

Antiprotozoal activity of Brazilian plant extracts from isoquinoline alkaloid-producing families[☆]

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Abstract

Leishmaniasis and Chagas disease afflict the poorest countries in the world. The Brazilian flora represents a rich source for the screening of potential antiparasitic compounds. In this work, we tested the total alkaloid and ethanol extracts of nine different plants from Brazilian families which produce isoquinoline alkaloids, to determine their *in vitro* antiparasitic effect against *L. chagasi* and *T. cruzi* parasites. Promastigotes of *L. chagasi* were shown to be susceptible only to the total alkaloid extracts of *A. crassiflora* (EC₅₀ value = 24.89 µg/ml), *A. coriacea* (EC₅₀ value = 41.60 µg/ml), *C. ovalifolia* (EC₅₀ value = 63.88 µg/ml) and *G. australis* (EC₅₀ value = 37.88 µg/ml). Except for the *G. australis* total alkaloids, all the three extracts presented a considerable activity when tested against intracellular amastigotes. The most effective alkaloid extracts were those from *A. crassiflora* and *C. ovalifolia*, which reduced the number of infected macrophages at 25 µg/ml by 86.1% and 89.8%, respectively. Among the 18 tested extracts, 16 showed anti-*Trypanosoma* activity. Eight extracts (*A. crassiflora*, *A. coriacea*, *C. ovalifolia*, *D. furfuracea*, *D. lanceolata*, *S. guianensis*, *X. emarginata* and *G. australis*) were the most effective against the trypomastigotes, killing approximately 100% of the parasites at the maximal concentration of 100 µg/ml. Cytotoxicity against mammalian cells was evaluated for all extracts, but potential ones showed little or no cytotoxicity and a considerable antiparasitic effect, including *D. furfuracea*, *D. lanceolata*, *G. australis*, *S. guianensis* and *X. emarginata*. Plants are a rich source of natural compounds, and a powerful tool for the development of new arsenals for the therapy of protozoan diseases.

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Introduction

Protozoan diseases constitute the world's most widespread human health problem. It is estimated that 3 billion individuals suffer from one or more parasitic infections, with the greatest causes of morbidity being attributed to the trypanosomatid and apicomplexan

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parasites (Martin et al., 2001). Diseases like Leishmaniasis are still common in rural areas and are endemic in most Central and South American countries, afflicting the world's poorest population in 88 countries, with 12 million cases in tropical and subtropical areas (Desjeux, 1992). For six decades, long parenteral courses of toxic pentavalent antimonials have been used to treat visceral and cutaneous leishmaniasis (Davies et al., 1992). The use of second-line drugs such as pentamidine and amphotericin B is limited because of the severity of side-effects and in-hospital administration (Balãna-Fouce et al., 1998).

Chagas disease or American trypanosomiasis, caused by infection with the parasite *Trypanosoma cruzi*, is a long-lived disease and a serious public health problem, affecting about 18 million people in the Americas (Almeida and Gazzinelli, 2001). The transmission of the disease occurs mainly by the vector (80–90%), but blood transfusion (5–20%) and congenital routes (0.5–0.8%) represent a high risk for the population (Coura and Castro, 2002). Old and ineffective chemotherapeutic agents were employed, such as quinolein derivatives, antimalarials, arsenobenzoles, arsenicals, phenantridines, salts of gold, bismuth and gentian violet. Developed empirically over three decades ago, the currently available drugs nitrofurans and nitroimidazoles, are unsatisfactory due to frequent toxic side-effects and limited efficacy, particularly in the prevalent chronic form of the disease. Furthermore, studies of their mechanism of action have shown that their antiparasitic activity is inextricably linked to mammalian host toxicity (Urbina, 2002). The severe side-effects of benznidazole and nifurtimox, including agranulocytosis, hypersensitivity reactions and anorexia, make them poorly tolerated (Almeida and Gazzinelli, 2001).

The high toxicity and resistance of current available drugs used for these parasitic diseases, is an impetus for the development of new therapeutic approaches. Plants have been used traditionally for the treatment of protozoan diseases and phytotherapy has received considerable recent attention in the search for alternative compounds with antiparasitic activity (Muelas-Serrano et al., 2000; Neves-Pinto et al., 2002). The Brazilian flora represents a rich source of components, and among these, the isoquinoline alkaloids have been showing promising results against protozoan parasites (Mahiou et al., 2000; Camacho et al., 2002). In this work, species were selected among the Annonaceae (Leboeuf et al., 1982), Menispermaceae (Thorner, 1970) and Siparunaceae (Leitão et al., 1999) families, which produce a wide range of isoquinoline alkaloids structures as benzylisoquinolines, bisbenzylisoquinolines, aporphines, proaporphines and oxoaporphines (Mahiou et al., 1994; Waechter et al., 1999). In the course of our systematic study of biological active compounds with antiparasitic activity from medicinal

plants, we have tested ethanol and total alkaloid extracts from 9 species of Brazilian plants that produce isoquinoline alkaloids active against *Leishmania (L.) chagasi* and *Trypanosoma cruzi* parasites. Using antiparasitic *in vitro* screening and mammalian cell cytotoxicity testing, we searched for the most promising species against Leishmaniasis and Chagas disease.

Material and Methods

Plant material

The nine tested plants, collected in São Paulo, Mato Grosso do Sul and Minas Gerais states (Brazil), are used in traditional medicine (Table 1). Voucher specimens were identified and deposited at the herbaria of the Instituto de Biociências of the University of São Paulo (SPF) and at the Instituto de Botânica de São Paulo (SP). Other collection data are available in Table 1. The leaves of the plants were separated and dried carefully by forced air at 60 °C.

Extraction

Powdered, air-dried leaves (50 g) were extracted with 96% ethanol (1.5 l) by cold percolation. The ethanol extract was concentrated to dryness *in vacuo* and dissolved with 10% phosphoric acid and then washed with *n*-hexane. After alkalization (pH 9) with ammonium hydroxide, the aqueous phase was partitioned with dichloromethane, up to negative Dragendorff's reagent. The dichloromethane extract was dried over sodium sulfate and concentrated under reduced pressure to dryness. Alkaloid extract yields were, respectively: *A. coriacea* 0.5%, *A. crassiflora* 0.45%, *C. ovalifolia* 0.53%, *D. furfuracea* 0.42%, *D. lanceolata* 0.34%, *G. australis* 0.29%, *S. apiosyce* 0.13%, *S. guianensis* 0.4%, *X. emarginata* 0.31%.

Parasites

Leishmania (L.) chagasi (M 6445 strain) was maintained in Golden Hamsters. Amastigotes were obtained from the spleen by differential centrifugation at 60–70th day post infection (Stauber et al., 1958). Promastigotes were obtained from the purified amastigotes maintained in M199 medium, supplemented with 10% fetal bovine serum, 5% male human urine at 24 °C. *Trypanosoma cruzi* (Y strain) trypomastigotes were maintained in LLC-MK2 cells in RPMI-1640 medium, without phenol red and supplemented with 2% fetal bovine serum at 37 °C, in a 5% CO₂ humidified incubator (Kesper et al., 2000).

Table 1. Classification and characteristics of the plants

Species (family), vernacular name, voucher number	Traditional use	References
<i>Annona coriacea</i> Mart. (Annonaceae), <i>araticum</i> , <i>marolo</i> , <i>araticum-liso</i> , Fischer 2(SPF)	Rheumatism (gn) Anti-helminthic (gn) Leishmaniasis (gn)	Cruz (1995) dos Santos and Sant'Ana (2000, 2001) Akendengue et al. (1999)
<i>Annona crassiflora</i> Mart. (Annonaceae), <i>anona</i> , <i>pinha</i> , <i>fruta-do-conde</i> , Fischer 7(SPF)	Diarrhea (sp) Anti-helminthic (gn), rheumatism (gn) Chagas disease	Rodrigues and Carvalho (2001) Cruz (1995) Queiroz et al. (1996)
<i>Cissampelos ovalifolia</i> DC (Menispermaceae), <i>orelha-de-onça</i> , <i>orelha de burro</i> , Fischer 6(SPF)	Fevers, Malaria (sp), Urinary disturbances (sp), Inflammations (sp), cough (sp)	Rodrigues and Carvalho (2001) Gessler et al. (1994) Cruz (1995), Estrella (1994)
<i>Duguetia furfuracea</i> (St. Hil.) Benth. et Hook. (Annonaceae), <i>marolinha-do-campo</i> , <i>araticum-do-campo</i> , Fischer 1(SPF)	Rheumatism(sp) Malaria (gn)	Rodrigues and Carvalho (2001) Muhammad et al. (2001)
<i>Duguetia lanceolata</i> St. Hil. (Annonaceae), <i>pindaíba</i> , <i>pindabuna</i> , Fischer 9(SPF)	Malaria (gn)	Muhammad et al. (2001)
<i>Guatteria australis</i> St. Hil. (Annonaceae), <i>cortiça</i> , Mello-Silva 1805 (SPF)	Leishmaniasis (gn) Gastric disturbances (gn) Chagas disease	Akendengue et al. (1999) Cruz (1995) Fournet et al. (1994)
<i>Siparuna apiosyce</i> (Mart.) DC (Siparunaceae), <i>limão-bravo</i> , <i>negamina</i> , <i>cidreira-do-mato</i> , SP: 309569	Bronchitis, cough and other Respiratory disturbances gastric disturbances (sp), rheumatism (sp) Fevers, malaria (gn)	Coimbra (1994) Occhioni and Lyra (1947) Jenett-Siems et al. (1999)
<i>Siparuna guianensis</i> (Aubl.) Tulasne (Siparunaceae), <i>limão-bravo</i> , <i>negamina</i> , Fischer 3(SPF)	Fevers, malaria (sp) Rheumatism (sp), inflammation, headache, digestive disturbances	Estrella (1994) Rodrigues and Carvalho (2001)
<i>X. emarginata</i> Mart.(Annonaceae), <i>pindaíba-reta</i> , <i>embira-preta</i> , <i>pindaíba</i> 101499 (SPF)	Fevers, malaria (gn)	Jenett-Siems et al. (1999)

sp: species, gn: genus, nd: no data.

Cells

Peritoneal macrophages were collected from the peritoneal cavity of female BALB/c mice by washing with RPMI-1640 without phenol red, supplemented with 10% fetal bovine serum and seeded into 24-wells plates containing glass coverslips (13 mm) for a period of 2 h for attachment, at 37°C in a 5% CO₂ humidified incubator. Non-adherent cells were removed by one step washing with medium and the subsequent incubation was performed for 24 h at the same temperature. Murine macrophages RAW 264.7 (ATCC TIB-71) and LLC-MK2 cells were maintained in RPMI-1640 medium without phenol red and supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ humidified incubator.

Determination of the Anti-Leishmania activity

The drug sensitivity assay was performed as previously described by Tempone et al. (2001). To determine the 50% Effective Concentration (EC₅₀ value) of the extracts against *Leishmania* promastigotes, compounds were dissolved previously in dimethyl sulphoxide (DMSO) and diluted with M199 medium in 96-well microplates to the highest concentration of 100 µg/ml. Each extract was tested twice at 8 concentrations prepared at two-fold dilution steps. Promastigotes were counted in a Neubauer haemocytometer and seeded at 1 × 10⁶/well with a final volume of 150 µl. Controls with DMSO and without drugs were performed. Pentamidine was used as a standard drug. The plate was incubated for 24 h at 24 °C and the viability of

promastigotes was verified by morphology in the light microscopy and the diphenyltetrazolium assay—MTT (Tada et al., 1986). Briefly, MTT (5 mg/ml) was dissolved in PBS, sterilized through 0.22 μm membranes and added, 20 μl /well, for 4 h at 37°C. Promastigotes were incubated without compounds and used as viability control. Formazan extraction was performed using 10% SDS for 18 h (100 μl /well) at 24°C and the optical density (OD) was determined in a Multiskan MS (UNISCIENCE) at 570 nm. The data analysis was done in Graph Pad Prism 3.0 software. 100% viability was expressed based on the OD of control promastigotes, after normalization. Those extracts presenting anti-Leishmania activity were tested against intracellular amastigotes of *L. chagasi*. Briefly, peritoneal macrophages were obtained as described previously and seeded for 24 h at 4×10^5 /well in 24-wells plates before infection with *Leishmania (L.) chagasi* amastigotes, which was made at a ratio 1:10 (macrophage/amastigotes) for 18 h at 37°C in a 5% CO₂ humidified incubator. Infected macrophages were incubated at 37°C with the test compounds at 25 $\mu\text{g}/\text{ml}$ for 120 h at the same conditions described above. Macrophages incubated without drugs were used for control (100% infected). Glucantime was used as a standard drug at two concentrations –25 and 300 $\mu\text{g}/\text{ml}$. At the end of the assay macrophages were fixed with methanol and stained with Giemsa. The parasite burden was determined by the number of infected macrophages (out of 200 cells in duplicate). The data were analyzed using Graph Pad Prism 3.0, which considered the mean of two performed assays in duplicate.

Anti-Trypanosome activity

In order to determine the antiparasitic effect of the test compounds against culture trypomastigotes of *T. cruzi*, the compounds were dissolved previously in DMSO and diluted in RPMI-1640 medium as described above for the anti-Leishmania assay. Free trypomastigotes obtained from LLC-MK2 cultures were counted in a Neubauer haemocytometer and seeded at 1×10^6 /well in 96-well microplates. Test compounds were incubated to the highest concentration of 100 $\mu\text{g}/\text{ml}$ for 48 h at 37°C in a 5% CO₂ humidified incubator. Benznidazole was used as standard drug at a fixed concentration of 100 $\mu\text{g}/\text{ml}$ and at different concentrations (two-fold dilutions) in order to determine the EC₅₀ value in our assay. The viability of the trypomastigotes was based in the cellular conversion of the soluble tetrazolium salt MTT into the insoluble formazan by mitochondrial enzymes (Lane et al., 1996). The formazan extraction was carried out with 10% SDS for 18 h (100 μl /well) at 24°C as described previously. The number of living trypomastigotes was determined indirectly by the OD at

570 nm. The data analysis was performed using Graph Pad Prism 3.0 software, which considered the mean of two performed assays in duplicate.

Cytotoxicity assay

Murine macrophages-RAW 264.7 cells (ATCC TIB-71) were seeded at 4×10^4 /well in 96-well microplates and incubated at 37°C for 48 h in the presence of the extracts, dissolved previously in DMSO and diluted in RPMI-1640 medium to the highest concentration of 120 $\mu\text{g}/\text{ml}$. The microplates were incubated for 48 h at 37°C in a 5% CO₂ humidified incubator. Control cells were incubated in the presence of DMSO and without drugs. The viability of the macrophages was determined with the MTT assay, as described above, and was confirmed by comparing the morphology of the control group via light microscopy. The sigmoid dose-response analysis was made using Graph Pad Prism 3.0 software, which considered the mean of two performed assays in duplicate.

Results

Anti-Leishmania activity

To determine the 50% Effective Concentration (EC₅₀ value) of the extracts, promastigotes of *Leishmania (L.) chagasi* were incubated with the compounds for 24 h at 24°C and the viability measured using MTT. We observed an anti-Leishmania effect only with the total alkaloids of *A. coriacea*, *A. crassiflora*, *C. ovalifolia* and *G. australis* (Table 2). The most effective total alkaloid extract was that from *A. crassiflora*, which showed an EC₅₀ value of 24.89 $\mu\text{g}/\text{ml}$ (95% Confidence Interval—95% CI—18.22–33.99 $\mu\text{g}/\text{ml}$). Comparing the 95% CI of all the extracts, we observed an intermediate dose-response among *G. australis*, *A. coriacea* and *C. ovalifolia*, with an EC₅₀ value of 37.88 $\mu\text{g}/\text{ml}$ (95% CI = 26.15–54.89 $\mu\text{g}/\text{ml}$), 41.60 $\mu\text{g}/\text{ml}$ (95% CI = 26.99–64.12 $\mu\text{g}/\text{ml}$) and 63.88 $\mu\text{g}/\text{ml}$ (95% CI = 38.66–105.5 $\mu\text{g}/\text{ml}$), respectively (Fig. 1). Pentamidine was used as a standard drug, resulting in an EC₅₀ value of 1.69 $\mu\text{g}/\text{ml}$ (95% CI = 14.16–20.27 $\mu\text{g}/\text{ml}$), and could be properly compared to the extracts in Fig. 1. Among the four total alkaloids extracts presenting the anti-Leishmania activity against promastigotes, three showed an inhibitory effect against the intracellular amastigotes. *C. ovalifolia* and *A. crassiflora* were the most effective, showing a reduction in the parasite burden by 89.8% (± 2.9) and 86.1% (± 5.0), respectively. *A. coriacea* showed an intermediate activity, reducing the number of infected macrophages by 27.2% (± 2.9) and *G. australis* was inactive against the intracellular amastigotes, resulting in the same number

Table 2. Effect of plant extracts on *Leishmania (L.) chagasi* and *Trypanosoma cruzi* and their toxicity against mammalian cells

Species	Extract	<i>L. chagasi</i> promastigotes EC ₅₀ value (µg/ml) (95% CI)	<i>L. chagasi</i> amastigotes % treated macrophages at 25 µg/ml (±SD)	<i>T. cruzi</i> % trypomastigotes survival at 100 µg/ml (±SD)	Cytotoxicity RAW 264.7 cells EC ₅₀ value (µg/ml) (95% CI)
<i>A. crassiflora</i>	ET	>100	Nd	97.77±8.3	98.48 (23.5–411.5)
	TA	24.89 (18.2–33.9)	86.1±5.0	0±0	23.79 (21.1–26.7)
<i>A. coriacea</i>	ET	>100	Nd	11.04±4.9	57.09 (44.4–73.3)
	TA	41.60 (26.9–64.1)	27.20±2.9	0±0	22.88 (4.8–108.6)
<i>C. ovalifolia</i>	ET	>100	Nd	86.04±2.7	50.96 (34.2–75.9)
	TA	63.88 (38.6–105.5)	89.8±2.9	0±0	42.52 (12.1–148–7)
<i>D. furfuracea</i>	ET	>100	Nd	91.57±1.5	>120
	TA	>100	Nd	0±0	58.01 (56.6–59.4)
<i>D. lanceolata</i>	ET	>100	Nd	0±0	>120
	TA	>100	0±0	34.01±12.1	>120
<i>G. australis</i>	ET	>100	Nd	15.21±0.4	>120
	TA	37.88 (26.1–54.8)	Nd	2.13±3.0	29.86 (13.0–68.2)
<i>S. apiosyce</i>	ET	>100	Nd	40.89±8.7	>120
	TA	>100	Nd	35.27±3.5	43.82 (20.8–91.9)
<i>S. guianensis</i>	ET	>100	Nd	100±18.63	>120
	TA	>100	Nd	0±0	54.79 (17.2–174.2)
<i>X. emarginata</i>	ET	>100	Nd	11.14±0.9	71.91 (36.0–143.4)
	TA	>100	Nd	3.77±5.3	89.65 (89.5–89.7)

*Glucantime*25 (µg/ml) = 10%±4.9; *Glucantime* 300 (µg/ml) = 100%±0; Toxicity ≥120 µg/ml; *Pentamidine* = 1.69 µg/ml (14.1–20.27), Toxicity = 18.83 µg/ml (13.1–26.9); *Benznidazole* (100 µg/ml) = 31.79%±1.7, Toxicity = 57.86 µg/ml (45.4–73.7). nd = not determined, ±SD = standard deviation; ET = ethanol extract; TA = total alkaloid extract.

of infected macrophages found in the non-treated group. The standard drug Glucantime resulted in a reduction of the parasite burden by 100% at 300 µg/ml and by 10% at 25 µg/ml.

Anti-*Trypanosoma* activity

The trypomastigotes of *T. cruzi* appeared to be more sensitive to the extracts than the promastigotes of *Leishmania chagasi*. When the extracts were incubated at 100 µg/ml for 48 h with trypomastigotes of *T. cruzi*, we observed anti-*Trypanosoma* activity in all the nine species. The increase in the OD obtained at 570 nm, was correlated to the number of living parasites and the data reported in Table 2 was expressed as percentage of cell survival, compared to the control group (trypomastigotes without drugs). The total alkaloids from 5 species (*A. coriacea*, *A. crassiflora*, *C. ovalifolia*, *D. furfuracea*, *S. guianensis*) were the most effective against trypomastigotes, killing 100% of the cells (Table 2). Two alkaloid extracts (*X. emarginata* and *G. australis*) showed a considerable antiparasitic effect, reducing parasite survival by approximately 97%. The only ethanol extract that was 100% effective was from *D. lanceolata*. Benznidazole was used as standard drug, which resulted in a reduction in trypomastigotes viability by 31.79%

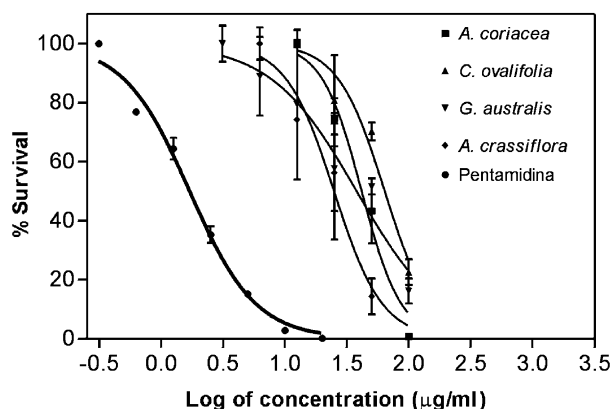


Fig. 1. Determination of the EC₅₀ value of plant extracts against *Leishmania (L.) chagasi* promastigotes. Promastigotes were counted in a Neubauer haemocytometer and seeded at 1×10^6 /well with a final volume of 150 µl. Ethanol and total alkaloid extracts were incubated with promastigotes in M199 medium at 24 °C for 24 h. Each extract was tested twice at 8 concentrations prepared at two-fold dilution steps. Pentamidine was used as standard drug (tick line). The number of living promastigotes was determined indirectly by the optical density (OD –570 nm) and correlated to the percentage of survival. The data analysis was performed using a sigmoid dose-response curve using Graph Pad Prism 3.0 software, which considered the mean of two performed assays in duplicate.

(± 1.7) at 100 $\mu\text{g/ml}$. The 50% Effective Concentration of benznidazole against trypomastigotes in the MTT assay was determined as 43 $\mu\text{g/ml}$ (95% CI = 36.87–50.15 $\mu\text{g/ml}$).

Toxicity for mammalian cells

The cytotoxicity of the extracts was determined in macrophages RAW 264.7, after 48 h incubation. Of the 18 plant extracts, six showed a lack of toxicity for mammalian cells at the maximal concentration used, 120 $\mu\text{g/ml}$ (Table 2). In general, the ethanol extracts were the least toxic for RAW cells. The comparison of the toxicity values from the total alkaloids and the standard drugs was presented in Fig. 2. Pentamidine and benznidazole were, in general, more toxic to mammalian cells than the plant extracts, showing an EC_{50} value of 18.83 and 57.86 $\mu\text{g/ml}$, respectively. This effect was also seen in the ethanol extracts (Fig. 2B). The standard drug Glucantime showed no toxicity at the tested concentration. All data obtained with the MTT assay were confirmed by inverted light microscopy, by comparing the morphology of the control group to macrophages incubated in the presence of the different compounds.

Discussion

In this study, nine species of Brazilian plants were screened against *Leishmania (L.) chagasi* and *Trypanosoma cruzi*, based on the antiparasitic activities of the majority of the plant genus and/or studied species (Table 1) and, additionally, on the presence of isoquinoline alkaloids in the Annonaceae (Leboeuf et al., 1982; Akendengue et al., 1999), Menispermaceae (Thorner, 1970; Akendengue et al., 1999) and Siparunaceae (Leitão et al., 1999) families. Furthermore, these compounds have been demonstrated a strong antiprotozoal action (Mahiou et al., 2000; Camacho et al., 2002; François et al., 1997).

Among the 18 extracts tested against *Leishmania* promastigotes, four total alkaloid extracts from *A. coriacea*, *A. crassiflora*, *C. ovalifolia* and *G. australis* were effective, killing from 80 to 100% of the parasites. Anti-helminthic (dos Santos and Sant'Ana, 2000, 2001) antileishmanial (Akendengue et al., 1999; Fournet et al., 1988) and antimalarial (Gessler et al., 1994) activities have been described for the genera and/or species of these plants. The most effective extract was found for *A. crassiflora* (total alkaloid), despite its 14-fold lower effectiveness when compared to the standard drug pentamidine. Comparisons should be drawn carefully between the pentamidine and the plant, as the former is a pure compound, while the latter are crude mixtures. Regarding the cytotoxicity of the effective extracts, it

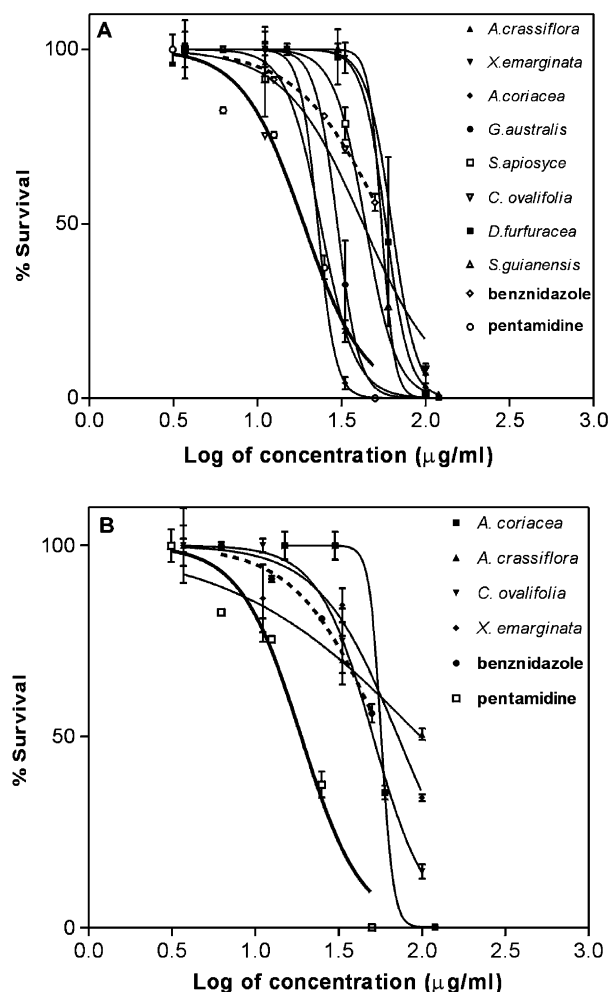


Fig. 2. Cytotoxicity of plant extracts. RAW 264.7 cells were seeded at 4×10^4 /well in 96-well microplates and incubated in RPMI-1640 medium at 37 °C for 48 h in the presence of the extracts. Control cells were incubated in the presence of DMSO and without drugs. The standard drugs pentamidine (tick continuous line) and benznidazole (tick non-continuous line) were used for comparison. A. Toxicity of total alkaloid extracts. B. Toxicity of ethanol extracts. The viability of the macrophages was determined using the MTT assay and was confirmed by comparing the morphology of the control group under light microscopy. The sigmoid dose-response analysis was performed using Graph Pad Prism 3.0 software, which considered the mean of two performed assays in duplicate.

was clear that the standard drug was at least 8-fold more toxic to mammalian cells. In our assay, only those compounds presenting activity against promastigotes were further evaluated against intracellular amastigotes. Three of the four total alkaloid extracts presented an anti-*Leishmania* effect against infected macrophages. *A. crassiflora* and *C. ovalifolia* showed similar inhibitory effects, reducing the parasite burden by approximately 90%, as compared to the non-treated group. Although the mammalian cytotoxicity of the standard drug

Glucantime could not be detected in the tested concentrations, this first line drug used in the therapy of Leishmaniasis resulted in only a 10% reduction in the number of infected macrophages at same concentration used for testing the plant extracts (25 µg/ml). The total alkaloid extract of *A. coriacea* poorly inhibited the intracellular amastigotes and *G. australis* showed no anti-parasitic effects, despite its moderate EC₅₀ value against promastigotes. Although promastigotes can be used for fast screenings of potential compounds, the clinically relevant form of the parasite is the amastigote form (Callahan et al., 1997), which shows metabolic differences from the extracellular forms. A variation in the sensitivity of the different extracts is expected between the two forms of the parasite and could be due to differences in the intracellular localization, rate of division, biochemical targets and drug metabolism (Escobar et al., 2002).

Isoquinoline alkaloids, which are produced by all three of the families considered in this work, are implicated strongly in the inhibition of an essential antioxidant enzyme of *Leishmania* and *Trypanosoma*, trypanothione reductase (Fournet et al., 2000). Trypanothione itself performs a variety of functions and protects the parasites from various reactive oxygen species generated by the host defense cells, being a good target molecule in Trypanosomes (Krauth-Siegel and Coombs, 1999). Although the molecular target of the selected extracts was not studied in *Leishmania* parasites, single or multiple compounds in the total alkaloid extracts could have contributed to the anti-*Leishmania* activity observed.

Many of the extracts ineffective against *Leishmania* parasites presented anti-*Trypanosoma* activity, which could be explained by the major metabolic and cellular differences found between the parasites. The *Leishmania* parasites live in parasitophorous vacuoles inside macrophages of pH ranging from 4 to 5 (Burchmore and Barret, 2001), while *T. cruzi* parasites are found free in the cytoplasm of many different cells (Coura and Castro, 2002). Of the 18 tested extracts, 16 were effective against trypomastigotes of *T. cruzi* (Table 2), but only eight extracts—from *A. crassiflora*, *A. coriacea*, *C. ovalifolia*, *D. furfuracea*, *D. lanceolata*, *S. guianensis*, *X. emarginata* and *G. australis*—killed approximately 100% of the living trypomastigotes. From the results obtained in this assay, it is clear that the anti-*Trypanosoma* activity was concentrated in the total alkaloid fractions. The effectiveness of the standard drug benznidazole against trypomastigotes of *T. cruzi* with the MTT test, corroborates data described elsewhere (Lane et al., 1996). Based on cytotoxicity data, we suggest that the most promising total alkaloid extracts against *T. cruzi* were those which showed little or no toxicity to mammalian cells at the highest tested concentrations, represented by *D. furfuracea*, *D. lanceolata*,

G. australis, *S. guianensis* and *X. emarginata*. The cytotoxicity levels considered in our data were correlated to the values found for the standard drug benznidazole against the mammalian cells. The Selectivity Index (Toxicity for mammalian cells/EC₅₀ value in parasites) of these extracts could be enhanced if, in a future isolation, the active alkaloids present less significant cytotoxicity, which could be evaluated easily by bio-monitored assays. Regarding the *in vitro* cytotoxicity of the standard drugs pentamidine and benznidazole, it was clear that the majority of the plant extracts were less toxic to mammalian cells, suggesting the considerable potential of these plants.

Our data support the antiparasitic activity of isoquinoline alkaloid-producing families. The lack of an effective chemotherapy for Chagas disease and Leishmaniasis, urges a wide investigation for active compounds, and among them, natural products have been successfully screened. Plant-derived compounds continue to provide key lead structures and therapeutic agents for the treatment of protozoan diseases (Camacho et al., 2002). Bioguided assays are in progress in order to identify the active alkaloids of the most promising tested species. Moreover, isolated compounds could work as template structures for further molecular modeling studies, which are a useful tool to improve new drug designs.

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