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Screening of six medicinal plant species for antileishmanial activity

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This study is aimed to investigate the *in vitro* antileishmanial activity of ethanolic, aqueous or dichloromethane extracts of leaves, flowers, fruits or roots, of six medicinal plant species, namely, *Nectandra megapotamica*, *Brunfelsia uniflora*, *Myrcianthes pungens*, *Anona muricata*, *Hymenaea stigonocarpa* and *Piper corcovadensis*. After isolation and analysis of chemical components by ultra-high performance liquid chromatography-high-resolution tandem mass spectrometry (UHPLC-HRMS/MS), the extracts were also tested for toxicity in J774.A1 macrophages and human erythrocytes. Phenolic acids, flavonoids, acetogenins, alkaloids and lignans were identified in these extracts. Grow inhibition of promastigotes forms of *Leishmania amazonensis* and *Leishmania braziliensis* and the cytotoxicity in J774.A1 macrophages were estimated by the XTT method. The most promising results for *L. amazonensis* and *L. braziliensis* were shown by the ethanolic extract of the fruits of *Hymenaea stigonocarpa* and dichloromethane extract of the roots of *Piper corcovadensis*, with IC_{50} of 160 and 150 μg mL^{-1}, resp. Ethanolic extracts of *A. muricata* (leaf), *B. uniflora* (flower and leaf), *M. pungens* (fruit and leaf), *N. megapotamica* (leaf), and aqueous extract of *H. stigonocarpa* (fruit) showed $IC_{50} > 170$ μg mL^{-1} for *L. amazonensis* and > 200 μg mL^{-1} for *L. braziliensis*. The extracts exhibited low cytotoxicity towards J774.A1 macrophages with CC_{50} > 1000 μg mL^{-1} and hemolytic activity from 0 to 46.1 %.

Keywords: *Piper corcovadensis*, *Hymenaea stigonocarpa*, antileishmanial, *Leishmania amazonensis*, *Leishmania braziliensis*, cytotoxicity, hemolysis

Leishmaniasis is an infectious disease caused by protozoa of the genus *Leishmania*. The clinical manifestations depend on the species of *Leishmania* and the host immune response. Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis and

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causes skin lesions, mainly ulcers on exposed body parts, leaving life-long scars and serious disability or stigma. *Leishmania braziliensis* is associated with the cutaneous and mucocutaneous form (MCL), whereas *Leishmania amazonensis* is related to the diffuse cutaneous forms (DCL) (1). MCL and DCL are the most severe forms due to the destructive disfiguring lesions, which can result in increased individual and social stigma as well as suicidal ideas (2). Approximately 95 % of cutaneous leishmaniasis occur in the American continent, Mediterranean bay, Middle-East and Central Asia. The World Health Organization has estimated that about 600 thousand to 1 million new cases occur worldwide a year, and more than 90 % of the cases of MCL occur in Bolivia, Brazil, Peru and Ethiopia (3).

For decades, the first choice to treat this disease has been the utilization of pentavalent antimony, and the second choice has utilized treatment drugs such as amphotericin B and pentamidine. However, these drugs present high toxicity, resistance and parenteral administration, high cost and relatively long treatment regimen (4). Other drugs have also been used as an alternative tool in cases of therapeutic failure, abandon and parasitic resistance, such as imidazole, miltefosine, paromomycin and liposomal amphotericin B. Nevertheless, not all the drugs are available or accessible to the most affected populations, making necessary to search for new sources of bioactive molecules essential to discover new medicines and alternative therapies (5).

Over the years, humans have been using nature to treat a broad spectrum of diseases, mainly infectious and parasitic ones (6). The need for new, safer and more effective treatments of leishmaniasis stimulated research with natural products obtained from plants with antileishmanial activity. A lot of studies have shown the potential of medicinal plants to treat several diseases, working as a base for the formulation of phytotherapeutic drugs or as a source to obtain active principles (7).

In this context, this study is aimed to chemically characterize nine extracts belonging to six different medicinal plants belonging to different families, to investigate their effects on the promastigote forms of *L. amazonensis* and *L. braziliensis*, and also, evaluate their cytotoxicity to J774.A1 macrophages and erythrocytes.

EXPERIMENTAL

Plant material

The exsiccata data and the location of the collection are presented in Table I for five native species of Brazil: *Brunfelsia uniflora (*Pohl.) David Don, *Hymenaea stigonocarpa* Martius ex. Hayne, *Myrcianthes pungens* (Otto Berg) Diego Legrand, *Nectandra megapotamica* (Spreng) Mez and *Piper corcovadensis* (Miq.) C. DC, and one introduced and well-acclimated species in Brazil, *Annona muricata* Linnaeus.

Crude extracts. – The crude extracts of *N. megapotamica* leaves, *B. uniflora* leaves and flowers, *M. pungens* fruits and leaves and *H. stigonocarpa* fruits were obtained by dynamic maceration utilizing 96 % (*V*/*V*) ethanol as solvent. For the fruits of *H. stigonocarpa,* an aqueous extract was prepared. The crude extract of *A. muricata* leaves was obtained in 95 % (*V*/*V*) ethanol. For the *P. corcovandesis*, roots were extracted in dichloromethane using a Soxhlet apparatus. All extracts were concentrated in a rotary evaporator (Tecnal®, TE-211, Brazil) at 40 °C to obtain the crude extracts.

Ultra-high performance liquid chromatography – high-resolution tandem mass spectrometry (UHPLC-HRMS/MS)

The extracts were analyzed by UHPLC (Nexera X2, Shimadzu, Japan) coupled with HRMS (QTOF Impact II, Bruker Daltonics Corporation, USA) equipped with an electro -spray ionization source. The capillary voltage was operated in negative and positive ionization mode, set at 4500 V, and with an endplate offset potential of –500 V. The dry gas parameters were set at 8 L min^{-1} at 200 °C with a nebulization gas pressure of 4×10^5 Pa. Data were collected from *m/z* 50 to 1300 with an acquisition rate of 5 spectra per second, and the ions of interest were selected by auto MS/MS scan fragmentation. Chromatographic separation was performed using a C18 column (75 × 2.0 mm i.d., 1.6 µm, Shim-Pack XR-ODS III, Shimadzu). The gradient mixture of solvents A (H_2O) and B (acetonitrile) was as follows: 5 % B 0–1 min, 30 % B 1–2 min, 95 % B 2–8 min, maintained at 95 % B 8–12 min, at 40 °C. The identification of the compounds was proposed from a review of secondary metabolites found in species belonging of the genus *Brunfelsia, Hymenaea, Myrcianthes, Nectandra, Piper* and *Annona*. In addition, the mass error value was considered (the difference between the calculated exact mass and the experimental exact mass) (8). The mass error was calculated using the following equation:

$$\text{Mass error} = \frac{(\text{exact} - \text{ion precursor})}{\text{ion precursor}} \times 10^6 \text{ in ppm (parts per million)}$$

Cultivation and maintenance of promastigote forms

Promastigote forms of *L. amazonensis* (MHOM/BR/1977/LTB0016) were provided by the *Leishmania* Collection from Oswaldo Cruz Institute (CLIOC, Rio de Janeiro, Brazil) and *L. braziliensis* (MHOM/BR/1987/M11272) were isolated from the patient carriers of American cutaneous leishmaniasis, attending the Laboratório de Ensino e Pesquisa em Análises Clínicas of the Maringá State University, Brazil, identified in the Instituto Evandro Chagas, Brazil (9). Promastigote forms were cultivated and kept at 25 °C through weekly transplants in cultivation medium 199 (Sigma-Aldrich, USA) supplemented with 10 % (*V*/*V*) fetal bovine serum (FBS, Cultilab, Brazil), 2 mmol L^{-1} L-glutamine and antibiotics (100 IU mL^{-1} penicillin and 0.1 µg mL^{-1} streptomycin (Sigma-Aldrich).

Antileishmanial activity

To evaluate the growth inhibition of promastigote forms of *Leishmania*, the parasites were cultivated until they reached the stationary phase. A suspension was prepared in RPMI 1640 medium to contain 4×10^7 mL^{-1} of parasites.

The parasite's viability was determined using the colorimetric method based on the reduction of the tetrazolium salt (XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) (Sigma-Aldrich) to formazan, a colored, water-soluble compound (10). Briefly, the extracts were dissolved in Tween 80 and serially diluted with RPMI 1640 (Sigma-Aldrich, USA) from 1000 to 1.5 µg mL^{-1} in 96-well culture plates (TPP, Switzerland). Amphotericin B (AmB, Laboratório Cristalia, Brazil) was diluted in RPMI 1640 from 125 to 0.24 µg mL^{-1}. A suspension in RPMI medium of *L. amazonensis* promastigote forms (100 µL) from a logarithmic growth phase (4×10^6 parasites per 100 µL) was added to each well.

After 24-h incubation at 25 °C, 100 µL of a solution containing XTT (0.2 mg mL^{-1}) and phenazine methosulfate (PMS, Sigma, USA) (0.2 mmol L^{-1}) were added to each well.

The plates were incubated for 3 h at 37 °C. The results were measured in a microplate reader (ASYS Expert Plus, ASYS Hitech GmbH, Austria) at 450/620 nm. Amphotericin B was utilized as a reference drug and positive control of the treatment (parasite death). The IC_{50} (minimum concentration for 50 % inhibition) was calculated from a polynomial regression of the data comparing it to the negative control (non-treated parasites). The tests were performed in duplicate and replicated at least three times.

Cytotoxicity against J774.A1 macrophages

J774.A1 macrophages were cultivated in RPMI 1640 medium and distributed in 96-well plates at the concentration of 1×10^6 cells mL^{-1} at 37 °C in an atmosphere containing 5 % of CO_2. Then, the cultivation medium was removed and the cells were washed several times with RPMI 1640 to remove non-adherent cells. The extract (1000 to 1.5 µg mL^{-1}) was added to each well. After 24 h of cultivation, 100 µL of XTT was added to each well. The plates were incubated for 3 h, and the absorbance was measured by a microplate reader at 450/620 nm. The cytotoxic concentration sufficient to inhibit macrophages by 50 % (CC_{50}) was calculated by the polynomial regression of data compared to the control of non-treated cells. The selectivity index (SI) was determined using the following equation:

$$\text{SI} = CC_{50} \text{ in cells}/IC_{50} \text{ in } \textit{Leishmania}$$

The tests were performed in duplicate and replicated at least three times.

Hemolytic activity

The toxicity to red blood cells was determined according to the method described by Valdez *et al*. (11) with modifications. Human blood samples were obtained by venipuncture from six healthy women donors, aged 21–60 years old, non-smokers, who were not on any medication, and suffered from no chronic diseases. The donors completed "Consent form" agreeing to participate in the research. Approval of the Ethics Committee has been obtained.

Briefly, a 6 % suspension of fresh defibrinated human blood was prepared in sterile 1 % glucose saline solution. The extracts were serially distributed into culture plates (96 wells) at concentrations ranging from 1000 to 1.5 µg mL^{-1}. In sequence, 100 µL of suspension of red blood cells was added. After 2 h of incubation at 37 °C, the culture plates were centrifuged at 3000 *g* for 5 min (Rotina 420, Hettich, Germany), and the hemolysis was measured in the supernatant using a microplate reader (Asys Hitech, Austria) at 550 nm. A 4 % solution of Triton X-100 (Sigma-Aldrich) was utilized as a positive control, whereas the suspension of non-treated cells was used as a negative control. The solvent used in the negative control was sterile 1 % glucose saline solution. The results were expressed in the hemolysis percentage. The tests were performed in duplicate and replicated at least three times.

Statistical analysis

The results were first analyzed by the Shapiro-Wilk test to verify the normality distribution of the data. Given the assumption of normality, the data were subjected to analysis

of variance by the *F*-test and the means were analyzed by the Scott-Knott test. Statistical differences were considered significant with *p*-value of less than 0.05.

RESULTS AND DISCUSSION

Chemical characterization

In this study, nine extracts obtained from plants belonging to six different botanical families were selected, and their botanical name, traditional use and biological activity described in the literature are presented in Table I. We have performed a qualitative analysis of these plants parts by UHPLC-HRMS/MS, in positive and negative mode (Table II), as well as the antileishmanial activity against promastigote forms of *L. amazonensis* and *L. braziliensis* (Table III).

The chemical analysis of the aqueous extract of *H. stigonocarpa* fruits identified epicatechin and flavonoid glycoside, nicotiflorin, and epicatechin in the ethanolic extract. Fig. 1 shows a representative chromatogram (negative mode) of the ethanolic extract (Fig. 1a) and aqueous extract (Fig. 1b) of *H. stigonocarpa* fruits. Epicatechin was one of the main compounds present in the sap extracted by the perforation of the trunk and stem bark of *H. stigonocarpa* in a study by Farias *et al*. (18). Veggi *et al*. (19) and Sasaki *et al*. (20) found epicatechin in *H. courbaril* bark extract. A study of the phytochemical profile of methanolic extract of stem barks and fruit pulp of *H. stigonocarpa* indicated the presence of phenolic compounds, mainly flavonoids and condensed tannins (21).

The compounds piperlonguminine, chingchengenamide, piperine and piperolide were identified in the dichloromethane extract from the roots of *P. corcovadensis* (Figs. 2 a,b). The piperlonguminine was also recognized by Costa and Mors (22) as a constituent of *P. corcovadensis* roots extract. Facundo *et al*. (23) isolated chingchengenamide. Piperine was isolated by Bezerra *et al*. (24) in *P. tuberculatum* root extract, whereas Mata *et al*. (25) identified piperolide in the extract of *P. sanctum*. Thus, the results found in the present work are in agreement with the ones previously reported in the literature regarding the composition of the dichloromethane extract from the roots of *P. corcovadensis*.

From the ethanolic extract of *A. muricata* leaves the chromatogram was obtained in the negative (Fig. 3a) and positive (Fig. 3b) ionization mode; it was possible to identify the presence of flavonoids (rutin, nicotiflorin and quercitrin), acetogenins (annomuricin A, annoreticuin-9-one and annonacin) and alkaloids (isoboldine and liriodenine). Rutin (26), nicotiflorin (27), quercitrin (28), annonacin and annomuricin (29), annoreticuin-9-one (30), liriodenine (31) and isoboldine (32) have already been isolated from *A. muricata* extract in the previous studies. Acetogenins were mainly obtained from leaves, roots and seeds and to a lower portion from the stem of *A. muricata* (33).

Phenolic compounds derived from cinnamic acid (caffeic acid, chlorogenic acid and ferulic acid), coumarin (scopoletin), an alkaloid (brunfelsamidine) and flavonoid (rutin) were identified in ethanolic extract of *B. uniflora* flowers (Fig. 4a,b), whereas scopoletin and brunfelsamidine were found in ethanolic extract of *B. uniflora* leaves (Fig. 5a,b). The presence of scopoletin isolated from the roots of *B. uniflora* ethanolic extract has been already reported (34), as well as caffeic and chlorogenic acid of the leaves (35). The alkaloid brunfelsamidine is present in the leaves, stems and roots of this same species (36).

Table I. Botanical and pharmacological data of the studied plants

Botanical name (family)	Popular name	Medicinal use (reference)	Location of collection (exsiccata number)
Annona muricata L. (Annonaceae)	Graviola, guanabana	Antioxidant, antimicrobial, anti-inflammatory, insecticide, larvicidal, cytotoxic for cancer cells (12)	Umuarama, Paraná, Brazil (282 HEUP)
Brunfelsia uniflora (Pohl.) D. Don (Solanaceae)	Manacá	Antimicrobial and antifungal (13)	Alto Paraná, Paraná, Brazil (2855 HEUP)
Hymenaea stigonocarpa Mart. ex. Hayne (Fabaceae)	Jatobá, jatoba-do-cerrado	Antiinflammatory (14)	Uberlândia, Minas Gerais, Brazil (43687 (HUFU)
Myrcianthes pungens (O. Berg) D. Legrand (Myrtaceae)	Guabiju, guabijueiro, guabira-guaçú, ibariú, ibaviú	Antioxidant (15)	Paranavaí, Paraná, Brazil (1721 HEUP)
Nectandra megapotamica (Spreng.) Mez (Lauraceae)	Canela-preta, canela-do-mato	Antiinflammatory (16)	Esperança Nova, Paraná, Brazil (8523 UNOP)
Piper corcovadensis (Miq.) C. DC (Piperaceae)	João brandinho, falsa Jaborandi	Antifungal (17)	Diamante do Norte, Paraná, Brazil (16706 HNUP)

HEUP – Educational Herbarium of the Universidade Paranaense, Umuarama, Paraná, Brazil, HNUP – Herbarium of the Nupelia, Universidade Estadual de Maringá, Maringá, Paraná, Brazil, HUEM – Educational Herbarium of the Universidade Estadual de Maringá, Maringá, Paraná, Brazil, HUFU – Herbarium Uberlandense, Universidade Federal de Uberlândia, Uberlândia, Minas Gerais, Brazil, UNOP – Herbarium of the Universidade Estadual do Oeste do Paraná, Cascavel, Paraná, Brazil.

Table II. Chemical constituents of plant extracts

Botanical name (type of extract)	Plant part	Compound	Molecular formula	Exact mass (*m*/*z*)	Ion precursor (*m*/*z*)	Mass error (ppm)	t_R (min)
Annona muricata (ethanolic extract)	Leaf	Rutin	$C_{27}H_{30}O_{16}[M-H]^-$	609.1450	609.1446	0.66	4.54
		Nicotiflorin	$C_{27}H_{30}O_{15}[M-H]^-$	593.1506	593.1504	0.37	4.79
		Quercitrin	$C_{21}H_{20}O_{11}[M-H]^-$	447.0918	447.0915	0.67	5.09
		Annomuricin A	$C_{35}H_{64}O_8[M-H]^-$	611.4520	611.4508	1.96	12.05
		Isoboldine	$C_{19}H_{21}NO_4[M+H]^+$	328.1540	328.1531	2.74	3.54
		Isocodein	$C_{18}H_{21}NO_3[M+H]^+$	300.1590	300.1592	0.66	3.74
		Annoreticuin-9-one	$C_{35}H_{62}O_7[M+H]^+$	595.4570	595.4567	0.50	8.48
		Annonacin	$C_{35}H_{64}O_7[M+H]^+$	597.4730	597.4721	1.51	9.21
		Liriodenine	$C_{17}H_9NO_3[M+H]^+$	276.0660	276.0660	0	4.27

Botanical name (type of extract)	Plant part	Compound	Molecular formula	Exact mass (*m/z*)	Ion precursor (*m/z*)	Mass error (ppm)	t_R (min)
Brunfelsia uniflora (ethanolic extract)	Leaf	Scopoletin	$C_{10}H_8O_4[M-H]^-$	191.0338	191.0343	2.62	5.07
		Brunfelsamidine	$C_5H_7N_3[M+H]^+$	110.0712	110.0712	0	0.67
Brunfelsia uniflora (ethanolic extract)	Flower	Caffeic acid	$C_9H_8O_4[M-H]^-$	179.0338	179.0343	2.79	4.06
		Rutin	$C_{27}H_{30}O_{16}[M-H]^-$	609.1450	609.1450	0	4.70
		Chlorogenic acid	$C_{16}H_{18}O_9[M-H]^-$	353.0867	353.0809	16.42	4.81
		Ferulic acid	$C_{10}H_{10}O_4[M-H]^-$	193.0495	193.0502	3.63	5.04
		Scopoletin	$C_{10}H_8O_4[M-H]^-$	191.0338	191.0344	2.64	5.99
		Brunfelsamidine	$C_5H_8N_3[M+H]^+$	110.0712	110.0711	0.91	0.65
Hymenaea stigonocarpa (ethanolic extract)	Fruit	Nicotiflorin	$C_{27}H_{30}O_{15}[M-H]^-$	593.1506	593.1528	3.71	4.81
		Epicatechin	$C_{15}H_{14}O_6[M-H]^-$	289.0706	289.0713	2.42	4.09
Hymenaea stigonocarpa (aqueous extract)	Fruit	Epicatechin	$C_{15}H_{14}O_6[M-H]^-$	289.0706	289.0711	1.73	4.14
Myrcianthes pungens (ethanolic extract)	Fruit	Gallic acid	$C_7H_6O_5[M-H]^-$	169.0131	169.0138	4.14	0.92
		Isoquercitrin	$C_{21}H_{20}O_{12}[M-H]^-$	463.0871	463.0878	1.51	4.78
Myrcianthes pungens (ethanolic extract)		Gallic acid	$C_7H_6O_5[M-H]^-$	169.0131	169.0138	4.14	1.00
		Isoquercitrin	$C_{21}H_{20}O_{12}[M-H]^-$	463.0871	463.0878	1.51	4.71
		Quercitrin	$C_{21}H_{20}O_{11}[M-H]^-$	447.0921	447.0936	3.35	5.11
		Quercetin	$C_{15}H_{10}O_7[M-H]^-$	301.0348	301.0352	1.32	6.12
Nectandra megapotamica (ethanolic extract)	Leaf	Epicatechin	$C_{15}H_{14}O_6[M-H]^-$	289.0706	289.0712	2.07	4.10
		Rutin	$C_{27}H_{30}O_{16}[M-H]^-$	609.1450	609.1471	3.44	4.67
		Nectandrin B	$C_{20}H_{24}O_5[M+Na]^+$	367.1515	367.1579	17.76	3.93
		Galgravin	$C_{22}H_{28}O_5[M+H]^+$	373.2009	373.1980	7.77	6.00
Piper corcovadensis (dichloromethane extract)	Root	Elemicin	$C_{12}H_{16}O_3[M-H]^-$	207.1015	207.1021	2.89	7.50
		Piperlonguminine	$C_{16}H_{19}NO_3[M+H]^+$	274.1433	274.1434	0.36	4.91
		Chingchengenamide	$C_{18}H_{23}NO_3[M+H]^+$	302.1737	302.1744	2.32	5.27
		Piperine	$C_{17}H_{19}NO_3[M+H]^+$	286.1437	286.1430	2.45	5.13
		Piperolide	$C_{15}H_{14}O_3[M+H]^+$	259.0964	259.0952	4.63	5.90

Compound data identified by UHPLC-HRMS/MS in negative and positive mode.
t_R – retention time

Table III. In vitro *antileishmanial activity, cytotoxicity and selectivity indices of plant extracts*

Botanical name	Plant part	Extract	Promastigotes IC_{50} (µg mL^{-1})		CC_{50} (µg mL^{-1})	SI		Hemolysis (%)
			Leishmania amazonensis	*Leishmania braziliensis*		*Leishmania amazonensis*	*Leishmania braziliensis*	
Annona muricata	Leaf	Ethanolic	270 ± 12^{d}	210 ± 10^{b}	560 ± 14	2.2	2.7	1.1 ± 0.0
Brunfelsia uniflora	Flower	Ethanolic	220 ± 5^{c}	460 ± 16^{d}	910 ± 11	4.3	2.0	1.1 ± 0.0
Brunfelsia uniflora	Leaf	Ethanolic	210 ± 8^{c}	480 ± 14^{d}	> 1000	>1	>1	0.02 ± 0.0
Hymenaea stigonocarpa	Fruit	Ethanolic	170 ± 11^{a}	160 ± 4^{a}	> 1000	>1	>1	0.0 ± 0.0
Hymenaea stigonocarpa	Fruit	Aqueous	190 ± 9^{b}	200 ± 8^{b}	> 1000	>1	>1	0.0 ± 0.0
Myrcianthes pungens	Leaf	Ethanolic	540 ± 11^{e}	260 ± 9^{c}	> 1000	1.9	4.0	0.8 ± 0.0
Myrcianthes pungens	Fruit	Ethanolic	180 ± 14^{b}	210 ± 5^{b}	400 ± 35	2.4	1.9	1.3 ± 0.0
Nectandra megapotamica	Leaf	Ethanolic	200 ± 11^{b}	760 ± 16^{e}	> 1000	>1	>1	0.2 ± 0.0
Piper corcovadensis	Root	Dichloromethane	150 ± 4^{a}	150 ± 14^{a}	> 1000	>1	>1	46.1 ± 0.1

IC_{50} – minimum concentration for 50 % inhibition, CC_{50} – cytotoxic concentration sufficient to inhibit macrophages by 50 %, SI – selectivity index (SI = CC_{50}/IC_{50})
Negative control for antileishmanial activity and cytotoxicity is RPMI 1640, for hemolytic activity 1 % glucose saline.
Results are expressed as mean ± standard error of the mean of three independent experiments.
The values of the averages that follow the same letter in the column did not differ significantly by the Scott-Knott test at 5 % probability.

The results obtained by phytochemical analysis of *M. pungens* indicated the presence of gallic acid and isoquercitrin in the ethanolic extract of the fruits (Fig. 6a) and gallic acid, isoquercitrin, quercetin and quercitrin in the ethanolic extract of the leaves of *M. pungens* (Fig. 6b). De Almeida *et al.* (37) identified the flavonoids quercetin and quercitrin in the leaves extract of *M. pungens*. In the extract of the fruit, Andrade *et al.* (15) found quercitrin and isoquercitrin, whereas Seraglio *et al.* (38) detected gallic acid and isoquercetrin, corroborating well to our results. Quercetin and quercitrin have also been previously reported to have *in vitro* antileishmanial activity against *L. amazonensis* (39, 40).

Epicatechin, rutin, nectandrin B and galgravin were identified in the ethanolic extract of *N. megapotamica* leaves (Fig. 7a,b). The tetrahydrofuran lignan known as nectandrin B and galgravin were isolated from crude ethanolic extract of the leaves of *N. megapotamica* in a study by Silva-Filho *et al*. (41), corroborating with our results. Garcez *et al*. (16) identified epicatechin in the extract of *N. megapotamica* stem bark.

Antileishmanial activity

This study demonstrated that extracts from several plant species inhibited the growth of both *L. amazonensis* and *L. braziliensis*. The ethanolic extract of *H. stigonocarpa* fruits and di-

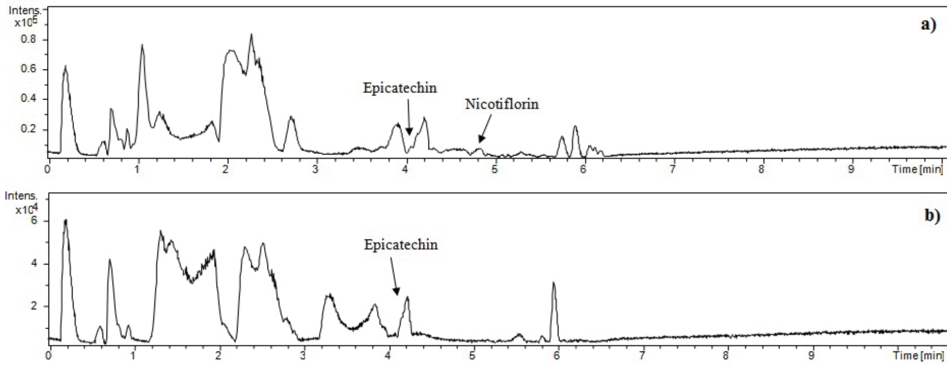

Fig. 1. Chromatographic profile of *Hymenaea stigonocarpa* fruit extracts obtained by UHPLC-HRMS/MS in negative mode: a) ethanolic extract, b) aqueous extract.

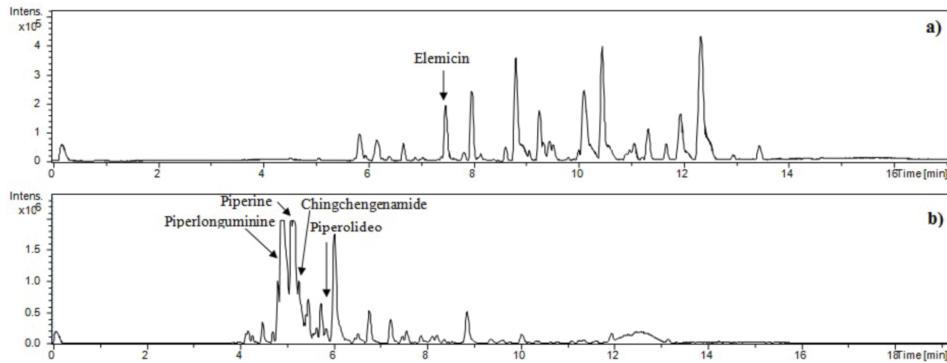

Fig. 2. Chromatographic profile of dichloromethane extract from the roots of *Piper corcovadensis* obtained by UHPLC-HRMS/MS: a) negative mode, b) positive mode.

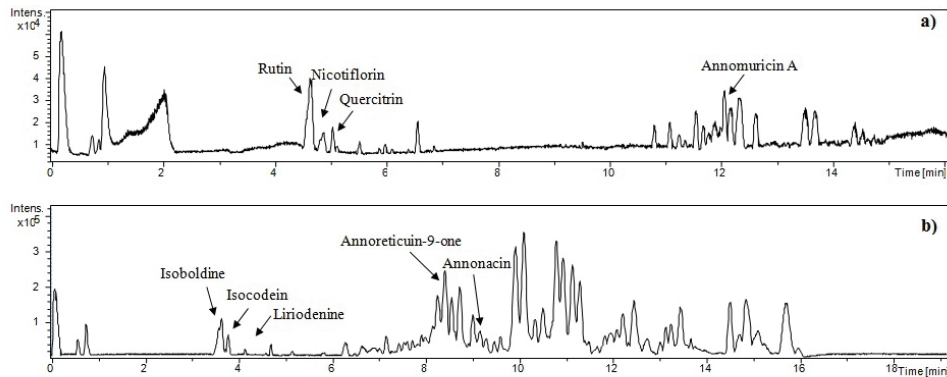

Fig. 3. Chromatographic profile of the ethanolic extract of the leaves of *Annona muricata* obtained by UHPLC-HRMS/MS: a) negative mode, b) positive mode.

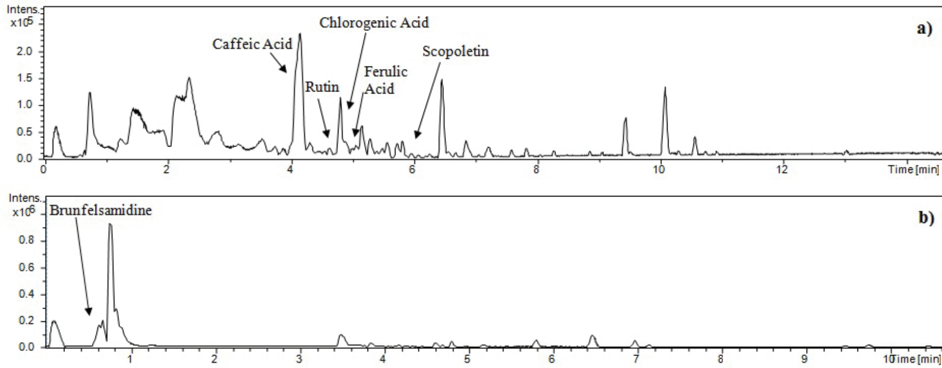

Fig. 4. Chromatographic profile of the ethanolic extract of the flower of *Brunfelsia uniflora* obtained by UHPLC-HRMS/MS: a) negative mode, b) positive mode.

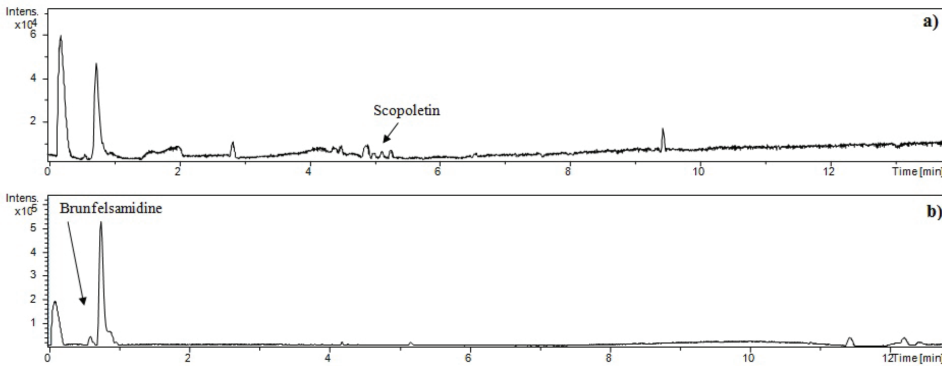

Fig. 5. Chromatographic profile of ethanolic extract of the leaves of *Brunfelsia uniflora* obtained by UHPLC-HRMS/MS: a) negative mode, b) positive mode.

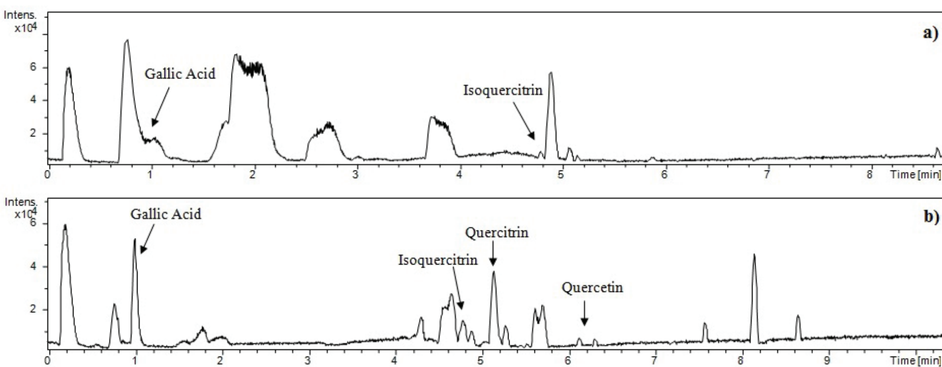

Fig. 6. Chromatographic profile of *Myrcianthes pungens* ethanolic extracts obtained by UHPLC-HRMS/MS in negative mode: a) fruits, b) leaves.

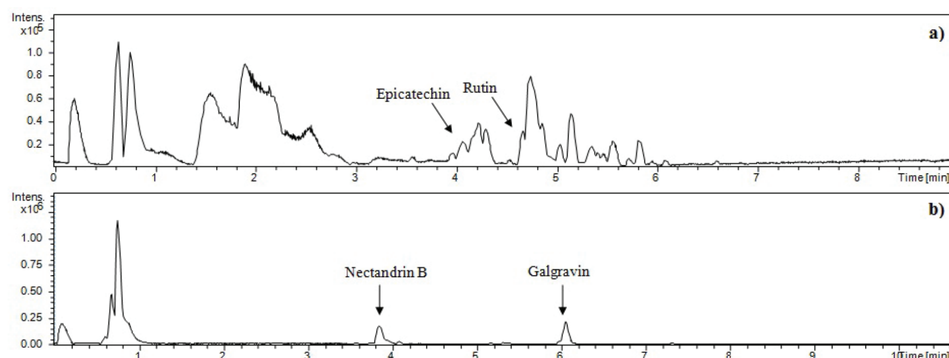

Fig. 7. Chromatographic profile of the ethanolic extract of *Nectandra megapotamica* leaves obtained by UHPLC-HRMS/MS: a) negative mode, b) positive mode.

chloromethane extract from the roots of *P. corcovadensis* were the most active against both protozoans, not differing statistically from each other (Table III).

The IC_{50} values for *H. stigonocarpa* ethanolic extract were 170 μg mL^{-1} for *L. amazonensis* and 160 μg mL^{-1} for *L. braziliensis*, whereas for the aqueous extract it was 190 and 200 μg mL^{-1}, resp. Ribeiro *et al*. (42) found IC_{50} of 199 μg mL^{-1} for hexane extract of *H. stigonocarpa* leaves against *L. amazonensis*, results similar to those found for *H. stigonocarpa* fruit extracts in our study.

Dichloromethane extract from the roots of *P. corcovadensis* presented IC_{50} of 150 μg mL^{-1} for both *L. amazonensis* and *L. braziliensis*. In studies with other *Piper* species, IC_{50} results were similar to those found in this paper. Nakamura *et al*. (43), in a survey with extract of *Piper regnellii* leaves, found IC_{50} of 167 μg mL^{-1} for hydroalcoholic extract, Sales *et al*. (44) found IC_{50} of 143 μg mL^{-1} for the essential oil of *Piper tuberculatum* Jacq. fruit against *L. braziliensis*. Piperlongumine, an alkaloid amide isolated from *P. tuberculatum*, showed antileishmanial activity against *L. infantum* and *L. amazonensis* (45).

The ethanolic extract of *A. muricata* leaves, ethanolic extract of *B. uniflora* leaves and flowers, the aqueous extract of *H. stigonocarpa* fruits, ethanolic extract of *M. pungens* leaves and fruits, and ethanolic extract of *N. megapotamica* leaves showed IC_{50} from 180 to 540 μg mL^{-1} against *L. amazonensis*, whereas against *L. braziliensis* it varied from 200 to 760 μg mL^{-1} (Table III). The IC_{50} shown by amphotericin B was 0.00053 μg mL^{-1} for *L. amazonensis* and 0.0015 μg mL^{-1} for *L. braziliensis*.

Acetogenins in the ethanolic extract of *A. muricata* leaves were probably responsible for the observed antileishmanial activity (46). Although a clear structure-activity relationship has not yet been established for acetogenins, their leishmanicidal activity can be attributed to the number of hydroxy groups or the presence of a single tetrahydrofuran ring in their structure (47).

Cytotoxicity in macrophages J774.A1

To assess the potential application of the tested extracts in the treatment of leishmaniasis, the absence of toxicological effects against the host is required (48). Therefore,

the possible cytotoxic effects of the extracts on J774.A1 macrophages and human red blood cells were evaluated. Our results indicated that the ethanolic and aqueous extracts of *H. stigonocarpa* fruits, dichloromethane extract from the roots of *P. corcovadensis*, ethanolic extract of *N. megapotamica* leaves and ethanolic extract of *B. uniflora* leaves showed low cytotoxicity for J774.A1 macrophages with CC_{50} > 1000 μg mL^{-1} (Table III). The selectivity index (SI) was higher than 1 for all extracts evaluated, indicating a greater selectivity against the parasite in relation to mammal cells. These results are supported by De Almeida *et al*. (49) and Tiuman *et al*. (50), who standardized the selectivity index over 1, suggesting that it indicates higher activity against protozoa and smaller activity against mammal cells.

Hemolytic activity

The extracts exerted low cytotoxicity in human red blood cells. The percentage of hemolysis ranged from 0 to 1.3 % at the highest concentration tested (1000 μg mL^{-1}), except for dichloromethane extract from the roots of *P. corcovadensis* which caused 46.1 % hemolysis (Table III). In a study by De Mello *et al*. (51) amphotericin B showed a strong hemolytic effect, causing 100 and 84.07 % hemolysis at 0.13 and 0.01 mmol L^{-1}, resp. Valdez *et al*. (11) also demonstrated a high percentage of hemolysis of amphotericin, 70 % at 0.01 mmol L^{-1}, concentration much lower than that used in our work.

CONCLUSIONS

In search of new substances with potential antileishmanial action, the extracts of *H. stigonocarpa* fruits and *P. corcovadensis* roots showed promising activity against *L. amazonensis* and *L. braziliensis*, and might be a potential source of active and less toxic compounds for the development of novel therapeutic agents to treat leishmaniasis. To the best of our knowledge, this is the first report on these species against two species of *Leishmania*. Additional experiments should be performed to isolate the active constituents of the most active plants, as well as to evaluate *in vivo* models for the treatment of these diseases.

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