Journal of Pharmacy and Pharmacology 5 (2017) 654-660
doi: 10.17265/2328-2150/2017.09.007

Anti-promastigote Activity of the Amazon Plants

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Abstract: This study evaluated in vitro activity of ethanol extract, fractions, and isolated substance from Amazon species against promastigotes of *L. amazonensis*. The ethanol extracts were concentrated and fractionation. The anti-promastigote activity was evaluated through the cell viability assessment method (MTT). The ethanol extract, fractions, and isolated substance from *Himatanthus articulatus* and *Parahancornia fasciculata* were inactive in promastigote of *L. amazonensis*, as the ethanol extract of *Physalis angulata.* The hexane fractions from different parts of *Montrichardia linifera* showed anti-promastigote activity probably due to the presence of steroids and terpenes. All species in studies were inactive, except of *M. linifera*. The few polar constituents can be responsible for the activity. Therefore, the isolation and purification of the active on *L. amazonensis* promastigotes are urgently required.

Key words: Leishmania, himatanthus articulatus, parahancornia fasciculata, montrichardia linifera, physalis angulata.

1. Introduction

Leishmaniasis is caused by the protozoan *Leishmania* which are transmitted by the bite of infected female phlebotomine sandflies. There are three main forms of the disease: VL (visceral leishmaniasis), CL (cutaneous leishmaniasis) and ML (mucocutaneous leishmaniasis). An estimated 900, 000-1.3 million new cases and 20,000 to 30,000 deaths occur annually. Cutaneous leishmaniasis is the most common. About 95% of CL cases occur in the Americas, the Mediterranean basin, the Middle East and Central Asia [1].

Pharmacological treatments for CL include pentavalent antimony, pentamidine and amphotericin B [2]. However, the increase of drug therapeutic failure has emerged as a serious problem. The therapeutic failure has been related to growing resistance to first-line drugs. The ABC (ATP-binding cassette) transporters have been associated with drug resistance. The *Leishmania* over expressing LABCG2 were resistant to antimony. The overexpressing LABCG2 reduced accumulation of Sb^{III} due to an increased in drug efflux [3].

Due to the toxicity and the failure of many drugs available, the search for alternative therapies for leishmaniasis is urgent. The plants used in traditional medicine can be a valuable source of study for the discovery of alternative treatments. In the Brazilian Amazon, distinct species are used to heal wounds or leishmaniasis. Among these stand out: *Montrichardia linifera* (Arruda) Schott [4], *Himatanthus articulates* (Vahl) Woodson [5, 6] and *Parahancornia fasciculata* (Poir.) Benoist [7].

The aqueous extract obtained from roots of *Physalis angulata* class was active in promastigote and amastigote forms [8]. Substances isolated from *P. angulata* showed activity against *L. major* and *L. amazonensis* [9]. This study evaluated the inhibitory activity of *Himatanthus articulatus, Parahancornia fasciculata, Montrichardia linifera* and *Physalis*

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angulata on the growth of promastigotes of *Leishmania amazonensis.*

2. Methods

2.1 Plant Material

Plants were collected at State of Pará, Brazil (Table 1). These were identified and voucher specimens were deposited in the herbarium "João Murça Pires" of the Museu Paraense Emílio Goeldi (MG).

2.2 Obtaining and Fractionation of Extracts

Plants were dried at room temperature for seven days and the material was powdered. The ethanol extracts were prepared by maceration, and concentrated in a rotary evaporator. The extracts were fractioned by extraction under reflux or column chromatographic, to obtain fractions of different polarities (hexane, dichloromethane, ethyl acetate and methanol). The hexane fraction of the different parts of *M. linifera* was fractionated by extraction under reflux and the methanol fraction of stem bark of *H. articulatus* was fractionated by chromatography column. The pure compounds were identified by NMR (nuclear magnetic resonance).

^{1}H-NMR and ^{13}C-NMR were obtained in equipment Bruker Avance DPX 200, at 25 °C. The NMR used deuterated methanol (Merck, Germany) solvent to solubilize the samples.

Plumeride: NMR^{1}H-5.3(d), 7.5(d), 3.9(m), 6.5(dd), 5.5(dd), 4.5(dd), 7.4(d), 1.4(d), 3.7(s), 4.7(d); NMR ^{13}C-94.1 (C1), 152.4(C3), 111.0(C4), 40.3(C5), 141.3(C6), 129.9(C7), 97.8(C8), 50.5(C9), 150.2(C10), 138.6(C11), 172.7(C12), 63.4(C13), 22.4(C14), 168.4(C15), 52.9(C16), 100.0(C1'), 74.6(C2'), 77.4(C3'), 71.2(C4'), 77.7(C5') and 62.5(C6').

Lupeol: NMR ^{1}H-3.15(dd), 2.37(dt), 4.61 and 4.5(d); NMR^{13}C-38.7 (C1), 27.4(C2), 79.0(C3), 38.8(C4), 55.3(C5), 18.35(C6), 34.3(C7), 40.9(C8), 50.4(C9), 37.2(C10), 20.9(C11), 25.2(C12), 38.1(C13), 42.8(C14), 27.5(C15), 35.6(C16), 43.0(C17), 48.0(C18), 48.3(C19), 150.99(C20), 29.9(C21),40.04(C22), 28.01(C23), 15.38(C24), 16.1(C25), 16.0 (C26), 14.6(C27), 109.34(C29) and 19.3(C30).

2.3 Phytochemical Screeenig

The extracts subjected to phytochemical screening were used for major secondary metabolites identification by Wagner et al. [10] methodology.

2.4 Antipromastigotes Activity of Leishmania Amazonensis

Leishmania (L.) amazonensis strains isolated from mucocutaneous leishmaniasis (ML, MHOM/BR/2009/M26361) were obtained from the Instituto Evandro Chagas, Ananindeua, Brazil.

The promastigotes were obtained after primary isolation on NNN blood slopes. The strains were sub-cultured, and adapted to RPMI. The MCL were cultivated at 26 ºC in RPMI 1,640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) [11].

Culture of promastigote forms in logarithm phase was adjusted to 5×10^{6} parasites/100 μL. The susceptibility

Table 1 Collection site and number of voucher specimen of the species included in the work.

Species	Collection site	Number of voucher specimen
Himatanthus articulatus	Terra do Meio, Pará State, Brazil S 41°10′86″; W 41°53′51.6″	MG 206619
Parahancornia fasciculata	Moju, Pará State, Brazil S 02°10′52.2″; W 048°47′43.9″	MG 20270
Montrichardia linifera	Right bank of Rio Guamá, Belém, Pará State, Brazil S 01°28′41.3″; W 48°47′29.0″	MG 188906
Physalis angulata	Universidade Federal do Pará, Belém, Pará State, Brazil S 1º 28′17″; W 48º26′54″	MG 203914

test was performed in 96-well plates. The extracts were tested in triplicate in a concentration gradient (200 to 3.125 μg/mL). The negative control contained only parasites and the incubation medium, and the positive control was made with amphotericin B (25-0.3906 μg/mL). After 24 hours of incubation at 26 ºC, it was addicted 10 μL of tetrazolium salt (5 mg/mL), and the parasites were quantified in enzyme-linked immunosorbent-assay plate reader. The IC_{50} was determined by linear regression (Graph Pad Prism version 5.04). The results were classified as: $IC_{50} \leq 100$ μg/mL was considered active, IC_{50} between 101 and 200 μg/mL was considered moderate active, and $IC_{50} \geq$ 200 μg/mL were considered to be inactive [11].

3. Results

3.1 Phytochemicals Studies

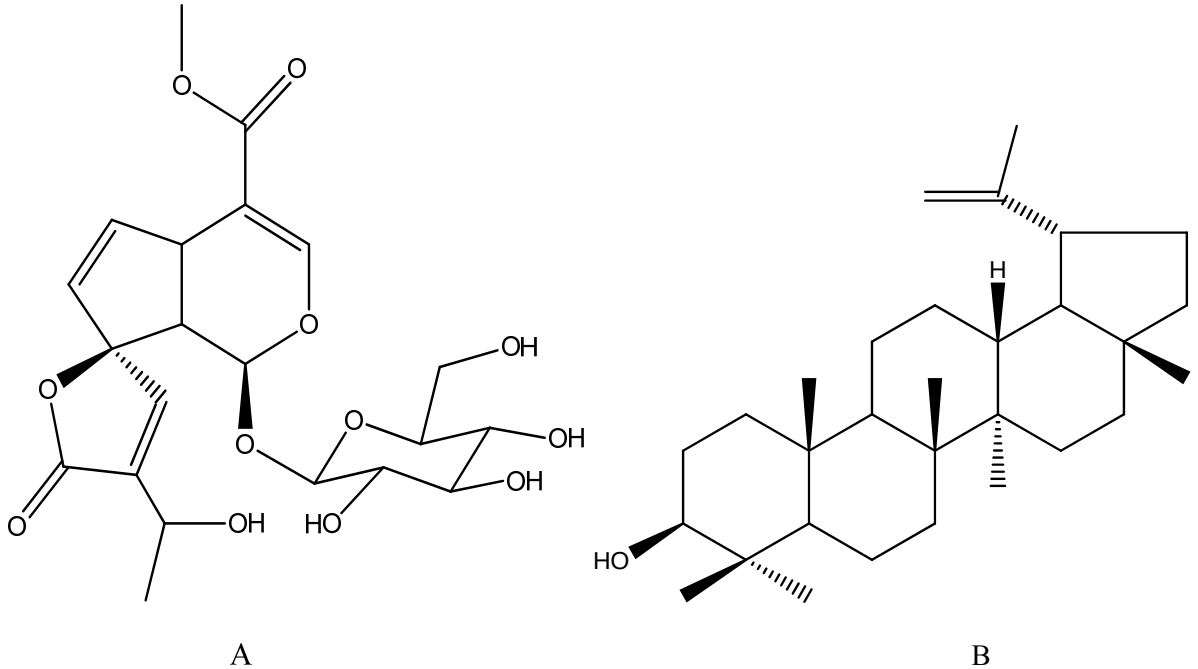
The methanol fraction was obtained by fractionation of ethanol extract from H. articulatus, and the plumeride (Fig. 1) was isolated from this fraction with the NMR results consistent with the literature data [12]. The ethanol extract from *P. fasciculata* was subjected to a reflux system, and the extract was separated into four increasingly polar fractions. The lupeol (Fig. 1) was extracted from the ethanol extract of *P. fasciculata* by fractionation in chromatographic column.

The *P. angulata* and *M. linifera* extracts were submitted preliminary to phytochemical analysis. *P. angulata* extracts probably contain phenols and tannins, steroids and triterpenes, coumarins, and alkaloids. The extract of *M. linifera* probably contains alkaloids, coumarins, steroids and triterpenes, flavonic heterosides, phenols and tannins, and saponinas (Table 2).

3.2 Anti-promastigote Activity

The ethanol extract of *H. articulatus* did not show activity against promastigotes of *L. amazonensis*. Fractionation did not contribute to anti-promastigote activity. Similarly, ethanol extract of *P. fasciculata* was inactive and fractionation did not interfere in the activity. The *P. angulata* extracts were not active in promastiogote (Table 3).

The extracts obtained from different parts of *M. linifera* were inactive. However, fractionation of these extracts resulted in active hexane fractions (Table 3). These fractions should contain steroids and terpenes.



A B

Fig. 1 Chemical structure of compouds plumeride (A) and lupeol (B).

Table 2 Phytochemical screening of *Himatanthus articulates, Parahancoria farciculata, Physalis angulata* and *Montrichardia linifera*.

Metabolites	*H. articulatus*	*P. fasciculata*	*P. angulata*	*M. linifera*					
	Ethanol extract	Ethanol extract	Ethanol extract	Ethanol Extract stem	Hexane Fraction stem	Ethanol Extract leaves	Hexane Fraction leaves	Ethanol Extract sheath	Hexane Fraction sheath
Alkaloids	+	-	+	-	-	-	-	-	-
Anthraquinone	-	-	-	U	U	U	U	U	U
Coumarins	-	-	+	-	-	-	-	-	-
Sterols and Triterpenes	+	+	+	-	+	-	+	-	+
Phenols and Tanines	+	+	+	+	_	+	-	+	-
Flavonoids	+	+	-	-	-	+	-	+	-

(-): Test negative; (+): Test positive; U: unrealized.

Table 3 Anti-promastigote activity of plants used in folk medicine Amazon.

Species	Extract, fraction or pure substance	IC_{50} ± SD (µg/mL)	Activity
Himatanthus articulatus (Apocynaceae)	Ethanol Extract stem bark	> 200	Inactive
	-Hexane Fraction	> 200	Inactive
	-Dichloromethane Fraction	> 200	Inactive
	-Ethyl Acetate Fraction	> 200	Inactive
	-Methanol Fraction	> 200	Inactive
	-Plumerídeo	> 200	Inactive
Parahancornia fasciculata (Apocynaceae)	Ethanol Extract stem bark	> 200	Inactive
	-Hexane Fraction	> 200	Inactive
	-Dichloromethane Fraction	> 200	Inactive
	-Ethyl Acetate Fraction	> 200	Inactive
	-Methanol Fraction	> 200	Inactive
	-Lupeol	> 200	Inactive
Montrichardia linifera (Araceae)	Ethanol Extract stem	> 200	Inactive
	-Hexane Fraction	18.99 ± 0.014	Active
	-Dichloromethane Fraction	> 200	Inactive
	-Ethyl Acetate Fraction	> 200	Inactive
	-Methanol Fraction	> 200	Inactive
	Ethanol Extract leaves	> 200	Inactive
	-Hexane Fraction	13.47 ± 0.024	Active
	-Dichloromethane Fraction	> 200	Inactive
	-Ethyl Acetate Fraction	> 200	Inactive
	- Methanol Fraction	> 200	Inactive
	Ethanol Extract sheath	> 200	Inactive
	- Hexane Fraction	100.9 ± 0.006	Active
	-Dichloromethane Fraction	> 200	Inactive
	-Ethyl Acetate Fraction	> 200	Inactive
	-Methanol Fraction	> 200	Inactive
Physalis angulata (Solanaceae)	Ethanol Extract Root	> 200	Inactive
	Ethanol Extract Stem	> 200	Inactive
	Ethanol Extract Leaves	> 200	Inactive

4. Discussion

Species of Apocynaceae family in general are rich in alkaloids [13]. Various alkaloids presented anti-leishmanial activity [14-16]. In the present study we selected two species of this family, but any alkaloid was detected or isolated. The absence of alkaloids may explain the lack of activity anti-promastigote of *H. articulatus* and *P. fasciculata* (Table 3).

H. articulatus showed inactive against promastigotes of *L. amazonensis*; however, in another study, the extract of this species was active in *L. amazonensis*. This activity was attributed to plumericine and isoplumericine [17] which are

compounds with nonpolar and hydrophobic character, crossing easily plasma membrane of the parasite [18]. The inactivity of *H. articulatus* extract and its fractions in promastigote may be related to the major iridoid plumeride [12]. The plumeride contains five OH groups which makes very polar difficult to pass through the membrane parasite.[18]

From another Apocynaceae, *P. fasciculata*, was isolated lupeol and obtained rich esters of lupeol fraction [8]. *Parahancornia amapa* (Huber) Ducke was isolated friedelin, lupeol, β-amyrin, α-amyrin and their acetyl derivatives, four 3b-*O*-acyl lupeol, two 3b-O-3'-hydroxiacyl lupeol and two 3b-O-3',5'-dihydroxyacyl lupeol in the bark and latex [19].

The ethanol extract of *P. fasciculata*, fractions and lupeol were inactive in promastigote (Table 3). Another study evaluated the anti-amastigote activity in *L. amazonensis* of lupeol, being observed significant inhibitory activity in 500 μg/mL [20]. These results suggest that lupeol and plants rich in lupeol are not promising as anti-leishmanial.

Ethanol extracts obtained from *M. linifera* were inactive in promastigotes as well. However, the fractionation resulted in active hexane fractions. Preliminary phytochemicals studies suggest that this activity may be related to steroids and triterpenes. There are few studies of phytochemicals from this species. A previous study found steroids in the hexane extract from leaves, [21] triterpenes and steroids in the ethanolic extract from leaves and stem [22, 23] and isolated *p*-hydroxybenzaldehyde [24]. This compound was shown to be active in *Plasmodium falciparum* [25-27] and perhaps has anti-leishmanial activity. The inhibiting activity of triterpenoids isolated is described for promastigotes [28, 29] and intracellular amastigotes of *Leishmania amazonensis* [28]. Terpenes are lipophilic hydrocarbon and this lipophilicity facilitates their penetration into the lipid bilayer of cell membranes that eventually leads to cell death, even in the case of promastigote forms of some *Leishmania* species [29, 30].

The anti-leishmanial activity of nerodiol, a sequiterpene, was evaluated against promastigote of *L. amazonensis*, *L. braziliensis* and *L. chagasi* and amastigote *L. amazonensis*. The nerodiol inhibited the growth of promastigote of *L. amazonensis*, *L braziliensis* and *L. chagasi* (IC_{50} = 85, 74 and 75 μM, respectively). The growth of amastigote *L. amazonensis* was inhibited (IC_{50} = 67 μM) [31]. The anti-leishmanial activity of hexane fractions from *M. linifera* may be related to sequiterpenes and other terpenes (Table 2 and Table 3).

Physalis angulata was isolated physalins [32], a alkaloid, phygrine [33] a flavonol glycoside, myricetin 3-*O*-neohesperidoside [34]. Physalins B and F were able to reduce the percentage of *Leishmania*-infected macrophages and the intracellular parasite number. The topical treatment with physalin F reduced the lesion size, the parasite load and histopathological alterations in BALB/c mice infected with *L. amazonensis* [9]. In this study, perhaps the content of the active substances in the extracts is low. In this case, the fractionation may contribute to antileishmanial activity. Another possibility is the selectivity of the substance, i.e., being active only for the amastigote form.

5. Conclusions

Although different species used for treating wounds, these species have different chemical characteristics. *H. articulatus* has iridoids, *P. fasciculata* has lupeol, *M. linifera* has steroids and terpenes, and *P. angulata* has phenols and tannins, steroids and triterpenes, azulenes, coumarins and alkaloids. Only *M. linifera* showed promise, and it seems the responsible for the activity has nonpolar characteristics. Therefore, the isolation and purification of the active on L. amazonensis promastigotes may be tools in further studies for the development of novel antileishmanial drugs.

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