Abstract
This paper aims to investigate, for the first time, in vitro antitubercular, antileishmanial and antiproliferative activities of essential oils (EOs) from S. odoratissima leaves and flowers – grown in midwestern Brazil – against Mycobacterium tuberculosis, promastigote forms of Leishmania amazonensis and human tumor cell lines. Antimycobacterial activity of EOs was evaluated in terms of the minimal inhibitory concentration (MIC). EOs from leaves and flowers showed to be active antimicrobials against M. tuberculosis, since MIC values were 150 µg/mL and 162.5 µg/mL, respectively. Both EOs exhibited significant activity against promastigote forms of L. amazonensis; IC\textsubscript{50} values (50% growth inhibition) were 14.36 ± 2.02 (EOs from leaves) and 19.89 ± 2.66 µg/mL (EOs from flowers). Antiproliferative activity in normal (GM07492A, lung fibroblasts) and tumor (MCF-7, HeLa and M059J) cell lines was performed by the XTT assay; results were expressed as IC\textsubscript{50} (50% cell growth inhibition) and the selective index was calculated. IC\textsubscript{50} values of EOs from leaves and flowers obtained in normal cell lines for were 502.97 ± 40.33 µg/mL and 370.60 ± 2.01 µg/mL, respectively. Antiproliferative activity was observed against human tumor cell lines, whose IC\textsubscript{50} values were significantly lower than those obtained in normal cell lines of MCF-7 cells (367.57 ± 4.46 µg/mL-EOs from leaves and 357.70 ± 1.85 µg/mL-EOs from flowers) and M059J cells (492.53 ± 56.67 µg/mL-EOs from leaves and 324.90 ± 6.72 µg/mL-EOs from flowers), thus, indicating selectivity. These in vitro results showed that EOs from S. odoratissima may be an antimycobacterial, antiparasitic and antitumor agent.

Key words: Leishmania amazonensis, medicinal plant, Mycobacterium tuberculosis, phytotherapy, tumor cells, β-caryophyllene.

Resumo
Este trabalho tem por objetivo investigar, pela primeira vez, as atividades antituberculose, antileishmaniana e antiproliferativa in vitro dos óleos essenciais (OEIs) obtidos de folhas e flores de S. odoratissima. Avaliamos a atividade antimicobacteriana em termos da Concentração Inibitória Mínima (CIM). Os OEIs mostraram ser antimicrobianos ativos contra M. tuberculosis, com valores de CIM de 150 µg/mL (folhas) e 162.5 µg/mL (flores). Os OEIs apresentam significativa atividade antiparasitária contra a forma promastigota de L. amazonensis, com valores de CI\textsubscript{50} (50% de inibição do crescimento) de 14,36 ± 2,02 (folhas) e 19,89 ± 2,66 µg/mL (flores). A atividade antiproliferativa em linhagem celular normal (GM07492A, fibroblastos de pulmão) e tumorais (MCF-7, HeLa e M059J) foi realizada utilizando o ensaio XTT; os resultados foram expressos a partir do cálculo da Concentração Inibitória de 50% do crescimento celular (CI\textsubscript{50}) e o índice de seleitividade foi calculado. OEIs de folhas e flores apresentaram um CI\textsubscript{50} de 502.97 ± 40.33 µg/mL e 370,60 ± 2,01 µg/mL para a linhagem GM07492A, respectivamente. Observou-se atividade antiproliferativa contra...
Introduction

Essential oils (EOs) are natural, complex, multi-component systems which are mainly composed of terpenes and some other non-terpene components. Specifically, EOs and their constituents exhibit different biological activities, such as antioxidant, antimicrobial, antifungal, anti-inflammatory and antitumor activities (Sharifi-Rad et al. 2017). They consist of mixtures of several lipid-soluble and volatile compounds, such as monoterpenes, sesquiterpenes and phenylpropanoids, that can easily diffuse across cell membranes, a major advantage with regard to interactions with intracellular targets. Besides, possible synergistic interactions among components of EOs are beneficial to their activities (Raut & Karuppayil 2014).

Spiranthera odoratissima A. St. Hil. (manacá), a species of the family Rutaceae, is widely found in the Brazilian Cerrado. In folk medicine, its leaves have been used for blood depuration and prevention of renal and hepatic diseases. They also have important anti-anxiety and anti-inflammatory activities; the latter has been directly related to the inhibition of phospholipase A2 activity. Its roots have been applied to the treatment of stomach diseases, muscle pain, headache, rheumatism and hepatic disorders, besides central nervous system depression (Chaibub et al. 2013; Souzs et al. 2015; Galdino et al. 2012).

In previous phytochemical studies, furoquinoline alkaloids and limonoids were isolated from S. odoratissima twigs, while ten compounds, i.e., two limonoids, three furoquinoline alkaloids, three β-indoloquinazoline, the coumarin auraptene and the phytosterol β-sitosterol were extracted from its roots (Matos et al. 2014).

Tuberculosis (TB), which is caused by the aerobic mycobacterium Mycobacterium tuberculosis (MT), is the main infectious disease caused by bacteria worldwide. The bacterium has the shape of a bacillus and may be disseminated by exteriorization of contaminated biological material, mainly from the lungs by spraying little droplets when sneezing and coughing. Since the lung tissue is rich in oxygen, it is very favorable for the development of this microorganism. However, MT is capable of remaining latent even in tissues with good oxygen concentration. As a result, it does not respond adequately to the anti-TB treatment and enables the disease to relapse. Even though lungs account for the highest number of cases, extrapulmonary TB may develop, and both may coexist. In this case, the mycobacterium may infect different human tissues, such as lymphatic, gastrointestinal, nervous and even bone ones, besides leading to the wide dissemination named miliary tuberculosis, which is extremely hazardous (Global Tuberculosis Report 2018).

The World Health Organization states that about one third of the world’s population is infected by MT and estimates that one out of 10 contaminated persons may develop TB. Its incidence was around 8.8 million new cases in 2010, when about 1.45 million people died; 25% of them were victims of TB and HIV coinfection. Even though Africa and Asia concentrate 86% of TB cases, Brazil ranks 17th among 22 countries which account for 80% of all cases. Brazil estimates that about 50 million people have been infected by the bacillus. This disease registered 71 thousand new cases and 4.6 thousand deaths in 2010 in the country, a fact that makes it the 4th main cause of death due to infectious diseases and the main one among HIV patients. The most alarming numbers belong to Rio de Janeiro and Amazonas, since both states have incidence rates which are similar to the ones found in Asia and Africa (Global Tuberculosis Report 2018).

Leishmaniasis is an infectious disease which is caused by a protozoan of the genus Leishmania. It may occur in different forms, i.e., visceral, cutaneous, mucocutaneous and diffuse cutaneous ones. The disease uses an invertebrate host, a phlebotomine, a sandfly that is called straw mosquito, which is responsible for transmitting...
promastigote forms to wild and domestic animals, besides humans. In vertebrate hosts, promastigote forms are brought into the cell by macrophages, transform themselves into amastigote forms and, thus, propagate the infection. In Brazil, the visceral form is caused by *L. donovani*, while both cutaneous and mucocutaneous ones are triggered by infections caused by *L. tropica*, *L. braziliensis* and *L. amazonensis* (Kauffman et al. 2017).

Since conventional medication which have been applied to treat certain diseases, such as TB and leishmaniasis, result in adverse side effects, the development of more efficient, safer, less toxic and less aggressive new treatments is urgent. Therefore, studies which focus on natural alternatives by investigating EOs that may reveal promising anti-*Leishmania amazonensis* and anti-*Mycobacterium tuberculosis* activities have been exhaustively carried out all over the world (Bernuci et al. 2016).

Estimates show that about 60% and 75% of pharmaceuticals which have been currently used for treating cancer and infectious diseases, respectively, derive from natural sources. Thus, studies of toxicity and mutagenicity are relevant because they contribute to their safe and effective use (Fachinetto & Tedesco 2009). As a result, plants keep drawing the attention of researchers and pharmaceutical companies in studies of prototypes that aim at the development of new medication based on natural compounds. From this perspective, EOs have shown promising biological activities, such as antibacterial, antiproliferative and antiparasitic ones, which have triggered several studies of this type of natural product (Estevam et al. 2017; Silva et al. 2019).

Considering the medicinal potential of *S. odoratissima*, besides the chemical and biological importance of EOs from plants that belong to the family Rutaceae (Liaqat et al. 2018), and the interest in deepening studies of EOs from *S. odoratissima* leaves and flowers that have been carried out by our research group (Cabral et al. 2019), this paper describes, for the first time, *in vitro* antitubercular, antileishmanial and antiproliferative activities of EOs extracted from *S. odoratissima* leaves and flowers.

**Materials and Methods**

**Plant material**

*Spiranthera odoratissima* leaves and flowers were collected in Iporá, GO, Brazil (16º24’11.2”S, 51º06’41.4”W) in November 2017. The plant was identified by the botanist Erika Amaral, M. Sc., and a voucher specimen (#1039) was deposited in the herbarium in Rio Verde, at the Instituto Federal Goiano (IFGOIANO).

Extraction of EOs

Fresh leaves and flowers were submitted to hydrodistillation in a Clevenger-type apparatus for 3 h (Cabral et al. 2019). To this end, 900 g plant material was divided into three 300-g samples and 1,000 mL distilled water was added to each sample. After manual collection, traces of water remaining in EOs were removed with the use of anhydrous sodium sulfate, which was followed by filtration. EOs were stored in an amber bottle and kept in a refrigerator at 4 ºC until further analysis. Yield of EOs was calculated from the weight of fresh leaves and flowers and expressed as the average of triplicate analysis.

**GC-FID and GC-MS analyses**

EOs were obtained by hydrodistillation for 3 h in a Clevenger-type apparatus and stored at 4 ºC up to GC-MS, GC-FID and bioassays. Gas chromatography-flame ionization detection and gas chromatography–mass spectrometry analyses were performed by Shimadzu QP2010 Plus and GCMS2010 Plus (Shimadzu Corporation, Kyoto, Japan) systems. GC-MS and GC-FID conditions and the identification of chemical constituents of EOs were carried out in agreement with the methodology proposed by Cabral et al. (2019).

**In vitro antitubercular assay**

The strain *M. tuberculosis* H37Rv (ATCC 27294) was obtained from the American Type Collection (ATCC) and maintained at -80 ºC. Antimycobacterial activity of EOs from *S. odoratissima* leaves and flowers was evaluated by the MIC broth microdilution method conducted on microplates. Resazurin was employed to reveal mycobacterial growth by the Resazurin Microtiter Assay (REMA) method (Palomino et al. 2002). EOs were serially diluted (two-fold) with Middlebrook 7H9 broth (Difco TM, Detroit, MI, USA). The mycobacterium inoculum was then added to obtain concentrations ranging from 150 to 162.5 µg/mL. Isoniazid was used as positive control at concentration from 1.0 µg/mL, whereas Middlebrook 7H9 broth and the inoculum were used as solvent and negative control, respectively.
**In vitro** antileishmanial assay

To evaluate antileishmanial activity, promastigote forms of *L. amazonensis* (MHOM/BR/PH8) were maintained in RPMI 1640 (Gibco) culture medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL). Subsequently, about 1 x 10^6 parasites were distributed on 96-well plates and EOs, which had previously been dissolved in 100% dimethylsulfoxide (DMSO, stock solution 100 mM) (Synth), were added to the cultures at concentrations which ranged from 3.12 to 50 µg/mL. Amphotericin B (Sigma Aldrich, 97 % purity), at concentrations ranging from 0.19 to 0.011 µg/mL, was added to cultures and used as positive control. Cultures were incubated in a BOD (Quimis) incubator at 25 °C for 24 h. Then, antileishmanial activity was determined by verifying whether the growth of promastigote forms had been inhibited. It was observed by counting the total number of live promastigotes in the Neubauer (Global Glass, Porto Alegre, BR) chamber on the basis of flagellar motility. RPMI 1,640 medium (Gibco) with 0.1% DMSO (Synth) (the highest concentration) was used. Results were expressed as the mean of the percentage of growth inhibition related to the negative control (0.1% DMSO). Experiments were performed in triplicate.

**In vitro** antiproliferative assay

In this study, the following three different tumor cell lines were used: human breast adenocarcinoma (MCF-7), human cervical adenocarcinoma (HeLa) and human glioblastoma (M059J). A normal human cell line (lung fibroblasts, GM07492A) was included to evaluate possible selective activity of the natural product under investigation. Different cell lines were maintained as monolayers in plastic culture medium (HAM-F10 + DMEM, 1:1, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Nutricell), antibiotics (0.01 mg/mL streptomycin and 0.005 mg/mL penicillin; Sigma-Aldrich) and 2.38 mg/mL Hepes (Sigma-Aldrich). Cells were incubated at 36.5 °C in humidified 5% CO₂ atmosphere. Antiproliferative activity was measured by the in vitro Toxicology Colorimetric Assay Kit (XTT; Roche Diagnostics), in agreement with the manufacturer’s instructions. In the experiments, cells (10^4 cells/well) were incubated on 96-well microplates. Each well was filled with 100 µL HAM-F10/DMEM medium which contained essential oil at concentrations ranging from 3.91 to 500 µg/mL. Negative (no treatment), solvent (0.4% DMSO, dimethylsulfoxide, Sigma-Aldrich) and positive (doxorubicin, DXR, Pharmacia Brazil Ltda.) controls were included. After incubation at 36.5 °C for 24 h, the culture medium was removed. Cells were washed with 100 µL PBS (phosphate buffered saline) to remove treatments and exposed to 100 µL culture medium HAM-F10 without phenol red. Then, 25 µL XTT was added and cells were incubated at 36.5 °C for 17 h. Sample absorbance was determined by a multi-plate reader (ELISA - Tecan - SW Magellan vs 5.03 STD 2P) at the wavelength of 450 nm and reference length of 620 nm. Antiproliferative activity was evaluated with the use of IC₅₀, the concentration capable of inhibiting 50 % of cell line growth as a response parameter, which was calculated by the GraphPad Prism program that plotted cell survival against concentrations of the natural product under investigation. One-way ANOVA was used for comparing means (P < 0.05). Experiments were performed in triplicate. The selectivity index was calculated by dividing the IC₅₀ value of the EOs obtained for GM07492A cells by the IC₅₀ value obtained for the cancer cell line.

**Results**

The major components of EOs were sesquiterpene hydrocarbons, followed by oxygenated sesquiterpenes. The three major components identified in EOs from leaves were β-caryophyllene (23.8%), bicyclogermacrene (10.8%) and δ-cadinene (7.1%), while the ones found in EOs from flowers were β-caryophyllene (14.1%), spathulenol (8.1%) and γ-cadinene (7.2%). Complete data on the chemical composition of EOs may be found in a paper that has just been published by the research group that is composed of the authors of this study (Cabral et al. 2019).

*In vitro* antimycobacterial activity of EOs against *M. tuberculosis* was investigated in terms of minimum inhibitory concentration (MIC) values, by comparison with isoniazid (positive control). Table 1 summarizes resulting MIC values. EOs furnished MIC values which ranged from 150 µg/mL (EOs-leaves) and 162.5 µg/mL (EOs-flowers) against the important causative agent of TB.

Regarding *in vitro* antileishmanial activity of EOs from *S. odoratissima* leaves and flowers, IC₅₀ values were 14.36 ± 2.02 (EOs-leaves) and 19.89 ± 2.66 µg/mL (EOs-flowers) (Tab. 2). EOs from *S. odoratissima* inhibited parasite growth...
Table 1 – Antibacterial activity of essential oils from *S. odoratissima* leaves and flowers against *M. tuberculosis*.

|          | MIC (µg/mL) |
|----------|-------------|
| EOs-Leaves | 150         |
| EOs-Flowers | 162.5      |
| Isoniazid* | 1.0         |

* Positive control

Table 2 – Antileishmanial activity of essential oils from *S. odoratissima* leaves and flowers.

| Samples       | % of lysis ± SD / Concentration (µg.mL⁻¹) | IC₅₀(µg/mL) |
|---------------|------------------------------------------|-------------|
|               | 50                                       | 25          |
| EOs-Flowers   | 100 ± 0.00                               | 60.24 ± 56.22 | 24.44 ± 32.80 | 7.26 ± 10.27 | 0.42 ± 0.60 | 19.89 ± 2.66 |
| EOs-Leaves    | 97.01 ± 4.22                             | 76.05 ± 33.14 | 39.69 ± 39.84 | 16.10 ± 4.66 | 12.48 ± 17.65 | 14.36 ± 2.02 |
| Amph. B       | 99.88 ± 0.60                             | 78.33 ± 24.43 | 68.74 ± 21.97 | 54.67 ± 17.77 | 42.44 ± 20.97 | 0.011 ± 0.34 |

Negative Control: RPMI Medium + 0.1% DMSO. Amph. B: Amphotericin B (positive control); SD: Standard Deviation.

Discussion

As previously mentioned, twenty-eight components were identified in oils from *S. odoratissima* A. St. Hil. leaves, which exhibited about 93.8% of the total composition of the oil, whereas twenty-nine components were identified in oils from its flowers, which showed about 94.4% of the total composition of the oil (Cabral *et al.* 2019).

MIC values of EOs from *S. odoratissima* against *M. tuberculosis* are quite promising, since some authors have considered that MICs ≤ 200 µg/mL indicate good activity against *M. tuberculosis* (Mota *et al.* 2018). In addition, according to Holetz *et al.* (2002), natural products with MIC values below 100 µg/mL, between 100 and 500 µg/mL, from 500 to 1,000 µg/mL and above 1,000 µg/mL exhibit good antimicrobial activity, moderate antibacterial activity, weak antibacterial activity and no antibacterial activity, respectively. Concerning chemical constituents, some terpenes have already had their antimycobacterial activity evaluated. Isoprenes, such as monoterpenes, sesquiterpenes and related alcohols and phenols, have been responsible for antimycobacterial activity of EOs (Baldin *et al.* 2018). It should be emphasized that the promising antitubercular activity of EOs from *S. odoratissima* may be attributed to β-caryophyllene, a terpene whose favorable biological activity has already been described in the literature, mainly its antioxidant, antitumor and antimicrobial properties (Dahham *et al.* 2015).

EOs from *S. odoratissima* flowers (IC₅₀ = 19.89 µg/mL) and leaves (IC₅₀ = 14.36 µg/mL) were considered active against *L. amazonensis*, since the literature has attributed the antiparasitic potential of EOs as follows: IC₅₀ < 10 µg/mL is highly active, IC₅₀ > 10 µg/mL is active, IC₅₀
Table 3 – Antiproliferative activity of essential oils from S. odoratissima flowers and leaves against different cell lines.

| Cell line | Treatment (µg/mL) | IC₅₀ | SI | IC₅₀ | SI | IC₅₀ | SI | DXR |
|-----------|------------------|------|----|------|----|------|----|------|
| GM07492A  | EO-flowers        | 370.60 ± 2.01  | -   | 502.97 ± 40.33 | -   | 0.5 ± 0.2 | -   | -    |
|           | EO-leaves        | 357.70 ± 1.85 | 1.03 | 367.57 ± 4.46* | 1.36 | 62.1 ± 2.0 | -   | -    |
| HeLa      | EO-flowers        | 376.47 ± 6.45  | -   | 523.37 ± 1.93  | -   | 5.3 ± 1.3  | -   | -    |
|           | EO-leaves        | 324.90 ± 6.72* | 1.14 | 492.53 ± 56.67* | 1.02 | 16.2 ± 2.5 | -   | -    |

*IC₅₀ values = 50% cell growth inhibition; SI = selectivity index; GM07492A = human lung fibroblasts; MCF-7 = human breast adenocarcinoma; HeLa = human cervical adenocarcinoma; M059J = human glioblastoma. Doxorubicin (DXR) was used as positive control. The selectivity index is the ratio between the IC₅₀ value of EOs from flowers and leaves obtained for GM07492A cells and the value found for the tumor cell line. Values are mean ± SD, n = 3. **Significantly different from the normal cell line (GM07492A) (P < 0.05).**

> 50 < 100 µg/mL is moderately active and IC₅₀ > 100 µg/mL is inactive (Estevam et al. 2017). The remarkable antileishmanial activity of OEs from S. odoratissima should be highlighted, by comparison with other OEs. For instance, OEs from S. odoratissima leaves and flowers exhibited lower IC₅₀ values, i.e., they were more active than the ones from Siparuna guianensis (IC₅₀ = 48.55 µg/mL), followed by Cinnamodendron dinisii (IC₅₀ = 54.05 µg/mL), Matricaria chamomilla (IC₅₀ = 60.16 µg/mL), Cordia verbenacea (64.75 µg/mL), Bulnesia sarmientoi (IC₅₀ = 85.56 µg/mL), Ferula galbaniflua (IC₅₀ = 95.70 µg/mL), Melissa officinalis (132.02 µg/mL), Myroxylon periferum (IC₅₀ = 162.25 µg/mL), Salvia sclarea (IC₅₀ = 325.92 µg/mL), Foeniculum officinalis (IC₅₀ = 328.28 µg/mL) and Pelargonium graveolens (IC₅₀ = 363.71 µg/mL) (Andrade et al. 2016). It should be mentioned that the promising leishmanicidal activity of OEs from S. odoratissima may be attributed to the high concentration of β-caryophyllene, a terpene that is active against L. amazonensis (Soares et al. 2013).

Results of antiproliferative activity highlight β-caryophyllene in OEs from S. odoratissima flowers and leaves. Several authors state that this compound is responsible for the antitumoral activity of OEs (Leandro et al. 2015). Germacrene D has also been described as a compound that may positively influence antiproliferative activity against human leukemia cell lines (HL-60). OEs from Casearia sylvestris leaves exhibited selective cytotoxicity against tumor cell lines HeLa, A-549 and HT-29, due to the presence of both terpenes β-caryophyllene and α-humulene (Leandro et al. 2015). Cytotoxic activity exhibited by OEs from Eperua duckeana may be caused by their major compounds (β-caryophyllene or germacrene D), by synergism between them or with other compounds found in OEs that may have additive effects and inhibit tumor cell growth (Leandro et al. 2015). Besides, Nascimento et al. (2018) had previously observed that the IC₅₀ value of spathulenol, a major constituent of OEs from S. odoratissima, was 49.3 µg/mL against tumor cell line MCF-7. As a result, their cytotoxicity may be attributed - at least, partially - to the antiproliferative activity of this sesquiterpene.

Two action mechanisms have been proposed by the literature to explain the biological activities of OEs. Both are associated with the hydrophobicity of monoterpens and sesquiterpenes, which are often their main chemicals. Hydrophobicity of terpenoids may enable them to permeate cell membranes easily, thus, causing parasite or microorganism death by affecting their metabolic pathways or organelles (Lemes et al. 2018). OEs could interact with the parasite membrane, cause drastic physiological changes, lead to reduced membrane permeability and result in cell death. On the other hand, considering the large number of chemical constituents and either synergistic or antagonistic interactions among them, OEs could also act on cellular targets other than cell membranes, such as lipids and proteins (Lemes et al. 2018).

OEs from S. odoratissima exhibit promising in vitro antitubercular, antileishmanial and antiproliferative activities. OEs under study stand out as promising sources of bioactive compounds which may be used for developing alternative treatments of neglected diseases, such as leishmaniasis. Another favorable result found by
this study is the activity of these EOs against the agent that causes TB. Besides, β-caryophyllene, which is the major constituent of EOs from *S. Odoratissima* leaves and flowers, may be the main responsible compound for their antiproliferative activity. In sum, it should be highlighted that other *in vivo* studies must be carried out to confirm these activities and to investigate the mechanisms of action of the EOs.

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