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Original Article

In vitro anti-*Leishmania infantum* activity of essential oil from *Piper angustifolium*

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ABSTRACT

Piper angustifolium Lam., Piperaceae, popularly known as "matito", "pimenta-de-macaco", "pimenta-longa" or "jagurandi" in Brazil, has been commonly used in the treatment of cutaneous leishmaniasis-associated lesions, but there are few studies on the activity against visceral leishmaniasis-associated species. This study demonstrates the first *in vitro* antileishmanial activity of the *P. angustifolium* essential oil, of which the phytochemical profile showed the presence of sesquiterpenes and monoterpenes. The main compounds were spathulenol (23.8%) and caryophyllene oxide (13.1%). *P. angustifolium* essential oil was highly active [the half maximum inhibitory concentration = 1.43 μg/ml] against intracellular amastigotes of *Leishmania infantum*, the etiological agent of visceral leishmaniasis in the New and Old World. Activity was obtained 24 h after addition of the oil (6.25–50 μg/ml), with a reduction of 100% in the infection index at concentrations of 25 and 50 μg/ml. *P. angustifolium* essential oil showed low cytotoxicity for mammalian fibroblasts and macrophages (the half maximum inhibitory concentration values of 31.67 and 48.22 μg/ml, respectively), and it was 33 and 22 times more toxic to amastigotes than to mammalian cells, as indicated by selectivity indexes. The results demonstrated that *P. angustifolium* essential oil is a promising alternative for the study of potential drugs for visceral leishmaniasis.

Introduction

Visceral leishmaniasis (VL) is a severe systemic chronic disease caused by the *Leishmania donovani* complex in East Africa and the Indian subcontinent, and *Leishmania infantum* in Europe, northern Africa and Latin America (Lukes et al., 2007). It is one of the most important neglected diseases today, given its high incidence and mortality, especially among untreated individuals and malnourished children. It is also considered a disease emerging in immunocompromised individuals (Jarvis and Lockwood, 2013). Even during treatment, a case-fatality ratio of 10–20% is estimated (Collin et al., 2004).

The first choice drugs in the treatment of leishmaniasis are the pentavalent antimonials (WHO, 2010). These drugs, however, present significant limitations concerning their therapeutic safety as they have a high level of toxicity. High frequency of adverse effects and teratogenic risk have been described (Miranda et al., 2006). Lipid formulations of amphotericin B, miltefosine and paromomycin have been approved, but the correct dose and efficacy of these drugs have not been proven in all endemic areas of the disease (WHO, 2010). Combinations of these drugs have been required in the cases of infections resistant to antimony (Seifert and Croft, 2006). Considering the scenario of resistance to the main drugs in use, researchers have been studying new, less toxic drugs, with greater availability and within reach of the underprivileged population affected by the disease (Freitas-Junior et al., 2012). In the search for new and better compounds, products of plant origin have been tested since they are easily obtained at low cost (Li and Vederas, 2009).

Piper is one of the genera of great ecological and economic importance within the Piperaceae family (Monzote et al., 2010). Several classes of compounds have been isolated from *Piper* species,

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including those with antileishmanial activity such as benzoic acids (Flores et al., 2007, 2009), dillapiole (Parise-Filho et al., 2012), and adunchalcone (Dal Picolo et al., 2014). Moreover, in recent years its essential oils have become an important target in the search for new therapeutic options against parasites (Antony et al., 2005).

Piper angustifolium Lam. (sin. = *Piper aduncum* L.), popularly known as "matito", "pimenta-de-macaco", "pimenta-longa" or "jagurandi" in Brazil, is a plant native to tropical regions such as the South and Central Americas, Asia, and the Pacific Ocean islands (Martínez et al., 2003). Its leaves are used in folk medicine for the treatment of stomatitis, vaginitis, erysipelas and liver disorders (Lorenzi and Matos, 2002) or as an antiseptic, antidiarrheal, tonic, astringent, and antirheumatic medicine, and also as an insect repellent (Schaus and Desmarchelier, 2000). In addition, it has been commonly used in the treatment of cutaneous leishmaniasis-associated lesions (Martínez et al., 2003), which motivated the development of some works on *Leishmania* species associated with this clinical form of the disease (Braga et al., 2007). In contrast, few studies have been conducted on the activity against LV-associated species and up to now, there has been no research on the essential oil extracted from this plant. Thus, the aim of this study was to characterize the chemical composition of the *P. angustifolium* essential oil (PAEO) and evaluate its activity against *L. infantum*.

Materials and methods

Plant material

Piper angustifolium Lam., Piperaceae, was collected in January 2014 from the Abobral Subregion of the Pantanal of Mato Grosso do Sul. After the identification performed by Dr. Geraldo Alves Damasceno Junior, a botanical voucher was deposited in the herbarium of Campo Grande/MS, Brazil, under number 20182.

Extraction and analysis of the essential oil

The *P. angustifolium* essential oil (PAEO) was extracted from the plant leaves by distillation in a Clevenger apparatus (Vidrolex). The oil was dried with anhydrous sodium sulfate (Vetec, Rio de Janeiro, Brazil) with yield of 0.41% (w/w), and stored at −5 °C in a sealed container until the time of analysis. The chemical composition was determined by gas chromatography–mass spectrometry (GC–MS) using a Shimadzu QP2010 Plus system with a RTX5MS capillary column (30 m × 0.25 mm × 0.25 μm). Nitrogen was applied as carrier gas using a flow rate of 1.13 ml/min. The constituents were confirmed by comparison with libraries and calculation of the Kovats index.

Parasites

The standard strain MHOM/BR/1972/BH46 of *L.* (*Leishmania*) *infantum* was used for *in vitro* assays of antileishmanial activity. The amastigotes were routinely isolated from Golden hamsters (*Mesocricetus auratus*) and maintained as promastigotes in Schneider's Insect Medium (Sigma) supplemented with 20% fetal bovine serum (FBS, Sigma) and 140 μg/ml gentamicin (Sigma) at 26 °C. On the 7th day of cultivation, promastigotes from up to three serial passages after isolation were used in the experiments.

Animals

BALB/c mice aged six weeks were used to obtain the peritoneal cells used in the antileishmanial activity assays. The animals were obtained from the central animal facility of the Center for Biological and Health Sciences (CCBS) of the Federal University of Mato Grosso do Sul (UFMS, Brazil) in good health and free of infections or parasites common to rodents, maintained in individually ventilated cages equipped with mini-isolators, and fed a balanced feed (Nuvilab CR-1, Nuvital®) with free access to water. The study was approved by the Ethics Committee on Animal Use – CEUA/UFMS, under protocol 432/2012.

Activity against intracellular amastigotes of L. infantum

Peritoneal macrophages from BALB/c mice were isolated after rinsing with RPMI 1640 medium (Sigma) and placed in a 24-well plate (1×10^5 cells/well) in RPMI 1640 medium (Sigma) supplemented with 10% FCS (Cultilab) and 140 μg/ml gentamicin (Sigma). After incubation at 37 °C for 1 h, cells were infected with *L. infantum* promastigotes (1×10^6 cells/well) and incubated at 35 °C for 4 h. PAEO was added at concentrations of 6.25–50 μg/ml in sets of sextuplicate experiments. Untreated infected cells and amphotericin B (Sigma) were used as negative and positive control, respectively. The cells were incubated at 37 °C in 5% CO_2, fixed and stained with Giemsa after 24 h. The percentage of infected macrophages and the total number of amastigotes were determined by counting 200 cells sixfold. The infection index was determined by multiplying the percentage of macrophages that had at least one intracellular parasite by the mean number of amastigotes per macrophage, as described by Paladi et al. (2012). A nonlinear dose–response regression curve was used to calculate the half maximum inhibitory concentration (IC_{50}). The results were expressed as the mean ± standard deviation (SD) and the data were analyzed using the Student's *t*-test. Differences were considered significant at $p < 0.05$ (represented by an asterisk).

Nitric oxide (NO) evaluation

To evaluate the production of NO by the infected peritoneal cells, supernatants of the aforementioned cultures (100 μl) were collected after 24 h of treatment and incubated with an equal volume of Griess Reagent (1% sulfanilamide/0.1% (naphthyl)ethylenediamine in 5% phosphoric acid) at room temperature for 10 min. The accumulation of nitrite was quantified according to Ding et al. (1988) and the absorbance was determined at 540 nm. Absorbance was converted to μM of NO_2^- by comparing the samples with a standard curve obtained with known concentrations (1–10 μM) of sodium nitrite diluted in RPMI medium. The results were expressed as the mean ± standard deviation (SD). The data were analyzed using the Student's *t*-test and differences were considered significant at $p < 0.05$ (represented by an asterisk).

Cytotoxicity assay

Fibroblast (NIH/3T3) and murine macrophage (J774.A1) cells purchased from the Rio de Janeiro Cell Bank (Brazil) were treated with PAEO at concentrations of 0.25–250 μg/ml in triplicate to estimate IC_{50}. PAEO was dissolved in DMSO (dimethyl sulfoxide sodium) and diluted in complete medium (the highest concentration of DMSO used in the test was 0.25%, and did not affect cell viability). Cells in culture medium were used as negative control and amphotericin B (0.025–25 μg/ml) as positive control. Cell viability was determined using the sulforhodamine B assay (Skehan et al., 1990). The percentage of growth of each test sample was calculated as described by Monks et al. (1991). The IC_{50} was determined by nonlinear regression (Microcal Origin Version 6.0 and Microsoft Office Excel 2007). Selectivity index (SI) was calculated by the following formula: IC_{50} on mammalian cells/IC_{50} on amastigotes (Tiuman et al., 2005).

Table 1
Chemical constitution of the essential oil from *Piper angustifolium*.

	Compound	%	Retention time	Theoretical KI	Calculated KI
1	α-pinene	5.87	4.64	939	932
2	camphene	0.53	5.05	946	947
3	cymene	2.77	7.10	1025	1032
4	limonene	4.27	7.20	1032	1037
5	limonene oxide	0.64	8.50	1137	1103
6	*cis*-verbenol	0.32	8.62	1135	1113
7	cryptone	1.75	9.67	1186	1193
8	cuminaldehyde	1.29	10.24	1242	1249
9	p-cymen-7-ol	0.70	10.71	1291	1297
10	δ-elemene	1.11	11.14	1339	1339
11	α-copaene	0.44	11.55	1393	1388
12	β-elemene	4.01	11.68	1389	1402
13	*trans*-caryophyllene	2.24	12.04	1444	1437
14	aromadendrene	1.80	12.25	1469	1455
15	longifolene	4.5	12.59	1487	1405
16	α-muurolene	3.56	12.91	1512	1500
17	γ-cadinene	3.74	13.13	1534	1526
18	*cis*-calamenene	0.50	13.24	1543	1535
19	nerolidol	5.80	13.63	1562	1561
20	isospathulenol	1.67	14.04	1589	1630
21	spathulenol	23.78	14.15	1578	1598
22	caryophyllene oxide	13.06	14.28	1613	1605
23	viridiflorol	0.51	14.41	1620	1610
24	torreyol	3.81	15.24	1643	1651
25	α-cadinol	4.37	15.52	1656	1665

KI, Kovats index.

Results and discussion

Twenty-five constituents were identified in the essential oil from *P. angustifolium*, representing 93.04% of the total compounds. The majority of these were sesquiterpenes and the two major compounds identified, spathulenol and caryophyllene oxide, corresponded to 36.8% of the total compounds (Table 1). The phytochemical profile of PAEO differed from that found by Almeida et al. (2009), who observed the predominance of a phenylpropanoid (dillapiole – 86.9%) in the essential oil from the aerial parts of the plant collected in Pará, Brazil. Parise-Filho et al. (2012) also found dillapiole as the major compound of essential oil from the leaves of the same plant collected in São Paulo, Brazil. Tirillini et al. (1996), who collected the same plant in Peru, near Cuzco, observed the monoterpenes camphor (25.3%) and camphene (22.4%) to be the main constituents.

The antileishmanial activity of several sesquiterpenes has been described in the literature (Mikus et al., 2000; Santos et al., 2008; Arruda et al., 2005). Valadeau et al. (2009) demonstrated the activity of sesquiterpene-rich ethanolic extract from *Piper dennisii*; Monzote et al. (2010) found an IC_{50} of 22.3 μg/ml for the essential oil from *P. auritum* against *L. donovani* amastigotes. The methanolic eugenol-rich extract from *Piper betle* was active against *L. donovani* amastigotes and promastigotes (Misra et al., 2009). Other sesquiterpenes with leishmanicidal activity have previously been reported, as shown by Marques et al. (2011), who identified *E*-nerolidol in the essential oil from *Piper claussenianum* (83%). When tested against *Leishmania amazonensis* arginase, it showed an enzyme inhibition of 62.2%. Oliveira et al. (2014) tested the essential oil from *Bocageopsis multiflora* with 16.2% of spathulenol against *L. amazonensis* promastigotes.

This study presents the first description of anti-*L. infantum* activity associated with the essential oil obtained from *P. angustifolium*. Our results demonstrated a dose-dependent increase in the inhibition of the intracellular amastigote proliferation 24 h after the oil was added to infected cells. The infection index decreased, in a range from 88.1 to 100% from the lowest to the highest concentration, in comparison with untreated infected cells (Fig. 1). The IC that reduced 50% of the intracellular forms of *L. infantum* (IC_{50}) was 1.43 μg/ml with low cytotoxicity to mammalian cells compared with amphotericin B (Table 2). With regard to SI, PAEO was 33 and 22 times more cytotoxic to intracellular amastigotes than to NIH/3T3 and J774.A1 cells, respectively (Table 2). Although amphotericin B was about four times more active than PAEO, this reference drug was not as selective as the oil, since it was more cytotoxic to mammalian cells (Table 2).

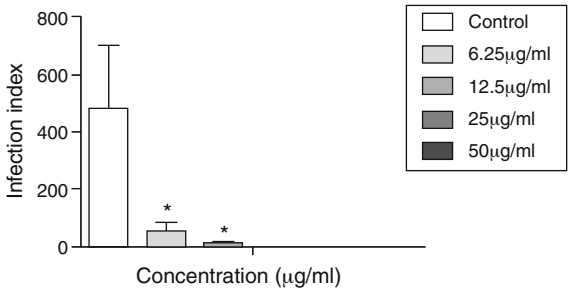

Fig. 1. Antileishmanial activity of PAEO on *L. infantum* intracellular amastigotes. Bars represent the mean ± SD of sextuplicates. *$p < 0.01$, for the different concentrations compared to untreated cells (control) (Student's *t*-test).

Activation of macrophages was investigated by NO release (Fig. 2). A significant increase ($p < 0.05$) in NO release was found after treatment with PAEO at concentrations of 6.25 and 12.5 μg/ml, suggesting that its activity may be associated with this antileishmanial mechanism (Stenger et al., 1994). At concentrations of 25 and 50 μg/ml, however, PAEO did not induce a significant increase in NO release, showing an atypical result that may be due to the presence of certain compounds in the oil. The literature has reported several compounds inducing this biphasic response (Nothnick and Soloway, 1998; O'Flaherty et al., 1989, 1990; Adedapo et al., 2009; Linner et al., 1993; Calabrese, 2005).

Natural products are an important source in the search for new therapeutic options. The antileishmanial activity of PAEO, coupled with low cytotoxicity to mammalian cells, makes it a promising natural product for the development of new drugs for the treatment of leishmaniases, especially the VL.

Table 2
Effect of PAEO on intracellular amastigotes of *Leishmania infantum*, cytotoxicity to mammalian cells and corresponding selectivity index (SI).

Test samples	Intracellular amastigotes	NIH/3T3		J774.A1	
	IC_{50} (μg/ml)[a]	IC_{50} (μg/ml)[a]	SI[b]	IC_{50} (μg/ml)[a]	SI[b]
PAEO	1.43	48.22	33.72	31.67	22.15
Amphotericin B	0.33	2.19	6.63	4.32	13.09

[a] IC_{50}, half maximum inhibitory concentration.
[b] SI, selectivity index: IC_{50} on mammalian cells/IC_{50} on intracellular amastigotes.

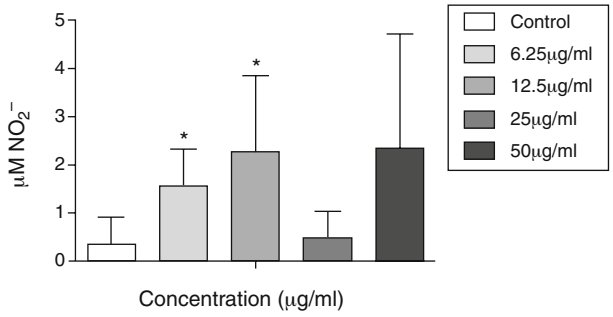

Fig. 2. Effect of addition of different concentrations of PAEO on the production of nitric oxide by peritoneal cells infected with *L. infantum*. Infected cells without treatment were used as controls. The data represent mean ± standard deviation of quadruplicates. $^{*}p < 0.05$ for the different concentrations of PAEO versus control (Student's *t*-test).

Authors' contributions

LSSB, YSR and MCC (MSc students) contributed to biological studies. DPD (PhD student) contributed by collecting plant samples, and performing chromatographic analysis. MCTK contributed to the NO evaluation test. MFCM contributed to the cytotoxicity test. MCSM contributed to critical reading of the manuscript. CCPA and CAC designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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