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Major Article

Evaluation of the antiplasmodial and leishmanicidal potential of *Myrciaria dubia* (Myrtaceae) extract

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Abstract

Introduction: Malaria and leishmaniasis are prevalent in tropical regions, which have environmental characteristics that are highly favorable to protozoa and vectors of these diseases; the transmission of these infections in sub-tropical regions, although recognized, represents only a small fraction of cases. Plants are constantly being used in the search for and acquisition of new drugs, and many compounds derived from them have been used to combat various diseases. In this study, we evaluated the action of the dichloromethanolic extract of *Myrciaria dubia* leaves against the protozoa *Plasmodium falciparum*, *Leishmania amazonensis*, *Leishmania braziliensis,* and *Leishmania chagasi* through bioassays. **Methods**: The extract from *M. dubia* was tested for its anti-*P. falciparum* activity in an anti-histidine-rich protein II immunosorbent assay. The antileishmanial assays were performed using the resazurin method, while cytotoxicity against human hepatoma (HepG2) strain was determined using the colorimetric MTT [3-(4, 5-dimethyl-2- thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide] method. **Results**: The *M. dubia* extract presented a half-maximal inhibitory concentration equal to 2.35 (1.05)µg/mL for *P. falciparum*, 190.73 (6.41) µg/mL for *L. amazonensis,* and greater than equal to 200µg/mL for *L. chagasi* and *L. braziliensis* strains. The cytotoxic concentration for 50% of the cells was above 500µg/mL for HepG2, indicating no toxicity and greater selectivity against parasites. **Conclusions**: The results obtained indicate the presence of antiplasmodial and leishmanicidal bioactive compounds in the dichloromethanolic extracts of *M. dubia* leaves, and point towards future studies to elucidate the mechanism of action for each physiological effect.

Keywords: *Plasmodium falciparum. Leishmania* spp. Camu-camu. Canguçu.

INTRODUCTION

Malaria is endemic in 95 countries, with high prevalence in mainly tropical and subtropical countries situated south of the Sahara desert, in Southeast Asia, Latin America, and the Caribbean, particularly those located in the Amazon Basin region[1]. It is a parasitic disease, with four causative agents, all of which are protozoa belonging to the genus *Plasmodium* (*Plasmodium vivax, Plasmodium ovale, Plasmodium malariae,* and *Plasmodium falciparum*)[2]. *P. falciparum* is the most widely studied since it causes the highest number of deaths. Natural malaria transmission occurs through the bite of female mosquitoes from the *Anopheles* genus, with the most frequent species in Brazil being *Anopheles darlingi*[3].

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Leishmaniasis is an infectious disease caused by protozoa of the genus *Leishmania*. It is endemic in 98 countries, with an estimated 50,000 deaths per year, ranking ninth among infectious diseases. It presents high levels of endemicity, morbidity, and mortality in populations in Africa, Asia, the Middle East, the Mediterranean region, and Latin America[4]. These diseases are transmitted to humans and other mammals through the bite of the female insect *Lutzomyia* spp[5].

Leishmaniasis may present diverse clinical manifestations, affecting the skin, mucous membranes, and internal organs. Cutaneous leishmaniasis may be caused by fourteen *Leishmania* species. Diffuse leishmaniasis is caused by *Leishmania (Leishmania) amazonensis,* the mucocutaneous form is mainly caused by *Leishmania (Viannia) braziliensis*, and visceral leishmaniasis is a parasitic disease involving the *Leishmania donovani* complex, caused by *Leishmania (Leishmania) chagasi* in Brazil[6].

Given the increasing need to obtain and develop new drugs for the treatment and control of diseases for which there is still

no treatment and/or satisfactory cure, natural plant products may be very useful[7]. In the Amazon, many plants are used for therapeutic purposes. These are sometimes the only source of medicine, particularly in isolated and distant places[8].

Camu-camu (*Myrciaria dubia* McVaugh) is a medicinal plant native to the Amazon rainforest, known for its high levels of ascorbic acid, carotenoids, potassium, and polyphenols, such as flavonoids and ellagitannins[9] [10] [11] [12]. The main economic characteristic of this fruit is related to its high ascorbic acid content, which helps combat and prevent the formation of free radicals, making it useful against colds, pulmonary disorders, bladder irregularities, low immunity, and early or natural aging[13] [14].

Myoda et al.[15] studied the effects of the methanol extract from the seeds and peel of *M. dubia* against the following microorganisms: *Staphylococcus aureus*, *Escherichia coli*, and *Saccharomyces cerevisiae*, proposing that the lipophilic and phenolic compounds present in the camu-camu extract are responsible for its antimicrobial activity. Given the phytotherapeutic potential of *M. dubia*, the objective of this study was to evaluate the action of the dichloromethanolic extract obtained from the leaves of *M. dubia* through bioassays against the protozoa *Plasmodium falciparum* and promastigote forms of *Leishmania amazonensis, Leishmania braziliensis,* and *Leishmania chagasi*.

METHODS

Collection of plant material

Samples of *M. dubia* were collected in August 2012, from along the banks of the river Javaés (09°32'S, 50°01'W), in the municipality of Pium, Tocantins, Brazil. The location of the samples was georeferenced by global positioning system (GPS), and the samples were preserved, identified, and registered (registration no. 10.592) in the Herbarium at the Federal University of Tocantins, Porto Nacional Campus.

Preparation and dilution of the *Myrciaria dubia* crude extract

The *M. dubia* extract was prepared using P.A. (Vetec) as a dichloromethane solvent, following the protocol proposed by Rosa et al.[16]. The extract was diluted in 0.5% dimethyl sulfoxide (DMSO) solvent, at varying concentrations (500-1.56µg/mL) for the bioassays.

Continuous cultivation of the erythrocytic phase of the parasite *Plasmodium falciparum*

In the antimalarial activity assays, sanguineous forms of a chloroquine (CQ)-resistant *Plasmodium falciparum* clone W2 were used. Parasites were cultured in O-type Rh^+ human red blood cells with 2% hematocrit and 50mg/mL albumax (Gibco) under the conditions established by Trager and Jensen[17]. To this end, the parasites were grown in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich) culture medium supplemented with 25mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) Hepes (Sigma-Aldrich), 21mM sodium bicarbonate (Sigma-Aldrich), 11mM glucose (Sigma-Aldrich) and 40µg/mL gentamicin, and then maintained at 37°C under a gaseous mixture containing 5% CO_2, 5% O_2 and 90% balanced N_2. The development of the parasites was monitored by daily observation of blood smears stained with Giemsa and analyzed using light microscopy.

Synchronization of parasites for use in *in vitro* tests

The cultures predominantly composed of rings (young trophozoites) used in chemotherapy trials were subjected to a synchronization process with sorbitol, as described by Lambros and Vanderberg[18].

Preparation of the plates for chemotherapy trials

Synchronized parasite cultures were distributed in 96-well microtiter plates by adding 180µL of RPMI culture medium containing 0.05% parasitemia and 1.5% hematocrit to each well. Initially, 20µL of the extract, in triplicate, and in serial concentrations from 100 to 0.097µg/mL were added to the culture plate. The control wells (six per assay) contained infected red blood cells with no added test compounds (negative control). The standard antimalarial drug, artemisinin (ART), was tested in parallel as the positive control in all the experiments in serial dilutions from 50 to 0.7812ng/mL.

The plates were maintained for 72h under ideal conditions for the development of *Plasmodium*. After this period the plates were frozen at -80°C and thawed twice to lyse the erythrocytes[19].

Anti-HRP II enzyme immunoassay

The anti- histidine-rich protein II (HRP II) enzyme immunoassay followed the protocol adopted by Noedl et al.[20]. In this test, the commercial monoclonal primary antibody (Mouse IgM anti-*Plasmodium falciparum* HRP II – Monoclonal) MPFM-55A (ICLLAB) was used at 1µg/mL to sensitize the plates (Maxysorp Nunc). After sensitization, 200µL of a blocking solution (2% PBS-BSA) was added to each well and the plate was incubated at room temperature for 2h. After washing thrice in PBS-Tween 20 (0.05% PBS-T), 100µL of hemolyzed *P. falciparum* culture samples was added to each well. The plate was incubated for 1h at room temperature in a humid chamber, then washed three times with PBS-T, and incubated with the secondary antibody MPFG55P (ICLLAB) (1:5.000) for 1h in the same conditions as mentioned above. Once again, the plate was washed thrice in PBS-T and the reaction was revealed using 100µL of 3.3', 5, 5'-tetramethylbenzidine (TMB) solution for 5 min. The reaction was stopped by adding 50µL per well of a 1M sulfuric acid solution. The optical density was determined using a microplate spectrophotometer (BIO-RAD Model 3550) with a wavelength of 450nm. Each assay was performed in triplicate, in three independent experiments.

Growth inhibition of 50% of the parasites (IC_{50}) was determined using dose-response curves, in non-linear regression function. The software Origin (OriginLab Corporation, Northampton, MA, USA) was used to determine the IC_{50} value. According to Reynertson et al.[21], samples with IC_{50} less than 50µg/mL are considered very active, 50-100µg/mL moderately active, 100-200µg/mL slightly active and a sample with a value above 200µg/mL is considered inactive.

Cultivation of HepG2 cells

The human hepatoma (HepG2) cell lines were cultured according to Calvo-Calle et al.[22]. They were maintained in culture bottles supplemented with RPMI containing 5% fetal bovine serum (FBS) (Gibco/Invitrogen) and 40mg/L gentamycin (Schering-Plough). The cells were maintained in an incubator with 5% CO_2 at 95% humidity and 37 °C. After a confluence of about 80% is attained, the cells were subcultured, or used to perform cytotoxicity assays.

Cytotoxicity test

Cytotoxicity was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method[23]. Cells were seeded at 2×10^4/well and after 24h of treatment with the *M. dubia* extract (500-7.8µg/mL), 20µL of MTT at a concentration of 5mg/mL in PBS (w/v) was added to each well. The plate was left for 4h in an incubator at 37°C. At the end of this period, the culture medium along with the excess MTT was discarded, and then 100µL of DMSO (Sigma-Aldrich) was added to each well. The optical density was read using a microplate spectrophotometer at a wavelength of 570nm. Cells with no added extract were used as negative control.

The 50% cytotoxic concentration (CC_{50}) of the cells was determined from nonlinear regression of the serial dilutions of the extracts using the program Origin.

Selectivity index

The selectivity index (SI) of the samples was evaluated by calculating the ratio of the CC_{50} value and the IC_{50} value. Values greater than 10 were considered to indicate lack of toxicity, while substances with values below 10 were considered toxic[24].

Cultivation of the parasite *Leishmania* spp

Strains of *Leishmania amazonensis* (IFLA/BR/67PH8), *Leishmania braziliensis* (IOCL 566) and *L. chagasi* (IOCL 579) were provided by *Leishmania* Collection from the Oswaldo Cruz Institute (CLIOC-RJ). *Leishmania* spp promastigote forms were cultured at 24°C in RPMI medium (SIGMA®, St. Louis, MO, USA) supplemented with 20% FBS, 2mM L-glutamine, 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and 40µg/mL of Gentamycin (Sigma).

The promastigote cultures were maintained *in vitro* using a parasitic aliquot in stationary growth phase, which was diluted in erythrosin B dye (0.04%). The concentration of the protozoa was estimated with the aid of a Neubauer chamber and adjusted to 1×10^6 promastigotes per mL. The parasites that were stained red were considered dead and those that were birefringent and mobile were considered alive. Only the living parasites were quantified. Consequently, the parasites were placed in RPMI/FBS culture medium and maintained at 24°C, and were subcultured every five days for up to eight passages.

Evaluation of the leishmanicidal potential of the crude extract of *Myrciaria dubia* using the resazurin method

Leishmania amazonensis, Leishmania braziliensis, and *Leishmania chagasi* promastigotes were plated (1×10^6 parasites/well) in 96-well plates and incubated with concentrations ranging from 200 to 1.56µg/mL of extract. Besides the control containing only parasites in culture medium, controls of the solvent 0.5% DMSO and the drug (3µg/mL pentamidine) were also used. The plate was incubated at 24°C for 72h. After this, 20µL of a 2mM resazurin solution was added to each well[25]. The plates were incubated for 4h, and the reduction of resazurin was determined by readings obtained from a fluorimeter (excitation wavelength of 485nm and emission wavelength of 590nm). The control value (RPMI blank) was subtracted from all obtained values. In all, three independent assays were performed and the samples were tested in triplicate. The results were expressed as growth inhibition (GI) rate according to the formula: $\% GI = 100 - [(A - a) / (B - a)] \times 100$. Where: A = Absorbance of treated cells; a = Absorbance of white cells and B = Absorbance of control cells.

RESULTS

Antiplasmodial activity

All assays were performed in triplicate and the results were expressed as the average of the repetitions. The *M. dubia* extract showed anti-*P. falciparum* (chloroquine-resistant strain W2) activity in an enzyme-linked immunosorbent assay (ELISA) using anti-HRPII monoclonal antibodies, involving a specific protein from the parasite, and the results of the bioassays are shown in **Table 1**.

TABLE 1

Evaluation of the dichloromethanolic extract from *Myrciaria dubia* in relation to the IC_{50} (growth inhibition of 50% of the parasites), CC_{50} (cytotoxic concentration for 50% of the HepG2 cells), and SI (selectivity index).

Protozoan	IC_{50} (σ) (µg/mL)	CC_{50} (µg/mL)	SI
Plasmodium falciparum	2.35 (1.05)	> 500	212.77
Leishmania amazonensis	190.73 (6.41)	> 500	2.6
Leishmania braziliensis	≥ 200	> 500	2.5
Leishmania chagasi	≥ 200	> 500	2.5

HepG2: human hepatoma**; IC_{50}:** 50% inhibitory concentration; **CC_{50}:** 50% cytotoxic concentration; **SI:** selectivity index.

The positive control used in the antiplasmodial bioassay was artemisinin, IC_{50} = 6.77ng/mL (3.45). The compound used as the extract solvent (0.5% DMSO) showed no antiplasmodial, leishmanicidal, or cytotoxic effect, excluding the possibility of the solvent acting as an inhibitory agent (data not shown).

Regarding the cytotoxic activity of the extracts on the cell line derived from human hepatoma (HepG2), assessed by the ability of cells to metabolize tetrazolium salt (MTT) to formazan, the cytotoxic concentration for 50% of the cell population (CC_{50}) was calculated based on a dose response. CC_{50} values above 500µg/mL were observed in all repetitions,

indicating that the compounds present in the plant extract did not present toxicity. The SI, considering the IC_{50} data for *P. falciparum,* was greater than 10, confirming that the extract is not toxic to HepG2 cells.

Leishmanicidal test

In the leishmanicidal tests, at a concentration of 200µg/mL, the *M. dubia* extract inhibited the growth of *L. amazonensis* and *L. chagasi* promastigotes up to 85% and 30%, respectively. This inference is based on the results obtained from the resazurin assay. The promastigote forms of *L. braziliensis* continued to proliferate in the presence of the extract (**Figure 1**).

The *M. dubia* extract presented greater inhibitory activity against the *L. amazonensis* strain than that against the other two strains of *Leishmania,* making it possible to calculate a dose response and IC_{50} values (**Table 1**). The positive control used in the anti-leishmanial bioassays was pentamidine, possessing IC_{50} value = 0.21µg/mL (0.09).

DISCUSSION

The results of this study show that *M. dubia* species could be added to the list of Amazonian plants with anti-*P. falciparum* W2 activity. Meneguetti et al.[26] reported that 76 species of plants distributed throughout 32 families in the Amazon presented antimalarial activity. Twenty six of the plant species mentioned previously showed *in vitro* activity against 3D7, W2, FcB1, K1, Dd2, D2, D6, F32, FcM29, and Nigerian strains of *P. falciparum,* particularly against *P. falciparum* and contained 66 active compounds.

Borges et al.[27] reported the antioxidant, antibacterial, and antifungal properties of the leaf, bark, and fruit extracts from plants of the *Myrciaria* genus; these properties are attributable to the presence of several active components including phenolic compounds such as tannins, flavonoids, ellagic acid, and anthocyanins.

Saraiva et al.[28] observed that the *Caesalpinia pyramidalis* Tull extract showed anti-staphylococcal activity attributable to the presence of quercetin, catechins, ellagic acid, flavonoids, proanthocyanidins, and gallic acid. Rauha et al.[29] showed that pure compounds such as flavone, quercetin, naringenin, morin, and kaempferol inhibited *Staphylococcus aureus*. Camu-camu fruits present high levels of ellagic acid, tannins, cyanidin, quercetin, catechin, rutin, and kaempferol[11][12][30][31], components that are related to its antimicrobial activity.

Yapu et al.[32] tested aqueous and ethanolic extracts from the peels of *M. dubia* cultivated in Peru against *P. falciparum*

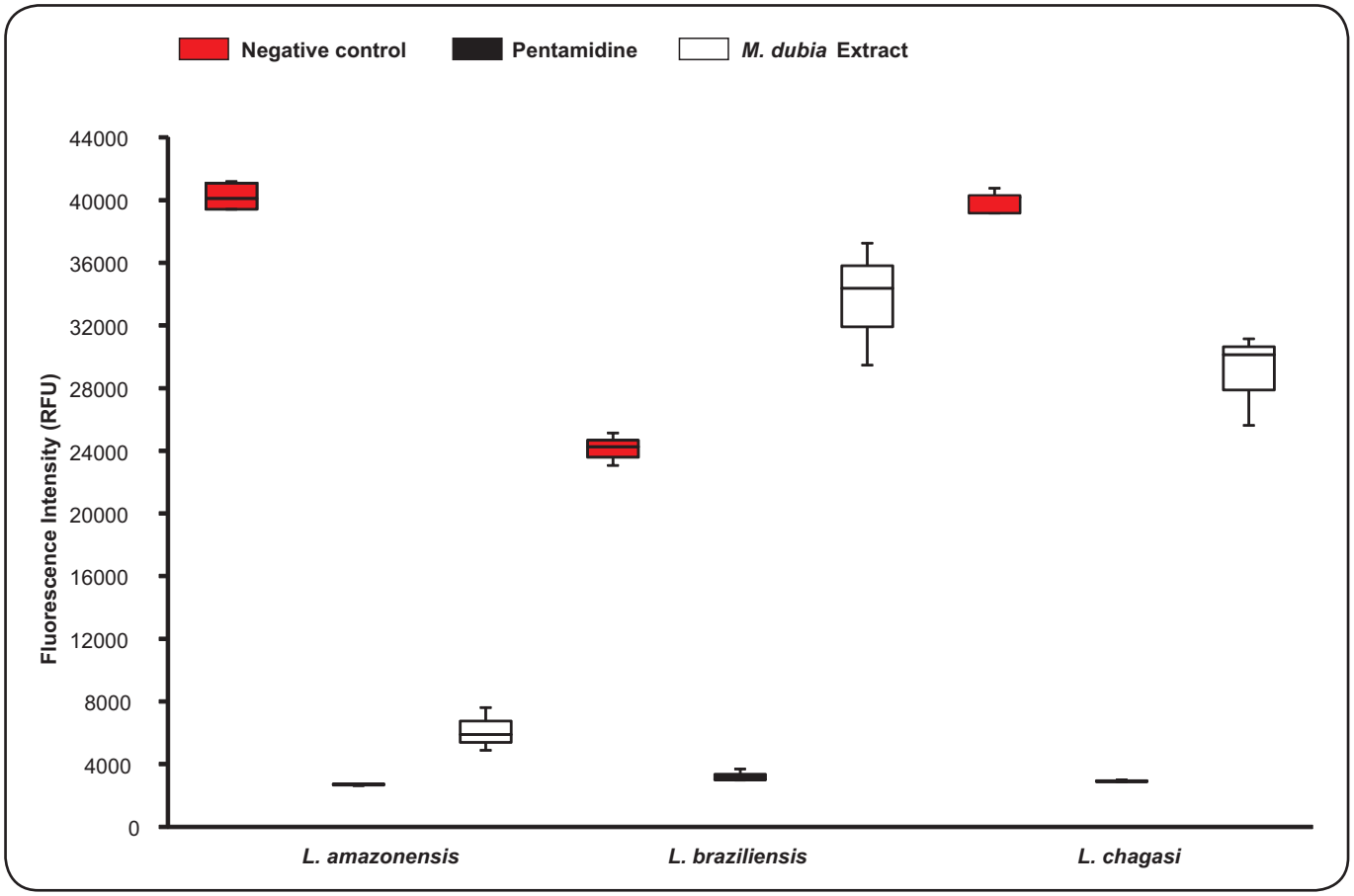

FIGURE 1. Viability of *Leishmania* spp promastigotes, after treatment with 200µg/mL of dichloromethanolic extract from *Myrciaria dubia* and 3µg/mL of pentamidine, as evaluated using the resazurin method. **RFU:** relative fluorescence Units; ***M.*:** *Myrciaria*; ***L.*:** *Leishmania*.

(FCR3 strain resistant to chloroquine), and obtained IC_{50} values equal to 3 and 6μg/mL, respectively. These values are close to those of other species that have activity against *P. falciparum,* such as *Remijia peruviana* (IC_{50} = 7.4μg/mL), *Cinchona officinalis* (IC_{50} = 4.2μg/mL), and *Pubescens cinchona* (IC_{50} = 1μg/mL), and to the value reported for *M. dubia* leaf extracts, 2.35 (1.05)μg/mL in this study.

Colarossi[33] analyzed the cytotoxicity of methanolic extracts from *M. dubia* seeds and pulp on Jurkat cells (immortalized human T lymphocytes). The CC_{50} for the seed extract and pulp extract was >800μg/mL and 524.37μg/mL, respectively, indicating that the methanolic extract of *M. dubia* is non-toxic.

Regarding its leishmanicidal potential, the *M. dubia* extract showed little leishmanicidal activity against *L. amazonensis,* displaying an IC_{50} value of 190.73 (6.41)μg/mL. Bezerra et al.[34] analyzed *Passiflora edulis* (Passifloraceae) and *Syzygium jambolanum* (Myrtaceae) extracts and observed leishmanicidal activity, as indicated by the IC_{50} values of 150.1 and 166.6μg/mL, respectively.

The leishmanicidal effect presented by *P. edulis* may be related to the presence of flavonoid and/or terpenoid compounds in the leaves of these plants[35]. The extract from the leaves of *S. jambolanum* exhibited a leishmanicidal effect, which could be explained by the presence of saponins and condensed and hydrolysable tannins in the leaves[36].

The SIs obtained in this study for *L. amazonensis*, *L. braziliensis,* and *L. chagasi* were less than 10. This result does not indicate the absence of antiparasitic activity, rather it shows that a high dose would be required to achieve the desired therapeutic effect[37], which is not feasible. The high IC_{50} values observed in this study may also be related to the antagonistic effect of other compounds present in the mixture, or to an insufficient amount of the active component responsible for the desired effect. A more detailed analysis of the phytochemical profile of this extract could reveal the existence of purified compounds with potential anti-leishmanial action.

The lack of leishmanicidal activity in inhibition tests against *L. braziliensis* may be attributed to changes in the genetic sequences responsible for promoting variability between species and phenotypically determining the resistance of these organisms, consequently affecting the efficacy of some compounds[38]. *L. braziliensis* is the most prevalent species in humans and is found in all endemic areas in the country. It is considered the most resistant species to diverse treatments[39].

Genomic plasticity can be observed in the *Leishmania* genus, which allows for the adaptation of the parasite to diverse environments, including different vertebrate hosts. This plasticity may influence their resistance to therapeutic drugs[40].

Meneguetti et al.[41] found 32 species of plants in the Brazilian Amazon with leishmanicidal activity distributed throughout 18 botanical families. Of these, 27 presented action against the species *L. amazonensis*, 6 against *L. braziliensis*, and 3 against *L. chagasi*.

Among the species studied for activity against *L. amazonensis*, the extracts with the optimal activity were the hexane and the methanolic ones from the leaves of the species *Lacistema pubescens*, with IC_{50} values of 3.5μg/mL and 3.9μg/mL, respectively. *Lacistema pubescens* possesses antioxidant properties, probably due to the phenolic compounds detected in this species[42], just like *M. dubia* which possesses these same chemical properties[11] [12].

Many plants have substances belonging to the compound classes alkaloids, terpenes, lignans, chalcones, flavonoids, and sesquiterpene lactones, all described in the literature as having effective leishmanicidal and antiparasitic activity[43] [44] [45] [46] [47] [48]. Flavonoids constitute one of the most abundant and widely distributed secondary metabolites in plants. The leishmanicidal activity of this class of metabolites has been observed in *L. peruviana*, *L. braziliensis*[49], and *L. infantum*[50].

The action mechanism of flavonoids on *Leishmania* species was proposed by Mittra et al.[51], who concluded that flavonoids cause apoptosis of the parasite by inducing cleavage of the kinetoplast. Cruz et al.[52] demonstrated that flavonoids, in addition to altering the arrangement of mitochondrial deoxyribonucleic acid (DNA), also inhibit the enzyme arginase, which participates in the parasite's polyamine biosynthesis. Fonseca-Silva et al.[53] demonstrated that treatment of *L. amazonensis* with quercetin, a flavonoid most commonly found in plants, increases the production of reactive oxygen species (ROS), promotes mitochondrial dysfunction in the parasite, and inhibits cell growth.

The leishmanicidal activity evaluated in this study may be related to the presence of both flavonoids and terpenoids in *M. dubia*, since Franco and Shibamoto[54] analyzed the volatile compounds in camu-camu fruits using gas chromatography-mass spectrometry (GC-MS) and identified 21 components. The majority of these were terpenes (98%), predominantly α-pinene (66%), *d*-limonene (24%), and β-caryophyllene (4.6%).

Meslin et al.[55] reported the involvement of terpenoids in the activation of programmed cell death in *Plasmodium* species, and in the interference of one or more substances in the process of cell differentiation of the parasite, which has proven to be an extremely complex process, with fine-tuned regulation of gene expression, resulting in a turnover of proteins and destruction of organelles present in *Plasmodium*, as well as other species of pathogenic protists[56].

To the best of our knowledge, this is the first study to evaluate the action of the dichloromethanolic extract obtained from the leaves of *M. dubia* through bioassays against *P. falciparum* and promastigote forms of *L. amazonensis*, *L. braziliensis,* and *L. chagasi*. This fact highlights the importance of this first report, since the results were satisfactory and contribute to the study of medicinal plants, and the biodiversity of the Amazon.

This study showed that *M. dubia* may be considered an important source for the development of novel drugs for diffuse cutaneous leishmaniasis and malaria treatment, since its extract was active against the parasite *P. falciparum*, presented moderate activity against the promastigote forms of *L. amazonensis,* and was non-toxic to HepG2 cells in *in vitro* tests. However, fractionation of the *M. dubia* extracts, and *in vivo* testing may help in the elucidation and characterization of

the chemical compounds responsible for the inhibitory action of these protozoa.

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Conflict of interest

The authors declare that there is no conflict of interest.

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