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Antileishmanial activity of *Melampodium divaricatum* and *Casearia sylvestris* essential oils on *Leishmania amazonensis*

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ABSTRACT

Leishmaniasis is a disease that affects millions of people and it is an important public health problem. The drugs currently used for the treatment of leishmaniasis present undesirable side effects and low efficacy. In this study, we evaluated the *in vitro* activity of *Melampodium divaricatum* (MD-EO) and *Casearia sylvestris* (CS-EO) essential oils (EO) against promastigote and amastigote forms of *Leishmania amazonensis*. Sesquiterpenes *E*-caryophyllene (56.0%), germacrene D (12.7%) and bicyclogermacrene (9.2%) were identified as the main components of MD-EO, whereas *E*-caryophyllene (22.2%), germacrene D (19.6%) and bicyclogermacrene (12.2%) were the main constituents of CS-EO. CS-EO and *E*-caryophyllene were active against promastigote forms of *L. amazonensis* (IC_{50} 24.2, 29.8 and 49.9 µg/mL, respectively). However, MD-EO, CS-EO and *E*-caryophyllene were more active against amastigote forms, with IC_{50} values of 10.7, 14.0, and 10.7 µg/mL, respectively. *E*-caryophyllene presented lower cytotoxicity against macrophages J774-A1 (CC_{50} of 62.1 µg/mL) than the EO. The EOs and *E*-caryophyllene should be further studied for the development of new antileishmanial drugs.

KEYWORDS: *Leishmania amazonensis*. *Melampodium divaricatum*. *Casearia sylvestris*. *E*-caryophyllene

INTRODUCTION

Leishmaniasis is an important public health problem and it is estimated that 2 million new cases occur each year, with at least 15-20 million infected people worldwide[1,2]. *Leishmania* is the protozoan parasite responsible for leishmaniasis which is manifested in visceral, mucocutaneous or cutaneous forms[2,3].

Drugs currently recommended for leishmaniasis treatment include compounds such as pentavalent antimonials, amphotericin B, lipid formulations of amphotericin B (treatment of visceral leishmaniasis) and miltefosine, which is the only orally administered drug. However, there are some limitations regarding their toxicity, lack of efficacy, need and cost of hospitalization cost[4-6]. Other problems such as re-emerging infectious diseases and resistance to currently used drugs are widely recognized as being of serious and immediate concern. Thus, it is imperative to search for new drugs against *Leishmania* parasites[7-9].

Plants and their metabolites are potential sources of new antiprotozoal drugs, and can contribute to overcome protozoan parasites drug resistance[10-12]. Many plant essential oils have demonstrated anti-Leishmania activity *in vivo* and *in vitro* against the promastigote and/or the amastigote forms[13-16].

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Asteraceae and Salicaceae species have attracted special attention due to their therapeutic properties, such as anthelmintic, antiinflammatory, astringent, antihemorrhagic, antimicrobial, diuretic, analgesic and antispasmodic effects[17-19]. Many species of these families, such as *Melampodium divaricatum* (Rich. ex Rich.) DC. (Asteraceae) and *Casearia sylvestris* Swartz (Salicaceae) are aromatic and their essential oils have a large chemical complexity. *M. divaricatum,* popularly known as 'falsa-calêndula', 'flor-amarela' or 'flor-de-ouro', occurs in Northeastern Brazil and is used in folk medicine as wound healing, diaphoretic and diuretic. The essential oil from the aerial parts of *M. divaricatum* showed antimicrobial activity against *S. aureus* and *B. subtilis*[16]. *C. sylvestris* occurs in Brazil and other countries of Latin America. In Brazil, it is known as 'guaçatonga', 'cafezinho-do-mato or 'erva-de-lagarto' and it is used in traditional medicine as antiophidic, antiulcer, anti-pyretic, anti-inflammatory and wound healing[20]. However, the antileishmanial activity of *M. divaricatum* and *C. sylvestris* essential oils has not been previously investigated to date. Thus, in this work, we report the *in vitro* antileishmanial activity of the essential oils obtained from the aerial parts of *M. divaricatum* and *C. sylvestris* leaves collected in Brazil and their main component *E*-caryophyllene.

MATERIAL AND METHODS

Plant material

The aerial parts of *Melampodium divaricatum* (Rich. ex Rich.) DC. (Asteraceae) and leaves of *Casearia sylvestris* Swartz (Salicaceae) were collected at "Medicinal Botanical Garden" of School of Pharmaceutical Sciences of Sao Paulo State University (UNESP), Araraquara, Sao Paulo State, Brazil (21º48'51.4'' S and 48º12'5.1'' W). A voucher specimen of *M. divaricatum* (HRCB 35294) and *C. sylvestris* (AGS 102) were deposited at the Herbarium Institute of Biosciences of the UNESP Rio Claro, Sao Paulo State, Brazil and Herbarium Maria E. P. Kauffman Fidalgo of the Botanical Institute of Sao Paulo, Brazil, respectively.

Essential oil extraction and chemicals

Casearia sylvestris essential oil was obtained by hydrodistillation of its leaves (150 g) for 4 h in a Clevenger-type apparatus according to the procedure described in the European Pharmacopoeia[21]. *M. divaricatum* essential oil was obtained by Moreira *et al*.[16]. The EOs were stored in amber bottles and kept in the refrigerator at 4 °C until further analysis. The yields (w/w) were calculated from the weight of the fresh aerial parts and leaves of *M. divaricatum* (0.4%) and leaves of *C. sylvestris* (0.3%). *E*-Caryophyllene was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

GC/MS analysis

GC-MS analyses were carried out on a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i auto sampler. The column consisted of Rtx5MS (Restek Co., Bellefonte, PA, USA) fused-silica capillary (length = 30 m, i.d. = 0.25 mm, and film thickness = 0.25 μm). The electron ionization mass spectrometry (EI-MS) mode at 70 eV was employed. Helium (99.99%) at a constant flow of 1.0 mL/min was the carrier gas. The injection volume was 0.1 μL (split ratio of 1:10). The injector and the ion source temperatures were set at 240 and 280 °C, respectively. The oven temperature program was the same as the one used for GC-FID. The mass spectra were registered with a scan interval of 0.5 s in the mass range of 40 to 600 Da. Essential oil was solubilized in hexane (chromatographic grade; Merck®, Darmstadt, Germany) 1:100 (v/v).

GC/FID analysis

The essential oil of *C. sylvestris* (CS-EO) was analyzed by gas chromatography (GC) on a Hewlett-Packard G1530A 6890 gas chromatograph fitted with FID and a data-handling processor. An HP-5 (Hewlett-Packard, Palo Alto, CA, USA) fused-silica capillary column (length = 30 m, i.d. = 0.25 mm, and film thickness = 0.33 μm) was employed. The column temperature was programmed to rise from 60 to 240 °C at 3 °C/min and then held at 240 °C for 5 min. The carrier gas was H_2 at a flow rate of 1.0 mL/min. The equipment was set to the injection mode; the injection volume was 0.1 μL (split ratio of 1:10). The injector and detector temperatures were 240 and 280 °C, respectively. The relative concentrations of the components were obtained by peak area normalization (%). The relative areas were the average of triplicate GC-FID analyses.

Compound identification

Compounds were identified on the basis of their retention indices relative to a homologous series of *n*-alkanes (C_8–C_{20}). To this end, an Rtx-5MS capillary column was employed under the same operating conditions as in the case of GC-FID. The retention index (RI) of each constituent was determined as described previously[22]. The chemical structures were computer-matched with the Wiley7, NIST08, and FFNSC1.2 spectral libraries of the

GC-MS data system; their fragmentation patterns were compared with the literature data[23].

Antileishmanial activity

Parasites and cell culture

Promastigote forms of *L. amazonensis* (WHOM/BR/75/Josefa), which were originally isolated by Cesar Augusto Cuba-Cuba (Universidade de Brasilia, Brazil) from a human case of diffuse cutaneous leishmaniasis. Parasites were cultured at 25 °C 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, USA) in a tissue flask. Macrophages (J774-A1) were maintained in tissue flasks with RPMI-1640 (pH 7.2) (Gibco Invitrogen, Grand Island, NY, USA), added with sodium bicarbonate and L-glutamine (As annex), and supplemented with 10% FBS (Gibco Invitrogen, Grand Island, NY, USA) at 37 °C in a 5% CO_2 atmosphere[24].

Antiproliferative activity studies on promastigote forms

For experiments, promastigote forms in the logarithmic phase (1 x 10^6 cells/mL) were cultured on a 24-well plate in Warren's medium supplemented with 10% of inactivated FBS in the presence or absence of different essential oils concentrations (1-100 µg/mL). Amphotericin B was used as positive control (0.01-10 µg/mL). After 72 h at 28 °C, cell growth was estimated using the colorimetric cell viability XTT assay (2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) (Sigma Chemical Co., St. Louis, MO, USA). All experiments were performed in duplicate, and the results expressed as log number cells/mL and as the percentage of growth inhibition concentration that inhibited cell growth in 50% (IC_{50}) was determined by nonlinear regression analysis[25].

Activity against intracellular amastigote forms

For the antiproliferative activity on intracellular amastigotes, peritoneal macrophages from healthy BALB/c mice were harvested and plated (3 x 10^5 cells/mL) in a 24-well plate with round coverslips using RPMI medium supplemented with 10% FBS to adhere for 2 h at 37 °C in 5% CO_2. Adhered macrophages were infected with promastigotes in the stationary growth phase using a ratio 1:7 at 34 °C for 4 h. Afterwards, non-interiorized parasites were removed and the infected culture was treated with different concentrations of essential oils (1 to 20 µg/mL) at 34 °C. Amphotericin B was used as a positive control (0.1-10 µg/mL). After 48 h, the coverslips were washed, fixed with methanol and stained with Giemsa. By counting 200 cells under a light microscope (Olympus CX 31) the percentage of infected cells and number of intracellular amastigotes were estimated. The survival index (percentage of infected cells x number of amastigotes per cell) was calculated and IC_{50} values were then determined by nonlinear regression analysis[25].

The activity against intracellular amastigote forms was compared with toxicity in macrophages, yielding the selectivity index (SI; ratio of the CC_{50} in J774-A1 and the IC_{50} in protozoa).

Cytotoxicity assay

A suspension of J774-A1 macrophages (5 x 10^5 cells/mL) was seeded in 96-well microplates. The cells were allowed to attach for 24 h at 37 °C in a 5% CO_2 atmosphere. The medium was then replaced by different concentrations of essential oil (10-1,000 µg/mL). Amphotericin B was also evaluated (0.1-10 µg/mL). Cytotoxicity in J774-A1 macrophages was evaluated after 48 h using the standard MTT colorimetric assay[26]. The mitochondrial-dependent reduction of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl-tetrazolium bromide (MTT) to formazan was used to assess the possible cytotoxic effects of the test compounds on murine peritoneal macrophages[26]. The cytotoxic concentration that reduced cell viability by 50% (CC_{50}) was estimated by nonlinear regression analysis.

Statistical analysis

One-way ANOVA and the Tukey's test were performed with GraphPad Prism 4 (GraphPad Software, San Diego, California, USA). Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Essential oil composition

The essential oils presented as main components sesquiterpene hydrocarbons (84.3% for MD-EO and 78.4% for CS-EO). *E*-caryophyllene (56.0%), germacrene D (12.7%), bicyclogermacrene (9.2%) and caryophyllene oxide (3.0%) were major components of MD-EO employed in this study, as previously reported[16]. The major components of CS-EO were *E*-caryophyllene (22.2%), germacrene D (19.6%), bicyclogermacrene (12.2%) and δ-cadinene (6.6%) (Figure 1 and Table 1). The sesquiterpenes α-zingiberene, *E*-caryophyllene, germacrene D and bicyclogermacrene have been previously reported as the main components in the essential oil of *C. sylvestris* collected in Brazil[11,27,28].

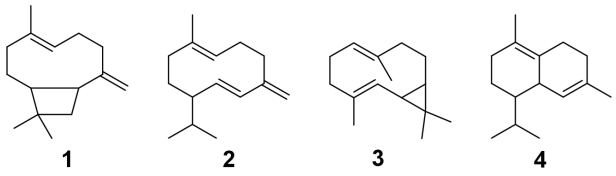

Figure 1 - Chemical structures of the major compounds of *M. divaricatum* and *C. sylvestris* essential oils: 1) *E*-caryophyllene; 2) germacrene D; 3) bicyclogermacrene; 4) δ-cadinene.

Antileishmanial activity

The *in vitro* antileishmanial activity of the MD-EO, CS-EO and *E*-caryophyllene are summarized in Table 2. The essential oils and *E*-caryophyllene were able to inhibit promastigote and amastigote growth. The 50% inhibitory concentration (IC_{50}) of 24.2, 29.8 and 49.9 µg/mL against promastigote forms of *L. amazonensis* were obtained

Table 1 - Composition of the essential oil of *C. sylvestris* leaves.

Compound name and class*)	*RT* [min][a])	RI_{exp} [b])	RI_{lit} [c])	Content [%][d])
δ-elemene	22.56	1332	1337	1.5
α-cubebene	23.00	1342	1345	2.4
α-longipinene	23.49	1354	1351	3.6
cyclosativene	24.22	1371	1371	0.3
isoledene	24.50	1378	1377	9.0
α-copaene	24.67	1382	1385	0.5
β-cubebene	25.00	1389	1389	0.2
cyperene	25.32	1397	1398	1.8
α-gurjunene	26.06	1415	1412	1.4
E-caryophyllene	26.58	1428	1428	22.2
α-*trans*-bergamotene	26.86	1436	1436	1.8
Z-β-farnesene	27.10	1442	1442	4.3
α-himachalene	27.29	1447	1447	0.5
α-guaiene	27.43	1450	1447	0.2
β-santalene	27.89	1462	1462	2.7
alloaromadendrene	28.18	1469	1465	0.3
γ-selinene	28.66	1481	1484	0.2
γ-muurolene	28.78	1484	1478	0.4
germacrene D	29.03	1491	1485	19.6
epizonarene	29.23	1496	1497	0.2
bicylogermacrene	29.92	1513	1514	12.2
α-selinene	30.06	1517	1517	1.3
hedycaryol	30.37	1525	1530	1.7
δ-cadinene	30.75	1534	1535	6.6
trans-γ-bisabolene	31.01	1541	1541	0.2
E-α-bisabolene	31.20	1546	1547	0.3
α-colocarene	31.44	1552	1548	0.4
trans-nerolidol	31.85	1562	1564	0.9
caryophyllene alcohol	32.11	1569	1568	0.2
longipinanol	32.41	1576	1575	1.0
viridiflorol	32.71	1584	1585	0.2
Sesquiterpene hydrocarbons:				78.4
Oxygenated sesquiterpenes:				18.4
Others:				0.8
Not identified:				2.4

[a]*RT*: Retention time determined on the *Rtx- 5MS* capillary column. [b]RI_{exp}: Retention index determined relative to *n*-alkanes (C_8–C_{20}) on the *Rtx-5MS* column. [c]RI_{lit}: Retention index. [d]Calculated from the peak area relative to the total peak area. Identification: RI, comparison of the retention index with the literature[29,30]; MS, comparison of the mass spectrum with the literature.

Table 2 - Antileishmanial and cytotoxic activity of essential oils from *M. divaricatum*, *C. sylvestris*, *E-caryophyllene* and amphotericin B.

Samples	*L. amazonensis* promastigotes IC_{50} (µg/mL)	*L. amazonensis* amastigotes IC_{50} (µg/mL)	SI	Macrophages J774-A1 CC_{50} (µg/mL)
M. divaricatum	24.2 ± 2.1	10.7 ± 2.6	ND	<10
C. sylvestris	29.8 ± 1.1	14.0 ± 5.9	2.9	40.8 ± 4.5
E-caryophyllene	49.9 ± 2.6	10.7 ± 0.6	5.8	62.1 ± 1.5
Amphotericin B	0.06 ± 0.0	0.42 ± 0.08	8.8	3.7 ± 0.3

Each value represents the mean ± S.E.M. for three experiments performed in duplicate. IC_{50}: inhibitory concentration for 50%; CC_{50}: cytotoxic concentration for 50%; SI: selective index (CC_{50} macrophages/IC_{50} against amastigotes).

for MD-EO, CS-EO and *E*-caryophyllene, respectively. Furthermore, MD-EO, CS-EO and *E*-caryophyllene were more active against the amastigote forms than the promastigote ones, displaying IC_{50} values of the 10.7, 14.0 and 10.7 µg/mL, respectively. Amphotericin B had an IC_{50} of 0.06 µg/mL and 0.42 µg/mL against *L. amazonensis* promastigote and amastigote forms, respectively, after 72h of treatment. These are promising results, given that the essential oils and *E*-caryophyllene proved to be more active against amastigote forms, the proliferative form found inside mammalian host cells.

Several authors showed the leishmanial effect of the essential oils[29,30-32] and different biological properties have been attributed to a group of sesquiterpenes present in these oils[33,34].

E-caryophyllene (a sesquiterpene hydrocarbon) was the main chemical constituent of the essential oils of *M. divaricatum* and *C. sylvestris*. According to Santos *et al.*[15], essential oils rich in sesquiterpenes, mainly *E*-caryophyllene, showed variable levels of activity against promastigote forms of *Leishmania* with IC_{50} values in the range between 5 and 22 µg/mL. Thus, our essential oils showed an antiparasitic potency similar to those described in the literature.

The antileishmanial activity of MD-EO and CS-EO may be associated with the presence of *E*-caryophyllene, as previously suggested in the literature for other essential oils in which *E*-caryophyllene was one of the main components[15,29,34,35]. *E*-caryophyllene could also interact with other minor components of MD-EO and CS-EO in a synergistic way.

Sesquiterpenes are considered potent skin permeation enhancers, and those with leishmanicidal activity would be very promising candidates for integration in nanocarriers to treat cutaneous leishmaniasis. A mechanism of action based on the *Leishmania* plasma membrane disruption is consistent with the reported broad spectrum of sesquiterpene activity against protozoa. It has been shown that the lipophilic components of essential oils may affect layers of polysaccharides, fatty acids, and phospholipids in plasma membranes of Leishmania spp. promastigotes. This then leads to cell lysis and release of macromolecules[36]. In the cytoplasm, these compounds can disrupt the specific metabolic pathways of lipids and proteins or stimulate the depolarization of mitochondrial membranes, which can lead to cell necrosis or apoptosis[37,38].

The cytotoxicity of MD-EO, CS-EO and *E*-caryophyllene to macrophages J774-A1 was also evaluated (Table 2). The cytotoxicity evaluation of natural products is very important for the development of new antiprotozoal agents[39]. Our results revealed that the cytotoxicity of MD-EO (CC_{50} < 10 µg/mL) was higher when compared with CS-EO (CC_{50} = 40.8 µg/mL). However, *E*-caryophyllene presented lower cytotoxicity to macrophages J774-A1 with CC_{50} value equal to 62.1 µg/mL.

The cytotoxicity and the antileishmanial activity were compared by using the selectivity index (SI) ratio (CC_{50} for J774-A1 cells/IC_{50} on intracellular amastigotes). Values greater than 1 are considered more selective for activity against parasites, and a value less than 1 is considered more selective for activity against cells. The SI ratio obtained for the CS-EO and *E*-caryophyllene was 2.9 and 5.8, in other words they are considered more selective to intracellular amastigotes than to mammalian host cells.

CONCLUSIONS

The essential oils of *M. divaricatum* and *C. sylvestris* displayed antileishmanial activity against both, promastigote and amastigote forms of *Leishmania amazonensis.* Their main component *E*-caryophyllene was more active against amastigote forms of *L. amazonensis* and showed lower toxicity, demonstrating a potential as a drug candidate against *Leishmania*.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

AUTHORS' CONTRIBUTIONS

Raquel Regina Duarte Moreira, Caio Humberto Perego, André Gonzaga dos Santos and Flavio Alexandre Carvalho performed and contributed to plant samples collection, extracts preparation and phytochemical study at Universidade Estadual Paulista (UNESP), *Faculdade de Ciências Farmacêuticas*, Araraquara. Eduardo José Crevelin and Antônio Eduardo Miller Crotti performed the analisys of GC at Universidade de São Paulo (USP), *Departamento de Química, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto*. Juliana Cogo, Mara Lane Carvalho Cardoso and Celso Vataru Nakamura performed the biological experiment at *Universidade Estadual de Maringá, Laboratório de Inovação Tecnológica no Desenvolvimento de Fármacos e Cosméticos e Laboratório de P&D de Fitoterápicos*. All authors contributed equally in analyzing the data and writing the article.

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