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Anti-leishmania activity of extract and fractions from the stem and leaf of *Montrichardia linifera* (Arruda) schott (Araceae) against *Leishmania amazonensis*

Atividade antileishmania do extrato e frações do caule e folha de *Montrichardia linifera* (Arruda) schott (Araceae) contra *Leishmania amazonensis*

Actividad antileishmania del extracto y fracciones del tallo y hoja de *Montrichardia linifera* (Arruda) schott (Araceae) contra *Leishmania amazonensis*

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Abstract

This study aimed to evaluate the anti-leishmania activity of extracts and fractions from stem and leaf of *Montrichardia linifera* against *Leishmania amazonensis*. The stem (EES) and leaf (EEL) extracts were obtained by maceration of powders with 96º GL alcohol. The extracts were subjected to exhaustive extraction using a reflux system and solvents of increasing polarity, obtaining 4 fractions for each extract: hexane, dichloromethane, ethyl acetate and methanol. The extracts and fractions were submitted to a phytochemical prospecting study. The antipromastigote activity and cytotoxicity in macrophages (J774) were performed using the cell viability test (MTT). In the extract and fractions of the stem, alkaloids, steroids, terpenes, flavonic heterosides, tannins, polyphenols and saponins were detected. In the extract and fractions of the leaves, coumarins, steroids, terpenes, flavonic heterosides, tannins, polyphenols and saponins were detected. In the anti-leishmania evaluation, the hexane fraction of the leaf

(HFL) showed promising activity (IC_{50}=38.56 µg/mL), and the hexane fraction of the stem (HFS) showed moderate activity (CI_{50}=179.3 µg/mL), the extracts and the other fractions were inactive (IC_{50}>200 µg/mL). In the cytotoxicity test, EES and HFS were cytotoxic (CC_{50} 54.82 µg/mL and 26.95 µg/mL, respectively). EEF and HFL showed moderate cytotoxicity (CC_{50} of 162.7 µg/mL and 347.1 µg/mL). As for the selectivity index, the HFL showed high selectivity (SI=90). In summary, fractionation contributed to increase anti-leishmania activity and the selectivity of HFS, such activity may be related to steroids or terpenes.
Keywords: Anti-leishmania; Aninga; Terpenes; Saponins.

Resumo

Este estudo teve como objetivo avaliar a atividade antileishmania dos extratos e frações do caule e da folha de *Montrichardia linifera* contra *Leishmania amazonensis.* Os extratos do caule (EEC) e da folha (EEF) foram obtidos a partir da maceração dos pós com álcool 96º GL. Os extratos foram submetidos à extração exaustiva utilizando um sistema de refluxo e solventes de polaridades crescentes, obtendo-se 4 frações para cada extrato: hexano, diclorometano, acetato de etila e metanol. Os extratos e frações foram submetidos ao estudo de prospecção fitoquímica. A atividade antipromastigota e a citotoxicidade em macrófagos (J774) foram realizados através do teste de viabilidade celular (MTT). No extrato e nas frações do caule, foram detectados alcaloides, esteroides, terpenos, heterosídeos flavônicos, taninos, polifenóis e saponinas. No extrato e frações das folhas, foram detectados cumarinas, esteroides, terpenos, heterosídeos flavônicos, taninos, polifenois e saponinas. Na avaliação antileishmania, a fração de hexano das folhas (FHF) apresentou promissora atividade (CI_{50}=38,56 µg/mL), e a fração de hexano do caule (FHC) apresentou moderada atividade (CI_{50}=179,3 µg/mL), os extratos e as demais frações foram inativos (CI_{50}>200 µg/mL). No teste de citotoxicidade, o EEC e a FHC foram citotóxicos (CC_{50} de 54,82 µg/mL e 26,95 µg/mL, respectivamente). O EEF e a FHF apresentaram moderada citotoxicidade (CC_{50} de 162,7 µg/mL e 347,1 µg/mL). Quanto ao índice de seletividade, a fração FHF apresentou alta seletividade (IS=90). Em síntese, o fracionamento contribuíu para o aumento da atividade antileishmania e da seletividade da FHF, tal atividade pode estar relacionada com esteroides ou terpenos.
Palavras-chave: Anti-leishmania; Aninga; Terpenos; Saponinas.

Resumen

Este estudio tuvo como objetivo evaluar la actividad antileishmania de los extractos y fracciones del tallo y hoja de *Montrichardia linifera* contra *Leishmania amazonensis*. Los extractos de tallo (EEC) y hoja (EEF) se obtuvieron de la maceración de los polvos con alcohol de 96º GL. Los extractos se sometieron a una extracción exhaustiva mediante un sistema de reflujo y disolventes de polaridad creciente, obteniendo 4 fracciones por cada extracto: hexano, diclorometano, acetato de etilo y metanol. Los extractos y fracciones se sometieron a un estudio de prospección fitoquímica. La actividad antipromastigota y la citotoxicidad en macrófagos (J774) se realizaron mediante la prueba de viabilidad celular (MTT). En el extracto y fracciones de tallo se detectaron alcaloides, esteroides, terpenos, heterósidos flavónicos, taninos, polifenoles y saponinas. En el extracto y fracciones de las hojas se detectaron cumarinas, esteroides, terpenos, heterósidos flavónicos, taninos, polifenoles y saponinas. En la evaluación de antileishmania, la fracción de hexano foliar (FHF) mostró actividad prometedora (IC_{50}=38.56 µg/mL) y la fracción de hexano de tallo (FHC) mostró actividad moderada ($CI5_0$=179.3 µg/mL), los extractos y las otras fracciones estaban inactivos (IC_{50}>200 µg/mL). En la prueba de citotoxicidad, EEC y FHC fueron citotóxicos (CC_{50} de 54.82 µg/mL y 26.95 µg/mL, respectivamente). EEF y FHF mostraron citotoxicidad moderada (CC_{50} de 162,7 µg/mL y 347,1 µg/mL). En cuanto al índice de selectividad, la fracción FHF mostró una alta selectividad (IS=90). En resumen, el fraccionamiento contribuyó al aumento de la actividad antileishmania y la selectividad de FHF, tal actividad puede estar relacionada con esteroides o terpenos.
Palabras clave: Anti-leishmania; Aninga; Terpenos; Saponinas.

1. Introduction

Leishmaniasis is an infectious disease caused by a variety of protozoan species of the genus Leishmania (Nord, 2019). The disease mainly affects Africa, Asia and Latin America, being associated with malnutrition, population displacement, poor housing conditions, weakened immune system and lack of resources (Who, 2018). There are two distinct clinical forms, visceral leishmaniasis (VL) and cutaneous leishmaniasis (CT), which are endemic in about 97 countries (Who, 2018), of which 18 countries are in America (Opas, 2018). In 2017, according to the World Health Organization (WHO), Brazil reported about 22,106 new cases of VL and CT.

In the treatment, pentavalent antimonials are used and alternatively amphotericin B; however, they are toxic, have low efficacy and high cost (Ponte-Sucre, et al., 2017). Another issue is the increasing in parasitic resistance, which makes treatment

more difficult and facilitates the spread of infection (Ponte-Sucre, et al., 2017). Therefore, the urgent search for new effective therapeutic alternatives with low toxicity is emphasized, and the study of medicinal plants used in traditional medicine should be prioritized (Who, 2015).

Montrichardia linifera (Arruda) Schott (Araceae), popularly known as Aninga, is found in tropical regions and grows in flooded ecosystems, being distributed in the Amazonian floodplains (Teixeira; Siqueira & Cattanio, 2014). It is a plant with a wide ethnopharmacological spectrum being used in traditional medicine mainly for its healing property (Amarante, et al., 2009). Phytochemical studies suggest that the stem and leaves of the species contain a diversity of secondary metabolites such as: alkaloids, flavonoids, tannins, triterpenes and steroids (Santos, et al., 2014).

Studies show that *M. linifera* has antiplasmodic (Amarante, et al., 2011), insecticide, antibacterial (Miranda, et al., 2015; Santos, et al., 2014), antipromastigote (Silva-Silva, et al., 2017) antioxidant, cytotoxic and healing properties (Santos, et al., 2014). The ethanolic extract of the stem showed antiplasmodic activity in a clone resistant to chloroquine and sulfadoxine (Dd2) with a 50% inhibitory concentration (IC_{50}) between 10 to 100 µg/mL (Amarante, et al., 2011).

Despite the important use in traditional medicine, little is known about the chemical composition of this species and there is a lack of studies about biological activities of extracts, fractions and isolated substances from *M. Linifera*. In this context, this study aimed to conduct a phytochemical prospecting study and evaluate the anti-leishmania activity against *Leishmania amazonensis* of extracts and fractions obtained from stem and leaf of *M. linifera*, in addition to evaluating the cytotoxicity profile of active samples in order to determine the selectivity index.

2. Methodology

2.1 Plant material

The stem and leaves of *M. linifera* were collected from the campus of the Federal University of Pará (UFPA), in Belém-PA, on the right bank of the Guamá River (01°28'41,3'' S; 48°47'29.0'' W), in April 2014. The species was identified by Dr. Alba Lins, from the Botanical Coordination of the Museu Paraense Emílio Goeldi (MPEG), exsicata MG188906.

2.2 Biological material

The parasite used was *Leishmania (L.) amazonensis*, isolated from a human case in Ulianópolis, State of Pará (MHOM/BR/2009/M26361). The murine macrophage cell line (J774.G8) was acquired from the Laboratory of Immunomodulation and Protozoology of the Oswaldo Cruz Institute - IOC/FioCruz by Profª. Dr. Katia da Silva Calabrese.

2.3 Extract obtaining and fractionation

The extract and fractions of *M. linifera* were obtained according to the method described by Silva-Silva et al. (2017). The plant materials, stem and leaf, were selected and cleaned with 70% alcohol, dried in an oven with air circulation around 45° C for 7 days, then the stem was crushed in a hammer mill and the leaves in a blender yielding 800g of stem powder and 620g of leaf powder. The ethanolic extracts were obtained from the powder of the stem (500 g) and leaves (500g) and subjected to extraction by maceration with ethanol (alcohol 96 ° GL). Subsequently, they were concentrated in a rotary evaporator under reduced pressure, until final residue, obtaining the ethanolic extracts of the stem and leaves (EES and EEL).

The extracts were subjected to exhaustive extraction using a reflux system and solvents of increasing polarity (hexane, dichloromethane, ethyl acetate and methanol; P.A. analytical grade of the Vetec brand). For this purpose, 5g of each ethanolic extract was placed in a round bottom flask, the solvent (100 mL) was added and heated (40°C) under reflux for 20 minutes. The procedure was repeated 3 times in each solvent. The fractions obtained were concentrated on a rotary evaporator.

2.4 Phytochemical study

For phytochemical prospecting, the stem and leaf extracts and their respective fractions were subjected to thin-layer chromatography (TLC). The samples were solubilized in methanol (1mg/mL, in Methanol) and applied to glass chromatoplates covered by Merck 60G F254 silica gel, in order to detect the presence of the following groups of secondary metabolites: alkaloids, coumarins, steroids and triterpenes, flavonoid genines, flavonic heterosides, tannins, polyphenols and saponins. For each group, an appropriate eluent system, specific reagents and reference samples (adapted from Wagner, et al., 1984) were used.

2.5 Anti-leishmania activity

The promastigotes of *L. amazonensis* were cultured at 26°C in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco®, Grand Island, NY, USA), penicillin (100U/mL) and streptomycin (100µg/mL; Mota, et al., 2015). The test was carried out in the logarithmic phase, using a suspension of 5 x 10^6 parasites/mL of the culture. These were distributed in 96-well plates previously dosed with stem and leaf extracts and fractions, in different concentrations (200 to 3.125 µg/mL). A culture medium solution with methanol and parasite suspension was used as a negative control, and the drug Amphotericin B, also added to the parasite suspension in concentrations of 25 to 0.3906 µg/mL, was used as a positive control. Then, the plates were incubated (26°C/24h). After 24h, the viability of promastigotes was evaluated by the colorimetric method of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrvoazole bromide (MTT 5mg/mL), with the addition of 10 µg/mL in each well (Mosmann, 1983). The results were expressed from the parasite growth inhibition concentration (IC_{50}). Inhibitory concentration (IC_{50}) was determined by linear regression (Graph Pad Prism version 5.04).

Antipromastigote activity was assessed using the following requirements: active (IC_{50}≤100 µg/mL); moderately active (IC_{50} between 101 and 199µg/mL); inactive (IC_{50}≥200 µg/mL; Mota, et al., 2015; Brígido, et al., 2020).

2.6 Cell viability assay and selectivity index

Cell viability was determined by the MTT method. The J774 macrophage lineage was cultivated in 25 cm^2 culture bottles containing 5 mL in total, using complete RPMI 1640 medium (10% fetal bovine serum). They were incubated in an oven at 37ºC in an atmosphere of 5% CO_2 at a density of 5x10^5/mL. The cells were seeded in the 96-well plates with the extracts (stem and leaf) and with the hexane fractions of the stem and leaf in different concentrations (between 500 and 7.8125 µg/mL). As a negative control, a culture medium solution with RPMI medium and cell suspension was used. After 72h of incubation (37ºC in a 5% CO_2 atmosphere) MTT (5.0 mg/mL) was added. The plate was incubated for another 4h at 37ºC and 5% CO_2. Dimethylsulfoxide (DMSO) was added to solubilize the formazan crystals. The reading on a spectrophotometer was performed at 490 nm. The values of the 50% cytotoxic concentration (CC_{50}) were calculated by linear regression using dose-response curves from three independent experiments. Finally, the calculation of cell viability was performed according to Galucio (2014).

The results of cytotoxicity were expressed as cytotoxic (CC_{50} ≤ 100µg/mL), moderately cytotoxic (CC_{50} between 101 and 500 µg/mL) and non-cytotoxic (CC_{50}≥500 µg/mL; Galucio, 2014, Brígido, et al., 2020). The selectivity index (SI) was calculated based on the ratio between the CC_{50} values of the cells and the IC_{50} of the protozoa (Nakamura, et al., 2006).

3. Results

In the phytochemical prospecting studies, alkaloids, steroids, terpenes, flavonic heterosides, tannins, polyphenols and saponins were found in the extract and fractions from the stem of *M. linifera*. In the extract and fractions from the leaves, coumarins, steroids, terpenes, flavonic heterosides, tannins, polyphenols and saponins were detected (Table 1).

Table 1. Phytochemical prospecting of extracts and fractions from the stem and leaves of *Montrichardia linifera*.

Samples	Secondary metabolites									
	Stem					Leaves				
	EES	HFS	DFS	EAFS	MFS	EEL	HFL	DFL	EAFL	MFL
Alkaloid	-	-	+	+	-	-	-	-	-	-
Coumarin	-	-	-	-	-	-	-	-	-	+
Steroids and Terpenes	-	+	-	-	-	-	+	-	-	-
Flavonic Genines	-	-	-	-	-	-	-	-	-	-
Flavonic Heterosides	-	-	-	+	-	+	-	-	-	+
Tannins and polyphenols	+	-	+	+	+	+	-	+	+	+
Saponins	+	-	+	+	+	+	-	+	+	+

Legend: (+) presence; (-) absence; EES - Ethanolic Extract of the Stem; HFS - Hexane Fraction of the Stem; DFS– Dichloromethane fraction of the stem; EAFS - Ethyl Acetate Fraction of the Stem; MFS - Methanol Fraction of the Stem; EEL - Ethanolic Extract of the Leaf; HFL - Hexane Fraction of the Leaf; DFL - Dichloromethane fraction of the Leaf; EAFL - Ethyl Acetate Fraction of the Leaf; MFL - Methanol Fraction Leaf.

In the antipromastigote activity against *L. amazonensis*, hexane fraction of the stem (HFS) showed moderate activity (IC_{50} = 179.3 µg/mL). The hexane fraction of the leaves, on the other hand, was active (IC_{50}=38.56 µg/mL). The stem and leaf extracts and their respective fractions did not present activity (IC_{50}> 200 µg/mL; Table 2).

The stem and leaf extracts (EES and EEL) and the fractions that showed activity against promastigotes of *L. amazonensis* were subjected to the cytotoxicity test against murine macrophage cell (J774.G8). Both the EES and the hexane fraction of the stem (HFS) showed toxicity (CC_{50} of 54.82 µg/mL and 26.95 µg/mL, respectively). The EEL and hexane fraction of the leaf (HFL) presented moderate cytotoxicity (CC_{50} of 162.7 µg/mL and 347.1 µg/mL, respectively; table 2).

In the selectivity index, the HFS showed low selectivity (SI=0.15). The HFL, on the other hand, was selective showing an SI of 90 (Table 2).

Table 2. Antipromastigote activity (*L. amazonensis*), cytotoxicity and selectivity index of the ethanolic extract and fractions from the stem and leaves of *Montrichardia linifera*.

Samples	*L. amazonensis*		Cytotoxicity (J774.G8)		Selectivity Index
	IC_{50} (µg/mL)	Results	CC_{50} (µg/mL)	Results	
EES	> 200	Inactive	54,82	Cytotoxic	ND
HFS	179,3	Moderate	26,95	Cytotoxic	0,15
DFS	> 200	Inactive	ND	ND	ND
ACFS	> 200	Inactive	ND	ND	ND
MFS	> 200	Inactive	ND	ND	ND
Amphotericin B	0,2763	Active	>100	Moderate	>362
EEL	> 200	Inactive	162,7	Moderate	ND
HFL	38,56	Active	347,1	Moderate	90
DFL	> 200	Inactive	ND	ND	ND
EAFL	> 200	Inactive	ND	ND	ND
MFL	> 200	Inactive	ND	ND	ND

Legend: IC_{50} - 50% inhibitory concentration; CC_{50} - Cytotoxic Concentration 50%; EES - Ethanolic Extract of the Stem; HFS - Hexane Fraction of the Stem; DFS– Dichloromethane fraction of the stem; EAFS - Ethyl Acetate Fraction of the Stem; MFS - Methanol Fraction of the Stem; EEL - Ethanolic Extract of the Leaf; HFL - Hexane Fraction of the Leaf; DFL - Dichloromethane fraction of the Leaf; EAFL - Ethyl Acetate Fraction of the Leaf; MFL - Methanol Fraction Leaf; Moderate – Moderately active; J774.G8 - murine macrophage cell line.

4. Discussion

Montrichardia linifera is widely used in traditional Amazonian medicine, mainly due to the healing properties of the sap and juice of this plant (Amarante, et al., 2009). In addition, it is used as antirheumatic (Macedo, et al., 2005), expectorant (Lins & Oliveira, 1994), diuretic, anti-inflammatory (Piedade; Schöngart & Junk, 2005), antiulcer (Plowman, 1969), in the treatment of persistent coughs (Rodrigues, 2007), diabetes, tuberculosis (Van Andel, 2000) and impingens (Amarante, et al.,

2009).

In this context, the plant species must contain biologically active substances. However, there is a lack of studies about the chemical composition and biological activities of the species that may eventually be useful against human infections caused by parasites, such as leishmaniasis, which is a serious public health problem in tropical and subtropical developing countries. (Moo-Puc; Robledo & Freile-Pelegrin, 2008).

In phytochemical prospection studies of the extract from the stem and leaves of *M. linifera*, the presence of alkaloids, flavonoids, tannins, steroids and triterpenoids were noted, and the absence of anthraquinones and saponins (Amarante, et al., 2011; Costa, et al., 2009). These results corroborate in part with the present study, the difference is that in our work, anthraquinones and saponins were also identified, this fact can be explained by the seasonality in the collection of plant material.

In the evaluation of anti-leishmania activity, the hexane fractions from the stem (FHC) and leaf (FHF) of *M. linifera* were the only samples that inhibited parasitic growth, with HFS showing moderate activity (IC_{50}=179.3 µg/mL) and HFL showed promising activity against promastigotes of *L. amazonensis* (IC_{50}=38.56 µg/mL). Such activity may be related to the chemical composition of these fractions, in both samples, steroids and terpenes were detected. Studies show that terpenes have antipromastigote and amastigote activity (Morales-Yuste, et al., 2010; Rosa, et al., 2003; Brandão et al., 2020). The lipophilic characteristic of terpenes facilitates their penetration into the lipid bilayer of cell membranes and can produce important changes in the mitochondrial membrane of different pathogens, modifying their integrity and permeability (Burt, 2004). In addition, terpenes can produce changes in the chromatin of the trypanosomatid kinetoplast, also generating an increase in the volume of the mitochondria, which can lead the parasites to death (Rosa, et al., 2003).

In the evaluation of cell viability, the ethanolic extract of the stem (EES) and the hexane fraction of the stem (HFS), were the only samples that presented cytotoxicity to macrophages (CC_{50} of 54.82 µg/mL and 26.95 µg mL, respectively), while the ethanolic extract of the leaves (EEL) and the hexane fraction of the leaf (HFL) showed moderate cytotoxicity (CC_{50} of 162.7 µg/mL and 347.1 µg/mL, respectively). These samples have saponins and terpenes, and the interaction of terpenes and saponins with biological membranes can justify the cytotoxicity of the extracts and their respective fractions, in addition, previous studies showed the cytotoxic activity of saponins against tumor cells (Bitchi, et al., 2019; Zhang, et al., 2019).

The effect of saponins may be related to different mechanisms that interfere with the process of cell homeostasis and depending on the chemical skeleton it is possible to differentiate saponin activity, which can generate mitochondrial damage (Jayatilake, et al., 2003), damage to the cellular membrane (Man, et al., 2010) and interfere with the progression of the cell cycle (Hsu; Kuo & Lin, 2004).

When we analyze the most promising sample in anti-leishmania activity, the HFL, there is a decrease in cytotoxicity compared to the extract (EEL), thus, the extract fractionation contributed to increase the anti-leishmnia activity and decreased toxicity against murine macrophages. Such result, directly inferred in the selectivity of this sample (SI = 90), the HFL has more specificity to cause damage to Leishmania than to macrophages. Therefore, HFL is a promising sample when we analyze the anti-leishmania activity, however, further studies for the isolation and identification of biologically active compounds are necessary, in order to seek even more selective compounds.

5. Conclusion

In summary, our results suggest that *Montrichardia linifera* is a promising species *in vitro* against *Leishmania amazonensis*. We observed that extracts fractionation contributed to leishmanicidal effect and increase selectivity, especially the hexane fraction of the leaf (HFL) that proved to be the most promising sample. The positive activity results were obtained

from fractions; therefore, further studies are needed to assist in isolate the substances responsible for anti-leishmania activity, which can improve the inhibitory effect against the parasite and consequently increase selectivity.

In addition, new studies can be carried out to evaluate the action against intracellular amastigote forms, in an attempt to obtain promising substances, necessary to investigate the effect of *M. linifera* against *Leishmania* sp. in animal models.

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