Atividade antileishmania e citotóxica in vitro de Annona mucosa (Annonaceae)

de Lima, J.P.S.; Pinheiro, M.L.B.; Santos, A.M.G.; Pereira, J.L.S.; Santos, D.M.F.; Barison, A.; Silva-Jardim, I.; Costa, E.V.*

Resumo

A atividade antileishmania dos extratos das folhas e sementes de Annona mucosa, e do alcaloide oxoaporfínico liriodenina isolado do extrato diclorometano das folhas foi avaliada in vitro contra formas promastigotas de três espécies de Leishmania, e formas amastigotas intracelulares de Leishmania amazonensis. A atividade citotóxica foi avaliada contra macrófagos peritoneais de camundongos. O extrato diclorometano das folhas foi o mais ativo contra Leshmania spp. apresentando valores de CI50 menores que 30 µg.mL-1. O alcaloide liriodenina foi o mais citotóxico contra macrófagos peritoneais. Os extratos hexânicos das sementes apresentaram maior indice de seletividade contra Leishmania spp. (IS = 5,93 a 1,54). Todas as amostras foram ativas contra formas amastigotas intracelulares, inibindo, depois de 96h, mais que 70% da replicação de amastigotas nos macrófagos infectados. A investigação fitoquímica do extrato diclorometano das folhas de A. mucosa levou ao isolamento dos alcaloides oxoaporfínicos atherospermidina (1) e liriodenina (2), identificados com base nos seus dados espectroscópicos, principalmente RMN 1D/2D, e comparação com os dados da literatura.

Palavras-chave: Atividade antileishmania; Annona mucosa; alcaloides oxoaporfínicos; Annonaceae.

IN VITRO ANTILEISHMANIAL AND CYTOTOXIC ACTIVITIES OF ANNONA MUCOSA (ANNONACEAE)

Abstract

The antileishmanial activity of extracts from the leaves and seeds of Annona mucosa, and of the oxoaporphine alkaloid liriodenine isolated from the dichloromethane extract of the leaves, were evaluated in vitro against promastigote forms of three Leishmania species and against intracellular amastigote forms of L. amazonensis. Cytotoxic activity was evaluated against peritoneal macrophages of mice. The dichloromethane extract from the leaves was the most active against Leishmania spp., showing IC_{50} values lower than 30 µg.mL^{-1}. Liriodenine was the most cytotoxic against peritoneal macrophages. Hexane extracts of seeds showed the highest selectivity index against Leishmania spp. (SI = 5.93 to 1.54). All samples were active against intracellular amastigote forms, after 96 h inhibiting more than 70% of amastigote replication in infected macrophages. Phytochemical investigation of the dichloromethane extract from the leaves of A. mucosa led to the isolation of the oxoaporphine alkaloids atherospermidine (1) and liriodenine (2), identified on the basis of their spectroscopic data, mainly 1D/2D NMR, and comparison with literature data.

Keywords: Antileishmanial activity; Annona mucosa; oxoaporphine alkaloids; Annonaceae.

*Departamento de Química, Universidade Federal do Amazonas, 69077-000, Manaus-AM, Brazil.
lbelem@ufam.edu.br

IN VITRO ANTILEISHMANIAL AND CYTOTOXIC ACTIVITIES OF ANNONA MUCOSA (ANNONACEAE)

Janaína P. S. de Limaa, Maria L. B. Pinheirob,*, Antonio Marcos Guimarães Santosb, José L. da S. Pereirab, Delvânia M. F. Santosc, Andersson Barisond, Izaltina Silva-Jardime and Emmanoel Vilaça Costaf

[a]Instituto de Educação, Agricultura e Ambiente, Universidade Federal do Amazonas, Campus Vale do Rio Madeira, Rua 29 de agosto, 786, Centro, 69800-000, Humaitá, AM, Brazil.

[b]Departamento de Química, Universidade Federal do Amazonas, 69077-000, Manaus, AM, Brazil.

[c]Laboratório de Quimioterapia, Instituto de Pesquisas em Patologias Tropicais de Rondônia, Fundação Oswaldo Cruz, Fiocruz Noroeste, Rua da Beira 7671, BR 364, Km 3,5, 78912-000, Lagoa, Porto Velho, RO, Brazil.

[d]Departamento de Química, Universidade Federal do Paraná, 81531-990, Curitiba, PR, Brazil.

[e]Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, 45662-900, Ilhéus, BA, Brazil.

[f]LABORGANICS, Departamento de Química, Universidade Federal de Sergipe, 49100-000, São Cristóvão, SE, Brazil.

* lbelem@ufam.edu.br

1. INTRODUCTION

Leishmaniasis is a group of tropical diseases caused by several species of protozoa parasites belonging to the genus Leishmania. Leishmaniasis affects more than 12 million people in 88 countries worldwide, constituting a growing public health problem in Africa, Asia and Latin America.[1] The existence of several different species of Leishmania makes it difficult to reduce the infection rate of the diseases caused by this parasite, in both tegumentary and visceral forms. The different epidemiological status of endemic regions and the trend toward urbanization require the adoption of different strategies for the control of leishmaniasis in Brazil.[2]

In the Brazilian Amazon, L. (Leishmania) amazonensis, L. (Viannia) braziliensis and L. (Viannia) guyanensis are the major causal agents of American Tegumentary Leishmaniasis (ATL). Three forms of the disease are described: cutaneous leishmaniasis, mucocutaneous leishmaniasis, and diffuse cutaneous leishmaniasis. Cutaneous leishmaniasis is usually caused by L. amazonensis, L. braziliensis or L. guyanensis. Mucocutaneous leishmaniasis is usually caused by L. braziliensis or L. guyanensis, following cure of the initial cutaneous leishmaniasis. Diffuse cutaneous leishmaniasis is caused by L. amazonensis.[3] Cutaneous forms may heal spontaneously within a few months, or may develop into the other tegumentary leishmaniasis, which can cause serious facial disfiguration, morbidity, or mortality.[4]

The current status of ATL, evaluated in Brazilian municipalities based on the records of the Health and Sanitation Department of the Ministry of Health,[3] revealed incidences ranging from 10.45 to 22.9 per 100,000 inhabitants, clearly indicating a geographical expansion of the disease. The Northern region has 34.9% of the total cases in the country and a global risk of acquiring the disease of 92.3 per 100,000 inhabitants, five times the national average.

Nowadays, chemotherapy for leishmaniasis is still based mainly on daily intramuscular injections of pentavalent antimonials (Glucantime® and Pentostam®), diamines (Pentamidine®) and an antifungal polyene (Amfotericine® B), which are toxic, expensive, generate resistance, and require long-term treatment. There is an urgent need to develop new, more-effective and safer therapeutic agents in the fight against leishmaniasis.[4]

Advances in combating leishmaniasis using natural products have been recently reviewed. Studies of plants producing alkaloids and acetogenins have yielded promising results against Leishmania spp., in particular extracts from species of the family Annonaceae.[6]

Annonaceae is a group of aromatic trees, shrubs, and lianas that occur mainly in tropical and subtropical regions. Previous chemical and pharmacological investigations on some species of this family have indicated the presence of important bioactive compounds with various pharmacological activities, including antiparasitic, in particular antileishmanial. [7-14]

Continuing our chemical and biological antileishmanial studies of members of the Annonaceae, we investigated the species Annona mucosa Jacq. [synonym Rollinia mucosa (Jacq.) Baill].[15] This species provides the delicious fruit biribá, and popular reports reveal the use of its leaf extracts for the elimination of fleas and other insects infesting domestic animals. Chemical and pharmacological

studies of species of Annonaceae have revealed the presence of alkaloids, acetogenins and lignoids, with proven antiprotozoal, antimicrobial and antifungal effects,[16-18] suggesting great chemical and pharmacological potential. In this study, extracts from A. mucosa (leaves and seeds), and the oxoaporphine alkaloid liriodenine, isolated from a dichloromethane extract of the leaves, were investigated in vitro against promastigote forms of L. amazonensis, L. braziliensis and L. guyanensis, and intracellular amastigote forms of L. amazonensis. The cytotoxicity to mice peritoneal macrophages was also evaluated.

2. EXPERIMENTAL

2.1. GENERAL

Melting points (m.p.) were measured using a Microquímica MQAPF 301 Model. 1D and 2D NMR experiments were acquired in $CDCl_3$ at 293 K on a Bruker AVANCE 400 NMR, operating at 400 and 100 MHz for ^1H and ^{13}C, respectively. The spectrometer was equipped with a 5-mm multinuclear direct detection probe with z-gradient. One-bond and long-range ^1H-^{13}C correlation (HSQC and HMBC) experiments were optimized for an average coupling constant $^1J_{(C,H)}$ and $^{LR}J_{(C,H)}$ of 140 and 8 Hz, respectively. All ^1H- and ^{13}C-NMR chemical shifts (δ) are given in ppm, with respect to the TMS signal at 0.00 ppm as internal reference and the coupling constants (J) in Hz. Silica gel 60 (70–230 mesh) was used for column chromatography, and silica gel 60 F_{254} was used for analytical (0.25 mm), and preparative (1.00 mm) TLC (thin layer chromatography). Compounds were visualized by exposure under $UV_{254/366}$ light, spraying p-anisaldehyde reagent followed by heating on a hot plate, as well as spraying with Dragendorff's reagent.

2.2. ANIMAL EXPERIMENTATION GUIDELINES

The experimental protocols conformed to the ethical principles in animal research adopted by the Comissão de Ética no Uso de Animais of the Instituto de Pesquisas em Patologias Tropicais (CEUA/IPEPATRO - 2011/01). Male BALB/c mice were housed in temperature-controlled rooms and provided water and food ad libitum until used.

2.3. PLANT MATERIAL

Leaves and fruits of A. mucosa were collected from the campus of the Universidade Federal do Amazonas (UFAM) [coordinates: S 03º 06' 2.4" W 59º 58' 27.7"], Manaus, Amazonas, Brazil, in September 2007. The specimen was identified by a plant specialist of the UFAM Department of Biology, and a voucher specimen has been deposited in the Herbarium of this University, with register number HUAM/UFAM 8148.

2.4. EXTRACTION AND ISOLATION PROCEDURES

Dried and powdered leaves (900 g) of A. mucosa were successively extracted with n-hexane, dichloromethane and methanol, to yield hexane (18.4 g), dichloromethane (42.4 g), and methanol (19.6 g) extracts, after removal of each solvent. A similar procedure was followed with the seeds from fruits (300 g), using only hexane and methanol as solvents to obtain hexane (5.0 g), and methanol (15.0 g) extracts, respectively. TLC investigations indicated that the dichloromethane extract of the leaves contained the highest concentration of alkaloids. A part of this extract (11.2 g) was initially subjected to an acid-base extraction[12] to give dichloromethane alkaloid (0.25 g) and dichloromethane neutral (6.0 g) fractions. The alkaloid fraction was subjected to silica gel column chromatography eluted with the gradient systems: hexane-dichloromethane from 100:0 to 10:90, followed by dichloromethane-methanol from 100:0 to 50:50, yielding 56 subfractions. The eluted subfractions were evaluated and pooled according to TLC analysis, to afford 6 groups of fractions (GF1-GF6). GF3 (20.0 mg) was purified by preparative TLC eluted with hexane-acetone (60:40, three times) affording **1** (2.0 mg) and **2** (10.0 mg) respectively.

Atherospermidine (1): Orange crystals (CH_2Cl_2:MeOH 3:1); mp 285–288 °C (lit. 286–287 °C); identified by comparison with literature data (co-TLC, mp, ^1H-NMR and ^{13}C-NMR).[19]

Liriodenine (2): Yellow crystals (CH_2Cl_2:MeOH 3:1); mp 279–280 °C (lit. 281–282 °C); identified by comparison with literature data (co-TLC, mp, ^1H-NMR and ^{13}C-NMR).[19]

2.5. IN VITRO ANTILEISHMANIAL ASSAY

2.5.1. Parasites

The antileishmanial activity was evaluated against promastigote forms of Leishmania (Leishmania) amazonensis (IFLA/BR/67/PH8) and Leishmania (Viannia) guyanensis (MHOM/BR/75/M4147) cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 50 μg.mL-1 gentamycin at 24 °C in a tissue culture flask, and the promastigote forms of Leishmania (Viannia) braziliensis (MHOM/BR/75/M2903) cultured in Schneider's Drosophila medium supplemented with 10% (v/v) heat-inactivated FBS, 5% (v/v) male human urine and 50 $\mu g.mL^{-1}$ gentamycin at 24 °C in a tissue culture flask.

2.5.2. Antileishmanial activity against promastigote forms

A modification of the method described by Camacho et al.[20] was used to assess the in vitro antileishmanial activity. The screening was performed in eppendorfs maintained at 24 °C. Promastigote forms of Leishmania spp. (5.0×10^5 parasites.mL-1) were exposed to six concentrations (6, 12, 25, 50 and 100 $\mu g.mL^{-1}$) of each extract and liriodenine. The samples were dissolved in DMSO. Up to 0.7% (v/v), DMSO had no effect on parasite growth. The activity of the samples was evaluated for six days by counting microscopically the number of live parasites that showed flagellar motility, using a Neubauer hemocytometer. The counts were compared with those of controls grown without a sample of extract, and with pentamidine isethionate (Sigma/P0547) used as the reference antileishmanial drug. Each concentration was assayed in duplicate. Results are expressed as concentrations inhibiting parasite growth by 50% (IC_{50}) after six days of incubation.

2.5.3. Antileishmanial activity against intracellular amastigote forms

The in vitro sensitivity of amastigote forms of Leishmania amazonensis (IFLA/BR/67/PH8) was determined in a mice peritoneal macrophage model. Peritoneal macrophages were collected from the peritoneal cavity of male BALB/c mice by washing with RPMI 1640 medium four days after an intraperitoneal injection of 2.0 mL of sodium thioglycolate. The macrophages were dispensed into flat-bottomed plastic 24-well tissue plates containing glass coverslips (13 mm), at a concentration of 5×10^5/well (500 $\mu L.well^{-1}$) in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 50 $\mu g.mL^{-1}$ gentamycin, and incubated at 37 °C in a 5% CO_2/air mixture. After 24 h, non-adherent cells were removed by washing with pre-warmed phosphate-buffered saline (PBS), and then the plated macrophages were infected with Leishmania amazonensis promastigotes, at a ratio of 10:1, and incubated at 34 °C in a 5% CO_2/air mixture. The culture medium was renewed after 24 h, and a new culture medium containing 25 $\mu g.mL^{-1}$ of each sample was added and the plates were incubated in the same conditions described above. Each extract and liriodenine were assayed in triplicate. The experiments were stopped at day 2, 3 or 5. The method described by Fumorola et al.[21] was used to make direct counts of the intracellular amastigotes. The infected macrophages from each sample were stained with May-Grunwald/Giemsa, and then evaluated microscopically to determine the percentage of infected macrophages and the number of amastigote forms per macrophage; 200 macrophages were inspected in each sample.[22]

2.5.4. Cytotoxicity in peritoneal macrophages

Cytotoxicity was studied after 96 h incubation of peritoneal macrophages with concentrations ranging from 6 to 100 $\mu g.mL^{-1}$ of each extract and liriodenine. Peritoneal macrophages were collected from the peritoneal cavity of male BALB/c mice, as described in section 2.5.3. The macrophages were plated into 24-well tissue plates and incubated at 37 °C in a 5% CO_2/air mixture. The viability of the macrophages was determined by direct counts, using a Neubauer hemocytometer. The counts were compared with those of controls incubated without a sample and with the antileishmanial reference drug pentamidine isethionate. Each concentration was assayed in triplicate. Results are expressed as 50% lethal concentrations (LC_{50}) after 96 h of incubation.

2.6. STATISTICAL ANALYSIS

All results were expressed as mean ±SEM for n experiments. Statistical evaluation was undertaken by analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons.

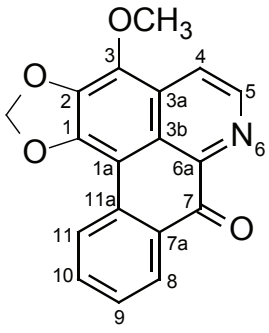
A P value of less than 0.05 was considered statistically significant.

The IC_{50} and LC_{50} values at the 95% confidence interval were calculated using sigmoid dose-response curves in Graph Pad Prism 5.01 software.

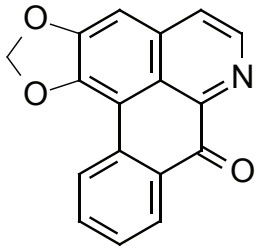
3. RESULTS AND DISCUSSION

Phytochemical investigation of the dichloromethane extract from the leaves of A. mucosa led to the isolation of the oxoaporphine alkaloids atherospermidine **(1)** and liriodenine **(2)** (Figure 1). The structures of these alkaloids were elucidated based on their spectroscopic data, mainly 1D and 2D NMR, and also comparison with literature data.[19] Atherospermidine **(1)** is reported for the first time in this species, and compound 2 has been previously described in the stem and fruits of this plant.[16] Atherospermidine **(1)** and liriodenine **(2)** are two oxoaporphine alkaloids that are widely found in almost all genera of Annonaceae, for which 2 is considered a chemotaxonomic marker. [23]

Figure 1. Alkaloids isolated from leaves of Annona mucosa.



(1)



(2)

The extracts and liriodenine isolated from A. mucosa were screened for antileishmanial activity against *L. amazonensis, L. braziliensis and L. guyanensis promastigote* forms. Because of the small yield of atherospermidine, this compound was not screened. The results of the evaluation of antileishmanial activity (IC_{50} values) of the seven samples are shown in Table 1. The IC_{50} values are the means of six determinations. The samples were classified as highly active (IC50 < 10 μg.mL^{-1}), active (10 < IC50 < 50 μg.mL^{-1}), moderately active (50 < IC_{50} < 100 μg.mL^{-1}) and non-active (IC_{50} > 100 μg.mL^{-1}). [20]

Table 1. In vitro antileishmanial activity forms of extracts and liriodenine from Annona mucosa on promastigotes of Leishmania spp.

Extract/fraction	P [a]	Leishmania sp.[b] IC_{50} (μg.mL^{-1}) ± SEM[c]	
		PH8	M2903
Hexane extract	L	24.24 ± 1.51	65.27 ± 1.20
Dichloromethane extract	L	9.32 ± 0.56	27.42 ± 5.42
Methanol extract	L	28.32 ± 1.15	44.74 ± 5.89
Hexane extract	S	44.22 ± 5.64	170.15 ± 1.46
Methanol extract	S	46.54 ± 4.95	133.17 ± 5.41
Compound		IC_{50} (μg.mL^{-1}) ± SEM [c]	
Liriodenine		1.43 ± 0.58	55.92 ± 3.55
Pentamidine[d]		0.07 ± 0.00	5.48 ± 0.00

[a]P (plant part); L (leaves); S (seeds). [b] PH8, Leishmania (Leishmania) amazonensis; M2903, Leishmania (Viannia) braziliensis; M4147, Leishmania (Viannia) guyanensis. [c]The IC_{50} values are expressed as mean ± SEM. d Standard antileishmanial drug. The data are the mean values ± SEM of six experiments.

The in vitro cytotoxic activities (LC_{50} values) of the extracts and liriodenine against peritoneal macrophages are shown in Table 2. The LC_{50} values are the means of three determinations. The samples were classified as highly toxic (LC_{50} < 10 μg.mL^{-1}), toxic (10 < LC_{50} < 100 μg.mL^{-1}), moderately toxic (100 < LC_{50} < 1000 μg.mL^{-1}) and potentially non-toxic (LC_{50} > 1000 μg.mL^{-1}). 20 The results showed that four samples were toxic to mice peritoneal macrophages, and two extracts were moderately toxic. Cytotoxic activity (LC_{50}) was related to antileishmanial activity by determining their corresponding selectivity index (SI = LC_{50}/IC_{50}) as shown in Table 2.

A value lower than 1 indicates that they are more toxic to peritoneal macrophages than to the parasites, and a value greater than 1 indicates higher selectivity against the parasites than against the peritoneal macrophages.

Table 2. Cytotoxicity and selectivity index of extracts and liriodenine from Annona mucosa.

Extract/fraction	P [a]	Cytotoxicity[b] LC^{50} (μg.mL^{-1}) ± SEM[c]	SI[d]	
			PH8	M2903
Hexane extract	L	62.63 + 4.10	2.58	0.95
Dichloromethane extract	L	24.07 + 0.72	2.58	0.87
Methanol extract	L	29.41 + 0.89	1.03	0.65
Hexane extract	S	262.33 + 5.81	5.93	1.54
Methanol extract	S	139.00 + 3.13	2.98	1.04
Compound				
Liriodenine		19.11 + 1.06	13.36	0.34
Pentamidine[d]		51.99 + 0.58	742.71	9.48

aP (plant part); L (leaves); S (seeds). [b] Cells, BALB/c mice peritoneal macrophages. [c] The LC_{50} values are expressed as mean ± SEM. d SI, selectivity index = LC_{50}/IC_{50}. e Standard antileishmanial drug. The data are the mean values + SEM of three mice.

The samples were also assayed on intracellular amastigote forms in three independent experiments. The percentage of infected macrophages (Figure 2A) and the parasite load (Figure 2B) decreased over time. The percentage of infected macrophages at 24, 48 and 96 hours showed that the activity reduction was maintained for the hexane and dichloromethane extracts from the leaves of A. mucosa and liriodenine. The observed lower activity of the alkaloid liriodenine than of the plant extracts over this period can be explained only after complete investigation of the chemical constituents and biological evaluation of these extracts.

The macrophages showed no apparent effect of toxicity such as rounding or detachment, even in the presence of the samples at 25 μg.mL^{-1}.

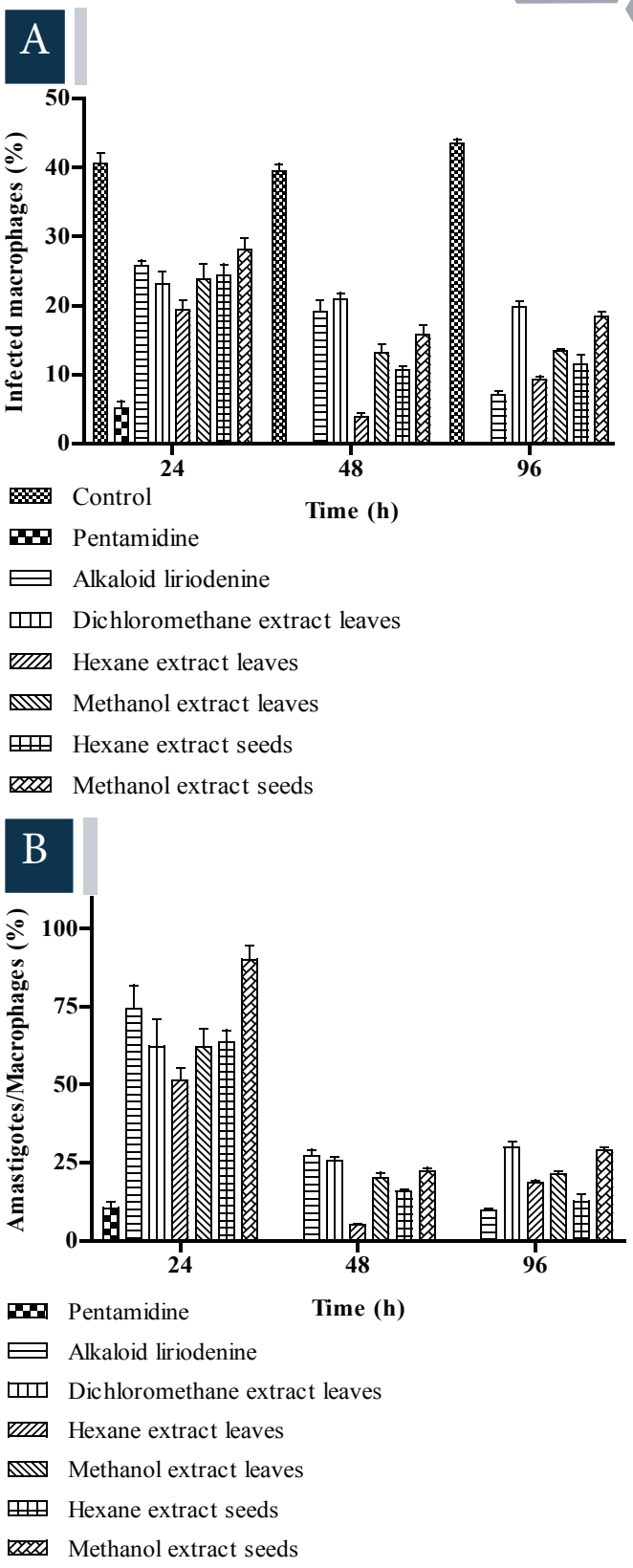


Figure 2. Antileishmanial activity of extracts and liriodenine from Annona mucosa on intracellular amastigotes.

(A) Percentage of macrophages infected. (B) Parasite load (number of amastigotes/macrophage) as a percentage of that in controls, taken as 100%. Controls averaged 4.86 amastigotes/macrophage incubated for 24 h, 8.28 amastigotes/macrophage incubated for 48 h, and 7.38 amastigotes/macrophage incubated for 96 h. The data are the mean values ± SEM of six mice.

Few studies have examined the effects of plant extracts on L. guyanensis, but some studies have shown antileishmanial activity of extracts of the genus Rollinia (presently merged in Annona) against promastigote forms of L. amazonensis, L.

braziliensis and L. donovani.[25,26] In our study, the dichloromethane extract of the leaves proved to be more active than the other extracts assayed, inhibiting the growth of promastigotes with IC_{50} values between 9.32 to 27.42 μg.mL^{-1}. It was classified as toxic with LC_{50} of 24.07 μg.mL^{-1}, and caused total lysis of macrophages at 100 μg.mL^{-1}. The results indicated that the hexane and methanol extracts of leaves were effective in inhibiting the growth of promastigote forms of L. amazonensis, with IC_{50} values from 24.24 to 28.32 μg.mL^{-1}, as well as promastigotes of L. braziliensis, with IC_{50} values from 44.74 to 65.27 μg.mL^{-1} and were classified as toxic with LC_{50} of 62.63 and 29.41 μg.mL^{-1}, respectively. The hexane and methanol extracts of the seeds were effective in inhibiting the growth of promastigotes of L. amazonensis, with IC50 values from 44.22 to 46.54 μg.mL^{-1} and were classified as toxic with LC_{50} of 262.33 and 139 μg.mL^{-1}, respectively. In the same test, the reference drug pentamidine had an IC_{50} value from 0.07 to 5.48 μg.mL^{-1} and was classified as toxic, with LC_{50} of 51.99 μg.mL^{-1}.

Some previous reports describe the antileishmanial activity of the alkaloid liriodenine.[12], [26,27] Antileishmanial activity has been reported to differ against promastigotes of L. amazonensis (IC_{50} 5 - 100 μg.mL^{-1}), L. braziliensis (IC_{50} 5 - 100 μg.mL^{-1}) and L. donovani (IC_{50} 3.12 - 100 μg.mL^{-1}). In our study, this compound inhibited the growth of promastigotes, showing activity against L. amazonensis with IC_{50} of 1.43 μg.mL^{-1}, L. braziliensis with IC_{50} of 55.92 μg.mL^{-1}and L. guyanensis with IC_{50} of 0.84 μg.mL^{-1}. It has been suggested[5] that the observed differences in the biological activity of this metabolite against the same species of Leishmania could be due to the use of biphasic or liquid media to evaluate antileishmanial activity. When tested for cytotoxicity, liriodenine was classified as toxic with LC_{50} of 19.11 μg.mL^{-1}, causing total lysis of the macrophages at 100 μg.mL^{-1} and was the most selective against L. guyanensis with SI of 22.75. Nevertheless, against L. braziliensis, liriodenine is non-selective, with SI of 0.34. This result suggests that cytotoxicity does not always correlate with antileishmanial activity. Leishmania spp. differ intrinsically in their drug sensitivity, as seen in Table 1. Croft and Coombs[28] suggested a possible explanation for this variation, that different Leishmania spp. not only reside in different macrophage types, but also have differing adaptations that facilitate intracellular survival.

The samples were active against intracellular amastigote forms of L. amazonensis at concentrations of 25 μg.mL^{-1}. In our study, pentamidine was highly active, and after 48 h of incubation, eliminated 100% of infected macrophages. Nevertheless, the toxicity of pentamidine makes it a second choice, for use when treatment with antimony compounds is not effective.[5] In the same conditions, the extracts of A. mucosa and liriodenine showed more than 80% eliminated infected macrophages and 70% eliminated intracellular amastigotes. Because the extracts are a complex mixture of substances, purification of the active compounds might considerably increase their antileishmanial activity. Additionally, in our assays, almost all the extracts showed antileishmanial activity, mainly the dichloromethane extract, which provided the cytotoxic and antileishmanial oxoaporphine alkaloids atherospermidine and liriodenine.[19] Therefore, these alkaloids may be responsible for the antileishmanial activity observed.

4. CONCLUSIONS

This study resulted in the isolation and identification of two oxoaporphine alkaloids, atherospermidine (1) and liriodenine (2), from the leaves of A. mucosa, 1 is reported for the first time in this species. These results contribute to the chemotaxonomic understanding of the family Annonaceae, especially the genus Annona. The results obtained in this study also confirm the importance of the selection of plant extracts used in folk medicine in screening programs in the search for new antileishmanial agents. Further investigation including the oxoaporphine alkaloid liriodenine and other constituents extracted from A. mucosa should focus on understanding their mechanisms of action, and encourage us to continue in vitro and in vivo investigations.

ACKNOWLEDGMENTS

The authors are grateful to FAPEAM, CNPq and FINEP for financial support and scholarships.

REFERENCES AND NOTES

[1] World Health Organization. The leishmaniasis and Leishmania/HIV co-infections. 2002. In: http://www.who. int/mediacentre/factsheets/fs116/ en/print.htm. Access: December 2011.

[2] Costa, J.M.L. Gaz. Méd. Bahia 2005, 75, 3.

[3] Sítio do Ministério da Saúde. In: <http://portal.saude.gov.br/portal/arquivos/pdf/manual_lta_2ed.pdf>. Access: December 2011.

[4] Teles, C.B.G.; Moreira, L.S.; Silva A de, A.E.; Facundo, V.A.; Zuliani, J.P.; Stabéli, R.G.; Silva-Jardim, I. J. Braz. Chem. Soc. 2011, 22, 936.

[5] Chan-Bacab, M.J.; Peña-Rodriguez, L.M. Nat. Prod. Rep. 2001, 18, 674.

[6] Rocha, L.G.; Almeida, J.R.G.S.; Macêdo, R.O.; Barbosa-Filho, J.M. Phytomedicine 2005, 12, 514.

[7] Rupprecht, J.K.; Hui, Y.H.; MacLaughlin, J.L. J. Nat. Prod. 1990, 53, 237.

[8] Queiroz, E.F.; Roblot, F.; Cavé, A.; Paulo, M.Q.; Fournet, A. J. Nat. Prod. 1996, 59, 438.

[9] Cavé, A.; Cortes, D.; Figadère, A.; Laurens, A.; Pettit, G.R. Acetogenins from Annonaceae, p 81-288; In: Herz, W.; Kirby, G.W.; Moore, R.E.; Steglich, W.; Tamm, C. Progress in the Chemistry of Organic Natural Products. Springer-Verlag: Vienna 1997, 70: 330 p.

[10] Boyom, F.F.; Ngouana, V.; Zollo, P.H.A.; Menut, C.; Bessiere, J.M.; Gut, J.; Rosenthal, P.J. Flavour Frag. J. 1996, 11: 333.

[11] Montenegro, H.; Gutiérrez, M.; Romero, L.I.; Ortega-Barria, E.; Capson, T.L.; Rios, L.C. Planta Med. 2003 69: 677.

[12] Costa, E.V.; Pinheiro, M.L.B.; Xavier, C.M.; Silva, J.R.A.; Amaral, A.C.F.; Souza, A.D.L.; Barison, A.; Campos, F.R.; Ferreira, A.G.; Machado, G.M.C.; Leon, L.L.P. J. Nat. Prod. 2006, 69, 292.

[13] Osorio, E.J.D.; Montoya, G.L.P.; Arango, G.J.A. Vitae (Medellín) 2006, 13, 61.

[14] Costa, E.V.; Pinheiro, M.L.B.; Silva, J.R.A.; Maia, B.H.L.N.S.; Duarte, M.C.T.; Amaral, A.C.F.; Machado, G.M.C.; Leon, L.L. Quím. Nov. 2009, 32, 78.

[15] Rainer, H. Naturlist Mus. Wien 2007, 108B, 191.

[16] Chen, Y.Y.; Chang, Fr.; Wu, Yc. J. Nat. Prod. 1996, 59, 904.

[17] Liaw, C.C.; Chang, F.R.; Chen, Y.Y.; Chiu, F.F.; Wu, M.J.; Wu, Y.C. J. Nat. Prod. 1999, 62, 1613.

[18] Alali, F.Q.; Liu, X.-X.; McLaughlin, J.L. J. Nat. Prod. 1999, 62, 504.

[19] Costa, E.V.; Pinheiro, M.L.B.; Souza, A.D.L.; Barison, A.; Campos, F.R.; Valdez, R.H.; Ueda-Nakamura, T.; Dias Filho, B.P.; Nakamura, C.V. Molecules 2011, 16, 9714.

[20] Camacho, M.D.R.; Phillipson, J.D.; Croft, S.L.; Solis, P.N.; Marshall, S.J.; Ghazanfar, S.A. J. Ethnopharmacol. 2003, 89, 185.

[21] Fumarola, L.; Spinelli, R.; Brandonisio, O. Res. Microbiol. 2004, 155, 244.

[22] Torres-Santos, E.C.; Moreira, D.L.; Kaplan, M.A.C.; Meirelles, M.N.; Rossi-Bergmann, B. Antimicrob. Agents Chemother. 1999, 43, 1234.

[23] Pinheiro, M.L.B.; Xavier, C.M.; de Souza, A.D.L.; Rabelo, D.M.; Batista, C.L.; Batista, R.L.; Costa, E.V.; Campos, F.R.; Barison, A.; Valdez, R.H.; Ueda-Nakamura, T.; Nakamura, C.V.; J. Braz. Chem. Soc. 2009, 20, 1095.

[24] Février, A.; Ferreira, M.E.; Fournet, A.; Yaluff, G.; Inchausti, A.; Arias, A.R.; Hocquemiller, R.; Waechter, A.S. Planta Med. 1999, 65, 47.

[25] Osorio, E.; Arango, G.J.A.; Jimenez, N.; Alzate, F.; Ruiz, G.; Gutierrez, D.; Paco, M.A.; Gimenez, A.; Robledo, S. J. Ethnopharmacol. 2007, 111, 630.

[26] Queiroz, E.F.; Roblot, F.; Cavé, A. J. Nat. Prod. 1996, 59, 438.

[27] Waechter, A.I.; Cavé, A.; Hocquemiller, R.; Bories, C.; Muñoz, V.; Fournet, A. Phytother. Res. 1999, 13, 175.

[28] Croft, S.L.; Coombs, G.H. Trends Parasitol. 2003, 19, 502.