the effects promoted by the extract at the ultrastructural level, the treated promastigotes, in the IC_{50} concentration, were evaluated by scanning and transmission electron microscopy. Figure 2 shows the scanning electron micrographs for the untreated and treated promastigotes with the extract and amphotericin B.

The microscopy technique is useful to evaluate changes in the surface of the parasite when treated with different extracts. Figure 2A shows the untreated promastigote form, showing a typical fusiform morphology of the parasite, in addition to a topologically normal surface and a long, free, preserved flagellum. Parasites treated with amphotericin B (Fig. 2B) and extract (Fig. 2C) showed alterations in cell topology, presenting a body with undulations/wrinkling,

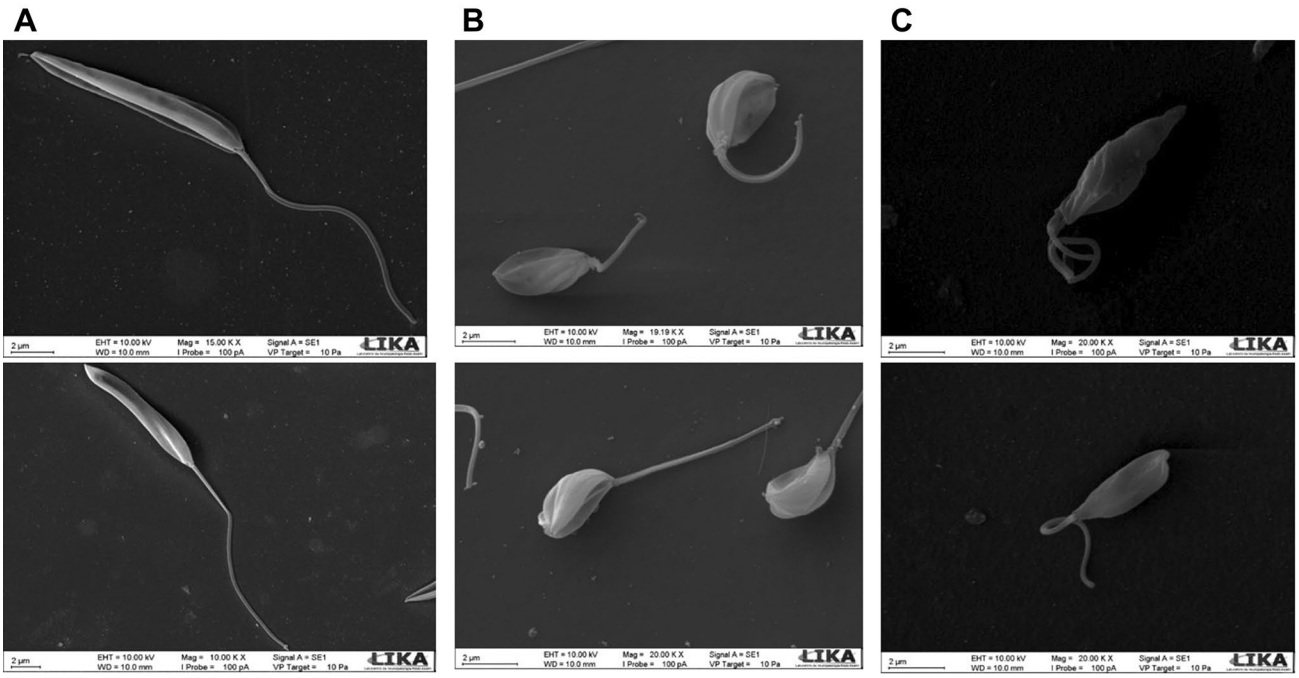


Fig. 2 Scanning electron micrographs for the promastigotes forms of *L. amazonensis* without treatment (**A**) and treated with amphotericin B (IC_{50} of 0.15 ± 0.01 μg/mL) (**B**) and with the hydroalcoholic extract of *C. racemosa* (IC_{50} of 95.7 ± 0.2 μg/mL) (**C**) for 72 h, respectively

morphology varying from fusiform to ovoid. In addition to evaluating the changes on the surface of the parasite, the effect of alterations at the level of organelles caused by the hydroalcoholic extract of *C. racemosa* and amphotericin B was evaluated through the analysis of transmission electronic microscopy (Fig. 3).

Ultrastructural analyzes using TEM showed that in the untreated group, the promastigotes of *L. amazonensis* showed normal cell shape and morphology, in addition to intact cytoplasm with well-preserved organelles. In the cells treated with amphotericin B (Fig. 3B) and with the hydroalcoholic extract of *C. racemosa*, it was possible to observe mitochondrial swelling, intense vacuolization of the cytoplasm, presence of electron-dense and electron-lucent structures. These alterations are associated with the presence of phenolic groups present in the extract; these groups are known to be potential leishmanicidal agents. These results indicate that the hydroalcoholic extract of *Clarisia racemosa* is a potential leishmanicidal agent against the promastigotes of *L. amazonensis*.

In vitro trypanocidal activity

For the in vitro trypanocidal activity assays, the trypomastigote forms of *T. cruzi* (infective form) were used as an experimental model. This form has an elongated morphology (it can appear as thin and wide forms), the kinetoplast has a rounded shape located in the region posterior to the nucleus. In addition to a flagellum emerging from the flagellar pocket (not visible under an optical microscope) which is located laterally, in the posterior region of the parasite (Magalhães et al. 2022; Martín-Escolano et al. 2022).

Like the promastigotes, of the genus leishmania, the trypomastigotes also have an affinity for the phagocytic system (Martín-Escolano et al. 2022). When trypomastigotes parasites infect host cells, they transform into the amastigote form (Magalhães et al. 2022). When the host cells are full of parasites, they again change into trypomastigotes. Due to the large, large number of parasites (inside the cells), the cells rupture and the protozoa reach the bloodstream, reaching other organs (Magalhães et al. 2022; Martín-Escolano et al. 2022). Therefore, the search for new anti-trypanocide components is an important strategy to fight the infection. Toxicity tests against *T. cruzi* trypomastigotes promoted by the hydroalcoholic extract of *C. racemosa* (Fig. S5 of the Supplementary Material) showed IC_{50} results of 84.59 ± 0.5 µg/mL and SI = 1.9 (CC_{50} of 160.5 µg/mL Albuquerque Nerys et al. (2022)). In addition, the extract was classified according to the adapted arbitrary scale proposed by Gouveia et al. (2022) and Mariano et al. (2022) as moderate compared to the trypomastigotes form. These results were lower when

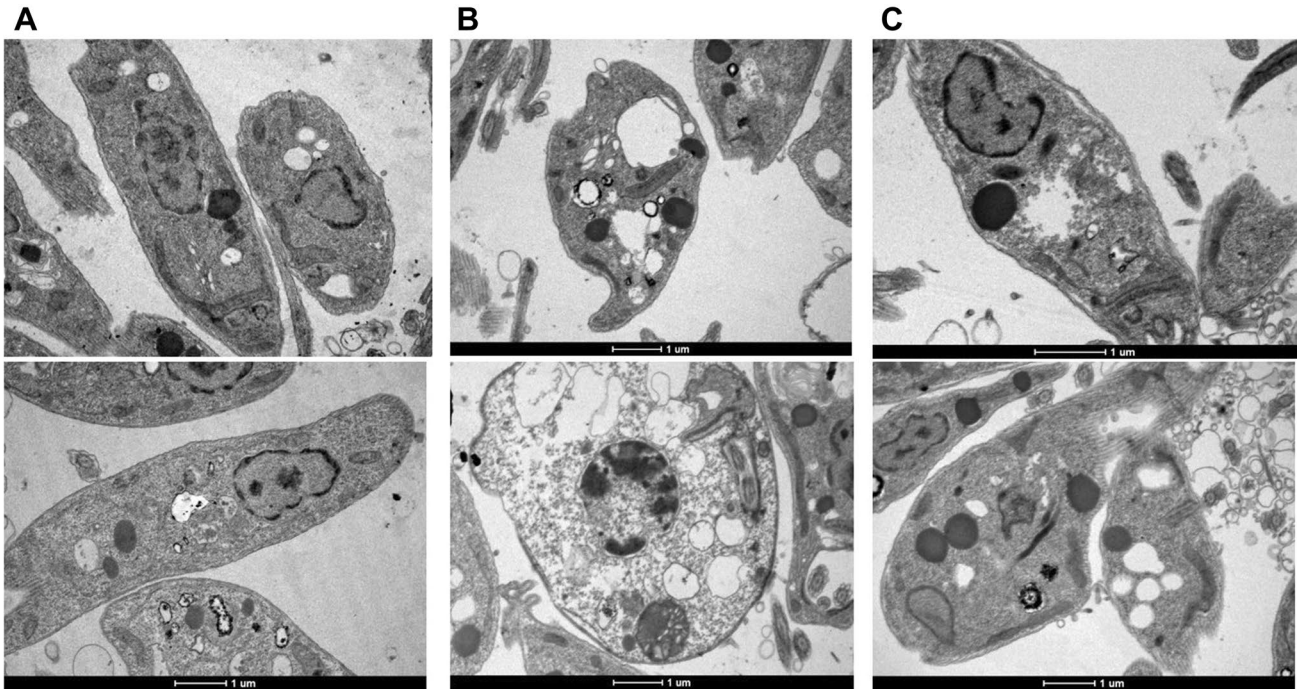


Fig. 3 Transmission electron micrographs for the promastigotes forms of *L. amazonensis* without treatment (**A**) and treated with amphotericin B (IC_{50} of 0.15 ± 0.01 µg/mL) (**B**) and with the hydroalcoholic extract of *C. racemosa* (IC_{50} of 95.7 ± 0.2 µg/mL) (**C**) for 72 h, respectively

Table 3 Motility scores of *S. mansoni* adult worms incubated with *C. racemosa* hydroalcoholic extract

Groups	Incubation time																			
	24 h				48 h				72 h				96 h				120 h			
	Motility score (%)																			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Control 1	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)
Control 2	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)
PZQ 10 µM	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)	–	–	–
C. racemosa hydroalcoholic extract																				
200 µg/mL	–	–	16 ± 0.0 (100%)	–	–	16 ± 0.0 (100%)	–	–	–	16 ± 0.0 (100%)		–	–	16 ± 0.0 (100%)	–	–	16 ± 0.0 (100%)	–	–	–
100 µg/mL	–	8 ± 1.82 (50%)	8 ± 1.82 (50%)	–	–	12 ± 0.0 (75%)	4 ± 0.0 (25%)	–	–	13 ± 1.41 (81.25%)	3 ± 1.41 (18.75%)	–		14 ± 1.41 (87.5%)	2 ± 2.82 (12.5%)	–	–	14 ± 1.41 (87.5%)	2 ± 2.82 (12.5%)	–
75 µg/mL		4 ± 0.0 (25%)	12 ± 0.0 (75%)	–	–	4 ± 1.41 (31.25%)	12 ± 2.82 (68.75%)	–	–	4 ± 1.41 (31.25%)	12 ± 2.82 (68.75%)	–		6.4 ± 1.1 (40%)	9.6 ± 1.5 (60%)	–	–	8 ± 1.82 (50%)	8 ± 1.82 (50%)	–

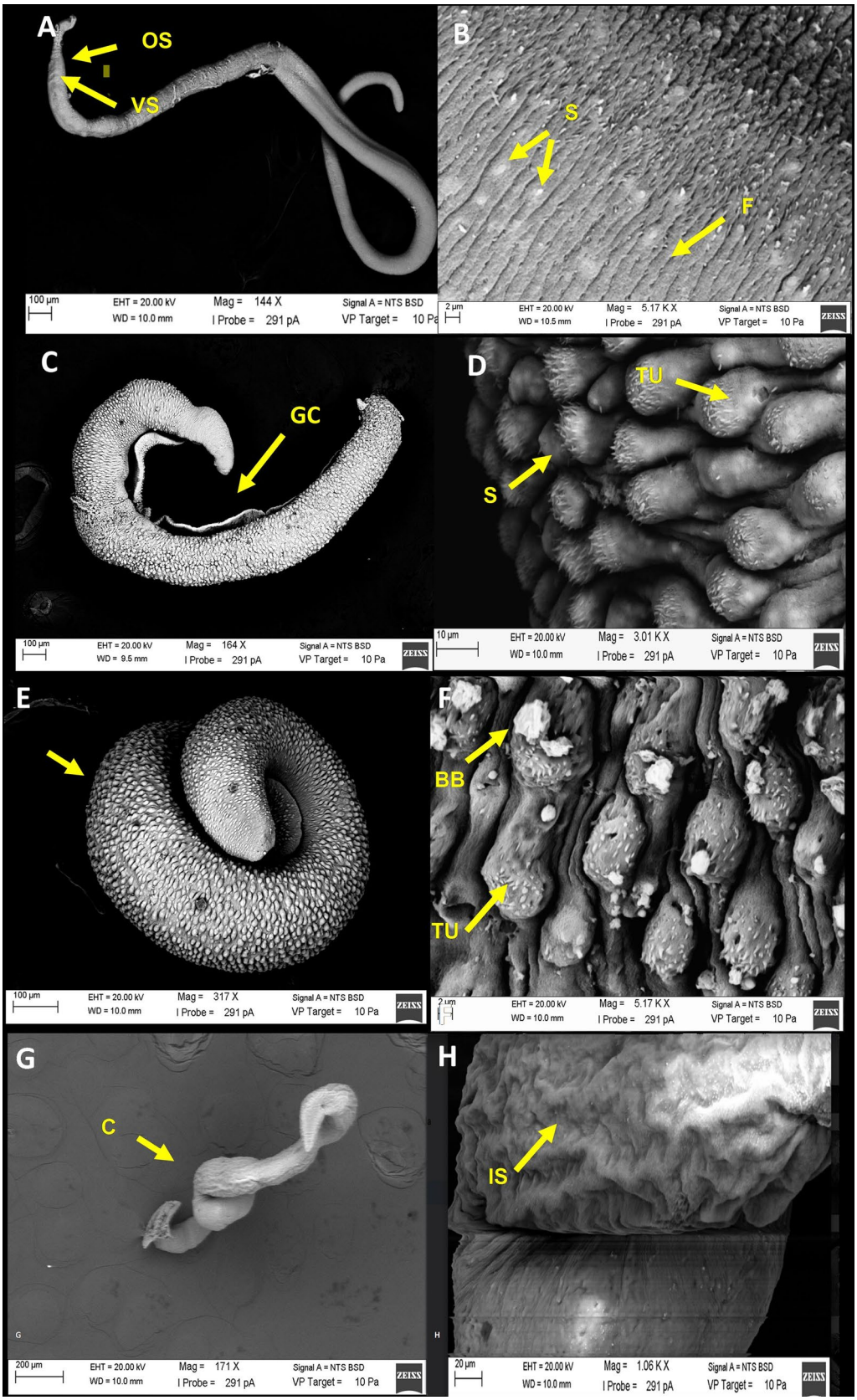
Average value of 16 worms (8 couples). Experiment in quadruplicate, totaling 32 couples of adult worms per concentration

Score 0 = complete absence of motions and integument with or without changes in coloration

Score 1 = present movements only at the extremities or at only one of the extremities (anterior and/or posterior regions), with absence of peristalsis of the internal organs and not adhered suckers

Score 2 = present reduced movements throughout the body, peristalsis of internal organs and suckers

Score 3 = present typical movements, exhibiting peristalsis of the internal organs, uckers in movement, adhering to the bottom or sides of the culture plate


A
OS
VS
100 µm EHT = 20.00 kV WD = 10.0 mm Mag = 144 X I Probe = 291 pA Signal A = NTS BSD VP Target = 10 Pa
B
S
F
2 µm EHT = 20.00 kV WD = 10.5 mm Mag = 5.17 K X I Probe = 291 pA Signal A = NTS BSD VP Target = 10 Pa
C
GC
100 µm EHT = 20.00 kV WD = 9.5 mm Mag = 164 X I Probe = 291 pA Signal A = NTS BSD VP Target = 10 Pa
D
TU
S
10 µm EHT = 20.00 kV WD = 10.0 mm Mag = 3.01 K X I Probe = 291 pA Signal A = NTS BSD VP Target = 10 Pa
E
100 µm EHT = 20.00 kV WD = 10.0 mm Mag = 317 X I Probe = 291 pA Signal A = NTS BSD VP Target = 10 Pa
F
BB
TU
2 µm EHT = 20.00 kV WD = 10.0 mm Mag = 5.17 K X I Probe = 291 pA Signal A = NTS BSD VP Target = 10 Pa
G
C
200 µm EHT = 20.00 kV WD = 10.0 mm Mag = 171 X I Probe = 291 pA Signal A = NTS BSD VP Target = 10 Pa
H
IS
20 µm EHT = 20.00 kV WD = 10.0 mm Mag = 1.06 K X I Probe = 291 pA Signal A = NTS BSD VP Target = 10 Pa

◂**Fig. 4** **A**–**D** SEM images of untreated *S. mansoni* adult worms after 24 h of observation. **A** Oral sucker (OS) and ventral sucker (VS) of adult female worm. **B** Parallel fissures (F) and spines **C** gynecophoric channel (GC) of adult male worm **D** Tubercles (TU) with spicules (S). SEM images of *S. mansoni* treated with praziquantel (10 µM) after 24 h of observation. **E** Adult male worms showing body with muscle contraction (arrow). **F** Severe damage to the integument, with the appearance of bursting blisters (BB) with loss of spicules. **G** Adult female worms showing contorted body (C), **H** Integument swelling (IS)

compared to the benznidazole standard, which presented an IC_{50} of 1.1 ± 0.01 µg/mL SI = 146, respectively.

The literature presents different results of trypomastigote activity in vitro. Quintero-Pertuz et al. (2022), evaluating the effect of the ethanolic extract and its fractions obtained from the leaves of *Castanedia santamartensis,* obtained IC_{50} values that ranged from 88.2 µg/mL to values greater than 200 µg/mL against the trypomastigote form. Castaneda et al. (2021), evaluating three different ethanol extracts from Colombian plants, obtained values ranging from 42.9 to values greater than 250 µg/mL. Meira et al. (2015), evaluating the concentrated ethanolic extract of *Physalis angulata* L., obtained IC_{50} values of 1.7 and 2.3 µg/mL for different strains of trypomastigotes.

These results show that the chemical composition of the extracts is an important parameter since different composition results promote different activity values. Furthermore, the in vitro results showed that the hydroalcoholic extract of *C. racemosa* is a potential trypanocidal agent.

Anti-*Plasmodium falciparum* activity in vitro

The in vitro anti-*Plasmodium falciparum* activity results showed that an extract was able to present low toxicity values. It is not possible to obtain IC_{50} values for any of the species at the evaluated concentrations. At the highest concentration, 45% inhibition was obtained for the chloroquine-sensitive *P. falciparum* (3D7) strain and 10.61% for the chloroquine-resistant *P. falciparum* (Dd2) strain. The inhibition curves are found in Fig. S6 of the Supplementary Material. These results were lower when compared to the drugs chloroquine (IC_{50} 156.1 ± 0.5 ng/mL) and dihydroartemisinin (IC_{50} 5.26 ± 0.01 ng/mL).

The literature presents different results for in vitro anti-*Plasmodium falciparum* activity. Kwansa-Bentum et al. (2019), evaluating different extracts obtained from the leaves of *Polyalthia longifólia* against the strain of *P. falciparum* (NF54), obtained IC_{50} results that ranged from 9.25 to 24 µg/mL (9250–24000 ng/mL). Djouwoug et al. (2021) found that hydroethanolic extract from the bark of Bridelia atroviridis müll. Arg. showed inhibition against the chloroquine-resistant *P. falciparum* strain Dd2.

Jansen et al. (2017), evaluating different extracts obtained from Mezoneuron benthamianum leaves, obtained results ranging from 6.4 to 44.3 µg/mL (6400–443000 ng/mL) against the chloroquine-sensitive *P. falciparum*(3D7) strain. Therefore, we can conclude that the anti-*Plasmodium falciparum* activity is directly related to the constituents present in the extract, in addition to the method of obtaining it.

In vitro schistosomicidal activity

Changes in motility and mortality of *S. mansoni* adult worm couples

The worms were incubated for 120 h with different concentrations 200, 100, and 75 µg/mL, and monitored every 24 h to assess general conditions, such as: motor activity, pairing and mortality rate (Table 3). All parameters were evaluated using a Leica DMIL inverted microscope.

The results in Table 3 show that for the positive control PZQ 10 µM, after 3 h of incubation, 100% of the worms were at motility score 0. The negative control groups, incubated in supplemented RPMI 1640 medium (controls 1 and 2), showed no differences in motility. These worms continued to show typical movements for score 3, such as movement of the suckers and their adhesion to the side or bottom of the Petri dish, in addition, they showed peristalsis of the internal organs. Thus, the worms incubated in controls 1 and 2 were chosen for negative motility control. The groups containing *C. racemosa* hydroalcoholic extract were divided into three concentrations 200, 100, and 75 µg/mL. It was observed that the hydroalcoholic extract of *C. racemosa* caused an effect on the pairing of adult worms of the parasite *S. mansoni* during the 24-h incubation period. All pairs of mated worms separated into male and female individuals after 24 h of incubation.

This fact allows the action of the extract on male and female worms, increasing the possibility of leading to the destruction of the integument of both parasites The effect of *C. racemosa* hydroalcoholic extract also affected the motor activity of both parasites (males and females) in just 24 h at a dose of 200 µg/mL in motility score 2. A total reduction in motility was observed after 120 h; the parasites had a score of 2, motility was 0. Decreased motor activity was marked by loss of natural movement and subsequent death. The worms gradually became inflexible, with rocking movement and without contraction of the suckers, showing weak motor activity until the complete loss of movement was observed.

The literature presents different results for the schistosomicidal activity. This difference is related to the complex chemical composition of the hydroalcoholic extracts. Silva Alves et al. (2020) evaluated the ethanolic extracts of *P. arboreum* and J. *gossypiifolia* at a concentration of 250 µg/mL. They were effective against adult worms, with 100% reduction in viability of male and female worms after

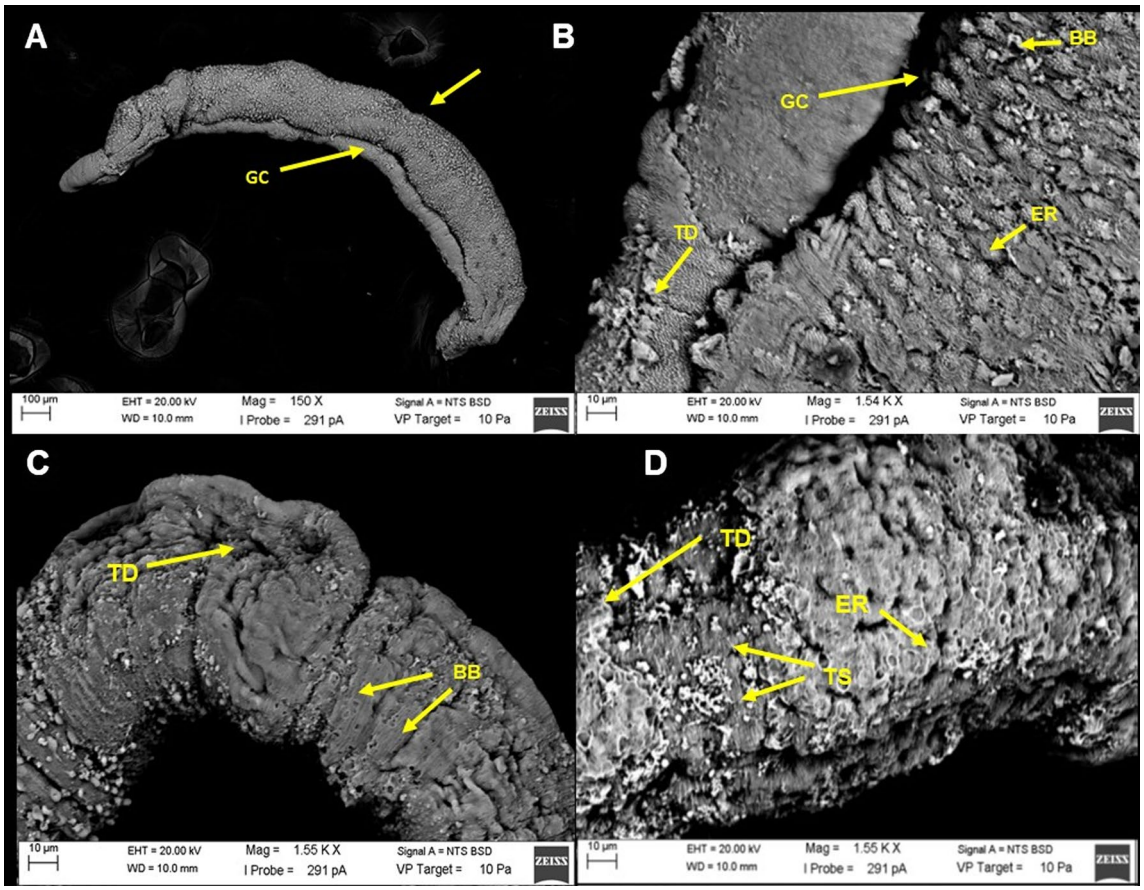


Fig. 5 **A**–**D** SEM images of adult worms of *S. mansoni* treated with hydroalcoholic extract of *C. racemosa* (200 µg/mL) after 24 h of observation. **A** Adult male worms with muscle contraction and gynecophoric canal (GC). **B** Gynecophoric channel (GC), extensive disintegration of the integument (TD), erosions (ER) with appearance and blisters (BU), throughout the body. **D** Female worms with extensive tegument disintegration (TD) and erosions (ER), with appearance and blisters (BB), subcutaneous tissue (TS)

12 and 24 h, respectively. *P. arboreum* and *J. gossypiifolia* were equally effective in inhibiting *S. mansoni* oviposition (93% reduction) and causing damage to internal and external structures in adult worms.

Khalil et al. (2016) evaluated the alcoholic extract of the plant *Calotropis procera* (6.25, 12.5, and 25 mg/L) applied against adult worms of the genus *S. mansoni*. of *C. procera* over a period of 48 h. The ethanolic extract of *C. procera* (at 25 mg/L) caused the death of all worms after only half an hour. However, concentrations of 12.5 and 6.25 mg/L caused the death of all tested worms after three hours of exposure to the ethanolic extract.

Almeida et al. (2012), evaluating the ethanolic, dichloromethane and hexane extracts from the branches of *Eremanthus erythropappus*, found that at concentrations of 100 and 200 µg/mL, they showed schistosomicidal activity, demonstrated by the analysis of various aspects such as darkening of the integument, lack of motility, inability to adhere to the culture plate, and absence of egg in the culture medium.

An important biological target in the study of schistosomicidal activity is the tegument of *S. mansoni* parasites. The degenerative changes in the teguments of the adult worms of the *S. mansoni* parasites, both male and mainly female, are useful to elucidate the mechanism of action. The integument that covers the surface of the parasites, since the physiology of the superficial membrane and the integrity of the integument are fundamental for the development of these parasites. These structures perform essential functions of immunity and nutrient absorption (Da Rocha et al. 2022). With the objective of verifying the superficial and ultrastructural alterations of adult worms treated with concentrations of 200 µg/mL of hydroalcoholic extract of *C. racemosa*.

With the objective of verifying the superficial and ultrastructural alterations of the adult worms treated with hydroalcoholic extract of *C. racemosa*, the parasites were treated, cut, and analyzed by microscopy (Fig. 4A–H).

Figure 4A–D shows the topographic surface of adult female and male parasites from the negative control after 24 h, when all parasites were alive. Observation of the

female's body shows that the oral sucker and the ventral suckers have tubercles, spines or sensory papillae (Fig. 4A) and fissures arranged parallel to the spines (Fig. 4B). Male parasites exhibit a normal parallel arrangement of their folds, a long body with a normal gynecophore canal (Fig. 4C), displaying tubercles with some projections, spicules (Fig. 4D).

After treatment with PZQ (10 µM), all parasites were dead after 24 h. It was noted that the topographic surfaces of these parasites showed some damage, such as the contracted body (males) (Fig. 4E) or contorted, presenting a spiral shape (females) (Fig. 4E and G), with ulcerations caused by bursting of bubbles and loss of tubercles and spicules (Fig. 4F). And females showed swelling and loss of parallel fissures (Fig. 4H).

The treatment of adult worms of *S. mansoni* parasites with the hydroalcoholic extract of *C. racemosa* (200 µg/mL) led to degenerative changes in the tegument. As the extract had an effect on the pairing of adult worms of the *S. mansoni* parasite during the 24 h incubation period, equally severe damage was observed in both male and female parasites, as extensive destruction is observed throughout the integument (Fig. 5A–D).

Adult male worms show muscle contraction (Fig. 5A). In Fig. 5B, the male worms show erosions or ruptures of the integument near the gynecophoric canal and many blisters in addition to the aggressive destruction of the integument. In female parasites, the disintegration of part of the integument and extensive erosions throughout the body (Fig. 5C), as well as desquamation in some areas (Fig. 5D), no longer allowing the display of the parallel fissure's characteristic of females, due to the swelling caused by the extract action.

Yones et al. (2016), evaluating the ethanolic extract, observed tegumentary morphological changes in adult males and females of *S. mansoni* after 12 h of in vitro incubation with concentrations of 100, 300, and 500 µg/mL. Abd-Allah et al. (2022), evaluating different extracts of red sea snail muscles, verified, by scanning electron microscopy, that at a concentration of 50 µg/mL, it alters the tegument of adult parasites. These findings indicate that *C. racemosa* hydroalcoholic extract has schistosomicidal potential.

Conclusion

The results obtained in this work showed that the *C. racemosa* extract has low toxicity; in addition, it can stimulate, activate, and promote the proliferation of immune cells. Furthermore, it was able to stimulate the production of anti-inflammatory cytokines. In antiparasitic assays, it inhibited the growth of all parasites evaluated here. However, it showed better results for the parasites *L. amazonensis*, *S. mansoni,* and *T. cruzi* and low results against strains of *P. falciparum.* This in vitro study showed that the evaluated extract can be used as a potential antiparasitic agent contributed as a new form of treatment of these evaluated parasites.

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Data availability The authors made available the data presented in this work.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could appear to influence the work reported in this article.

Ethical approval The study was approved by the UFPE Animal Ethics Committee (n° 0060/2019 CEUA/UFPE).

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