

# ACTA PROTOZOLOGICA

## ***In vitro* Antileishmanial Activity of Hydroalcoholic Extract, Fractions, and Compounds Isolated from Leaves of *Piper ovatum* Vahl against *Leishmania amazonensis***

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**Summary.** We assessed the biological activity of a crude extract, a mixture of several fractions, and a pure compound obtained from *Piper ovatum* Vahl against promastigote and amastigote forms of *Leishmania amazonensis*. The medicinal plant *P. ovatum* is used popularly as an anesthetic and anti-inflammatory. This study included the extraction process and bioassay-guided fractionation by the adsorption chromatography and Sephadex LH-20 method. A progressive increase in the antileishmanial effect was observed in the course of fractionation. The 50% inhibitory concentration (IC<sub>50</sub>) for dichloromethane-ethyl acetate (1:1 v/v) fraction was 2.1 µg/ml and 24 µg/ml; mixture of piperovatine: piperlongumune (2:3) 0.9 µg/ml and 24 µg/ml; piperovatine (1) 9.5 µg/ml and 10 µg/ml; and piperlonguminine (2) 2.5 µg/ml and 9.0 µg/ml, for promastigote and amastigote forms, respectively. Cytotoxicity analysis indicated that these toxic concentrations were much higher for J774G8 macrophages and Vero cells than for the protozoans. The mixture of piperovatine: piperlongumune (2:3) showed important antiprotozoal activity against the amastigote and promastigote forms of *L. amazonensis*, and it produced morphological changes in promastigotes and amastigotes at 0.9 µg/ml and 24 µg/ml (50% growth inhibition concentration), respectively, including intense cytoplasmic vacuolization, mitochondrial swelling, and mitochondrial damage, as revealed by transmission electron microscopy.

**Key words:** Antiprotozoan activity, ultrastructure alterations, *Leishmania amazonensis*, *Piper ovatum*.

## INTRODUCTION

Leishmaniasis is an insect-borne protozoan infection that affects an estimated 12 million people worldwide, causing significant morbidity and mortality in Africa,

Asia and Latin America (WHO 2001). Pentavalent antimonials are the first-line treatments, but their irregular effectiveness makes them sometimes disappointing because of antimonial resistance and relapse following Amphotericin B therapy. Second-line drugs, such as Amphotericin B, are more toxic, and Amphotericin B's lipid formulation is too expensive for routine use in underdeveloped countries (Murray 2001). There is at present no effective vaccine for humans (Handman 2001). Several new alkylphosphocholine derivatives with amide or ester bonds in the alkyl chain have been

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synthesized that show antileishmanial activity. More recently, these compounds have shown great promise in the treatment of parasitic infections, in particular those caused by *Leishmania* spp., and n-hexadecylphosphocholine (miltefosine) has been approved in several countries for clinical use in the treatment of leishmaniasis (Obando *et al.* 2007).

The family Piperaceae contains 2000 species (Wanke *et al.* 2006, Quijano-Abril *et al.* 2006) and *Piper* is the most important genus of the family. Species of *Piper*, which are widely distributed in the tropics and subtropics, are used medicinally in various ways. Plants of the genus *Piper* are well known in Indian Ayurvedic medicine for their medicinal properties, and also in folk medicine of Latin America and the West Indies (Parmar *et al.* 1997). The leaves of two species of the family Piperaceae (*Piper hispidum* Sw. and *Piper strigosum* Trel.) show good leishmanicidal activity, with IC<sub>50</sub> < 10 g/ml against amastigotes (Estevez *et al.* 2007). The biological activity of the crude extract and several fractions obtained from *Piper regnellii* var. *pallescens* was assessed against the growth of promastigote forms of *Leishmania amazonensis* (Nakamuara *et al.* 2006).

*Piper ovatum* Vahl, an herbaceous plant found throughout Brazil, is popularly known as “joão burandi” or “anestésica,” and is used in traditional medicine for the treatment of inflammation and as an analgesic (Correa 1984). The hydroalcoholic extract, fractions, and a mixture of piperovatine and piperlonguminine in a proportion of 2:3 obtained from *P. ovatum* were assayed for anti-inflammatory activity by means of carrageenan-induced pleurisy in rats and croton oil-induced ear edema in mice (Rodrigues Silva *et al.* 2008). Piperovatine isolated from *Ottonia martina* Miq. showed satisfactory activity against *Cylindrocladium spathulatum*, *Fusarium oxysporum*, *Colletotrichum acutatum*, and *Rhizoctonia* sp., by inhibiting the germination process (Cunico 2006). Other activities of piperovatine are still little known. It can stimulate intense salivation, has an anesthetic effect in mice, and is toxic to fish (Makapugay *et al.* 1983, McFerren and Rodriguez 1998). Piperovatine isolated from *Piper piscatorum* is a potent stimulator of neuronal intracellular calcium, and is considered clinically to be a local anesthetic (McFerren *et al.* 2002). Extraction of the roots of *P. piscatorum* with MeOH and subsequent bioassay-guided fractionation using the guppy *Girardina guppia* yielded the active piperovatine (McFerren and Rodriguez 1998). The amide constituent (piperlonguminine) from the fruit of *Piper chaba* caused adipogenesis of 3T3-L1 cells (Zangh *et al.* 2008).

*Piper* species such as *Piper kadsura* and *Piper boehmeriifolium* var. *tonkinense* are used to treat inflammatory diseases in traditional Chinese medicine. The *n*-hexane extract of this species inhibits the formation of leukotrienes, and prostaglandin synthesis and amides were identified in the *n*-hexane extract by analytical investigation (Stohr *et al.* 2001). In the present study, amides including piperovatine and piperlonguminine obtained from this species were purified by bioassay-guided chemical fractionation and identified. Their antiproliferative effects on the amastigote and promastigote forms of *Leishmania amazonensis* and the ultrastructural changes that they produced in amastigote and promastigote forms were evaluated. In addition, the cytotoxicity of the compounds to macrophages was tested.

## MATERIALS AND METHODS

### General experimental procedures

The NMR spectra were obtained in Bruker DRX-400 (8.4 T) and VARIAN GEMINI 300 (7.05T) spectrometers, using deuterated solvent, TMS as the internal standard, and a constant temperature of 298K. Low-resolution electrospray data were acquired in the negative ion mode, using a Micromass Quattro-LC instrument. Silica gel 60 (70–230 and 230–400 mesh); TLC: silica gel plates F<sub>254</sub> (0.25 mm thickness).

### HPLC analysis

The hydroalcoholic extract of *Piper ovatum* leaves (HE) was analyzed by HPLC using a Shimadzu HPLC system consisting of an LC-10AD solvent delivery pump, and an SPD-10Avp UV detector. The system was controlled by CLASS LC-10 software. The column was Metasil ODS (150 × 4.6 mm; 5 μm particle size). Elution was done in step isocratic mode, with CH<sub>3</sub>CN/H<sub>2</sub>O 60-40 (0–12 min.) at a flow rate of 1.0 ml min.<sup>-1</sup> and the eluted compounds were monitored at 280 nm. The HE extract (1 mg) was dissolved in 1 mL of CH<sub>3</sub>CN/H<sub>2</sub>O (60–40), filtered in a Millex® filter, and injected (20 μL) into the HPLC system.

### Plant material

*Piper ovatum* Vahl leaves were collected in November 2004, in Monte Formoso, state of Minas Gerais, Brazil, and were identified by Dra. Elsie Franklin Guimarães. A voucher specimen was deposited in the herbarium of the Department of Botany, University of Maringá (HUM 10.621).

### Plant extraction and purification

Leaves were dried at room temperature, and powdered (100 g). The extract was prepared by exhaustive maceration in ethanol:water (9:1 v/v) at room temperature, filtered, and concentrated under vacuum at 40°C to obtain an hydroalcoholic extract (HE), which

was lyophilized, yielding 23 g. The hydroalcoholic extract (14 g) was chromatographed in a vacuum silica-gel column and eluted with gradients of hexane, dichloromethane-ethyl acetate (1:1 v/v), ethyl acetate and methanol, which afforded F1 (2.19 g), F2 (3.83 g), F3 (2.91 g) and F4 (3.60 g). The dichloromethane-ethyl acetate fraction F2 (3.1 g) was rechromatographed on a silica gel 60 (70–230 mesh) column chromatograph eluted with gradients of hexane, hexane/dichloromethane (98:2, 95:5, 90:10, 80:20 and 50:50 v/v), dichloromethane, dichloromethane/ethyl acetate (98:2, 95:5, 90:10, 80:20 and 50:50 v/v), Ethyl acetate and methanol afforded 108 fractions. Fractions 23–38 (258.2 mg) were identified as a mixture of piperovatine and piperlonguminine (**M**). This subfraction was rechromatographed on a Sephadex LH 20, and the column chromatograph was eluted with ethyl acetate, obtaining 50 fractions. Fractions 15–32 (20 mg) and 42–50 (40 mg) were isolated and identified as piperovatine (**1**) and piperlonguminine (**2**) respectively, by analyses of spectral data of  $^1\text{H}$  and  $^{13}\text{C}$  and by comparison of data from the literature (McFerren and Rodriguez 1998; Wu *et al.* 2004).

### Preparation of stock solutions

The crude extracts and fractions were dissolved 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.

### Parasites

Promastigote forms of *L. amazonensis* (MHOM/BR/75/Josefa), originally isolated from a human case of diffuse cutaneous leishmaniasis, were maintained by weekly transfers in Warren's medium (brain-heart infusion plus haemin and folic acid) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen Corporation, New York, U.S.A.) at 25°C in a tissue flask.

Axenic amastigote forms of *L. amazonensis* were obtained by *in vitro* transformation of infective promastigotes (Ueda-Nakamura *et al.*, 2001), and maintained by weekly transfers in Schneider's Insect medium (Sigma Chemical Co., St. Louis, Missouri, USA) at pH 4.6, supplemented with 20% heat-inactivated FBS at 32°C in a tissue flask.

### Cells

J774G8 murine macrophages were maintained in tissue flasks in RPMI 1640 medium (Gibco Invitrogen Corporation, New York, USA) with sodium bicarbonate and L-glutamine and supplemented with 10% fetal bovine serum at 37°C in a 5% CO<sub>2</sub>-air mixture.

### Antileishmanial activity

Promastigote forms of *L. amazonensis* ( $1 \times 10^6$  parasites/ml) were added to a 24-well plate in Warren's medium supplemented with 10% inactivated FBS containing different concentration (of the hydroalcoholic extract, hexane dichloromethane-ethyl acetate (1:1 v/v), ethyl acetate, methanol fractions (were performed with 1000 – 1 µg/ml); mixture amides and pure isolated compounds (at concentrations 100 – 1 µg/ml), incubated at 25°C for 72 h. Axenic amastigotes were incubated in Schneider's Insect medium supple-

mented with 20% inactivated FBS containing the crude extract, fractions, mixture amides or pure compounds at 32°C, for 72 h. The cell density for each treatment was determined daily in a hemocytometer (Improved Double Neubauer) with an optical microscope. In all tests, 0.5% dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, Missouri), a concentration that was used to dissolve the highest dose of the compounds but that had no effect on cell proliferation, and medium alone were used as controls. Each experiment was performed triplicate on three different occasions. The 50% Inhibitory Concentration (IC<sub>50</sub>) and the 90% Inhibitory Concentration (IC<sub>90</sub>) were determined by logarithm regression analysis of the data obtained, used with the positive control, Amphotericin B.

### Cytotoxicity assay

A suspension of  $5 \times 10^4$  J774G8 macrophage cells in RPMI-1640 medium or Vero cells in DMEM supplemented with 10% FBS was added to each well in 96-well microplates. The plates were incubated in a 5% CO<sub>2</sub>-air mixture at 37°C to obtain confluent growth of the cells. After 24 h, the medium was removed and several concentrations of purified compound and fractions (0.1 to 1000 µg/ml) were added to each well containing the cells, and the plates were incubated for 48 h. The nonadherent cells were removed by washing with the medium, and the adhered cells were fixed with 50 µl/well of 10% trichloroacetic acid at 4°C for 1 h; after that, the well plates were washed with water, and 50 µl/well of sulforhodamine B (0.4% w/v in 1% acetic acid solution was added); the microplate was then maintained at 4°C for 30 min. Next, the sulforhodamine B was removed and the microplate was washed 5 times with 1% acetic acid, then 150 µl/well of 10 mM unbuffered Tris-base solution (Sigma) was added, and this was homogenized for 15 min. The absorbance of each individual well, minus the blank value, was calculated automatically. Each experiment was performed in triplicate on three different occasions, and the percentage of viable cells was calculated in relation to controls cultured in medium alone. The 50% Cytotoxicity Concentration (CC<sub>50</sub>) was determined by logarithm regression analysis of the data.

### Ultrastructural analysis

Promastigote and amastigote forms of *L. amazonensis* treated with the isolated compound (IC<sub>50</sub> and IC<sub>90</sub>), 0.5% DMSO, or medium alone were collected by centrifugation after 72 h incubation, washed in 0.01 M phosphate-buffered saline at pH 7.2, and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C. The cells were postfixed in a solution containing 1% osmium tetroxide, 5 mM CaCl<sub>2</sub> and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer, washed in the same buffer, dehydrated in acetone, and embedded in Epon® resin. Ultrathin sections obtained in a Reichert Ultracut E ultramicrotome were stained with uranyl acetate and lead citrate and examined in a Zeiss 900 transmission electron microscope (TEM).

### Statistical analysis

All experiments were performed in triplicate on three different occasions. The means and standard deviations of at least three experiments were determined. The differences between mean values obtained for experimental groups were analyzed by means of Student's-t test. *P* values of 0.05 or less were regarded as significant.

## RESULTS

### Structure elucidation

The leaves of *Piper ovatum* afforded a hydroalcoholic extract, and this extract was chromatographed in a vacuum silica-gel column, which yielded hexane, dichloromethane-ethyl acetate (1:1, v/v), ethyl acetate and methanol fractions. The dichloromethane-ethyl acetate fraction showed the highest antiprotozoal activity against the amastigote and promastigote forms of *L. amazonensis*. Fractionation of this fraction led to purification of the compounds 1 and 2, which were identified by analysis of their spectroscopic data and comparison with literature (McFerren and Rodriguez 1998, Wu *et al.* 2004). By analysis of spectral data of the  $C^{13}$  signal, it was determined that piperlonguminine was the majority substance in the mixture (2:3) (Rodrigues-Silva *et al.* 2008). Figure 1 shows the chromatographic profile of the crude extract. Two major peaks indicate piperovatine at a retention time of 3.9 min., and piperlonguminine at 4.65 min. (Fig. 1).

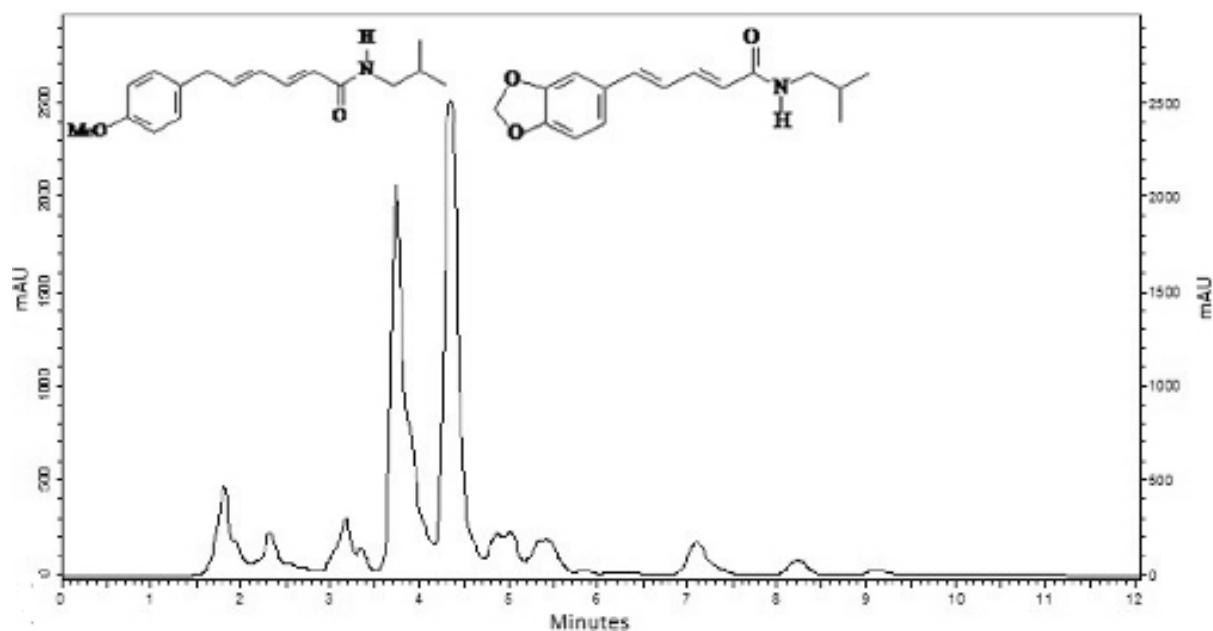
### Antileishmanial activity

The effects of the hydroalcoholic extract from *P. ovatum* leaves on the growth of *L. amazonensis* were

tested. This extract inhibited growth of the promastigote and amastigote forms, with  $IC_{50}$  of 60.0 and 78.5  $\mu\text{g/ml}$ , respectively, after 72 h of incubation. The  $IC_{50}$  values of these fractions are listed in Table 1. The dichloromethane-ethyl acetate fraction showed the most activity, with  $IC_{50}$  of 2.1  $\mu\text{g/ml}$  and 24  $\mu\text{g/ml}$  for promastigote and amastigote forms, respectively, after 72 h of incubation. Fractionation of the dichloromethane-ethyl acetate fraction led to purification of the compounds piperovatine and piperlonguminine and a mixture of compounds 1 and 2, which showed  $IC_{50}$  values of 9.5 and 10  $\mu\text{g/ml}$ ; 2.5  $\mu\text{g/ml}$  and 9  $\mu\text{g/ml}$ ; and 0.5  $\mu\text{g/ml}$  and 24  $\mu\text{g/ml}$  against promastigote and amastigote forms, respectively, after 72 h of treatment. The dilution agent (0.5% DMSO) had no effect on the parasite proliferation and cellular morphology. Amphotericin B showed  $IC_{50}$  of 0.058 and 0.231  $\mu\text{g/ml}$  against promastigote and amastigote forms, respectively, after 72 h of treatment.

### Cytotoxicity assay

J774G8 murine macrophages and Vero cells were treated with increasing concentrations of the hydroalcoholic extract, hexane, dichloromethane-ethyl acetate, ethyl acetate, methanol fractions or the pure isolated compounds piperovatine and piperlonguminine and a mixture of both (2:3), to evaluate the safety of this



**Fig. 1.** Chromatogram of the hydroalcoholic extract of *Piper ovatum* leaves [piperovatine (1) and piperlonguminine (2)]. *Chromatographic conditions:* Metasil ODS column; mobile phase: acetonitrile/water (60:40, v/v) with 2% acetic acid; flow-rate: 1.0 ml/min; room temperature; detection: 280 nm.

**Table 1.** Effects of the hydroalcoholic extract (HE); hexane (F1), dichloromethane-ethyl acetate (1:1, v/v) (F2), ethyl acetate (F3), methanol (F4) fractions, piperovatine (1), piperlonguminine (2), and mixture of both (M) on the growth of *L. amazonensis* promastigote forms (PRO), amastigote forms (AMA), cytotoxicity to Vero cells (VERO) and Macrophages (MF).

	IC50 (µg/ml)		CC50 (µg/ml)		SI			
	PRO	AMA	VERO	MF	VERO (µg/ml)		MF(µg/ml)	
HE	60	78.5	520	722	8.7		12.03	
F1	4.7		212	72.5	45.1		15.42	
F2	2.1	24	39.1	73.0	18.6	3.04	34.8	4.9
F3	101		>1000	>1000	> 9.9		> 9.9	
F4	>1000		>1000	>1000				
1	9.5	10	41.8	>1000	4.4	4.2	105.3	> 100
2	2.5	9.0	326	839	130	36.22	335.6	93.22
M(1+2)	0.9	24	53.7	679.5	59.66	2.24	755	28.3

IC<sub>50</sub> – 50% inhibitory concentration.

CC<sub>50</sub> – 50% cytotoxicity concentration.

SI – Selectivity index (CC50/IC50).

compound for mammalian cells After 48 h of treatment, cell viability was checked by a sulforhodamine B colorimetric assay. The CC50 values of piperovatine, piperlonguminine and the mixture of both (2:3) on macrophages and Vero cells were 41.8 and > 1000 µg/ml; 326 and 839 µg/ml; and 53.7 and 679.5 µg/ml, respectively. In order to evaluate the effectiveness of the fractions and pure compounds, the selectivity index (SI) was determined (IC50 for J774G8 macrophages or Vero cells/IC50 for protozoans). When this value is greater than 1, the compound is more selective for activity against parasites than macrophages or Vero cells; when the value is less than 1, the compound is more selective for activity against macrophages or Vero cells. All fractions and pure compounds were more selective against parasites than mammalian cells, as seen in Table 1.

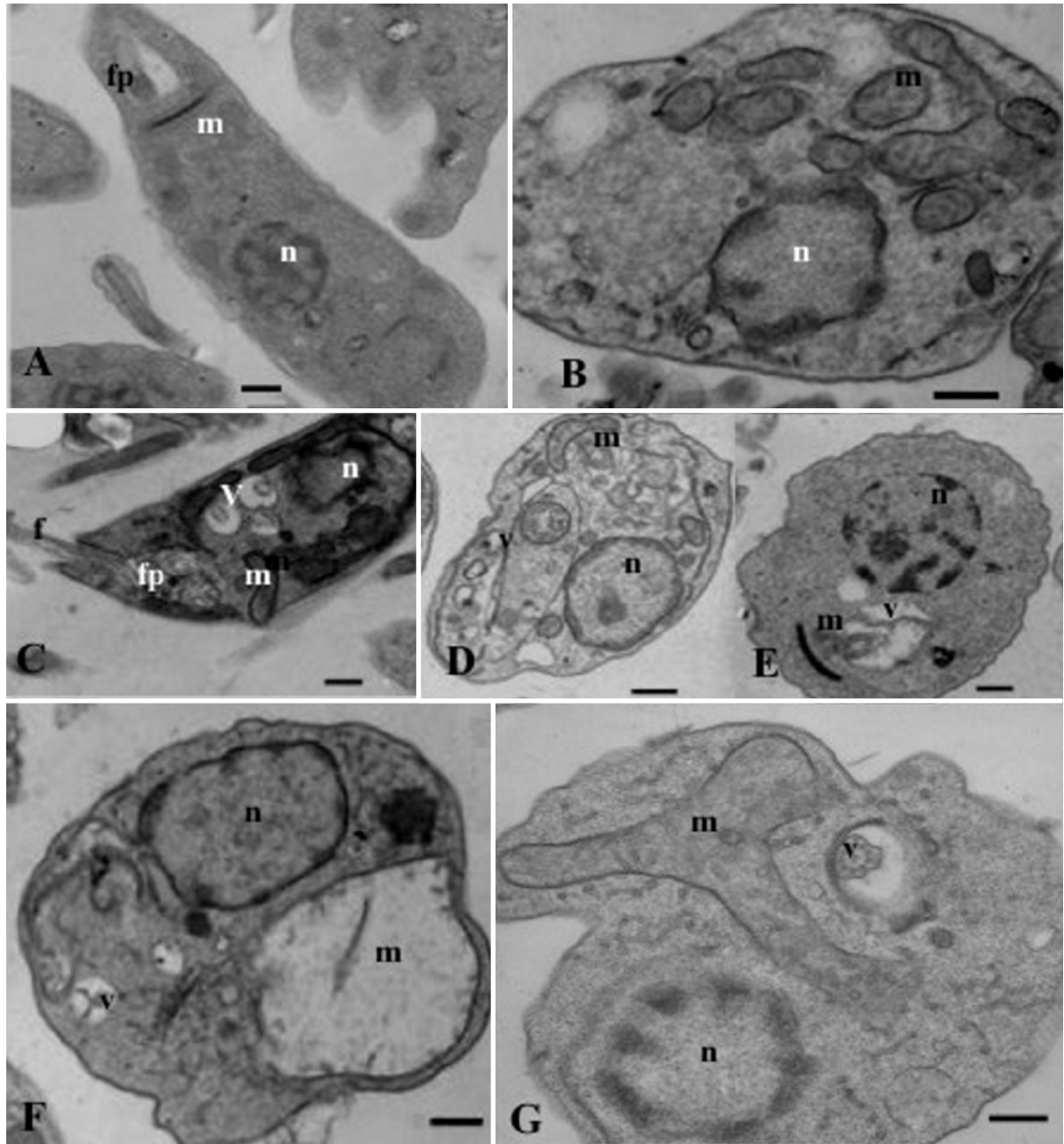
### Transmission electron microscopy

Promastigote and amastigote forms of treated and untreated *L. amazonensis* were examined by transmission electron microscopy to assess ultrastructural changes caused by the mixture of piperovatine:piperlonguminine (2:3). The promastigote forms were treated at 0.9 µg/ml (IC<sub>50</sub>; Fig. 2B – C); and 70 µg/ml (IC<sub>90</sub>; Fig. 2D). Amastigote forms were treated with this mixture at 24 µg/ml (IC<sub>50</sub>; Fig. 2F–G). The photomicrographs in Fig. 2 show parasites with different degrees of damage after 72 h of incubation. The promastigote and amastigote

forms treated with IC<sub>50</sub> of the mixture showed significant morphological alterations: the appearance of multiple cytoplasmic vacuolization (Fig. 2C) and considerable mitochondrial swelling (Fig. 2B, C, F, G). These features indicate damage and significant changes in this organelle. The promastigote forms treated with IC<sub>90</sub> of the mixture showed significant morphological alterations, as well as the appearance of multiple cytoplasmic vacuolization (Fig. 2C, d) and mitochondrial swelling (Fig. 2D). No structural changes were observed in untreated promastigote and amastigote forms or in cells cultured with 0.5% DMSO, which showed normal morphology (Fig. 2A, E).

### DISCUSSION

All chemotherapeutic agents for the treatment of leishmaniasis have serious side effects, and resistance has become a severe problem. Therefore, new drugs are urgently required (Croft *et al.* 2005). Natural products have potential in the search for new and selective agents for the treatment of important tropical diseases caused by protozoans. Alkaloids, terpenes, quinines, and other compounds well illustrate the diversity of antiprotozoal compounds found in higher plants (Wright and Phillipson 1990).



**Fig. 2.** Transmission electron microscopy of *L. amazonensis*: promastigote and amastigote forms cultured in medium alone. Promastigote Control (A) promastigotes treated with M(1+2) at 0.9 µg/ml (IC<sub>50</sub>) (B and C); promastigote treated with 70 µg/ml (IC<sub>90</sub>) (D); amastigote control (E); amastigote treated with M(1+2) at 24 µg/ml (IC<sub>50</sub>) (F and G). (A) amastigotes showing normal morphology; (B, C, and D) parasites showing some alterations in the mitochondrion, such as intense swelling; (E) amastigotes showing normal morphology; and (F and G) parasites showing some alterations in the mitochondrion, such as intense swelling. (C and D) parasites showing multiple cytoplasmic vacuolization; n – nucleus, f – flagellum, fp – flagellar pocket, k – kinetoplast, m – mitochondrion, v – vacuole. Scale: 1 µm.

The leaves of two species of a genus of the family Piperaceae (*Piper hispidum* and *Piper strigosum*) show good leishmanicidal activity against amastigotes (Estevez *et al.* 2007). According to Kapil (1993), the amide

piperine is a potent inhibitor of *Leishmania donovani* promastigotes *in vitro*. Luize *et al.* (2005) showed that extracts of leaves of *P. regnellii* possess activity against *Leishmania amazonensis* and *Trypanosoma cruzi*. Na-

kamura *et al.* (2006) evaluated the antileishmanial activity of crude extracts from leaves of *P. regnellii* var. *palescens* against promastigotes of *L. amazonensis*. Some bioactive substances have previously been isolated from species of the genus *Piper*, such as alkaloids, lignans, neolignans, terpenes, and benzoic acid (Zacchino *et al.* 1997, Park *et al.* 2002, López *et al.* 2002, Martins *et al.* 2003).

In the present study, we report antileishmanial activity of the dichloromethane-ethyl acetate (1:1 v/v) fraction obtained by a hydroalcoholic extract of leaves of *Piper ovatum*. This fraction showed IC<sub>50</sub> of 2.1 µg/ml and 24 µg/ml for promastigote and amastigote forms, respectively. Fractionation of the dichloromethane-ethyl acetate (1:1 v/v) fraction led to purification of piperovatine and piperlongumine-type amides. The dichloromethane-ethyl acetate fraction, pure isolated compounds piperovatine (1) and piperlongumine (2), and a mixture of piperovatine:piperlongumine (2:3) showed significant activity against promastigote and amastigote forms of *L. amazonensis in vitro* (Table 1). Our observations suggest that the piperovatine and piperlongumine present in this fraction were responsible for the antileishmanial activity. Both the mixture of two isolated compounds and the dichloromethane-ethyl acetate fraction were more active than piperovatine (1) and piperlongumine (2) for promastigote forms, but the opposite was true for the amastigote forms (Table 1). The difference in activity can be observed by pattern substitution of an aromatic ring with methoxyl group in piperovatine, and methylenedioxy in piperlongumine. The hydroethanolic extract, fractions, mixture, and compounds tested were also evaluated for their potential toxic effects in Vero cells and macrophages treated with different concentrations; the isolated compound showed less cytotoxicity than did the dichloromethane-ethyl acetate (1:1 v/v) fraction on Vero cells and macrophages. *In vitro* tests with macrophages and Vero cells showed that all the samples were more selective for *L. amazonensis*. This is demonstrated by their SI ratio values > 1 (Table 1).

Toxicity tests for medicinal plants are essential because of the growing interest in alternative therapies and the therapeutic use of natural products (Rates 2001). In this context, both the efficacy and the safety of natural products require investigation. Significant mitochondrial swelling was observed at the ultrastructural level when the promastigote and amastigote forms of *L. amazonensis* were treated for 72 h with IC<sub>50</sub> values of a mixture of piperovatine:piperlongumine (2:3) at 0.9

µg/ml and 24 µg/ml, respectively. Also, intense cytoplasmic vacuolization was observed when promastigote forms were treated with IC<sub>90</sub> values of a mixture of piperovatine:piperlongumine (2:3) at 70 µg/ml for 72 h. Similar mitochondrial swelling has been reported for *L. amazonensis* treated with 22,26 azasterol, an inhibitor of ergosterol synthesis (Rodrigues *et al.* 2002), ketoconazol and terbinafine (Vannier-Santos *et al.* 1995), and other compounds, derivatives of azasterol (Lorente *et al.* 2004) which induced a marked alteration. Previous studies have demonstrated that the presence of ergosterol and its analogs is essential for maintenance of the normal structural organization of the mitochondrial membrane in trypanosomatids (Rodrigues *et al.* 2001). Biochemical studies have shown that, contrary to what is known for mammalian cells, there are large amounts of endogenous and exogenous sterols in the mitochondrial membranes of trypanosomatids (Vannier-Santos *et al.* 1995 and Rodrigues *et al.* 2001). This indicates that the mitochondrion of trypanosomatids is an important target in leishmaniasis chemotherapy. Previous studies have demonstrated ultrastructural changes in mitochondrial morphology of promastigote forms of *L. amazonensis* treated with different leishmanicidal agents, such as purified chalcone from *Piper aduncum* inflorescences (Torres-Santos *et al.* 1999), a purified indole alkaloid obtained from the stem of *Peschiera australis* (Delorenzi *et al.* 2001), eugenol-rich essential oil from *Ocimum gratissimum* (Ueda-Nakamura *et al.* 2006), and a coumarin from *Calophyllum brasiliense* leaves (Brenzan *et al.* 2007, 2008). No ultrastructural changes were observed in untreated promastigote and amastigote forms, or in cells cultured with 0.5% DMSO, which showed a normal mitochondrial profile containing the kinetoplast (promastigote), and the nucleus and flagellum with normal morphology (Fig. 2 A and E). The TEM analysis contributed significantly to elucidating the mechanism of action of the mixture on the parasites, detecting significant alterations. However, these studies should be expanded. This drug may eventually be shown to have good therapeutic potential for the treatment of American cutaneous leishmaniasis.

These results are an exciting advance in the search for novel antileishmanial agents from natural sources, since they demonstrated significant and important effects against the intracellular stage of the protozoan. This plant showed significant activity against *Leishmania* pathogens, but further synthesis and *in vitro* studies are indicated in order to validate the results.

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