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Article

Antileishmanial *in vitro* activity of essential oil from *Myrciaria plinioides,* a native species from Southern Brazil

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In South American folk medicine members of the genus *Myrciaria* are used for the treatment of malaria, diarrhoea, asthma, inflammation and post-partum uterine cleansing. The aim of this work was to evaluate its antileishmanial properties (*in vitro)* of essential oil derived from leaves of *Myrciaria plinioides* D. Legrand, a plant species that is native in South of Brazil. The essential oil was obtained by hydro-distillation using fresh leaves of *M. plinioides*. The chemical composition of this essential oil (MPEO, *M. plinioides* essential oil) was determined by gas chromatography coupled to mass spectrometry (GC-MS). MPEO was assayed *in vitro* for antileishmanial properties against promastigotes of *Leishmania amazonensis* and *Leishmania infantum*, and for cytotoxicity against murine peritoneal macrophages. The MPEO comprised 66 components and was rich in oxygenated sesquiterpenes (82.66%) containing spathulenol (21.12%) as its major constituent. The MPEO was effective against *L. amazonensis* with IC_{50} value of 14.16 ± 7.40 µg/mL, while against *L. infantum* the IC_{50} value was higher with 101.50 ± 5.78 µg/mL. The MPEO showed significant activity against *L. amazonensis*, and presented a selectivity index (SI) of 6.60. The results suggest that the essential oil from leaves of *M. plinioides* is a promising source for new antileishmanial agents against *L. amazonensis*.

Keywords: Antileishmanial activity. *Myrciaria plinioides.* Myrtaceae. *Leishmania amazonensis. Leishmania infantum.*

INTRODUCTION

Leishmaniasis, a parasitic infection caused by protozoa of the genus *Leishmania,* rates as one of the most pernicious of neglected tropical diseases. Some 350 million people worldwide are at risk of contracting one of the forms of the disease, and around 2 million new cases occur annually, mainly within the poorest populations in developing countries. Various factors have contributed to the increase in the number of cases of the disease, especially the difficulties associated with vector control and the lack of a vaccine (Freitas-Junior *et al.,* 2012). Moreover, drugs such as meglumine antimoniate and pentamidine isethionate that are commonly employed in the treatment of leishmaniasis are of somewhat limited application because of issues relating to routes of administration, adherence to treatment, resistance, toxicity and/or teratogenicity (Buckner, Waters, Avery, 2012).

Alternative therapies, including miltefosine and paromomycin, and new formulations of older medications such as amphotericin B, have been introduced but most are restricted in their use and none provide a satisfactory treatment of the disease (Freitas-Junior *et al.*, 2012). In this context, medicinal plants that have been applied in traditional remedies often represent promising sources of lead compounds for the development of new drugs (Oliveira *et al.*, 2011). Within the last few years, considerable research interest has focused on screening plant extracts as potential sources of drugs for the treatment of leishmaniasis (Vila-Nova *et al.*, 2011; Cota *et al.*, 2012;

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Ramírez-Macías *et al.*, 2012; Vila-Nova *et al.*, 2012; Santos *et al.*, 2013).

Members of the family Myrtaceae are ubiquitous in Brazil, and the presence of around 1000 species in discrete biomes, principally the Atlantic forest, *restinga* and *cerrado*, suggests an ecological importance. *Myrciaria* is a genus of large shrubs and small trees belonging to the myrtle family, and various species are used in traditional medicine (Souza, Lorenzi, 2012).

The shrub *Myrciaria plinioides* D. Legrand, popularly known as *camboim*, *cambuim* or *cambuí*, is native to the state of Rio Grande do Sul in southern Brazil. Despite the medicinal potential of this species, very few reports are available concerning its pharmacological activities. For example, tea prepared from the leaves of *Myrciaria tenella*, popularly known as *vassourinha*, is employed in the Amazonian region as a post-partum uterine cleansing agent (Coelho-Ferreira, 2009), while the volatile oil obtained from leaves of this species is rich in α-pinene and β-pinene and exhibits antimicrobial activity against *Enterobacter* spp. and *Shigella flexneri* (Schneider *et al.*, 2008). Additionally, the leaves and trunk bark of *M. cauliflora* (popular name *jabuticaba*) are used to treat diarrhoea, asthma, and throat inflammation (Albuquerque *et al.*, 2007), while ethanolic extracts of the leaves exhibit inhibitory action against *Candida* and *Streptococcus* cultures derived from dental plaque (Carvalho *et al.*, 2009; Diniz *et al.*, 2010). Of particular interest is the report (Ruiz *et al.*, 2011) that the edible fruits of *M. dubia* are employed by Indigenous and *Mestizo* populations living on the banks of the Nanay river in the Loreto region of Peru as a traditional remedy for the treatment of malaria, which is also a neglected protozoan infection.

In consideration of the above, we have assessed the *in vitro* activities of essential oil of *M. plinioides* against *Leishmania infantum* (syn. *L. chagasi*), which is the causal agent of visceral leishmaniasis, and against *L. amazonensis*, a species that has been associated with various clinical forms of the disease including cutaneous, mucosa, diffuse cutaneous and visceral leishmaniasis (Leon *et al.*, 1990). In addition, we have determined the composition of the essential oil derived from this native Brazilian species.

MATERIAL AND METHODS

Plant material

Leaves of *Myrciaria plinioides* D. Legrand were collected in Lajeado, RS, Brazil during July 2012. The plants were authenticated by the botanist Dr. Elisete Maria de Freitas (Centro Universitário UNIVATES) and a voucher specimen was deposited at the Herbário do Vale do Taquari, Museu de Ciências Naturais UNIVATES under the registration number HVAT1066.

Preparation of essential oil

Fresh leaves (200 g) of *M. plinioides* were subjected to hydro-distillation for 3.5 h in a Clevenger-type apparatus. The essential oil (MPEO) was dried over anhydrous sodium sulphate, transferred to amber glass bottles and stored at −20 °C, until required for chemical analysis and bioassay.

Chemical analysis of the essential oil

Samples of MPEO were analysed by gas chromatography coupled to mass spectrometry (GC-MS) at the Instrumental Analysis Laboratory, Food Processing Development Centre - FPDC, Univates. Analyses were performed on a Shimadzu GC2010 Plus system, comprising a model AOC-5000 Plus auto injector and a model QP2110 Ultra mass detector, using a Restek Rtx®-5MS fused silica capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness). The chromatographic conditions were: carrier gas - helium at a flow rate of 1.00 mL/min; oven temperature - initially at 50 °C and increased at 4 °C/min to 290 °C; injector temperature - 240 °C; injection mode - split with 1:20 ratio and 3 mL/min purge; MS interface temperature - 280 °C; ion source temperature - 260 °C; ionisation energy - 70 eV. Oil samples (15 mg) were dissolved in 1.5 mL of purified ethyl acetate and aliquots in the order of 1 µL were injected for analysis. GC analyses with flame ionisation detection (FID) were carried out using an Agilent J & W HP-5 MS column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) with helium as carrier gas, an FID temperature of 260 °C and the oven temperature program as described for the GC-MS procedure.

Separated components were identified initially from their Kováts retention indices (RI), determined by reference to a series of *n*-alkanes, and their identities confirmed by comparison of mass spectral data with those obtained using pure standards together with values quoted in the literature (Adams, 2009) and those stored in the Wiley 8 and NIST11 spectral libraries of the analytical system. The relative compositions of the oils were calculated from the peak areas (uncorrected for specific response factors) of the separated components.

Cultivation of Leishmania promastigotes

Promastigotes of *L. amazonensis* MHOM/BR/77/LTB0016 were grown at 26 ºC in Schneider's Drosophila medium (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and adjusted to pH 7.2. Promastigotes of *L. infantum* MCAN/BR/97/P142 were cultivated at the same temperature and pH, but in this case the medium was supplemented with 20% (v/v) FCS, 2% (v/v) human urine, 100 µg/mL streptomycin and 100 U/mL penicillin. Promastigotes were harvested on day 4, when the percentage of infective metacyclic forms was found to be high, and counted in a Neubauer chamber. Parasite suspensions were adjusted to a concentration of $1x10^7$ promastigotes/mL using the supernatant of the respective culture as diluent.

Determination of antileishmanial activity *in vitro*

Appropriate amounts of MPEO or pentamidine isethionate (as reference drug) (Sideron®) were dissolved in aqueous dimethyl sulphoxide (DMSO; 10 mg/mL) to yield solutions containing analytes in the concentration range of 0.156 to 80 µg/mL. The level of DMSO in each assay solution was below 1.4%, which is the highest concentration that is not hazardous to the parasites.

Suspensions of late log phase promastigotes suspended in Schneider's Drosophila medium were seeded in Corning™ 96-well flat bottom tissue culture tested plates (1×10^7 promastigotes/200 µL/well). Aliquots of freshly prepared MPEO and pentamidine were added to the wells and the plates were incubated for 24 h at 26 °C. Promastigote viability was evaluated using a modified version of the dye-reduction assay employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Dutta *et al.*, 2005). Briefly, MTT reagent was added to each well and incubation was continued in the dark for an additional 4 h. After this time, an 80 µL aliquot of DMSO was added to each well and the optical density of the assay solution was determined at 570 nm using a BioTek µQuant™ microplate spectrophotometer. The specific absorbance associated with the formazan so-produced was determined by subtracting the background absorbance from the total absorbance, and the mean percentage viability was calculated from:

$$\frac{\text{Mean specific absorbance of treated parasites}}{\text{Mean specific absorbance of untreated parasites}} \times 100$$

Values for IC_{50}, i.e. the concentration that inhibited parasite growth by 50%, were determined.

Assessment of cytotoxicity

Peritoneal macrophages from BALB/c mice were obtained by using the lavage technique, counted in a Neubauer chamber and adjusted to a concentration of $2\ x10^6$ cells/mL. Macrophages were transferred to Corning 96-well flat bottom tissue culture tested plates and incubated for 24 h at 37 ºC under a 5% CO_2 atmosphere. Freshly prepared solutions, in aqueous DMSO, containing MPOE or pentamidine isethionate at concentrations range 0.156 to 80 µg/mL were then added to the wells. Macrophage viability was evaluated using a modified version of the dye-reduction assay employing MTT. In order to assess macrophage viability, 22 µL of MTT solution (5 mg/mL) was added to each well and the plates were incubated for an additional 2 h. After this time, an 80 µL aliquot of DMSO was added to each well and the optical density of the assay solution was determined at 540 nm using a BioTek µQuant™ microplate spectrophotometer. This study was approved for The Animal Ethics Committee of the Institute Oswaldo Cruz/IOC - FIOCRUZ (license number L-026/2015).

Statistical analysis

Assays were carried out in three independent experiments and each was performed in triplicate. Values of IC_{50} and CC_{50} were determined by logarithmic regression analysis using GraphPrism 5 software. Values for *in vitro* antileishmanial activity and *in vitro* cytotoxicity were expressed as mean ± standard deviation. The significant differences between samples were evaluated by analysis of variance (ANOVA) and the Tukey test using BioEstat 5.0 software with the alpha level set at 0.05.

RESULTS AND DISCUSSION

Analysis of the essential oil from fresh leaves of *M. plinioides*

The yield of essential oil obtained from fresh leaves of *M. plinioides* was 0.05% (w/w). According to the GC analyses, the oil comprised 66 components (Table I) and was particularly rich in oxygenated sesquiterpenes (82.66%) and sesquiterpene hydrocarbons (11.05%). The principal volatile components of the oil were the sesquiterpenes spathulenol (21.12%), caryophyllene oxide (15.20%), α-isolongifolan-7-ol (9.84%), mustakone

(5.60%), α-cadinol (5.40%), *cis*-isolongifolanone (3.38%) and α-copaene (3.27%).

Members of the family Myrtaceae are commonly rich in essential oils, many of which possess biological activity (Tietbohl *et al.*, 2012; Borges, Conceição, Silveira, 2014). In the case of *M. floribunda*, popularly known as *camboin amarelo*, monoterpenes predominated in the oils derived from leaves and flowers, with 1,8-cineole as the major component accounting for 38.4% of the leaf oil and 22.8% of the flower oil (Tietbohl *et al.*, 2012). In contrast, the stem oil contained mainly sesquiterpenes, of which (2*E*,6*E*)-farnesyl acetate represented the major component accounting for 19.9% of the oil.

Oxygenated sesquiterpenes have been identified as major constituents of leaf oils from a number of *Myrciaria* species (Apel *et al.*, 2006). Thus, the oil of *M. cauliflora*, similar to that of *M. plinioides*, contained mainly spathulenol (27.2%) and caryophyllene oxide (21.6%), while β-caryophyllene, caryophyllene oxide and spathulenol predominated in the essential oil of *M. edulis*. Conversely, the major components of *M. trunciflora* leaf oil were globulol, bicyclogermacrene and γ-muurolene, while the essential oil of *M. cordifolia* was rich in α-bisabolol oxide A, α-bisabolol oxide B, α-bisabolol and β-caryophyllene. The oxygenated sesquiterpenes spathulenol and caryophyllene oxide have been identified as major constituents of the essential oils of other members of the Myrtaceae, including *Eugenia brasiliensis* (Magina *et al.*, 2009), *E. calycina* (Sousa *et al.*, 2015), *Eucalyptus camaldulensis* (Verdeguer *et al.*, 2009) and *Callistemon citrinus* (Petronilho *et al.*, 2013).

In vitro antileishmanial activity and cytotoxicity of the essential oil from fresh leaves of *M. plinioides*

The essential oil derived from leaves of *M. plinioides* was effective against *L. amazonensis* promastigotes (Table II) and presented IC_{50} value of 14.16 ± 7.40 μg/mL, while the standard drug pentamidine isethionate presented IC_{50} value of 23.22 ± 9.04 μg/mL. However, activity against *L. infantum* promastigotes were less pronounced, presented an IC_{50} value of 101.50 ± 5.78 μg/mL (Table II).

American tegumentary leishmaniasis (ATL) affects populations in various regions of the world, including an area extending from southern USA to northern Argentina, with the exception of Chile and Uruguay. The disease can present with diverse clinical forms described as cutaneous, diffuse cutaneous or mucocutaneous. Moreover, ATL can cause injury way beyond its deforming effects, thereby raising issues concerning possible psychological damage and, consequently, social and economic losses (Amato Neto *et al.*, 2008; Garcia *et al.*, 2011).

Antileishmanial activity has been demonstrated for essential oils from a number of plant species including *Lippia origanoides* (Escobar *et al.*, 2010), *L. sidoides* (Medeiros *et al.*, 2011; Farias-Junior *et al.*, 2012) and *Lantana camara* (Machado *et al.*, 2012). In the case of *M. plinioides*, it is likely that the antileishmanial activity of the leaf oil is associated with the presence of the sesquiterpenes spathulenol and caryophyllene oxide, which represent 36.32% of the total components.

In this context, various studies have demonstrated that terpenes can cause alterations in the mitochondrial membrane potential, modification of the redox index, inhibition of cellular isoprenoid biosynthesis and changes in the plasma membrane (Santos *et al.*, 2008; Rodrigues *et al.*, 2013; Monzote *et al.*, 2014). According to Oliveira *et al.* (2014), the essential oil of *Bocageopsis multiflora* is also rich in spathulenol and exhibits *in vitro* antileishmanial activity against promastigotes of *Leishmania amazonensis*. Additionally, spathulenol and caryophyllene oxide have been identified as the principle components of the essential oil of *Piper angustifolium* (Bosquiroli *et al.*, 2015), which also exhibits significant *in vitro* antileishmanial activity against *L. infantum* amastigotes.

Monzote *et al.* (2014) carried out a comparative study of the essential oil of *Chenopodium ambrosioides* and its major constituents, namely ascaridole, carvacrol and caryophyllene oxide, and found that the natural mixture of the oil was potentially more active than the isolated components. Caryophyllene oxide, for example, exhibited non-specific activity and presented similar IC_{50} values against *L. amazonensis* and macrophages. Santin *et al.* (2009) reported an analogous situation for the essential oil of *Cymbopogon citratus* in which the principal constituent, citral, exhibited greater toxicity than the natural oil mixture. These results demonstrate the importance of complementary studies to determine whether the leishmanicidal activity against *Leishmania* promastigotes and cytotoxicity observed for *M. plinioides* is related to a specific component or mixture thereof.

Pentamidine isethionate and other drugs used in the treatment of leishmaniasis are toxic and their application is limited owing to issues associated with high cost, acquired resistance, routes of administration and difficulties of adherence to treatment (Bucknerm Waters, Avery, 2012). The MPEO showed significant activity against *L. amazonensis*, and presented selectivity index (SI) of 6.60 (Table II). The activity against *L. infantum* promastigotas form of sample was less significant, besides that the SI data demonstrated considerable toxicity.

TABLE I - Composition of the essential oil from fresh leaves of *M. plinioides*

Compound	RI_{EXP} [a]	RI_{LIT} [b]	Relative composition (%)
NI[c]	1049	-	0.05
α-Terpineol	1194	1189	0.11
α-Ylangene	1373	1375	0.08
α-Copaene	1378	1376	3.27
β-Bourbonene	1386	1388	1.24
β-Elemene	1394	1391	0.26
β-Ylangene	1421	1421	0.09
β-Copaene	1431	1432	0.10
Aromadendrene	1440	1441	0.17
α-Humulene	1455	1455	0.08
allo-Aromadendrene	1462	1460	0.83
γ-Muurolene	1479	1480	0.67
cis-Eudesma-6,11-diene	1487	1490	0.11
trans-Muurola-4(14),5-diene	1496	1494	0.10
α-Muurolene	1503	1500	0.31
δ-Amorphene	1522	1512	0.55
trans-Calamenene	1535	1529	0.15
(*E*)-*γ*-Bisabolene	1554	1531	0.06
β-Vetivenene	1559	1533	0.84
Silphiperfol-5-en-3-ol B	1575	1535	1.53
Selina-3,7(11)-diene	1595	1546	0.65
1-*nor*-Bourbonanone	1601	1563	0.14
Spathulenol	1606	1578	21.12
Caryophyllene oxide	1610	1583	15.20
β-Copaen-4-*α*-ol	1613	1591	1.25
Khusimone	1617	1604	2.61
Curzerenone	1619	1606	1.22
β-Atlantol	1623	1608	0.43
cis-Isolongifolanone	1626	1613	3.38
Isolongifolan-7-*α*-ol	1631	1619	9.84
Junenol	1637	1619	0.82
2,(7*Z*)-Bisaboladien-4-ol	1642	1619	0.99
1,10-Di-*epi*-cubenol	1646	1619	2.13
trans-Isolongifolanone	1649	1626	0.24
β-Cedren-9-one	1652	1631	0.33
epi- *α*-Muurolol	1657	1642	2.65
α-Muurolol	1661	1646	1.54
Vulgarone B	1663	1651	0.70
α-Cadinol	1667	1654	5.40
Selin-11-en-4-*α*-ol	1669	1659	0.63
cis-Calamenen-10-ol	1670	1661	0.26
14-Hydroxy-(*Z*)-caryophyllene	1673	1667	0.24
trans-Calamenen-10-ol	1677	1669	0.19
14-Hydroxy-9-*epi*-(*E*)-caryophyllene	1679	1669	0.22
Cadalene	1682	1676	1.49
Mustakone	1685	1677	5.60
Khusinol	1690	1680	1.60
5-*neo*-Cedranol	1695	1685	0.36
Germacra-4(15), 5, 10(14)-trien-1- *α*-ol	1702	1686	0.19
10-*nor*-Calamenen-10-one	1704	1702	0.45
Mayurone	1707	1704	0.43
E-Apritone	1711	1708	0.24
Longifolol	1716	1714	0.14
E-Nerolidyl acetate	1720	1717	1.70
iso-Longifolol	1728	1729	0.60
Vetiselinenol	1730	1731	0.10
Eremophilone	1735	1736	0.78
E-*β*-Santalol	1741	1739	0.41
8-*α*-11-Elemodiol	1748	1747	0.33
NI	1751	-	0.15
α-Bisabolol oxide A	1753	1749	0.45
β-Acoradienol	1767	1763	0.64
β-Costol	1772	1767	0.69
Khusinol acetate	1820	1823	0.18
NI	1839	-	0.43
8*S*,13-Cedranediol	1904	1897	0.22
Total constituents identified			**99.37**
Oxygenated monoterpenes			**0.11**
Sesquiterpene hydrocarbons			**11.05**
Oxygenated sesquiterpenes			**82.66**

[a] Experimental retention Index. [b] Literature retention Index (Adams, 2007). [c] Not identified.

The results obtained in this study reveal that the MPEO is promising as a source for new antileishmanial agents against *L. amazonensis*. However, more studies are necessary in order to determine the constituents

TABLE II - IC_{50} (µg/mL) value of essential oil of *M. plinioides* against promastigotes of *L. amazonensis* and *L. infantum* and CC_{50} (µg/mL) value of cytoxicity against murine peritoneal macrophages.

Extract	Antiparasitic Activity IC_{50} (µg/mL)		Toxicity CC_{50} (µg/mL)	SI
	L. amazonensis	*L. infantum*	Macrophages	
MPEO	14.16 ± 7.40[a]	101.50 ± 5.78[a]	93.50 ± 9.10	6.60
Pentamidine*	23.22 ± 9.04[a]	34.20 ± 2.50[b]	61.21 ± 1.40	2.63

*Reference drug. ** Selective Index: ratio CC_{50}/IC_{50} (*L. amazonensis*). Data are expressed as mean values ± standard error. Within each column, values followed by dissimilar upper case superscript letters are statistically different ($p > 0.05$).

responsible for the antileishmanial activity and the mechanism of action involved. It stands out that the assay against promastigote forms is a preliminary screening to identify possible novel antileishmanial compounds, as it is a low cost and easy to handle method like in amastigotes (Siqueira-Neto *et al.*, 2010). Even so, it is essential to evaluate the activity of MPEO against amastigote forms.

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