Chemistry, cytotoxicity and antileishmanial activity of the essential oil from *Piper auritum*

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Leishmaniasis is one of the most important parasitic infections, but current treatments are unsatisfactory due to their toxicity, cost and resistance. Therefore, the development of new antileishmanial compounds is imperative. Many people who live in endemic areas use plants as an alternative to treat the disease. In this paper, we characterised the essential oil from Piper auritum*, evaluated its cytotoxicity and determined its antileishmanial activity. The chromatogram obtained by gas chromatography revealed 60 peaks and we found that safrole was the most abundant compound, composing 87% of the oil. The oil was active against the promastigotes of* Leishmania major*,* Leishmania mexicana*,* Leishmania braziliensis *and* Leishmania donovani *with a favourable selectivity index against peritoneal macrophages from BALB/c mice. The* Piper-*oil inhibited the growing of intracellular amastigotes of* L. donovani *with an* IC_{50} *value of 22.3 ± 1.8 µg/mL. This study demonstrates the usefulness of the essential oils as a promising alternative to treat leishmaniasis.*

Key words: *Leishmania* - *Piper auritum* - essential oil

Leishmaniasis is caused by parasites of the *Leishmania* genus and affects more than 12 million people in 88 countries (Desjeux 2001). Antimonial derivatives are still the main compounds used for treatment, but they cause drug resistance in the parasite, severe toxicity in the patient and require parenteral administration. Second-line drugs, such as amphotericin B and pentamidine, are options in combined therapy or in cases of antimony treatment failures. In addition, clinical studies have identified the anticancer drug miltefosine as an effective antileishmanial agent. However, gastrointestinal toxicity and teratogenicity have been reported in association with this drug (Garnier & Croft 2002, Davies et al. 2003). Therefore, the development of new antileishmanial compounds is imperative. Many people who live in endemic areas use plants as an alternative to treat the disease. Given that the present treatment situation is unsatisfactory, the study of plant-derived drugs for leishmaniasis treatment is highly needed (Brenzan et al. 2007).

Piper plants are considered an economically and ecologically important genus in the family Piperaceae and consist of about 1,000-2,000 species. Although the largest numbers of *Piper* species are found in the Americas (about 700 species) and Southern Asia (about 300 species), smaller numbers of species exist in the South Pacific (about 40 species) and Africa (about 15 species). Among them, *Piper auritum* Kunt is easily recognised by its large (20-50 cm) leaves, which are unequally lobed at the base and the very characteristic sarsaparilla or anise-like odour of its crushed leaves. These plants grow to about 6 m in height with a single main stem that often has small prop roots near the base. The large leaves are borne in two alternate ranks and are often held horizontally on upper branches forming a broad light-intercepting crown with relatively few large leaves (Berger 1983).

P. auritum has been used for a number of different culinary and medicinal purposes. Medicinal applications include uses as a sudorific, diuretic and stimulant in cases of fever, erysipelas, gout and angina, a local aesthetic, as treatment for gonorrhoea and colic, headache and wound poultice and as both a repulsive and a digestion stimulant (Joly 1981). Anti-inflammatory, antibacterial and antifungal activities have also been reported (Gupta et al. 1985, Hernández et al. 2003).

Studies in the literature have reported that *Piper* species have antileishmanial activity (Caio et al. 1999, Flores et al. 2007, Sarkar et al. 2008) and their essential oils have become an important target in the last few years in the search for new antiparasitic treatments (Antony et al. 2005). The aims of this study were to perform the chemistry characterisation of the essential oil from *P. auritum* and to evaluate its cytotoxicity and antileishmanial activity.

MATERIALS AND METHODS

Essential oil from P. auritum - *P. auritum* (Piperaceae) was collected in the Pharmacy and Foods Institute, Havana's University, Cuba, in July 2001. A voucher specimen (4622) is kept at the Experimental Station of Medicinal Plants Dr. Juan Tomás Roig, Cuba. The essential oil was obtained by distillation, under laboratory conditions, of the aerial parts of the plant using a Clevenger's apparatus according to the NR309 Regulation (Cuba 1992). The composition of the essential oil was further analysed by high resolution gas chromatography-mass spectrometry (HRGC-MS) using a QP5050-A

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Shimadzu DZU system equipped with flame ionisation detection (280ºC), a 30 m column, a 0.25 µm film thickness and a 0.25 mm fused silica capillary column SPB-1. The flow rate was 1.5 mL/min, with a temperature program from 50ºC at a rate 5ºC/min to 280ºC for 30 min and the injection port was in split mode 1:40 at 280ºC. The mass spectrometer had an ionisation energy of 70 eV and an ion source at 280ºC.

Parasite culture - Strain MRHO/SU/59/P (LV39) of *Leishmania major,* MNYC/BZ/62/M379 of *Leishmania mexicana,* MHOM/BR/75/M2903 of *Leishmania braziliensis* and MHOM/ET/67/L82 of *Leishmania donovani* were maintained as promastigotes at 26ºC in Schneider's medium (SIGMA, St. Louis, MO, USA) containing 10% heat-inactivated foetal bovine serum (HFBS) (SIGMA, St. Louis, MO, USA), 100 µg of streptomycin/mL and 100 U of penicillin/mL.

Laboratory animals - Female BALB/c mice, with a body weight of approximately 20-22 g, were obtained from The National Centre for Laboratory Animals Production (CENPALAB). The maintenance and care of mice were followed according to guidelines from the Ethical Committee for the Human Use of Laboratory Animals.

Reference drug - As a drug of reference, we used amphotericin B (IMEFA, Havan City, Cuba) at a concentration of 2 mg/mL. The drug was diluted in sterile distilled water.

Antipromastigote activity - Eleven concentrations of the essential oil were assayed in quadruplicate. Exponentially growing cells (10^5 promastigotes/mL in 199 µL) were distributed in 96-well plates. One microlitre of essential oil dissolved in DMSO or 1 µL of DMSO for control was added and then incubated at 26ºC for 72 h. After three days of exposure, the parasites were incubated at 37ºC for 3 h with p-nitrophenyl phosphate (20 mg/mL) dissolved in 1 M sodium acetate buffer (BDH, Poole, England) at pH 5.5, with 1% Triton X-100 (BDH, Poole, England). The absorbance was determined in an EMS Reader MF Version 2.4-0 at a wavelength of 405 nm. The 50% inhibitory concentration (IC_{50}) was obtained from dose-response curves fit to data by means of the equation for the sigmoidal E_{max} model (Bodley et al. 1995).

Cytotoxicity assay - Resident macrophages were collected from peritoneal cavities of normal BALB/c mice in ice-cold RPMI 1640 medium (SIGMA, St. Louis, Mo, USA), supplemented with antibiotics, seeded at 30,000 cells/well and allowed to adhere for 2 h at 37ºC in 5% CO_2. After non-adherent cells were removed by washing with PBS, dilutions of the essential oil, in 1 µL of DMSO, were added in 200 µL of medium with 10% HFBS and antibiotics. The macrophages were treated with six concentrations of the product and cultures treated with 1 µL of DMSO were included as controls. The cytotoxicity was determined after three days of incubation using the colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (SIGMA, St. Louis, MO, USA). MTT solutions were prepared at 5 mg/mL in PBS, filtered and sterilised at the time of use and 15 µL of each solution were added to each well. After incubation for an additional 4 h, the formazan crystals were dissolved by addition of 100 µL of DMSO. The optical density was determined using an EMS Reader MF Version 2.4-0 at a test wavelength of 560 nm and a reference wavelength of 630 nm (Sladowski et al. 1993). The 50% cytotoxic concentration (CC_{50}) was obtained from dose-response curves fit to data by means of the equation for the sigmoidal E_{max} model. Selectivity indices (SI) were then calculated by dividing the CC_{50} for peritoneal macrophages of BALB/c mice by the IC_{50} for *Leishmania* promastigotes (Tiuman et al. 2005).

Antiamastigote activity - Resident macrophages were collected from peritoneal cavities of normal BALB/c mice in ice-cold RPMI 1640 medium (SIGMA, St. Louis, Mo, USA) and supplemented with antibiotics. The peritoneal macrophages were plated at 10^6 cells/mL in 24-Well Lab-Tek plates (Costar®, USA) and incubated for 2 h at 37ºC under an atmosphere of 5% CO_2. Non-adherent cells were removed by washing with pre-warmed phosphate-buffered saline (PBS). Stationary-phase *L. donovani* promastigotes were added at a 10:1 parasite/macrophage ratio and the cultures were incubated for another 24 h. The cell monolayers were washed three times with pre-warmed PBS to remove free parasites and 999 µL of RPMI completed medium and 1 µL of the essential oil dissolved in DMSO, were added in duplicate and incubated for an additional 48 h (Espuelas et al. 2000). The cells and parasites were then fixed in absolute methanol, stained with Giemsa and examined under light microscopy. The number of intracellular amastigotes was determined by counting the amastigotes in 100 macrophages of each sample and the results were expressed as a percent of the reduction of the infection rate (% IR) compared to that of the controls. The IRs were obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophage. The IC_{50} value was determined from the lineal concentration-response curves (Croft et al. 1996).

Statistical analysis - The data were analysed by outliers proof. The Mann-Whitney test was performed to find significance ($p < 0.05$) between the groups. The analysis was carried out with STATISTICA Programme for Windows, Version 4.5, 1993.

RESULTS

The HRGC-MS analysis of the essential oil revealed 60 peaks (Fig. 1). The retention times as well as some of the other characteristics of the 32 most important components are shown in Table I. The chromatogram demonstrated that safrole (86.91%) was the most abundant compound in the essential oil (Fig. 2).

The essential oil, at concentrations lower than 100 µg/mL, decreased the viability of promastigotes after 72 h of incubation (Table II). The *Piper*-oil had a CC_{50} value of 106.4 ± 3.4 µg/mL against peritoneal macrophages from BALB/c mice and amphotericin B showed an IC_{50} value of 5.8 ± 0.5 µg/mL. Out of all of the species of *Leishmania* evaluated, the oil had the highest activity ($p < 0.05$) against *L. donovani*.

The *Piper*-oil exhibited a concentration-dependent inhibitory effect against intracellular amastigotes of *L. donovani* (Fig. 3). The essential oil had an IC_{50} of 22.3 ± 1.8 µg/mL against amastigotes of *L. donovani* and the amphotericin B had an IC_{50} of 0.03 ± 0.002 µg/mL.

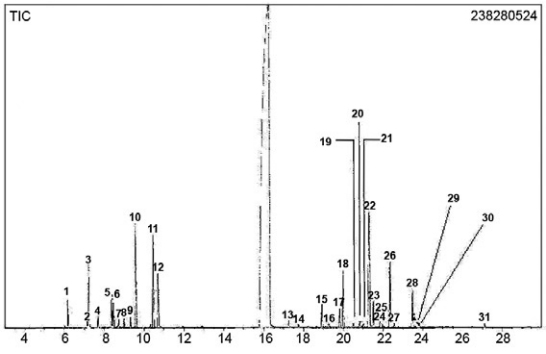

Fig. 1: chromatogram obtained by high resolution gas chromatography-mass spectrometry analysis of the essential oil from *Piper auritum* grown in Cuba.

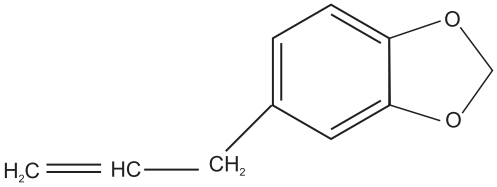

Fig. 2: chemical structure of safrol, main component of the essential oil from *Piper auritum*.

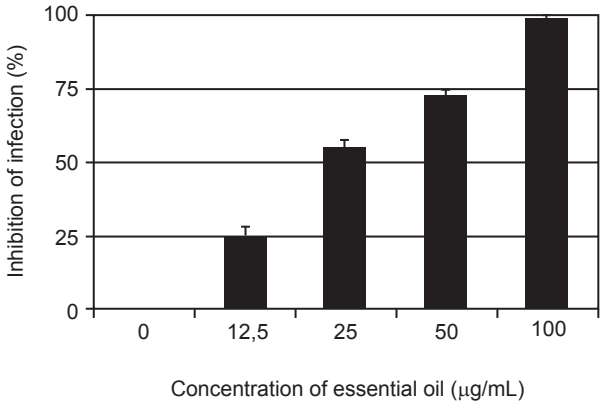

Fig. 3: effect of essential oil from *Piper auritum* on amastigote survival *Leishmania donovani*-infected mouse peritoneal macrophage. Results are from three experiments in duplicate and are shown as percentages ± standard deviations. The control showed 7.6 ± 1.8 average of amastigote per macrophage and 86 ± 6% of macrophage infected.

DISCUSSION

Piper species have been reported to have activity against *Leishmania*. For example, an ethanolic extract of leaves of *Piper betle* (Sarkar et al. 2008), the 2',6'-dihydroxy-4'-methoxychalcone isolated from the inflorescence of *Piper aduncum* (Caio et al. 1999) and kavapyron from leaves of *Piper rusbyi* (Flores et al. 2007) all showed in vitro activity against *Leishmania*. However, previous studies have reported that an aqueous and methanolic extract from the leaves of *P. auritum* did not show any activity against promastigotes of *L. mexicana* and *L. braziliensis* (Navarro et al. 2003). Although people commonly use infusion or beverages derived from herbs, there has been a resurgence of interest in essential oils, which are oily liquids obtained from plant material. Essential oils are a mixture of volatile aromatic compounds that have shown antimicrobial properties and have been used in pharmacology, phytopathology and food preservation (Bakkali et al. 2008). Furthermore, it has also been reported that various essential oils have inhibitory actions against diverse human parasites (Antony et al. 2005).

Recently, several essential oils extracted from plants have shown antileishmanial activity: *Croton cajucara* (Rosa et al. 2003), *Ocimum gratissimum* (Ueda-Nakamura et al. 2006), *Copaifera cearensis* (Santos et al. 2008), *Chenopodium ambrosioides* (Monzote et al. 2006), *Cymbopogon citratus* and *Lippia sidoides* (Oliveira et al. 2009). These studies have demonstrated that essential oils can be a promising source of new antileishmanial drugs. In this view, we have been exploring the activity of essential oil from *P. auritum* against *Leishmania* parasites.

It is well known that the main component of *P. auritum* is safrole (Gupta et al. 1985, Hernández et al. 2003, García et al. 2007), which is an oxygenated monoterpene. In the essential oil studied here, this compound represented 86.9% and could be the major component responsible for the antileishmanial activity. We also found other components, such as monoterpenes and sesquiterpenes, which have been previously described in the literature (Vizoso et al. 1999, Hernández et al. 2003, García et al. 2007). In addition, safrole has been found in other plants and is the major component of the essential oil (85%) of *Sassafras albidum* (Lauraceae) (Kamdem & Gage 1995, Simić et al. 2004), which has shown antifungal activity (Simić et al. 2004).

There are no previous reports about the antileishmanial activity of *Piper*-oil. In our study, this essential oil inhibited the growth of promastigotes in all species of *Leishmania* used, with IC_{50} values between 12.8-63.3 µg/mL. In vitro studies show that amphotericin B is usually more active than the essential oil. However, the IC_{50} values described here for the essential oil are similar to those of commonly used antileishmanial drugs, such as pentostam and aminosidine, which showed an IC_{50} of 10,000 and 54 µg/mL, respectively, against *L. mexicana* (Callahan et al. 1997). It is interesting to mention that, in our study, the essential oil was active against *L. braziliensis*, the main causal agent of mucocutaneous leishmaniasis, which has shown low sensitivity when treated

TABLE I

Principal components of essential oil from *Piper auritum* obtained by high resolution gas chromatography-mass spectrometry analysis

Peak	t_R	Compound	MM	MF	%[a]
1	6.180	α-pinene (4,7,7-trimethylbicyclo[3,1,1]-3-heptene)	136	$C_{10}H_{16}$	0.33
2	7.150	+ sabinene (1-methylene-4α-(2-propyl)-bicyclo[3,1,0]-hexene)	136	$C_{10}H_{16}$	0.07
3	7.229	β-pinene (4-methylene-7,7-dimethylbicyclo[3,1,1]-3-heptene)	136	$C_{10}H_{16}$	0.74
4	7.691	Myrcene (2-methyl-6-methylene-2,7-octadiene)	136	$C_{10}H_{16}$	0.13
5	8.375	α-terpinene (1-methyl-4-[2-propyl]-cyclohexa-1,4-diene)	136	$C_{10}H_{16}$	0,36
6	8.463	p-cymene (1-methyl-4-[2-propyl]-bencene)	134	$C_{10}H_{14}$	0.30
7	8.731	Limonene (1-methyl-4-[2-propenyl]-cyclohex-1-ene)	136	$C_{10}H_{16}$	0.09
8	8.996	β-ocimene (2,6-dimethyl-2E,5Z,7-octanotriene)	136	$C_{10}H_{16}$	0.11
9	9.311	α-ocimene (2,6-dimethyl-2Z,5Z,7-octanotriene)	136	$C_{10}H_{16}$	0.13
10	9.594	γ-terpinene (1-methyl-4-[2-propyl]-1,4-cyclohexadiene)	136	$C_{10}H_{16}$	1.32
11	10.469	Terpinolene (1-methyl-4-propylidene-1-cyclohexene)	136	$C_{10}H_{16}$	1.11
12	10.707	l-linalool (2,6-dimethyl-6-hydroxy-2,7-octadiene)	154	$C_{10}H_{18}O$	0.66
MP	16.000	Safrole (1,2-methylenedioxy-4-[1-(2-propenyl)]-bencene)	163	$C_{10}H_{11}O_2$	86.91
13	17.262	Methyl decanoate	186	$C_{11}H_{22}O_2$	0.08
14	17.750	Eugenol (1-hydroxy-2-methoxy-4-[1-(2-propenyl)]bencene)	164	$C_{10}H_{22}O_2$	0.04
15	18.931	α-copaene	204	$C_{15}H_{24}$	0.28
16	19.280	β-elemene	204	$C_{11}H_{22}O_2$	0.08
17	19.824	Methyl 2,4-decadienoate	182	$C_{11}H_{18}O_2$	0.23
18	20.004	Trans-caryophyllene	204	$C_{15}H_{24}$	0.75
19	20.512	Z3,Z6,E8-dodecatrien-1-ol	180	$C_{11}H_{18}O_2$	0.49
20	20.838	α-humulene	204	$C_{11}H_{18}O_2$	0.09
21	21.033	Methyl Z3,Z6,E8-dodecatrienoate	208	$C_{11}H_{18}O_2$	0.49
23	21.525	Germacrene	204	$C_{11}H_{18}O_2$	0.37
24	21.858	Aromadendrene	204	$C_{11}H_{18}O_2$	0.68
25	21.917	Myristicine	192	$C_{11}H_{18}O_2$	1.59
26	22.366	Tetradecane	204	$C_{15}H_{24}$	0.93
27	22.560	δ-cadinene	204	$C_{15}H_{24}$	0.05
28	23.479	Nerolidol (10-hydroxy-2,6,10-trimethyl-2,6,11-dodecatriene)	222	$C_{15}H_{16}O$	0.55
29	23.811	Caryophyllene oxide	220	$C_{15}H_{24}O$	0.07
30	27.115	Heptadecane	240	$C_{17}H_{36}$	0.05

the related compounds are the 99.08% of the essential oil. *a*: % from total area; MF: molecular formule; MM: molecular mass; MP: main pick; t_R: retention time.

TABLE II

Effect of the essential oil from *Piper auritum* on promastigotes of *Leishmania* spp and corresponding selectivity index

Species	Essential oil from *Piper auritum*		Amphotericin B	
	IC_{50} ± SD (μg/mL)[a]	SI[b]	IC_{50} ± SD (μg/mL)	SI
Leishmania major	29.1 ± 1.4[c]	4	0.022 ± 0.001	264
Leishmania mexicana	63.3 ± 2.6	2	0.0135 ± 0.002	430
Leishmania braziliensis	52.1 ± 3.1	2	0.035 ± 0.004	166
Leishmania donovani	12.8 ± 2.8[d]	8	0.03 ± 0.003	193

a: concentration of drug that caused 50% of inhibitory growth of promastigotes; *b*: selectivity index: CC_{50} against macrophage/ IC_{50} against promastigotes. The CC_{50} against macrophage of the essential oil from *P. auritum* and amphotericin B were of 106.4 and 5.8 μg/mL, respectively; *c*: significant differences ($p < 0.05$) compared with the IC_{50} values of essential oil against *Leishmania mexicana* and *Leishmania braziliensis*, using the Mann-Whitney test; *d*: significant differences ($p < 0.05$) compared with the other groups, using the Mann-Whitney test.

with derivatives of antimonies (Bailey & Lockwood 2007) and miltefosine (Yardley et al. 2005). Additionally, the essential oil is a complex mixture of substances and the purification of active compounds might result in a considerable increase of their antileishmanial activity. Amphotericin B is a pure compound and constitutes the most active antileishmanial drugs. Nevertheless, the toxicity of amphotericin B makes it a second-option treatment behind antimony in areas of refractory leishmaniasis (Garnier & Croft 2002, Guerin et al. 2002). *Piper*-oil was more active against promastigotes of *L. chagasi* (IC_{50} < 63 μg/mL) than other essential oils recently studied, such as *O. gratissimum* (IC_{50} = 75 μg/mL) and *L. sidoides* (IC_{50} = 89 μg/mL) (Oliveira et al. 2009).

In parallel, different activities were observed between the species of *Leishmania* and consequently with their SIs. Several differences in the susceptibility of *Leishmania* strains have been described and are dependent upon the species of parasite (Croft et al. 2002). In this study, the oil showed a better SI against *L. donovani*, which is the causal agent of visceral leishmaniasis. *L. donovani* is the fatal form of the disease and causes about 500,000 deaths per year if left untreated (Guerin et al. 2002). For this reason, further pharmacological studies in animal models of visceral leishmaniasis should be performed.

Piper-oil inhibited the growth of intracellular amastigotes of *L. donovani* at non-toxic concentrations, with values lower than those reported for meglumine antimoniate (IC_{50} = 80 μg/mL), which is a drug of first line use (Ephros et al. 1999). It was also more effective than other natural products such as the hydroalcoholic extract from *Austroplenckia populnea* (IC_{50} = 52 μg/mL) (Andrade et al. 2008).

Plants are an important source in the search for new and selective agents for the treatment of tropical diseases caused by protozoan. In conclusion, this study is a part of a continuing search for new drugs (with high activity and low side effects) to treat protozoa parasites, such as *Leishmania,* and demonstrates the usefulness of the essential oils as a promising alternative. Further evaluation of the essential oil from *P. auritum* against visceral murine leishmaniasis will be performed.

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