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* (IC₅₀ 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 IC₅₀ values of 10.7, 14.0, and 10.7 µg/mL, respectively. *E*-caryophyllene presented lower cytotoxicity against macrophages J774-A1 (CC₅₀ 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 worldwide². *Leishmania* is the protozoan parasite responsible for leishmaniasis which is manifested in visceral, mucocutaneous or cutaneous forms³.⁴.

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 cost⁴-⁶. 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* parasites⁷-⁹.

Plants and their metabolites are potential sources of new antiprotozoal drugs, and can contribute to overcome protozoan parasites drug resistance¹⁰-¹². Many plant essential oils have demonstrated anti-Leishmania activity *in vivo* and *in vitro* against the promastigote and/or the amastigote forms¹³-¹⁶.
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. 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 ‘falsacalêndula’, ‘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*. *C. sylvestris* occurs in Brazil and other countries of Latin America. In Brazil, it is known as ‘guacatonga’, ‘cafezinho-do-mato or ‘erva-de-lagarto’ and it is used in traditional medicine as antiophidic, antiulcer, anti-inflammatory and *erva-de-lagarto* 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*. *C. sylvestris* occurs in Brazil and other countries of Latin America. In Brazil, it is known as ‘guacatonga’, ‘cafezinho-do-mato or ‘erva-de-lagarto’ and it is used in traditional medicine as antiophidic, antiulcer, anti-inflammatory and wound healing. 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 (21°48’51.4” S and 48°12’5.1” 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. *M. divaricatum* essential oil was obtained by Moreira et al. 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 RtxSMS (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 (C₈–C₂₀). 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. The chemical structures were computer-matched with the Wiley7, NIST08, and FFNSC1.2 spectral libraries of the
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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% CO₂ atmosphere.

Antiproliferative activity studies on promastigote forms
For experiments, promastigote forms in the logarithmic phase (1 x 10⁶ 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). Values of p < 0.05 were considered significant.

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 10⁵ 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% CO₂. 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 IC₅₀ values were then determined by nonlinear regression analysis.

The activity against intracellular amastigote forms was compared with toxicity in macrophages, yielding the selectivity index (SI; ratio of the CC₅₀ in J774-A1 and the IC₅₀ in protozoa).

Cytotoxicity assay
A suspension of J774-A1 macrophages (5 x 10⁶ cells/mL) was seeded in 96-well microplates. The cells were allowed to attach for 24 h at 37 °C in a 5% CO₂ 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. 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. The cytotoxic concentration that reduced cell viability by 50% (CC₅₀) 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. 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.
Table 1 - Composition of the essential oil of C. sylvestris leaves.

| Compound name and class* | RT [min] | RI<sub>exp</sub> | RI<sub>lit</sub> | 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          |
| isoleadene               | 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

*RT: Retention time determined on the Rtx-5MS capillary column.  
<sup>2</sup>RI<sub>exp</sub>: Retention index determined relative to n-alkanes (C<sub>8</sub>-C<sub>20</sub>) on the Rtx-5MS column.  
<sup>3</sup>RI<sub>lit</sub>: Retention index.  
<sup>4</sup>Calculated from the peak area relative to the total peak area. Identification: RI<sub>lit</sub>, comparison of the retention index with the literature<sup>29,30</sup>; MS, comparison of the mass spectrum with the literature.

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<sub>50</sub>) of 24.2, 29.8 and 49.9 µg/mL against promastigote forms of *L. amazonensis* were obtained
Table 2 - Antileishmanial and cytotoxic activity of essential oils from *M. divaricatum*, *C. sylvestris*, *E*-caryophyllene and amphotericin B.

| Samples            | *L. amazonensis* promastigotes IC₅₀ (µg/mL) | *L. amazonensis* amastigotes IC₅₀ (µg/mL) | SI    | Macrophages J774-A1 CC₅₀ (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. IC₅₀: inhibitory concentration for 50%; SI: selective index (CC₅₀ macrophages/IC₅₀ 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 IC₅₀ values of the 10.7, 14.0 and 10.7 µg/mL, respectively. Amphotericin B had an IC₅₀ 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 oils29,30-32 and different biological properties have been attributed to a group of sesquiterpenes present in these oils33-34.

*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 IC₅₀ 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 components15,29,34,35. *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 macromolecules36. 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 apoptosis37,38.

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 agents39. Our results revealed that the cytotoxicity of MD-EO (CC₅₀ < 10 µg/mL) was higher when compared with CS-EO (CC₅₀ = 40.8 µg/mL). However, *E*-caryophyllene presented lower cytotoxicity to macrophages J774-A1 with CC₅₀ value equal to 62.1 µg/mL.

The cytotoxicity and the antileishmanial activity were compared by using the selectivity index (SI) ratio (CC₅₀ for J774-A1 cells/IC₅₀ 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 analysis 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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