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Potential Antileishmanial Activity of Essential Oils of Native Species from Southern Brazil

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Abstract

Leishmaniasis are a neglected tropical diseases that affecting 98 countries on three continents. Every year, 1.3 million of people are infected with the disease and 50.000 persons die because of this. The aim of this work was to evaluate antileishmanial activities *in vitro* from native species of South of Brazil belonging to the Myrtaceae family. The essential oils from leaves of *Calyptranthes grandifolia*, *Calyptranthes tricona*, *Eugenia anomala*, *Eugenia arenosa*, *Eugenia pyriformis*, *Myrrhinium atropurpureum* and *Psidium salutare* were analyzed *in vitro* for antileishmanial activity against promastigotes of *Leishmania amazonensis*, employed MTT assay. The essential oils from leaves of *C. grandifolia*, *C. tricona*, *E. arenosa* and *E. pyriformis* presented IC_{50} values of 31.27 ± 6.40 µg/mL, 26.13 ± 8.60 µg/mL, 13.72 ± 8.65 µg/mL and 19.73 ± 5.40 µg/mL, respectively, and not are statistically different from pentamidine (IC_{50} = 23.22 ± 9.04 µg/mL), the reference drug. The results show the potential of essential oils from leaves of *C. grandifolia*, *C. tricona*, *E. arenosa* and *E. pyriformis* as antileishmanial, as well as the importance of continuing studies to in order to advance in the search and development of new therapeutic options from of brazilian flora sources.

Keywords: antileishmanial activity, essential oil, *Leishmania amazonensis*, Myrtaceae

1. Introduction

Leishmaniasis are considered a neglected tropical diseases (NTDs), affecting 98 countries on three continents, with Brazil among the countries where visceral leishmaniasis (VL) cases, cutaneous leishmaniasis (CL) and mucocutaneuos leishmaniasis (MCL) are more frequent, ie, it is considered an endemic area. NTDs are generally infections diseases, prevalent in tropical or subtropical regions, which typically affect the poorest populations. Annually 300.000 new cases de VL and one million of new cases of CL are registrated, totalizing 1.3 million people stricken by illness, besides up to 50.000 deaths per year. The areas of disease transmission has been enlarged and, consequently, the reported cases increase exponentially. Among the causes related to the propagation of this NTD can be cited unfavorable socioeconomic conditions, malnutrition, climate and environmental changes, increased of migration populational, conflicts, coinfection with HIV that generates immunosuppression, accelerated urbanization (World Health Organization [WHO], 2015; WHO, 2016).

Medications for the treatment of NTDs are generally not research targets of the pharmaceutical industries, and the people affected by these diseases suffer from the deficiency of effective and safe pharmacotherapy. In addition, there are no vaccines available for leishmaniasis and vector disease control is also deficient. Thus, the solution for NTDs is complex, is necessary to improve people's living conditions, increase investment in research and development, as well as provide access to preventive or curative therapy for all populations, especially in developing countries (Brasil, 2010; Garcia, De Magalhães, Aurea, Dos Santos, & De Almeida, 2011; Boechat & Magalhães, 2012; L. C. Dias, Dessoy, Guido, Oliva, & Andricopulo, 2013).

The World Health Organization (WHO) recommends the use of plants as a therapeutic resource, in addition, Brazil is renowned for its biodiversity (Brasil, 2009), and thus the search for new therapeutic agents from plants becomes an attractive alternative. Among the botanical families present in our flora, the Myrtaceae family stands out, for its wide distribution and number of species, adding to the fact that many of these, despite the popular usage reporting, not present studies on its effectiveness or safety.

About 1.000 species from Myrtaceae family are found in Brazil, distributed among different biomes, especially the Atlantic Rain Forest, the Restinga and the Cerrado. Species from family are used in spice industry (*Syzygium aromaticum*), in the wood industry and paper production (*Eucalyptus* spp.), pharmaceutical and cosmetic industries (*Pimenta racemosa*, *Melaleuca* spp., *Callistemon* e *Leptospermum*). The family also is a source of edible fruit, like guava (*Psidium guajava*), jabuticaba (*Myrciaria cauliflora*), cherry (*Eugenia uniflora*), guabiroba (*Campomanesia* spp.), araçá (*Psidium cattleyanum*), jambo (*Syzygium* spp.) and jambolão (*Syzygium cumini*) (Govaerts et al., 2008; Souza & Lorenzi, 2012). Species from Myrtacea family are used popularly in treatment of diarrhea and inflammations, as *Myrciaria cauliflora* and *Eugenia uniflora*, while *Myrciaria dubia* is employed as antimalarial (Simões, Mentz, Schenkel, Irgang, & Stehmann, 1989; Boscolo & Valle, 2008; Ruiz et al., 2011). In addition, studies have shown the potential biological of species from family (Schneider et al., 2008; Diniz, Macêdo-Costa, Pereira, Pereira, & Higino, 2010; Magina et al., 2012; Ferreira et al., 2014; Sousa et al., 2015).

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). The screening for new antileishmanial compounds in endemic areas is suggested by employing assays against promastigotes forms of *Leishmania* spp., because these tests are easier and cheaper compared to analyses against amastigotes forms. Also, for small molecule, as terpenes, results in assays against promastigotes forms have demonstrate similar to those in amastigote forms (Siqueira-Neto et al., 2010). Thus, considering the problem of leishmaniasis as NTDs and the need to develop new therapeutic alternatives for this disease, this study aimed to evaluate the antileishmanial activity *in vitro* of native species of the State of Rio Grande do Sul, southern Brazil, belonging to Myrtaceae family, against promastigote forms of *Leishmania amazonensis*.

2. Materials and Methods

2.1 Plant Materials

Leaves samples from native species of Myrtaceae family were collected in various municipalities of the State of Rio Grande do Sul, Brazil, during June and July 2012, and identified by the botanist Dr. Elisete Maria de Freitas. The GPS data are listed in Table 1.

Table 1. Sources of Myrtaceae species employed in the study.

Species	Popular name	Source	GPS Data (WGS84)
Calyptranthes grandifolia O.Berg	guamirim[a]	Lajeado	Lat.: -29.443333 Long.: -51.956389
Calyptranthes tricona D.Legrand	guaburiti[a]	Lajeado	Lat.: -29.443333 Long.: -51.956389
Eugenia anomala D.Legrand	not available	Alegrete	Lat.: -29.657122 Long.: -55.403270
Eugenia arenosa Mattos	not available	Alegrete	Lat.: -29.657122 Long.: -55.403270
Eugenia pyriformis Cambess.	uvaia[a]	Cruzeiro do Sul	Lat: -29.988861 Long: -52.054722
Myrrhinium atropurpureum Schott	carrapato, pau-ferro[a]	Canudos do Vale	Lat.: -29.309402 Long.: -52.256820
Psidium salutare var. *sericeum* (Cambess.) Landrum	araçá-do-campo[b]	Alegrete	Lat.: -29.657122 Long.: -55.403270

[a] Sobral et al., 2013.

[b] Flora Digital, 2010.

2.2 Preparation of Essential Oil

Fresh leaves from each species of Myrtaceae family were subjected to hydrodistillation for 3.5 h in a Clevenger-type apparatus. The essential oil was dried over anhydrous sodium sulfate, transferred to amber glass bottles and stored at −20 °C, until required for chemical analysis and bioassay.

2.3 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 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.

2.4 Determination of Antileishmanial Activity in vitro

Appropriate amounts of samples or pentamidine isethionate (as reference drug) were dissolved in aqueous dimethyl sulfoxide (DMSO; 10 mg/mL) to yield solutions containing analytes in the concentration range 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 ($1x10^7$ promastigotes/200 µL/well). Aliquots of freshly prepared analyte solutions 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, Bandyopadhyay, Mandal, & Chatterjee, 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 (1):

$$\frac{-Mean\ specific\ absorbance\ of\ treated\ parasites}{Mean\ specific\ absorbance\ of\ untreated\ parasites}\ x\ 100 \quad (1)$$

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

2.5 Statistical Analysis

Assays were carried out in three independent experiments and each was performed in triplicate. Values of IC_{50} were determined by logarithmic regression analysis using GraphPrism 5 software. Values for in vitro antileishmanial activity were expressed as mean ± standard deviation. Results were analyzed by Student's T using BioEstat 5.0 software, and differences were considered significant when $p < 0.05$.

3. Results and Discussion

The yield of essential oils obtained from fresh leaves of *Calyptranthes grandifolia* O.Berg, *Calyptranthes tricona* D.Legrand, *Eugenia anomala* D.Legrand, *Eugenia arenosa* Mattos, *Eugenia pyriformis* Cambess., *Myrrhinium atropurpureum* Schott and *Psidium salutare* var. sericeum (Cambess.) Landrum were, respectively, 0.096%, 0.150%, 0.070%, 0.078%, 0.052%, 1.120%, 0.980% (w/w).

In Brazil, Myrtaceae constitutes one of the main families of national flora, with 22 genera and about 1000 species, specifically in the southern, species are found in areas of Atlantic Forest as in Pampa biome. Members of the genus *Eugenia* are of particular interest because produce edible fruits and many are used in popular medicine, especially in the treatment of gastrointestinal disorders and infectious diseases. Species from *Psidium* genus have attractives fruits with high concentration of vitamin C. While, leaves from *Myrrhinium atropurpureum* are employed popularly in the treatment of hypercholesterolaemia and diabetes. Still, the omnipresence of Myrtaceae species in threatened biomes suggests an ecological importance for family (Govaerts et al., 2008; Franzon, Campos, Proença, & Silva, 2009; Souza & Lorenzi, 2012; Sobral et al., 2013).

Pentamidine 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 (Buckner, Waters, & Avery, 2012). Pentamidine isethionate was used as drug reference in the assay and showed IC_{50} values of 23.22 ± 9.04 µg/mL (Table 2). The essential oils from leaves of *Calyptranthes grandifolia*, *Calyptranthes tricona*, *Eugenia arenosa* and *Eugenia pyriformis* were effective against *L. amazonensis*

promastigotes, and presented IC_{50} values of 31.27 ± 6.40 μg/mL, 26.13 ± 8.60 μg/mL, 13.72 ± 8.65 μg/mL and 19.73 ± 5.40 μg/mL, respectively, that were not significantly different ($p > 0.05$) from that of the standard drug pentamidine isethionate. While essential oils from leaves of *Eugenia anomala* and *Psidium salutare* showed moderate activity against *L. amazonensis* promastigotes, with IC_{50} values of 62.88 ± 3.19 μg/mL and 69.71 ± 2.30 μg/mL, respectively. However, essential oil from leaves of *Myrrhinium atropurpureum* was inactive, with IC_{50} values of 154.1 ± 8.14 μg/mL (Table 2).

Antileishmanial activity is demonstrated for essential oils from various species. The terpenes cause alterations in the mitochondrial membrane potential, modification of the redox index, inhibition of cellular isoprenoid biosynthesis and changes in the plasma membrane, that can explain the antileishmanial activity (Santos et al., 2008; Rodrigues et al., 2013; Monzote et al., 2014). Essential oil from leaves of *Annona foetida* showed activity against promastigotes forms of *L. guyanensis* (Costa et al., 2009), while essential oil of *Annona coriacea* showed activity against promastigotes forms of *L. chagasi* (Siqueira et al., 2011). The essential oil of *Lippia origanoides* demonstred activity against promastigotes of *L. chagasi*, with IC_{50} value of 4.4 μg/mL, and showing no toxicity to mammalian cells (Escobar, Leal, Herrera, Martinez, & Stashenko, 2010). Antileishmanial activity in vitro against promastigotes forms of *L. chagasi* was checked for the essential oil extracted from leaves of *Lippia sidoides* (Farias-Junior et al., 2012). For the essential oil from leaves of *Lantana camara* L., Verbenaceae, was demonstrated significant antileishmanial activity in vitro against promastigotes of *L. amazonensis*, with IC_{50} value of 0.25 μg/mL (Machado et al., 2012). The essential oil of *Piper auritum* proved to be active in vitro against promastigotes forms of *L. major*, *L. mexicana*, *L. braziliensis* and *L. donovani* (Monzote, García, Montalvo, Scull, & Miranda, 2010).

Table 2. IC_{50} (μg/mL) value of essential oil from fresh leaves of Myrtaceae species employed in the study against promastigotes of Leishmania amazonensis.

Species	IC_{50} (μg/mL) *L. amazonensis*
Calyptranthes grandifolia O.Berg	31.27 ± 6.40
Calyptranthes tricona D.Legrand	26.13 ± 8.60
Eugenia anomala D.Legrand	62.88 ± 3.19*
Eugenia arenosa Mattos	13.72 ± 8.65
Eugenia pyriformis Cambess.	19.73 ± 5.40
Myrrhinium atropurpureum Schott	154.1 ± 8.14*
Psidium salutare var. *sericeum* (Cambess.) Landrum	69.71 ± 2.30*
Pentamidine isethionate [a]	23.22 ± 9.04

[a] Reference drug.

Data are expressed as mean values ± standard error.

*Values are statistically different from reference drug ($p \leq 0.05$).

Essential oils obtained from species of the Myrtaceae family have also presented antileishamanial activity. The essential oil from leaves of *Eugenia uniflora* showed antileishmanial activity *in vitro* against promastigotes (IC_{50}=3.04 μg/mL) and amastigotes (IC_{50}=1.92 μg/mL) forms of *L. amazonensis*, and preferential toxicity to amastigotes compared to macrophages. The activity was related to activation of macrophages, evidenced by the increase in phagocytic capacity and lysosomes activity (Rodrigues et al., 2013).

Essential oil from leaves of *Eugenia jambolana* presented IC_{50} values of 60.0 μg/mL and 43.9 μg/mL, respectively, against promastigotes and amastigotes of *L. amazonensis*. Interestingly, α-pinene, the main constituent of essential oil, showed higher activity with IC_{50} values of 19.7 μg/mL and 16.1 μg/mL, respectively, against promastigotes and amastigotes of *L. amazonensis* (C. N. Dias et al., 2013; Rodrigues et al., 2015).

Others species from Myrtaceae family were evaluated about potential antileishmanial activity. Bioguided study with fractions isolated from the crude extract of leaves from *Blepharocalyx salicifolius*, a Brazilian native species, showed the potential *in vitro* of eight fractions against amastigotes forms of *L. amazonensis* at concentrations ranging 19 a 29 μg/mL (Siqueira et al., 2010). Antileishmanial activity *in vitro* was observed for the hexanic extract

of fruits of *Eugenia umbelliflora*, that showed IC_{50} values of 14.3 μg/mL and 5,7 μg/mL against promastigotes forms of *L. amazonensis* and *L. brasiliensis*, respectively (Cechinel Filho et al., 2013).

The antileishmanial activity *in vitro* of aqueous and hidroethanolic extracts from leaves of *Campomanesia eugenioides*, native species from brazilian flora, was evaluated against promastigotes forms of *L. amazonensis* and showed IC_{50} values of 388 ± 53 μg/mL and 555 ± 64 μg/mL, respectively (Moura-Costa et al., 2012). The extracts from bark and leaves of *Myrcia linearifolia*, native species of Brazilian Cerrado, not presented considerable activity *in vitro* against promastigotes of *L. amazonensis*, with IC_{50} values higher than 100 μg/mL (Costa et al., 2014). The hidroethanolic extracts from leaves of *Psidium guajava*, in concentration of 100 μg/mL, inhibited the growth of amastigotes and promastigotes of *L. amazonensis* in 65.4% ± 5.4 and 52.0% ± 2.1, respectively (Luize et al., 2005).

In Brazil, *L. amazonensis* cause skin ulcerations and in mucous membranes, and has been associated with various clinical forms of the disease including cutaneous, mucosa, diffuse cutaneous and visceral leishmaniasis (Leon, Machado, Paes, Grimaldi Jr., 1990; WHO, 2010). The results obtained for the essential oils of species *Calyptranthes grandifolia*, *Calyptranthes tricona*, *Eugenia arenosa* and *Eugenia pyriformis* are promising compared to activity demonstrated for essential oil from *E. jambolana* and comparable to those observed with extracts of *Blepharocalyx salicifolius and Eugenia umbelliflora*. Still, the results are hopeful, because these four essential oils showed activity comparable to pentamidine against this parasite.

The chemical composition of essential oils from leaves of the species analyzed in this study was previously determined by other authors, with exception of *Eugenia anomala*. However, edaphic conditions, climate, extraction procedure, between other factors, influence the constitution of oils and, consequently, qualitative and quantitative differences in composition of essential oils from the same plant species can be observed (Simões, et.al., 1999). The essential oil from leaves of *C. grandifolia* was rich in pinenes (55.9%) and beta-caryophyllene (10.5%) (Menut et al., 1997), while in the essential oil of *C. tricona* predominated chromenes derivatives (5,7-dimethoxy-2-methyl-2H-benzopyran and 5,7-dimethoxy-2,8-dimethyl-2H-benzopyran) and *cis*-β-farnesene (Menut et al., 2000). The sesquiterpenes farnesyl acetate (70.4%) and aromadendrene (11.7%) were the main constituents in the essential oil from leaves of *Eugenia arenosa* (Apel et al., 2004a), and in the essential oil from leaves of *Eugenia pyriformis* predominated δ-cadinene (12.4%), T-cadinol (11.9%) and α-cadinol (14.0%) (Apel et al., 2004b). The major components identified in the essential oil of *Myrrhinium atropurpureum* were α-pinene (12.2%), limonene (35.0%) and 1,8-cineole (23.4%) (Limberger, Moreno, Farias, Sobral, & Henriques, 2001). Caryophyllene oxide (39.8%) and ar-turmerone (17.3%) were the main constituents identified in the essential oil from leaves of *Psidium salutare* (Pino, Bello, Urquiola, & Agüero, 2003). Thus, the identification of chemical composition of essential oil from leaves of the species with better results, as well as the standardization of the collect conditions and the extraction procedure are necessary for ensure consistent outcomes.

The assay *in vitro* against promastigotes forms of *L. amazonensis*, which is easy to perform and inexpensive, allowed to define native species of Myrtaceae family with the greatest potential antileishmanial. Thus, the results showed the potential of essential oils from leaves of *C. grandifolia*, *C. tricona*, *E. arenosa* and *E. pyriformis* as antileishmanial, as well as the importance of continuing studies to in order to advance in the search and development of new therapeutic options from of brazilian flora sources. In this sense, studies to evaluate the chemical constitution of essential oils and their cytotoxicity are important. In addition, further study of bioguided form is important to optimize the process and rationalize cost.

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