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Studies on the Effectiveness of *Tanacetum parthenium* Against *Leishmania amazonensis*

Tatiana Shioji TIUMAN[1], Tânia UEDA-NAKAMURA[3], Benedito Prado DIAS FILHO[3], Diógenes Aparício Garcia CORTEZ[2], and Celso Vataru NAKAMURA[3]

[1]Programa de Pós-graduação em Ciências Farmacêuticas; [2]Departamento de Farmácia e Farmacologia; [3]Departamento de Análises Clínicas, Laboratório de Microbiologia Aplicada aos Produtos Naturais e Sintéticos, Universidade Estadual de Maringá, Maringá, Paraná, Brasil

Summary. We assessed the biological activity of a plant powder, crude extracts, and several fractions obtained from *Tanacetum parthenium* on *Leishmania amazonensis*. The medicinal plant *T. parthenium* is indicated for prevention of migraine headache crises, and several investigations have already demonstrated its anti-inflammatory activity. This study included the extraction process and bioassay-guided fractionation by the adsorption chromatography method. A progressive increase in the antileishmanial effect was observed in the course of the purification process. The plant powder (PTP) had a 50% inhibitory concentration (IC_{50}) at 490 µg/ml, whereas the dichloromethane fraction (DF) showed an IC_{50} of 3.6 µg/ml against the growth of promastigote forms after 48 h of culturing. In axenic amastigote forms, the IC_{50} of the PTP and DF were 74.8 µg/ml and 2.7 µg/ml, respectively. Cytotoxicity analysis indicated that the toxic concentrations of the PTP, ethyl-acetate crude extract (ECE), and DF were much higher for J774G8 macrophages than for the protozoans. Haemolytic experiments were performed, and the ECE and DF did not cause lysis at concentrations higher than the IC_{50} for promastigotes.

Key words: antileishmanial activity, cytotoxicity, *Leishmania amazonensis*, medicinal plants, *Tanacetum parthenium*.

INTRODUCTION

Leishmaniasis is a major global public-health problem, with 1.5-2 million humans affected by the disease annually. Approximately 350 million people in 88 countries are estimated to be threatened by the disease (World Health Organization 2001). This parasitosis is caused by organisms of the genus *Leishmania*, and has three clinical forms: visceral, cutaneous, and mucocutaneous. *Leishmania amazonensis* is one of the principal agents of diffuse cutaneous leishmaniasis, but visceralisation of strains has often been observed in patients with *Leishmania*-human immunodeficiency virus coinfection (Alvar *et al.* 1997). Despite the tremendous progress made in the understanding of the biochemistry and molecular biology of the parasite, the first-choice treatment for the several forms of leishmaniasis still relies on pentavalent antimonials developed more than 50 years ago (Croft and Coombs 2003). These drugs are potentially toxic and often ineffective (Berman 1996, 1997; Grevelink and Lerner 1996), and second-

Adress for correspondence: Celso Vataru Nakamura, Universidade Estadual de Maringá, Departamento de Análises Clínicas, Laboratório de Microbiologia Aplicada aos Produtos Naturais e Sintéticos, Bloco I-90 Sala 123 CCS, Avenida Colombo, 5790; BR-87020-900, Maringá, PR, Brazil; Fax: +55 44 261-4860; E-mail: cvnakamura@uem.br

choice drugs such as amphotericin B and pentamidine may be even more toxic (Grevelink and Lerner 1996, Croft and Coombs 2003). The spread of drug resistance, combined with other shortcomings of the available antileishmanial drugs, emphasises the importance of developing new, effective, and safe drugs against leishmaniasis.

Plants provide unparalleled chemical diversity and bioactivity, which has led to the development of hundreds of pharmaceutical drugs (Shu 1998). The species *Tanacetum parthenium* (Asteraceae), popularly known as feverfew, is a traditional remedy used in the prophylactic treatment of migraine. Its effects have been attributed to the plant´s content of sesquiterpene lactones, notably parthenolide (Martindale 1999). Several studies have also shown that feverfew is effective as an anti-inflammatory and antinociceptive agent (Heptinstall *et al.* 1985, Murphy *et al.* 1988, Sumner *et al.* 1992, Jain and Kulkarni 1999, Williams *et al.* 1999, Piela-Smith and Liu 2001).

The study reported here was undertaken to examine the antileishmanial activity of *T. parthenium*, which has not been tested previously against trypanosomatids. The cytotoxic activity against J774G8 macrophages and sheep blood cells was also determined.

MATERIALS AND METHODS

Plant material. Powder of aerial parts of *T. parthenium* (Lot 166871) (PTP) was kindly furnished by the Herbarium Laboratório Botânico Ltda (Colombo, Paraná, Brazil).

Extraction and fractionation. The plant material was sequentially extracted by exhaustive maceration at room temperature in the dark in ethanol:water (90:10). The supernatants were filtered, evaporated under vacuum and lyophilised. The powder resulting from lyophilisation was termed the aqueous crude extract, and the residue was dissolved in ethyl acetate or hexane and was named according to the solvent used. The aqueous (ACE), ethyl-acetate (ECE), and hexane (HCE) crude extracts were directly assayed against *L. amazonensis*. Subsequently, the ethyl-acetate extract was chromatographed on a silica-gel column using hexane, dichloromethane, ethyl acetate, methanol, and methanol:water (90:10). Each fraction (hexane, HF; dichloromethane, DF; ethyl acetate, EF; methanol, MF; methanol:water, MWF) was tested for antiprotozoal activity.

Parasite culture. Promastigote forms of *Leishmania amazonensis* (WHOM/BR/75/Josefa) were cultured at 28°C in Warren´s medium (brain-heart infusion plus haemin and folic acid) supplemented with 10% heat-inactivated fetal bovine serum in a tissue flask. Axenic amastigote cultures, obtained by *in vitro* transformation of infective promastigotes (Ueda-Nakamura *et al.* 2001), were maintained in Schneider's insect medium (Sigma Chemical Co., St. Louis, Missouri, USA), pH 4.5, with 20% fetal bovine serum at 32°C.

Cells. Murine macrophage J774G8 cells were maintained in tissue flasks in medium composed of RPMI 1640 medium (Gibco Invitrogen Corporation, New York, USA), sodium bicarbonate, L-glutamine, and heat-inactivated fetal bovine serum (10%). The cultures were maintained at 37°C in 5% CO_2-air mixture.

Preparation of stock solutions. The PTP and the crude extracts and fractions were solubilised in DMSO prior to adding them to the appropriate culture medium. The final concentration of DMSO in the test medium never exceeded 0.5%, a concentration which has no effect on the growth of parasites. The stock solution of Amphotericin B (AMPB) (Cristália Produtos Químicos Farmacêuticos Ltda, Itapira, São Paulo, Brazil) was prepared in phosphate-buffered saline.

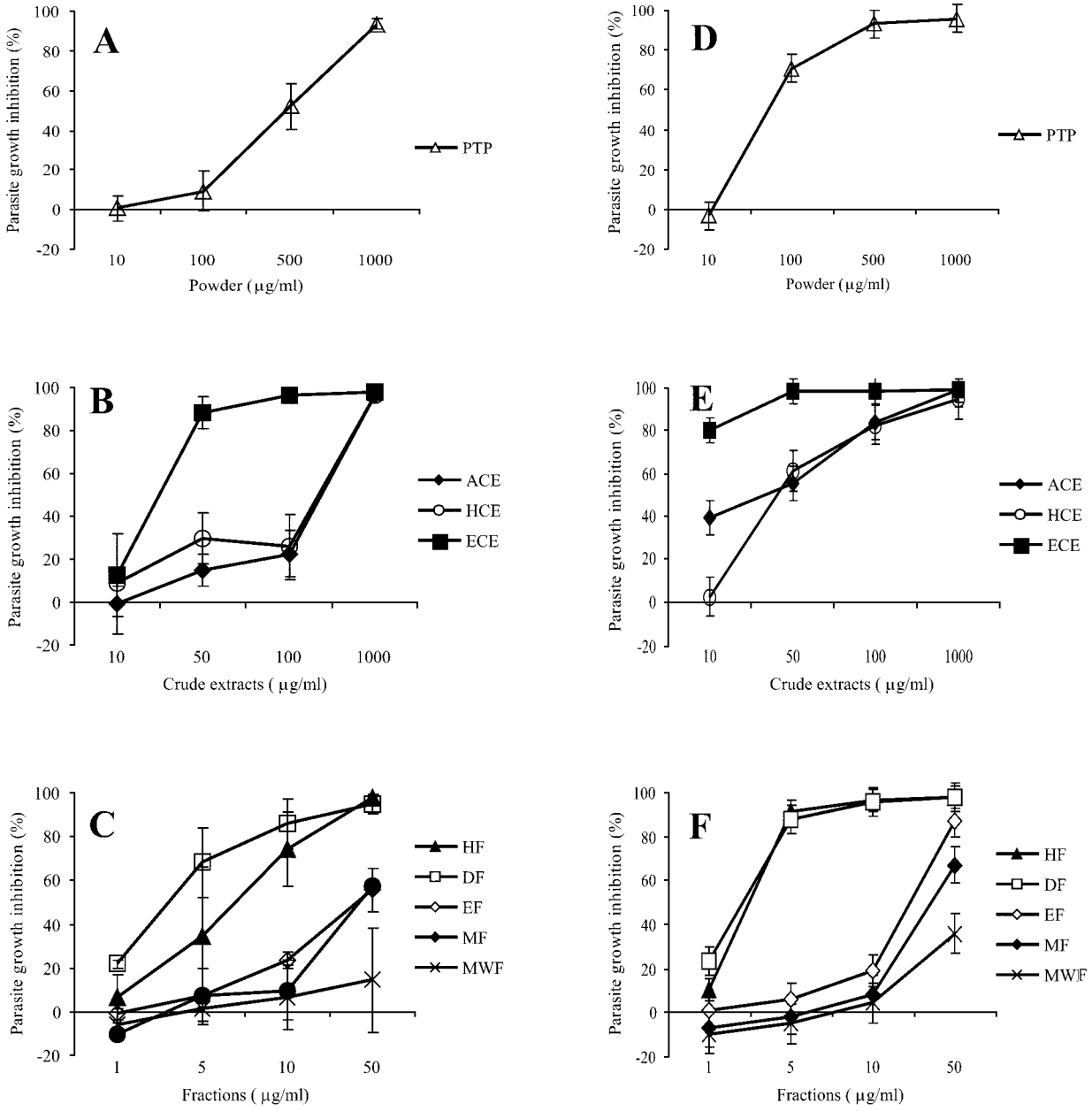
Antileishmanial assays. The effects of the PTP as well as the crude extracts and fractions were evaluated in 24-well microtitre plates. Cultures of promastigotes and amastigotes in the logarithmic phase (10^6 cells/ml) were incubated in appropriate medium and heat-inactivated fetal bovine serum in the presence of different concentrations of the extracts. In all tests, 0.5% DMSO and medium alone were used as controls. AMPB was used as the reference drug. Parasites were counted daily for 5 days in a haemocytometer with a light microscope, and the results were compared with those from the controls. Each assay was performed in duplicate and repeated in separate experiments.

Cytotoxicity assay against J774G8 cells. The cytotoxic effect of the plant, expressed as cell viability, was assessed on macrophage strain J774G8. The test was carried out in 24-well microtitre plates. A suspension of 5×10^5 macrophages was added to each well. The cells were grown as monolayers in culture medium at 37°C and 5% CO_2-air mixture. Confluent cultures were treated with medium containing different concentrations of PTP, ECE, DF, and AMPB for 48 h. Next the supernatant cells were homogenised with adhered cells, equal volumes of cell suspension and 0.4% erythrosin B (vital dye) were mixed, and at least 200 cells were counted and evaluated by light microscopy.

Red blood cell lysis assay. This experiment also evaluated the cytotoxicity of *T. parthenium*. A 4% suspension of freshly defibrinised sheep blood was prepared by adding blood to sterile 5% glucose solution. One ml of red-blood-cell suspension was added to each test tube, and different concentrations of ECE, DF, and AMPB (a reference drug, which may cause anaemia) (Bennett 1996) were added, gently mixed, and incubated at 37°C for 120 minutes. Samples were centrifuged at 1,000 g for 10 min. Absorbance of the supernatant was determined at 540 nm. The inhibition of haemolysis was calculated according to the equation: Inhibition (%) = (Ap-As)/(Ap-Ac) × 100; where Ap, As, and Ac are the absorbance of the positive control, test sample, and negative control, respectively. The negative control was the red-blood-cell suspension alone or with 0.5% DMSO, and the positive control was Triton X-114. These tests were performed in duplicate on separate occasions.

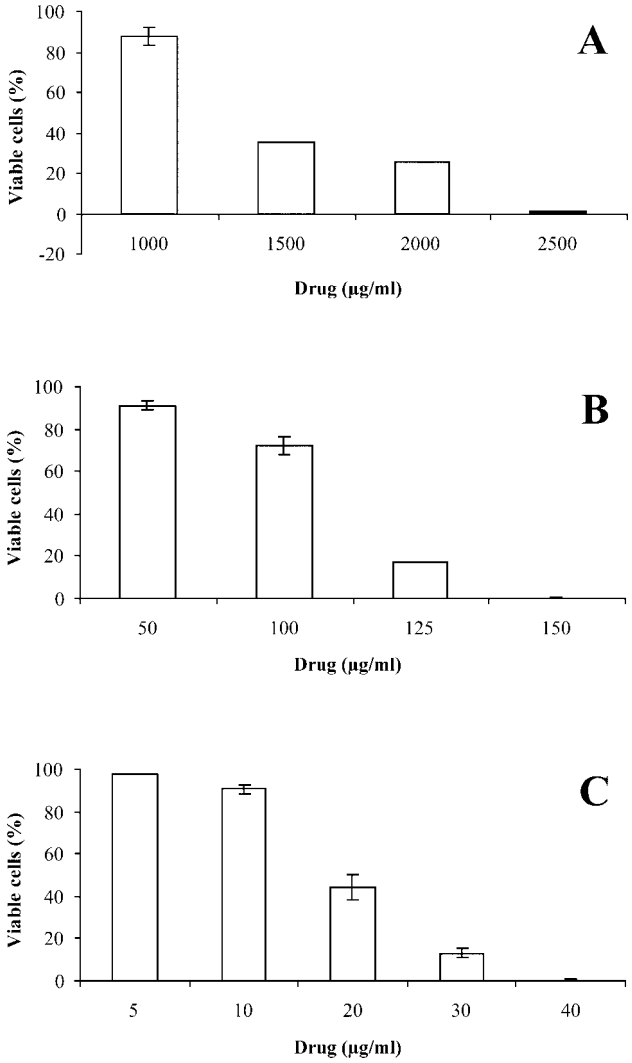
RESULTS

Antileishmanial assays. PTP inhibited the growth of promastigote and axenic amastigote forms of *L. amazonensis in vitro* after 48 h of incubation. At 1000 µg/ml it inhibited 93.6% of the promastigotes,



Figs 1A-F. Effects of *Tanacetum parthenium* against promastigote (**A-C**) and axenic amastigote (**D-F**) forms of *Leishmania amazonensis* cultured for 48 h in the presence of the indicated concentrations. **(A, D)** powder (PTP), **(B, E)** crude extracts (ACE, ECE, and HCE), and **(C, F)** fractions (HF, DF, EF, MF, and MWF). The results are from two experiments in duplicate and are shown as percentages ± standard deviations of growth inhibition in relation to untreated control protozoans.

growth and 95.9% of the amastigotes, and had a 50% inhibitory concentration (IC_{50}) of 490 µg/ml (Fig. 1A) and 74.8 µg/ml (Fig. 1D) respectively. ACE at 1000 µg/ml inhibited 96.6% of the promastigote forms and 98.9% of the amastigote forms, and had an IC_{50} of 434 µg/ml and 36.7 µg/ml respectively. HCE gave a similar result, with 96.6% growth inhibition of promastigotes and 95% of amastigotes, and an IC_{50} of 409 µg/ml and 42.4 µg/ml respectively. ECE was more effective, with an inhibition of 96.2% at 100 µg/ml for promastigote forms and 98.8% at 50 µg/ml for amastigote forms; it had an IC_{50} of 29 µg/ml and <10 µg/ml respectively



Figs 2A-C. Effects of *Tanacetum parthenium* powder (PTP) **A** - the ethyl-acetate crude extract (ECE) **B** - and the dichloromethane fraction (DF) **C** - on the survival of J774G8 macrophages with different concentrations of the drugs after 48 h of incubation. The results are from two experiments in duplicate and are shown as percentages ± standard deviations of viable cells in relation to the untreated control.

(Figs 1B, F). This last extract showed the highest antileishmanial activity, and was submitted to chromatography on a silica-gel column. Five fractions were obtained: HF, DF, EF, MF, and MWF. The HF and DF at a concentration of 50 µg/ml inhibited promastigote growth by 96% and amastigote growth by 98% after culturing for 48 h. Under the same conditions, the EF and MF inhibited promastigote growth by 56%. For amastigotes, EF showed 87%, and MF 67% growth inhibition. The MWF showed the lowest activity of all, with 14.4% and 36% inhibition for promastigote and

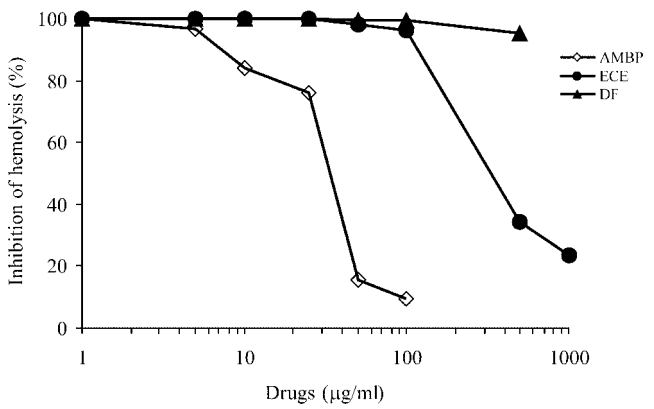


Fig. 3 Haemolytic properties of ethyl-acetate crude extract (ECE) and the dichloromethane fraction (DF) obtained from aerial parts of *Tanacetum parthenium*. Amphotericin B (AMPB) was included in the assay as a reference drug.

Table 1. Comparison of values of CC_{50} for J774G8 macrophages and IC_{50} for promastigote forms of *Leishmania amazonensis*, and their respective selectivity indexes (SI).

	J774G8 CC_{50}	Promastigote IC_{50}	SI
	µg/ml		
PTP	1400	490	2.9
ECE	110	29	3.8
DF	19	3.6	5.3

SI = CC_{50} J774G8 / IC_{50} promastigotes

amastigote forms respectively (Figs 1C, F). The IC_{50} values for promastigotes observed for the HF, DF, EF, MF, and MWF were 7.0, 3.6, 43.1, 43.8, and >50.0 µg/ml, and for amastigotes were 2.9, 2.7, 28.3, 48.4 and >50 µg/ml respectively. A concentration of 0.058 µg/ml of the reference drug AMPB inhibited 65.8% and 2.9% of promastigote and amastigote growth respectively (data not shown).

Cytotoxicity assay against J774G8 cells. J774G8 macrophages treated with 1000, 1500 and 2000 µg/ml of the PTP, showed 88.5, 35.2 and 25.6% viable cells, respectively (Fig. 2A). The toxicity to J774G8 cells and the antiprotozoal activity were compared using the selective index (SI) ratio: CC_{50} J774G8 cells/IC_{50} protozoans, where CC_{50} is 50% of the cytotoxic concentration. A value greater than 1 is considered to be more selective

against protozoans, and a value lower than 1 is considered to be more selective to the cells. In this case, the SI was 2.9, demonstrating that the PTP is less toxic to the cells than to the protozoans (Table 1). For macrophages treated with the ECE at 50 µg/ml, 91.1% of the cells remained viable. However, a concentration of 125 µg/ml of ECE showed only 17% viable cells, after 48 h of incubation (Fig. 2B). The SI for the ECE was 3.8 (Table 1). J774G8 macrophages treated with 5 µg/ml of the DF showed 97.6% viability. At concentrations of 20 and 30 µg/ml, 44.5% and 12.9% of the cells remained viable, respectively (Fig. 2C). In this case, the fraction was 5.3 times more selective against the parasites than the macrophages (Table 1). The CC_{50} for AMPB was 13.9 µg/ml (data not shown).

Red blood cell lysis assay. The red-blood-cell control did not show haemolysis, nor did the 0.5% DMSO control. On the other hand, the positive control TritonX-114 showed 100% haemolysis. Fig. 3 shows that red blood cells treated with 100 µg/ml of ECE caused only 4.0% lysis after 120 min of incubation. EC_{50} (50% effective concentration) of haemolysis was 397 µg/ml, a value 13.7-fold higher than the IC_{50} for promastigote forms. The DF added to a suspension of red blood cells caused 4.6% haemolysis at 500 µg/ml (the highest concentration tested), which is 138.9-fold higher than the IC_{50} for promastigote forms. At 100 µg/ml, this fraction showed only 0.5% haemolysis. Cells treated with AMPB at 6.25 µg/ml showed 3.9% lysis, while at 100 µg/ml they showed 90.5% lysis. The haemolysis EC_{50} was 36 µg/ml.

DISCUSSION AND CONCLUSION

There is a general lack of effective and inexpensive chemotherapeutic agents for the treatment of leishmaniasis. Pentavalent antimonial drugs are the first-line treatment for this disease, with amphotericin B and pentamidine being used as alternative drugs. All of these have serious side effects, and resistance has become a severe problem; therefore, new drugs are urgently required. Natural products have potential in the search for new and selective agents for the treatment of important tropical diseases caused by protozoans. Alkaloids, terpenes, quinones, and miscellaneous compounds well illustrate the diversity of antiprotozoal compounds found in higher plants. Some of these have been shown to act on systems present in parasites but which are absent or different from those in the host, and these merit further study in order to improve their activity and lessen toxic effects (Wright and Phillipson 1990).

Plants belonging to the genus *Tanacetum* are reputed to have excellent medicinal value, and a large number of sesquiterpenoids and sesquiterpene lactones, which are typical constituents of these drugs, have been isolated from *Tanacetum* species. These compounds might be responsible for the effects exhibited by the plants (Abad *et al.* 1995). The species *Tanacetum parthenium* has been used in folk medicine for treatment of migraine, tinnitus, giddiness, arthritis, fever, menstrual disorders, difficulty in labor, stomachache, toothache, and insect bites (Newall *et al.* 2002).

We demonstrated that crude extracts and fractions prepared from *T. parthenium* showed excellent antileishmanial activity. A progressive increase in the antileishmanial effect was observed in the course of the purification process. Bioassay-guided fractionation led us to a dichloromethane fraction which was 136-fold and 28-fold more active against promastigote and amastigote forms respectively, than the crude plant powder from aerial parts. Several studies have shown that crude extracts from different species of plants show antileishmanial activity. Screening of extracts from Bolivian plants against *L. braziliensis*, *L. amazonensis* and *L. donovani* demonstrated that five plants used in folk medicine to treat cutaneous leishmaniasis (*Bocconia integrifolia, Bocconia pearcei, Ampelocera edentula, Galipea longiflora*, and *Pera benensis*) showed antileishmanial activity (Fournet *et al.* 1994). Another study with extracts from Colombian plants found that four species used traditionally for treatment of leishmaniasis (*Conobea scoparioides, Hygrophila guianensis, Otoba novogranatensis* and *Otoba parviflora*) showed activity against promastigote forms of *Leishmania* spp. at 100 µg/ml (Weniger *et al.* 2001). Salvador *et al.* (2002) demonstrated that aqueous and ethanol crude extracts of roots from *Blutaparon portulacoides* showed antileishmanial activity against axenic amastigote forms of *L. amazonensis*. Additionally, crude extracts from *Alstonia macrophylla, Rhazya stricta, Swietenia macrophylla, Stephania dinklagei, Triclisia patens,* and *Cephaelis camponutans*, selected either from ethnobotanical or chemotaxonomic data, showed strong activity against promastigote forms of *L. donovani,* with IC_{50} values below 10 µg/ml. These extracts had stronger cytotoxic effects on the parasite than on mammalian cells (Camacho *et al.* 2003). The linalol-rich essential oil from the leaves of *Croton cajucara* showed excellent inhibition of the growth of promastigote and amastigote

forms of *L. amazonensis* (Rosa *et al.* 2003). Recently, Mendonça-Filho *et al.* (2004) demonstrated that the polyphenolic-rich extract from *Cocos nucifera* at 10 µg/ml inhibited the growth of promastigote forms of *L. amazonensis* and reduced by approximately 44% the association index between peritoneal mouse macrophages and this protozoan. Experiments with axenically cultured amastigotes should be of interest for developmental investigations of the disease-maintaining stage of this parasite (Pan *et al.* 1993).

A progressive decrease in the cytotoxic effect was observed in the course of the purification process. *In vitro* tests with J774G8 cells showed that the IC_{50}s of the PTP, ECE, and DF were more toxic to the protozoans. This is demonstrated by their SI ratio values of >1 (Table 1). Also, cytotoxicity studies with *T. parthenium* demonstrated that toxic concentrations for red blood cells are higher than for promastigote forms. Toxicity tests for medicinal plants are essential because of the growing interest in alternative therapies and the therapeutic use of natural products. This interest in drugs of plant origin is due to several reasons, e.g., conventional medicine can be inefficient, synthetic drugs may have side effects, or folk medicine suggests that natural products are harmless (Rates 2001). However, traditional use is no guarantee of safety (Edzard 1998), and relatively few drugs have been evaluated scientifically to prove their safety, potential benefits, or effectiveness (Calixto 2000).

Natural products can be lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development, and the discovery of new therapeutic properties not yet attributed to known compounds (Hamburguer and Hostettmann 1991). Considering that the Brazilian flora is very rich, reliable scientific knowledge is required for the transformation of medicinal plants into industrialised medicines (Rates 2001). Natural products have made, and are continuing to make, an important contribution to this area of therapeutics; perhaps their future potential will be even greater.

In this study we report the biological effect of crude extracts and fractions obtained from *T. parthenium* on promastigote and amastigote forms of *L. amazonensis*. This activity represents an exciting advance in the search for novel antileishmanial agents from natural sources, since a significant and important effect against the intracellular stage of the protozoan was demonstrated. This plant showed significant activity against *Leishmania* pathogens, but further synthesis and *in vitro* studies are indicated to validate these results. Therefore, the most promising fraction was given priority for further phytochemical analysis of the isolation and identification of the particular compounds which have antiprotozoal activity.

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