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BIOMEDICAL SCIENCES

In vitro evaluation against *Leishmania amazonensis* and *Leishmania chagasi* of medicinal plant species of interest to the Unified Health System

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Abstract: Leishmaniasis is a disease of public health relevance that demands new therapeutic alternatives due to the toxicity of conventional treatments. In this study, 27 plants of interest to the Unified Health System (SUS) were evaluated for cytotoxicity in macrophages, leishmanicidal activity and production of nitric oxide (NO). None of the species demonstrated cytotoxicity to macrophages (CC_{50} >100 µg/mL). Extracts from *Chenopodium ambrosioides*, *Equisetum arvense*, *Maytenus ilicifolia* showed greater efficacy in inducing the death of *Leishmania amazonensis* amastigotes with IC_{50} of 68.4, 82.3, 75.7 µg/mL, respectively. The species *Cynara scolymus*, *Punica granatum* and *Passiflora alata* were the most effective in inducing an increase in the indirect concentration of NO (41.31, 29.30 and 28.86 µM, respectively) in cultures of macrophages infected with *L. amazonensis*. Furthermore, *Punica granatum* was also the most effective species in inducing an increase in NO in macrophages infected by *Leishmania chagasi* (19.90 µM). The results obtained so far support the continuation of studies, with the possibility of developing safer and more effective treatments for leishmaniasis, using natural products. The identification of plants that stimulate the production of NO in macrophages infected by *Leishmania* opens doors for more detailed investigations of the mechanism of action of these natural products.

Key words: Leishmanicidal agents, nitric oxide, phytotherapy, treatment with natural extracts, MTT assay.

INTRODUCTION

Leishmaniasis is part of a complex of parasitic diseases caused by protozoa of the genus *Leishmania*, transmitted by the bite of sandflies, which are small insect vectors. *Leishmania* parasites have two main parasitic forms that change depending on the host. Promastigote forms are found in the digestive tract of sandflies (Serafim et al. 2021). When transmitted to mammalian hosts, promastigotes are targeted by the monocytic phagocytic system, differentiate into amastigotes and multiply within phagocytic cells, causing the symptoms of leishmaniasis (Loría-Cervera & Andrade-Narvaez 2020).

Depending on the causative species, leishmaniasis can cause different forms of disease, with different clinical aspects and manifestations. Manifest leishmaniasis (CL) is the most common disease and manifests itself

with skin lesions. Mucocutaneous leishmaniasis (CML) affects the skin and mucous membranes, which can lead to deformities and breathing difficulties (Hoyos et al. 2019). Visceral leishmaniasis (VL) or kala-azar is a more serious form and mainly affects the liver, spleen and bone marrow, if not specifically treated it can be fatal (Van-Griensven & Diro 2019).

Leishmaniasis is an endemic disease in 98 countries and territories, with a prevalence of 12 million cases per year, an annual incidence of 1 million new cases and 30 thousand deaths per year. In addition, about 1 billion people live in areas at high risk of infection from the disease, being classified by the World Health Organization (WHO) as one of the five largest parasitic diseases in the world (Georgiadou et al. 2015, WHO 2020).

However, despite its epidemiological importance, the therapeutic arsenal for the treatment of leishmaniasis is limited and highly toxic, being considered an extremely neglected disease by the pharmaceutical industry (Jain & Jain 2018, Efstathiou & Smirlis 2021). The drugs of the first choice for the treatment of the disease are pentavalent antimonials since the 1950s. The second line of treatment consists of amphotericin B (conventional or liposomal), pentamidine and miltefosine, the latter being the only drug on the market that can be administered orally (Mcgwire & Satoskar 2014, Uliana et al. 2018).

Therefore, there is an urgent need to search new therapeutic options for the treatment of leishmaniasis, as well as an urgent need to develop new prototypes that are easy to administer (preferably orally active), selective and with a high therapeutic index, capable of properly activate the immune system of the infected host, with leishmaniasis being one of the neglected diseases whose development of new therapies is considered a priority by the WHO (Croft & Coombs 2003, Vijayakumar & Das 2018, Van-Griensven & Diro 2019).

Given this context, there is a growing interest in the Ministry of Health of Brazil about the study of natural products and medicinal plants, evidenced by the dissemination of the Brazilian National List of Medicinal Plants of Interest to the Unified Health System (known in Brazil as RENISUS) in 2006, through the inclusion of herbal medicines in the list of medicines available for pharmaceutical assistance in the basic health network since 2006, such as due to the creation of National Program of Medicinal Plants and Herbal Medicines in 2009 (BRASIL 2009, Marmitt et al. 2016).

Keeping in mind the potential of natural products to develop new therapeutic alternatives for several diseases, including leishmaniasis, this work seeks to investigate the leishmanicidal activity of 27 commercially acquired medicinal plants and is listed in RENISUS. The approach described in this study aims to meet the need for research on new therapeutic alternatives based on medicinal plants useful for the possible development of new drugs for the treatment of the disease.

MATERIALS AND METHODS

Plant material and preparation of aqueous extracts

The species of plants were obtained from commercial sources from Erva Doce & Doce Erva (Maceió, AL, BRA). The aqueous extracts were prepared according to De Queiroz et al. 2014. Briefly, the aerial parts of *Allium sativum, Anacardium occidentale, Bauhinia forficata, Chenopodium ambrosioides, Copaifera langsdorffii, Curcuma longa, Cynara scolymus, Equisetum arvense, Eugenia uniflora, Glycine max, Matricaria recutita, Maytenus ilicifolia, Mentha piperita, Morus alba, Passiflora alata,*

Persea americana, Phyllanthus niruri, Polygonum acre, Psidium guajava, Punica granatum, Ruta graveolens, Syzygium jambolanum, Vernonia spp., *Zingiber officinale,* were dried in an oven at 40 ºC for 96h, pulverized, and processed with watch by infusing. Fresh succulent leaves of *Aloe vera* were crushed in an electric grinder, and the resultant slurry was used as the aqueous extract from this plant. The stem bark of *Stryphnodendron adstringens and Schinus terebinthifolia* were also dried in an oven at 40 ºC for 96h, pulverized, and processed with watch by decoction. The solutions were filtered and sterilized by filtering through sterile 0.22 µm membranes. For the experiments, the dry weight of each aqueous extract per mL was measured to determine the amount of solution required to achieve a given concentration in each well. The aqueous extracts obtained were subjected to *in vitro* activity assays.

Parasite culture

Promastigotes of *L. amazonensis* (MHOM/BR/77/LTB0016) were obtained from Dr. Eduardo Caio Torres dos Santos (Oswaldo Cruz Institute - Fiocruz). Promastigotes of *L. chagasi* (MCAN/BR/89/BA262) were obtained from Dr. Valéria de Matos Borges (Gonçalo Moniz Research Center, Fiocruz-BA). The parasites were maintained *in vitro* in Schneider's medium, supplemented with 10% FBS and 2% human urine at 27ºC in a BOD incubator.

J774.A1 murine macrophage culture

The adherent-phenotype murine macrophage line, J774.A1, was cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma) supplemented with 10% FBS at 37ºC in 95% humidity and 5% CO_2.

Cytotoxicity assay

To evaluate the cytotoxic activity against the J774.A1 cell line, the host cells were plated in 96-well vessels at 2 x 10^5 cells per well in complete culture medium 10% FBS at 37 ºC. After 1 h, the wells were washed with warm HBSS to remove non-adherent cells, leaving approximately 10^5 adherent macrophages. All cultures were done in complete RPMI supplemented with 10% FBS. Aqueous extracts and reference drugs were added in serial concentrations (0.1, 1, 10 and 100 µg/mL for plant extracts, pentamidine and miltefosine; and 0.1, 1, 10, 100 and 300 µM for meglumine antimoniate). The cells were also cultured with medium free from compounds or vehicles (basal growth control) or in media with DMSO 0.1% (vehicle control). Positive control (dead cells) was obtained by cellular lysis with 1% of Triton 100X in DMEM complete. After 48 h, the cytotoxicity was evaluated by the MTT assay (Mosmann 1983). Data obtained from experiments were expressed as the mean ± standard error of the mean (Mean ± S.E.M.) and statistical differences between the treated and the vehicle groups of experiments were evaluated by ANOVA and Tukey's post-hoc tests. The cytotoxic concentration for 50% of the cells (CC_{50}) was calculated by linear regression analysis from the Kc values at the concentrations used. This constant corresponds to the slope resulting from the graphical representation of the logarithm of growth measurement versus time for each drug concentration.

Antileishmanial assay

Initially, it was realized as a screening test against intracellular amastigotes of *L. amazonensis* and *L. chagasi*. To assess the activity of the test compounds against the amastigote stages of *L. amazonensis* and *L. chagasi*, a cell model of infection was generated on coverglass (Nunes et al. 2005). The murine macrophages (J774.A1

cell line) were prepared in 24-well vessels at $2x10^5$ adherent cells/well, subsequently infected with $2x10^6$ promastigotes on glass coverslips and placed in 1 ml of culture for 24 h. After the infection period, the cells received treatment with aqueous extracts of medicinal plants or reference medicines in serial concentrations (0.1, 1, 10 and 100 µg/mL for plant extracts, pentamidine and miltefosine; and 0.1 , 1, 10, 100 and 300 µM) and maintained for another 24h at 37 °C, 5% CO_2. Subsequently, the coverslips were washed, stained with Giemsa-MayGrünwald, and the intracellular amastigotes were counted in 100 macrophages. The data obtained from *in vitro* experiments were expressed as the means ± S.E.M. of duplicate cultures of representative assays. Significant differences between the treated and control groups were evaluated using ANOVA and Tukey's post-hoc tests. Differences with a p value <0.05 or lower were considered significant. The 50% inhibitory concentration (IC_{50}) was calculated by linear regression analysis from the Kc values at the concentrations used. This constant corresponds to the slope resulting from the graphical representation of the logarithm of growth measurement versus time for each drug concentration.

Nitric oxide dosage

The supernatants of the *Leishmania*-infected macrophages cultures, and treated or not with the aqueous extracts from medicinal plants (100 µg/mL) or pentamidine (100 µM), were collected and kept at -20 ºC until the moment of use. The production of NO will be evaluated indirectly by measuring the production of nitrite (NO_2^-) in the culture supernatant. The nitrite concentration was determined through the Griess reaction, according to (Ding et al. 1988). Briefly, 50 µl of the Griess reagent was added to 50 µl of the supernatant, and after 10 min at room temperature, the absorbance was determined (540nm filter) in a microplate reader. The data were expressed as the mean ± standard error ofthe mean (Mean ± S.E.M.) and significant differences between the treated and vehicle groups were evaluated using ANOVA and Tukey's post-hoc tests.

Statistical analysis

Data obtained from *in vitro* experiments were expressed as the mean ± standard error of the mean (Mean ± S.E.M.) of triplicate cultures of representative assays. Statistical analysis was performed by the program GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA Statistical). Differences between the treated and the control groups were evaluated by ANOVA and Tukey's post-hoc tests. Differences with a p-value less than 0.05 or lower were considered significant.

RESULTS

Cytotoxicity against host cells

A criterion to be evaluated when researching compounds that may have leishmanicidal activity, is the absence of toxicity of these compounds against host cells, in this case, macrophages. Compounds that inhibited 50% or more of macrophage viability were considered cytotoxic. The active compounds were submitted to the determination of their CC_{50} (Table I).

Determined by the MTT assay, the cell viability of the cultures treated with the plants in question was compared to the death pattern obtained in the control cultures (Mosmann 1983). The results of this study showed that none of the 27 plant species showed a cytotoxic effect for macrophages of the J774.A1 strain, evidenced by the viability of the cells in the treatments of 0.1, 1, 10, and 100 µg / mL after 48 hours of incubation. Regarding the drugs used in the standard treatment, at the concentrations

Table I. Determination of the cytotoxicity of the aqueous extracts from 27 medicinal plants listed in RENISUS against macrophages (J774.A1).

Treatment	J774.A1 cell line IC_{50} [a]
Pentamidine	35.5 ± 3.4 µM
Miltefosine	>100 µM
Meglumine antimoniate	>300 µM
Allium sativum	>100 µg/mL
Aloe vera	>100 µg/mL
Anacardium occidentale	>100 µg/mL
Bauhinia forficata	>100 µg/mL
Chenopodium ambrosioides	>100 µg/mL
Copaifera langsdorffii	>100 µg/mL
Curcuma longa	>100 µg/mL
Cynara scolymus	>100 µg/mL
Equisetum arvense	>100 µg/mL
Eugenia uniflora	>100 µg/mL
Glycine max	>100 µg/mL
Matricaria racutita	>100 µg/mL
Maytenus ilicifolia	>100 µg/mL
Mentha piperita	>100 µg/mL
Morus alba	>100 µg/mL
Passiflora alata	>100 µg/mL
Persea americana	>100 µg/mL
Phyllanthus niruri	>100 µg/mL
Polygonum acre	>100 µg/mL
Psidium guajava	>100 µg/mL
Punica granatum	>100 µg/mL
Ruta graveolens	>100 µg/mL
Schinus terebinthifolius	>100 µg/mL
Stryphnodendron adstringens	>100 µg/mL
Syzygium jambolanum	>100 µg/mL
Vernonia spp.	>100 µg/mL
Zingiber officinale	>100 µg/mL

[a]Concentration required to give 50% death of cells (IC_{50}) was calculated by linear regression analysis from the culture growth constant (Kc) values at employed concentrations (100, 30, 10, 3, 1, 0.3 and 0.1 µM or µg/mL). This constant corresponds to the slope resulting from plotting the log of the growth measurement versus time for each drug concentration.

tested, pentamidine was the only one to induce cytotoxicity during the assay, with an inhibitory concentration (CC_{50}) of 35.5 ± 3.4 µM.

In vitro leishmanicidal activity

The evaluation of leishmanicidal activity analyzes the infection rate and proliferation of the evolutionary forms of the parasite (amastigotes) found in host cells (macrophages), by counting intracellular parasites and calculating the 50% inhibitory concentration (IC_{50}) and effect maximum (EM). Determining the cytotoxicity of plants against host cells allows evaluating their tropism against the parasites, so it is preferable that the toxicity is selective only against *Leishmania*, without affecting, therefore, the host cells (De Queiroz et al. 2014).

As shown in Table II, of the 27 plants evaluated, five plant species exhibited significant activity against amastigote forms of *L. amazonensis*, highlighting *Aloe vera* and *Stryphnodendron adstringens* which up to the maximum concentration tested exhibited an EM of 40.4 and 53.0%, respectively. *Chenopodium ambrosioides* (EM of 60.6% and IC_{50} of 68.4 µg/mL), *Equisetum arvense* (EM of 51.8% and IC_{50} of 82.3 µg/mL) and *Maytenus ilicifolia* (EM 59.4% and IC_{50} of 75.7 µg/mL) also exhibited leishmanicidal activity. On the other hand, none of the plant extracts tested showed leishmanicidal activity against *L. chagasi*.

Figure 1 graphically shows the *in vitro* leishmanicidal activity of medicinal plants listed in RENISUS and standard drugs pentamidine, miltefosine and meglumine antimoniate against that were active against amastigote forms of *L. amazonensis*.

Modulation of NO production by medicinal plants

NO plays a fundamental role in the immune system in the defense against bacteria, parasites,

Table II. ***In vitro*** **leishmanicidal activity of 27 medicinal plants listed in RENISUS against amastigote forms of** ***Leishmania amazonensis*** **and** ***Leishmania chagasi*****.**

Treatment	*Leishmania amazonensis*		*Leishmania chagasi*	
	IC_{50} [a]	Maximum effect[b] (% ± S.E.M.)	IC_{50} [a]	Maximum effect[b] (% ± S.E.M.)
Pentamidine	19.4 ± 0.4µM	68.4 ± 4.4 ***	65.1 ± 4.9 µM	66.4 ± 3.7 ***
Miltefosine	22.1 ± 1.8µM	91.0 ± 0.4 ***	13.8 ± 1.7 µM	72.4 ± 0.7 ***
Meglumine antimoniate	251.3 ± 3.7µM	66.9 ± 1.4 ***	> 300µM	41.5 ± 2.6 ***
Allium sativum	>100 µg/mL	NA	>100 µg/mL	NA
Aloe vera	>100 µg/mL	40.4 ± 0.3 **	>100 µg/mL	NA
Anacardium occidentale	>100 µg/mL	NA	>100 µg/mL	NA
Bauhinia forficata	>100 µg/mL	NA	>100 µg/mL	NA
Chenopodium ambrosioides	68.4 ± 1.9 µg/mL	60.6 ± 1.3 ***	>100 µg/mL	NA
Copaifera langsdorffii	>100 µg/mL	NA	>100 µg/mL	NA
Curcuma longa	>100 µg/mL	NA	>100 µg/mL	NA
Cynara scolymus	>100 µg/mL	NA	>100 µg/mL	NA
Equisetum arvense	82.3 ± 1.0 µg/mL	51.8 ± 0.3 ***	>100 µg/mL	NA
Eugenia uniflora	>100 µg/mL	NA	>100 µg/mL	NA
Glycine max	>100 µg/mL	NA	>100 µg/mL	NA
Matricaria racutita	>100 µg/mL	NA	>100 µg/mL	NA
Maytenus ilicifolia	75.7 ± 9.6 µg/mL	59.4 ± 3.0 ***	>100 µg/mL	NA
Mentha piperita	>100 µg/mL	NA	>100 µg/mL	NA
Morus alba	>100 µg/mL	NA	>100 µg/mL	NA
Passiflora alata	>100 µg/mL	NA	>100 µg/mL	NA
Persea americana	>100 µg/mL	NA	>100 µg/mL	NA
Phyllanthus niruri	>100 µg/mL	NA	>100 µg/mL	NA
Polygonum acre	>100 µg/mL	NA	>100 µg/mL	NA
Psidium guajava	>100 µg/mL	NA	>100 µg/mL	NA
Punica granatum	>100 µg/mL	NA	>100 µg/mL	NA
Ruta graveolens	>100 µg/mL	NA	>100 µg/mL	NA
Schinus terebinthifolius	>100 µg/mL	NA	>100 µg/mL	NA
Stryphnodendron adstringens	>100 µg/mL	53.0 ± 4.0 ***	>100 µg/mL	NA
Syzygium jambolanum	>100 µg/mL	NA	>100 µg/mL	NA
Vernonia spp.	>100 µg/mL	NA	>100 µg/mL	NA
Zingiber officinale	>100 µg/mL	NA	>100 µg/mL	NA

[a]IC_{50} is the concentration required to give 50% death of parasites, calculated by linear regression analysis from the Kc values at employed concentrations (100, 30, 10, 3, 1, 0.3, and 0.1 µg/mL or µM). This constant corresponds to the slope resulting from plotting the log of the growth measurement versus time for each drug concentration. [b]Maximum effect is expressed as mean ± standard error of maximum toxicity average of triplicates of a representative experiment. The values of maximum effect were considered significant when **p < 0.01, ***p < 0.001 compared to the vehicle group. Abbreviation: NA, not active.

viruses and tumors, as it has a toxic effect against these microorganisms and tumor cells. In leishmaniasis, NO acts as an inflammatory mediator produced by activated macrophages in an attempt to defend against parasites, however, these pathogens can acquire resistance, inhibiting the microbicidal activity mediated by the host (Henard et al. 2014). In this context, researching and evaluating immunomodulatory substances capable of inducing an increase in NO is of paramount importance and may be one of the mechanisms of action against parasites of the *Leishmania* genus. Thus, by analyzing the culture supernatant of macrophages infected with *L. amazonensis* and *L. chagasi*, the effect of plants and the drugs pentamidine, miltefosine and meglumine antimoniate on NO production was measured. Macrophages were infected with 10 promastigotes of *L. amazonensis* or *L. chagasi* per cell. Cells were kept either without treatment or treated with reference drugs (100 μM) or aqueous extracts of medicinal plants

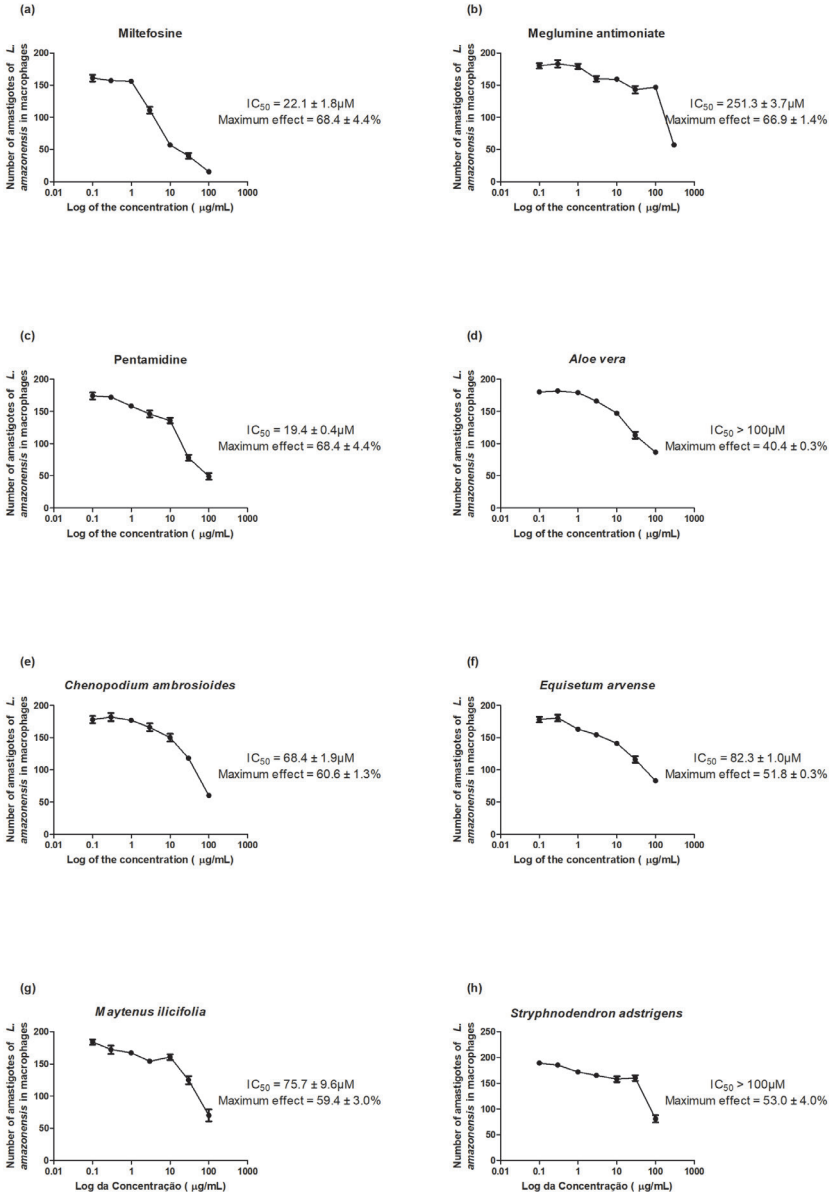

Figure 1. ***In vitro*** **leishmanicidal activity of miltefosine (a), meglumine antomoniate (b), pentamidine (c) as standard drugs; *Aloe vera* (d), *Chenopodium ambrosioides* (e), *Equisetum arvense* (f), *Maytenus ilicifolia* (g), and *Stryphnodendron adstrigens* (h), which are medicinal plants listed in RENISUS against amastigote forms of *Leishmania amazonensis.***

(100 µg/mL). The supernatants were collected and nitrite concentrations were evaluated by Griess reaction at 24 h post-infection (Table III).

According to the results, it is possible to observe that the species *Copaifera langsdorffii* (18.52 µM), *Cynara scolymus* (41.31 µM), *Matricaria recutita* (26.75 µM), *Mentha piperita* (25.30 µM), *Passiflora alata* (28.86 µM), *Phyllanthus niruri* (27.85 µM), *Polygonum acre* (24.30 µM), *Punica granatum* (29.30 µM), *Maytenus ilicifolia* (22.52 µM) and *Stryphnodendron adstringens* (25.19 µM) induced a significant increase in NO production concentration of 100 µg/mL in macrophages infected with *L. amazonensis* when compared to standard drugs. The species *Aloe vera* (7.58 µM), *Anacadium occidentale* (7.48 µM), *Chenopodium ambrosioides* (11.15 µM), *Equisetum arvense* (10.84 µM), *Stryphnodendron adstringens* (7.89 µM), *Eugenia uniflora* (17.15 µM), *Punica granatum* (19.90 µM) and *Syziguim jabolana* (10.84 µM) induced an increase in NO in cultures of macrophages infected with *L. chagasi.*

DISCUSSION

The plants used in the study are low cost, easily accessible and some species already have records in the literature for not showing cytotoxicity, as is the case of *Chenopodium ambrosioides* (De Queiroz et al. 2014). Kwon et al. 2016 demonstrated that the aqueous extract of *Morus alba* did not affect the cell viability of RAW 264.7 macrophages. Kumpunya & Praputbut 2014 demonstrated that at concentrations of 62.5-500 µg/mL the aqueous extract of *Vernonia cinerea* had no effect on macrophage cell viability.

There are few reports of cytotoxicity tests of aqueous extracts, however, records of species belonging to the same genus or other types of extracts were found, whose results may be similar to those found in the present work. Borges et al. 2012 showed that the essential oil of *Chenopodium ambrosioides* showed one of the lowest toxicities found, with a cytotoxic concentration (CC_{50}) of 275.6 lg/mL against macrophages, therefore, it did not show toxicity against mammalian cells. De Araújo Delmondes et al. 2014 demonstrated that hydroalcoholic extracts from the leaves, bark, seeds and stem of *Passiflora cincinnata* did not induce a cytotoxic effect on fibroblasts. *Cynara scolymus* glycolic extract did not induce cytotoxicity against RAW 264.7 macrophages up to the maximum concentration tested, 3.13 mg/ml (Higa et al. 2019).

Leishmaniasis has distinct clinical manifestations depending on the species causing the infection and the immune response developed during an infection. The phylogenetic difference between the species ends up differing in the drug susceptibility profile, as observed in the results of this work (Fotouhi-Ardakani et al. 2016).

Recently, Tariq et al. 2019 found that at a concentration of 50 µg/mL, different fractions of *Aloe vera* leaf extract induced the mortality of *Leishmania tropica* promastigotes by up to 92%. De Queiroz et al. 2014 observed that the aqueous extract of *Aloe vera* inhibited the growth of *L. amazonensis* promastigotes by 75.6% at 100 µg/mL and 26.1% at 10 µg/mL for amastigote forms. Dutta et al. 2007 demonstrated that the leishmanicidal effect of *Aloe vera* leaf exudate on *Leishmania donovani* promastigotes, is associated with programmed cell death.

According to Dutta et al. 2007, in the leaves of *Aloe vera,* the presence of alkaloids, triterpenes, cyanidins, proanthocyanidins, tannins and saponins can be identified. The alkaloids, triterpenes and saponins metabolites have leishmanicidal activity individually or together, acting synergistically. In addition, the leishmanicidal activity of *Aloe vera* seems to

Table III. Effect of 27 medicinal plants listed in RENISUS and the drugs pentamidine, miltefosine and meglumine antimoniate on NO production by *Leishmania*-infected macrophages.

Treatment	Sodium nitrite concentration (μM) in culture supernatant from infected macrophages	
	L. amazonensis	***L. chagasi***
Meio	2.94 ± 0.55	0.30 ± 0.10
DMSO 0.1%	2.87 ± 0.85	0.61 ± 0.17
Pentamidine	1.89 ± 0.44	1.09 ± 0.23
Miltefosine	4.38 ± 3.33	1.53 ± 0.28
Meglumine antimoniate	2.53 ± 1.46	0.62 ± 0.15
Allium sativum L.	6.61 ± 2.99	1.08 ± 0.62
Aloe vera (L.) Burm.f.	11.37 ± 2.71	7.58 ± 0.69 **
Anacardium occidentale L.	6.29 ± 1.47	7.48 ± 2.51 *
Bauhinia forficata Link	10.13 ± 2.85	0.76 ± 0.07
Chenopodium ambrosioides L.	4.94 ± 1.83	11.15 ± 1.76 ***
Copaifera langsdorffii Desf.	18.52 ± 1.71 *	0.49 ± 0.22
Curcuma longa L.	6.59 ± 3.89	0.48 ± 0.16
Cynara scolymus L.	41.31 ± 5.44 ***	0.54 ± 0.11
Equisetum arvense L.	11.08 ± 3.19	10.84 ± 1.73 ***
Eugenia uniflora L.	4.32 ± 1.02	17.15 ± 1.66 ***
Glycine max (L.) Merrill	1.43 ± 1.07	0.67 ± 0.12
Matricaria recutita L.	26.75 ± 4.15 ***	0.53 ± 0.14
Maytenus ilicifolia mart.ex reissek	22.52 ± 5.67 ***	4.43 ± 1.23
Mentha piperita L.	25.30 ± 3.70 ***	0.37 ± 0.05
Morus alba L.	7.29 ± 2.38	0.57 ± 0.17
Passiflora alata Curtis	28.86 ± 3.70 ***	3.03 ± 1.01
Persea americana Mill	2.25 ± 0.54	5.65 ± 1.75
Phyllanthus niruri L.	27.85 ± 3.27 ***	3.87 ± 2.28
Polygonum acre Lam.	24.30 ± 8.46 ***	1.95 ± 1.24
Psidium guajava L.	14.48 ± 1.59	5.55 ± 0.78
Punica granatum L.	29.30 ± 4.67 ***	19.90 ± 0.37 ***
Ruta graveolens L.	15.21 ± 1.40	0.73 ± 0.18
Schinus terebinthifolia Raddi	16.41 ± 4.66	0.41 ± 0.15
Stryphnodendron adstringens (Mart) Coville	25.19 ± 4.96 ***	7.89 ± 0.91 **
Syzygium jambolanum Lam.	13.19 ± 3.06	10.84 ± 1.54 ***
Vernonia spp.	15.63 ± 6.62	1.72 ± 1.18
Zingiber officinale Roscoe	12.09 ± 0.96	0.70 ± 0.19

Results are expressed as mean ± SEM from three independent experiments, each performed in duplicate. Data were analyzed using a one-way ANOVA test with Tukey's post-hoc test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

be closely linked to the anthraquinones found in this plant (Dalimi et al. 2015, Andima et al. 2022). Emodin is an anthraquinone found in *Aloe vera* exudate, the compound has antifungal, antibacterial, antiviral and anti-inflammatory activity (Shi et al. 2013). Dalimi et al. 2015 observed that aloe emodin inhibited the growth of *Leishmania major* amastigotes. *In vivo*, the emodin-based ointment reduces the size of the ulcer and may be a promising agent for clinical trials. Anthraquinone alloenin was also active against antimony sensitive *L. donovani* promastigotes (IC_{50} 26 ± 6.5 μM), with low toxicity against RAW264.7, monocyte and macrophage murine cells (Andima et al. 2022).

Chenopodium ambrosioides was the most potent species in inhibiting amastigotes of *L. amazonensis.* Bezerra et al. 2006 observed the leishmanicidal effect of the hydroalcoholic extract of *Chenopodium ambrosioides* in *L. amazonensis* promastigotes. De Queiroz et al. 2014 also evaluated the leishmanicidal effect of the aqueous extract of *Chenopodium ambrosioides*, however, on promastigote forms of *L. amazonensis*, verifying direct activity of the extract on the extracellular forms of the parasite, inhibiting the growth of the parasites by 87.4% at a concentration of 100 μg/mL.

The mechanism of action of *Chenopodium ambrosioides* is not yet fully understood, but it is known that there is an association with the immunomodulatory effect. *In vivo* tests demonstrated that the crude hydroalcoholic extract of this species promoted an increase in the recruitment of cells to secondary lymphoid organs, in addition to stimulating the macrophage activity evidenced by the increase in the cell's phagocytic capacity (Cruz et al. 2007). Several bioactive compounds are found in *Chenopodium ambrosioides,* including phenolic compounds, unsaturated fatty acids, tocopherols and some sugars (Barros et al. 2013). In addition, this species is abundant in flavonoids, rutin, quercetin and chrysin (Jesus et al. 2018). Previous studies show that rutin and quercetin are active against *L. amazonensis*, through the inhibition of arginases. Arginase is an enzyme responsible for the conversion of arginine into ornithine, which in turn is used in the polyamine pathway essential for the proliferation of *Leishmania* parasites (Da Silva et al. 2019).

In the northeast region of Brazil, the use of *Chenopodium ambrosioides* leaves on the lesions caused by cutaneous leishmaniasis is quite common (Moreira et al. 1998). Patrício et al. 2008 verified that intralesional treatment with crude hydroalcoholic extract of *Chenopodium ambrosioides* was efficient in murine infection by *L. amazonensis*. Therefore, the results obtained in the present study seem to reaffirm and justify the topical use of the plant popularly used in the treatment of ulcers caused by *Leishmania*.

Saeed et al. 2014 also identified leishmanicidal activity of the aqueous extract of *Equisetum arvense*, however, on the promastigote forms of *L. tropica*, showing an IC_{50} of 1.5 μg/mL. In addition, the authors identified a reduction in the concentration of proteins, carbohydrates and total nucleic acids in *L. tropica* promastigotes when treated at a concentration of 1.5 μg/mL. Species of the *Equisetum* genus are rich in minerals and consist mainly of silicon, representing about 15% of total constituents, in addition they have tannins, saponins, alkaloids, flavonoids and essential oils (Husby 2013). The main phenolic compounds found in the extract of *Equisetum arvense* are the various mono-, di- and triglycosides of caempferol, quercetin (isoquercitrin), apigenin and others (Gründemann et al. 2014). The isoquercitrin are uncompetitive inhibitors of *L. amazonensis* arginase (Jesus et al. 2018). This inhibition

may explain the mechanism of action of the *Equisetum arvense* extract shown in this work.

Dos Santos et al. 2013, observed the leishmanicidal effect of two quinonemetide triterpenes isolated from *Maytenus ilicifolia*, maytin and pristimerin. Against the promastigote forms of *L. amazonensis*, maytin and pristimerin presented IC_{50} of 0.09 nM and 0.05 nM and against amastigotes of *L. amazonensis* 0.47nM and 0.88 nM, respectively. This same study also evaluated the leishmanicidal activity of these metabolites in *L. chagasi*. The authors observed that *L. amazonensis* is up to eight times more sensitive to both metabolites when compared to *L. chagasi*, due to the known biochemical and molecular differences between the different *Leishmania* species. This may explain why the *Maytenus ilicifolia* extract was not active against *L. chagasi* amatigotes.

Ribeiro et al. 2014 when evaluating the leishmanicidal activity of fractions of the extract of *Stryphnodendron obovatum* and some of its isolated compounds, observed that gallic acid was the most effective compound against *L. amazonensis* promastigotes, presenting an IC_{50} of 1.7 ± 0.7 µg / mL. In parallel with these results, Lopes et al. 2009, when quantifying the tannins present in the species *Stryphnodendron polyphyllum*, *Stryphnodendron obovatum* and *Stryphnodendron adstringens*, found that in the latter species gallic acid was present in a proportion greater than 60%. High concentrations of gallic acid present in the species *Stryphnodendron adstringens* may justify the activity of this species against *L. amazonensis* amastigotes.

Macrophages are the key cells for the development of an innate response, in which they differ in M1 and M2 phenotypes, thus acting in the control of infection by the parasite. Through a TH1 response, M1 macrophages produce NO in order to cause the death of *Leishmania* species, since such response is mainly involved in the elimination of intracellular pathogens (Lopes et al. 2014). Thus, it is assumed that the induction of NO increase is an alternative mechanism to fight the parasite.

There are few reports in the literature about the immunomodulatory potential of aqueous extracts from these plants, however, the literature reports an increase in NO in other parts of the plants, such as the degalactosylated xyloglucans from *Copaifera langsdorffii* seeds, whose NO production is dose dependent (Do Rosário et al. 2017). In addition, polysaccharides from another species of the genus *Passiflora* demonstrated immunomodulatory activity, promoting an increase in NO production in a concentration-dependent manner, on macrophages RAW264.7 (Song et al. 2020). Likewise, the aqueous extract of *Phyllanthus niruri* induced an increase in the release of NO in a dose-dependent manner in mononuclear cells of the peripheral blood (Putri et al. 2018).

Given these results, the species *Maytenus ilicifolia* and *Stryphnodendron adstringens* stand out, because in addition to the production of NO, they also induce leishmanicidal activity against *L. amazonensis*. Therefore, it can be inferred that the production of NO constitutes a possible mechanism of action of these plants, but not unique, since NO alone is not able to induce the death of the parasites, as shown by the other plants which also induced an increase in NO, but were not active against parasites.

An example of this is what happens with the compound S-nitrosoglutathione (GSNO), belonging to the family of NO donors called S-nitrosothiols. Despite being a NO donor, the effect of GSNO on promastigote forms of *L. amazonensis* is due to S-trans nitrosation of parasite proteins and not to the release of NO (De Souza et al. 2006). An interesting fact is that the plants *Aloe vera*, *Chenopodium*

ambrosioides, *Equisetum arvense* and *Stryphnodendron adstringens*, totaling four of the five species active against *L. amazonensis*, induced an increase in NO for macrophages infected with *L. chagasi*, however none of them have leishmanicidal activity for this species. Previous studies have shown that *Aloe vera* leaf exudate induced a dose-dependent increase in NO in macrophages infected with *L. donovani* (Dutta et al. 2007). The treatment of macrophages with crude hydroalcoholic extract of the leaves of *Chenopodium ambrosioides* induced a significant increase in NO in a dose and time dependent manner (Cruz et al. 2007).

It is known that, although belonging to the same genus, the more than 20 species of *Leishmania* described in the literature differ in biochemical and molecular aspects, which may imply the sensitivity of different species of *Leishmania* to the same compounds, including sensitivity to NO (Croft et al. 2006). The role of NO is not yet fully elucidated, because, despite being a key molecule in the defense of macrophages against *Leishmania* parasites, this molecule is also produced by the parasites themselves. However, its concentration, production time and exposure time seem to be determining factors for the cytotoxic activity of NO (Wink & Mitchell 1998, Acuña et al. 2017, Muxel et al. 2017). The combination of all these factors may explain the fact that, even in the presence of NO, additional pathways are necessary to induce leishmanicidal activity, especially in *L. chagasi*.

CONCLUSIONS

According to the in vitro tests carried out with aqueous extractive solutions from 27 medicinal plants present in RENISUS, it was observed that none of the plants showed cytotoxic potential for macrophages of the J774.A1 strain. However, the species *Aloe vera*, *Chenopodium ambrosioides*, *Equisetum arvense*, *Maytenus ilicifolia* and *Stryphnodendron adistringens* induced a significant leishmanicidal effect against amastigote forms of *L. amazonensis*. Next, it was observed that of the 27 plant species tested, ten induced an increase in NO levels in macrophages infected with *L. amazonensis*, with emphasis on *Maytenus ilicifolia* and *Stryphnodendron adstringen*s, which were active against *L. amazonensis* amastigotes. In addition, eight plant species induced an increase in NO in macrophages infected with *L. chagasi*. The plant species that induced leishmanicidal and immunomodulatory activity can be considered promising and open to further studies. As evidenced, five of them were able to directly inhibit intracellular parasites and 16 induced an increase in NO, which can facilitate the death of the parasite. These results open perspectives for a more detailed investigation on the mechanism of action of these natural products and the feasibility of phytochemical and biological studies for the isolation and identification of new molecular skeletons that may be useful in the design of active molecules against *Leishmania* spp.

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