Leishmaniasis is a tropical disease caused by protozoa of the *Leishmania* genus. These protozoa cause a disease with different clinical forms, among them cutaneous, hyperergic, mucocutaneous, and anergic diffuse leishmaniasis (Leon et al. 1990a). The disease is endemic in some geographical areas of Brazil, where it constitutes a serious health problem (Lonardoni et al. 1999, Leon et al. 2000). The drugs of choice for the treatment of leishmaniasis are the pentavalent antimonials (SbV), but they present renal and cardiac toxicity. A second choice for the treatment of the disease is a diamidine (pentamidine isethionate), which also has serious side effects (Korolkovas & Burckhalter 1988). However already in some trials alternative pharmaceutical formulations have been used to reduce the toxicity of these drugs (Frezard et al. 2000).

The lack of an effective anti-leishmanial drug led a renewed interest in the study of traditional remedies as sources for the development of new chemotherapeutic compounds with better activity and less toxicity. Several plants have been used for the treatment of parasitic diseases (Araujo et al. 1998). In order to find new drugs against leishmaniasis, we have studied alkaloidal extracts of Brazilian plants (Oliveira et al. 2002). The aim of the present study is to investigate the anti-leishmanial activity of alkaloidal extracts from the stem bark of *A. ramiflorum* against *L. (V.) braziliensis* and *L. (L.) amazonensis*. Based on these in vitro results against *L. (L.) amazonensis* new studies should be made to find the compounds with anti-leishmanial activity.

**Key words:** Apocynaceae - anti-leishmanial activity - Brazilian trees - monoterpenoid indolic alkaloids - *Aspidosperma ramiflorum* - *Leishmania amazonensis* - *Leishmania braziliensis*

**MATERIALS AND METHODS**

**Plant materials** - *A. ramiflorum* Muell. Arg. was collected in the Horto Florestal de Maringá, July 2000, in Maringá, state of Paraná, Brazil. The plant was collected and identified by Prof. Dr Ismar Sebastião Moscheta and an exsiccatum deposited and authenticated at the Herbarium of the State University of Maringá, Maringá, Brazil.

**Extraction of plant materials** - Air-dried stem bark (1 kg) was extracted with 70% ethanol at room temperature. After removal of the ethanol, the crude extract was added to a 10% acetic acid solution (v/v) and kept at 5oC overnight. After filtration, the aqueous phase was first extracted with chloroform (acid extract), then the pH raised to 10 and the resulting solution re-extracted with chloroform (basic extract). The two chloroform extracts were concentrated under reduced pressure, and then lyophilised yieldings the acid (7.7 g) and basic fractions (11.6 g), which were both analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The main bulk of the alkaloids was in the basic fraction which was called the alkaloidal extract and which was used for the assays.

**Results**

In order to find new drugs against leishmaniasis, we have been studying extracts of Brazilian trees. In the present study, we have evaluated the effectiveness of an alkaloid extract of *Aspidosperma ramiflorum* Muell. Arg. (Apocynaceae), against the extracellular forms promastigotes of *L. (L.) amazonensis* and *L. (V.) braziliensis*. The alkaloid extract of *A. ramiflorum* was much more effective against *L. (L.) amazonensis* (LD50 < 47 µg/ml) than *L. (V.) braziliensis*. Based on these in vitro results against *L. (L.) amazonensis* new studies should be made to find the compounds with anti-leishmanial activity.
Characterization of the alkaloidal extract - The alkaloidal extract from A. ramiflorum was analyzed and compared with isolated samples of alkaloids from the stem bark of the plant using TLC on silica gel GF254, developed with CHCl₃:AcOEt:Triethylamine (49.5:49.5:1.0) in an NH₃ atmosphere. For HPLC analysis, the crude extract was dissolved in CH₂Cl₂:MeOH (80:20) and 10 µl were injected onto a Waters µ-Bondapak RP-18 (reverse phase, 4.6 mm x 250 mm) column at 40°C. Solvent A was 100 mmol l⁻¹ ammonium formate in 0.12% octanesulfonic acid (v/v), formic acid and acetonitrile (88:4:8, v/v), while solvent B consisted of 100 mmol l⁻¹ aqueous ammonium formate containing 0.12% octanesulfonic acid (v/v)/formic acid/acetonitrile (64:4:32, v/v). The separation was carried out using a mixture of solvent A and, a progressively increasing amount of B (0, 10, 40, 90, 100%) during 60 min. The flow rate was 1.3 ml min⁻¹. The effluent was monitored with a photodiode-array detector with windows at 222 nm and 254 nm and also by mass spectral analysis of isolated eluates.

Culture and maintenance of the parasite - L. (V.) braziliensis and L. (L.) amazonensis, MHOM/BR1987/M11272 and MHOM/BR/1977/LTB0016 promastigotes, were grown at 25°C in Schneider's Drosophila medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). Cells were harvested in the late log phase, resuspended in fresh medium, counted in Neubauer’s chamber and adjusted to a concentration of 4 x 10⁶/ml.

Anti-leishmanial in vitro assay with L. (V.) braziliensis and L. (L.) amazonensis promastigotes - The alkaloidal extract was added to the promastigote cultures, at 4 x 10⁶/ml, as above, for screening (from 320 mg/ml to 0.125 mg/ml of extract) solubilized in dimethylsulfoxide (DMSO) (the highest concentration used was 1.6%, v/v) and incubated at 25°C. After 24 h of incubation, the surviving parasites were counted in a Neubauer’s chamber and compared with controls, which only had DMSO. All tests were done in triplicate and pentamidine isethionate (Eurofarma) was used as reference drug (Leon et al. 2002). The LD₅₀/24 values were determined by linear regression analysis from this inhibition percentage using statistic error limits up 10%.

RESULTS AND DISCUSSION

The anti-leishmanial activity of plant extracts has been attributed to compounds belonging to diverse chemical groups, such as isoquinoline alkaloids, indole alkaloids, quinones, and terpenes (Araujo et al. 1998).

The alkaloidal extract of A. ramiflorum was chosen for assays because of the presence of bisindole monoterpenoid alkaloids (Fig. 1) with structures similar to alkaloids from Strychnos usambarensis, which have been reported to possess antiprotozoal activity (Angenot et al. 1991).

The major constituents of A. ramiflorum alkaloidal extracts are: ramiflorine A (1) and ramiflorine B (2), whose presence was monitored by HPLC (Fig. 2) and TLC (Oliveira et al. 2002).

In the present study, we evaluated the effectiveness of a crude alkaloid extract of A. ramiflorum against the extracellular form (promastigotes) of L. (L.) amazonensis and L. (V.) braziliensis. The alkaloid extract was more effective against the L. (L.) amazonensis (LD₅₀ < 47 µg/ml) than L. (V.) braziliensis.
Although the mode of action of these alkaloids is not known, the fact that they are similar in structure to usambarine (3) and usabarensine (4), makes it possible that their modes of action may be similar, that is, they would acting as inhibitors of protein synthesis (Angenot et al. 1991).

This preliminary positive result suggests further work with isolated compounds to evaluate the individual activity of ramiflorine A (1) and ramiflorine B (2), as part of a continued search for new drugs with high activity and low side effects against diseases associated with protozoan parasites, such as leishmaniasis.

REFERENCES


