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Full Paper

Anticariogenic and Antimycobacterial Activities of the Essential Oil of *Siparuna guianensis* Aublet (Siparunaceae)

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Abstract: *Siparuna guianensis* is a Brazilian plant with extensive ethnobotanical indication and identified as one of the priority species that should be preserved in the Brazilian Cerrado. This work aimed to investigate the chemical composition and the antibacterial effects of the essential oil from leaves of *S. guianensis* (SG-EO) grown in southeastern Brazil against a representative panel of oral pathogens and mycobacteria. Anticariogenic and antimycobacterial activities of SG-EO were evaluated in terms of their minimum inhibitory concentrations (MICs). The essential oil from leaves of *S. guianensis* was analyzed by gas chromatography coupled to mass spectrometry (GC-MS). Forty one compounds were identified, accounting for 92.7 % of the SG-EO composition. *E,E*-farnesol (18.0 %), β-myrcene (16.0 %), germacrene-D (10.0 %) and siparunone (14.6 %) were the major SG-EO constituents. SG-EO showed the strongest anticariogenic activity against the aerobic bacterium *Streptococcus mutans* (MIC of 50 µg/mL). SG-EO was also evaluated for its antimycobacterial activity, and showed MIC values of 250 µg/mL against *Mycobacterium avium* and 500 µg/mL against *M. tuberculosis* and *M. kansasii*. These results imply that *S. guianensis* may be a new alternative source of substances of medicinal interest. This is the first report of anticariogenic and antimycobacterial activities of essential oil of *S. guianensis*.

Keywords: antimycobacterial activity; cariogenic bacteria; essential oil; *Mycobacterium tuberculosis*; *Siparuna guianensis*; *Streptococcus mutans*

1. INTRODUCTION

Tooth decay is a major public health problem affecting a large number of people in several countries. More than 700 bacterial species have been detected in the oral cavity, among which *Streptococcus* and *Lactobacillus* stand out as genera that cause tooth decay and other periodontal diseases [1].

The most efficient procedure to prevent tooth decay is to remove the biofilm by daily brushing and flossing. However, most people cannot keep dental plaque control through mechanical removal [2], and therefore, the use of oral care products containing antimicrobial ingredients has become a complementary and necessary measure since they act by decreasing the biofilm on tooth surfaces [3].

Chlorhexidine is currently considered a standard anticariogenic agent and has received the approval of the Council on Dental Therapeutics from the American Dental Association. However, the regular use of oral care products containing chlorhexidine often incurs other effects, such as increase in staining of teeth and other oral surfaces, increase in calculus formation, and alteration in taste perception [4]. Thus, the search for new chemotherapeutic agents to be added into oral care products has increased over the last few years [5].

Essential oils from different plant sources

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exhibit several biological activities, such as antibacterial, anticancer, anti-inflammatory, antimutagenic, antifungal, antioxidant and antiprotozoal. Thus, the vast arsenal of bioactive compounds found in essential oils has increasingly drawn intensive attention from researchers [6].

The number of studies on the antimicrobial potential of essential oils extracted from plans against oral pathogenic agents increased in the last decade [7]. Essential oils are mixtures of volatile compounds, including monoterpenes, sesquiterpenes, and phenylpropanoids which may easily percolate through cell membranes, a great advantage in terms of interaction with intracellular targets. It is also known that many biological activities may be the result of synergistic interaction among components found in essential oils [8].

Tuberculosis belongs to a group of infectious diseases which together account for 90 % of human deaths all over the world. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, infects about three million people every year [9]. The World Health Organization (WHO) has declared tuberculosis a global emergency and pointed out that it requires a long course of treatment. The fact that many patients have limited access to their diagnoses is significant since *M. tuberculosis* strains may develop resistance. Besides, there has been a meaningful increase in the number of nontuberculous mycobacteria (NTM) such as *Mycobacterium kansasii* and *M. avium*, which also affect the lungs, lymph, skin and joints, thus, leading to severe sequels in case they are not treated properly. Therefore, the search for new active compounds against mycobacteria has become a universal demand [10].

The species *Siparuna guianensis*, which belongs to the family Siparunaceae, is a medicinal and aromatic plant with comprehensive ethnobotanical recommendations and has been widely used in the Neotropics. This shrub is known by several popular names depending on the country and/or region where it grows. In the Cerrado of Minas Gerais state, it is called *negramina*, *folha-santa* and *marinheiro* [11].

Previous studies have shown that the essential oil of *S. guianensis* has a complex mixture of monoterpenes, sesquiterpenes, aliphatic ketones and fatty acids [12]. Its physico-chemical characterization has also been reported [13]. In the literature, studies on the essential oil of *S. guianensis* have shown its biological activities, including antioxidant, antibacterial, antifungal, trypanocidal, insecticidal and repellent [14-16]. However, the evaluation of anticariogenic and antimycobacterial activities of *S. guianensis* essential has not been reported in the literature. Therefore, the present work aims at describing the chemical composition and the anticariogenic and antimycobacterial activities of the essential oil from fresh leaves of *S. guianensis* Aublet grown in the south of Minas Gerais state, Brazil.

2. MATERIAL AND METHODS

Plant material

Leaves of *S. guianensis* were collected in Machado, Minas Gerais, in May 2016. The plant material was identified by the botanist Walnir G. F. Júnior, Ph.D., and a specimen voucher was taken to the Herbarium at the Instituto Federal do Sul de Minas Gerais – *Campus* Machado (registration GERAES 02).

Extraction of volatile oil

The essential oil was extracted from fresh leaves of *S. guianensis* (100 g) by hydrodistillation (2 h) using a modified Clevenger-type apparatus. The oil was separated and dried over anhydrous sodium sulfate, stored in hermetically sealed glass containers and kept under refrigeration at 5 °C until GC/MS analysis and anticariogenic and antimycobacterial assays. Total oil yield was expressed as percentage (g/100 g fresh plant material). All experiments were carried out in triplicate.

Identification of chemical components

A gas chromatography-mass spectrometry (GC-MS) analysis was carried out by a Shimadzu QP2010 with an AOC-20i auto-injector and a DB-5MS column (30 m x 0.25 mm, 0.25 mm in thickness). The carrier gas was He with pressure of 57.4 kPa and flow rate of 1.00 mL/min. The split ratio was 1/30, the injector temperature was 250 °C and the injected volume was 1 µL. Temperature programming was the following : 60 – 240 °C, increasing 3 °C/min. MS were recorded on the electron ionization (EI) mode, with ionization energy of 70 eV (scan time: 2 scans/s). Identification of the constituents was based both on the retention indices (RI) determined with reference to a homologous series of *n*-alkanes (C-9 to C-22) and on the fragmentation pattern observed in the mass spectra, which were compared to results

found in the literature and the Wiley 7 and Nist 62 mass spectral library data [17].

Anticariogenic activity

Minimum inhibitory concentrations (MICs) of the crude essential oil were determined by the broth microdilution method in 96-well microplates with adaptations [18]. The following standard strains from the American Type Culture Collection (ATCC) were used: *Streptococcus salivarius* (ATCC 25975), *Streptococcus sobrinus* (ATCC 33478), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556) and *Lactobacillus casei* (ATCC 11578). Individual 24-h colonies on blood agar (Difco Labs, Detroit, USA) were suspended in 10.0 mL tryptic soy broth (Difco). The standardization of each microorganism suspension was carried out by adjusting the transmittance at λ 625 nm to 96, equivalent to 0.5 McFarland scale (1.5 x 10^8 CFU/mL), by a Femto spectrophotometer. Suspensions were then diluted to a final concentration of 5 x 10^5 CFU/mL. Samples were dissolved in DMSO (Merck, Darmstadt, Germany) at a concentration of 1 µg/mL and were then diluted with tryptic soy broth to obtain concentrations ranging from 50 to 400 µg/mL. The final DMSO concentration was 5 % (v/v) and concentrations ranging from 1 to 5 % were used as negative control. An inoculated well was included to control the adequacy of the broth for organism growth whereas a non-inoculated well – free of any antimicrobial agent – was included to ensure medium sterility. Two-fold serial dilutions of chlorhexidine (Sigma, St. Louis, MO, USA) made in tryptic soy broth to achieve concentrations ranging from 59.0 to 0.115 µg/mL were used as positive control. Microplates (96 wells) were sealed with Parafilm and incubated at 37 °C for 24 h. After incubation, 30 µL of 0.02 % aq. soln of resazurin (Sigma, St. Louis, MO, USA) was added to each microplate well to indicate microorganism viability [19]. The MIC value was determined as the lowest concentration of the sample capable of inhibiting microorganism growth. Three replicates were performed for each sample and microorganism.

Antimycobacterial activity

Mycobacteria *M. tuberculosis* H37Rv (ATCC 27294), *M. kansasii* (ATCC 12478) and *M. avium* (ATCC 25291) were obtained from American Type Collection (ATCC) and maintained at -80 °C. The antimycobacterial activity of the essential oil was evaluated by the MIC broth microdilution method conducted in microplates; resazurin was employed to reveal mycobacterial growth using the method of Resazurin Microtiter Assay (REMA) [20]. The essential oil was serially diluted (twofold) with Middlebrook 7H9 broth (DifcoTM, Detroit, MI, USA). The mycobacterium inoculum was then added to obtain concentrations ranging from 250 to 500 µg/mL. Isoniazid was used as positive control at concentrations ranging from 0.06 to 1.0 µg/mL whereas Middlebrook 7H9 broth and the inoculum were used as solvent and negative control, respectively.

3. RESULTS AND DISCUSSION

The yield of essential oil extracted from fresh leaves of *S. guianensis* was 0.8 %. Forty one constituents were identified in the essential oil from the fresh leaves; the main ones were hydrocarbon sesquiterpenes, oxygenated sesquiterpenes and hydrocarbon monoterpenes (Table 1). The following main constituents were identified: the acyclic monoterpene β-myrcene (**1**, 16.0 %) and the sesquiterpenes germacrene-D (**2**, 10.0 %), *E*,*E*-farnesol (**3**, 18.0 %), and siparunone (**4**, 14.6 %) (Figure 1). Table 1 shows the compounds identified in the essential oil, the retention indices and the relative percentages (%).

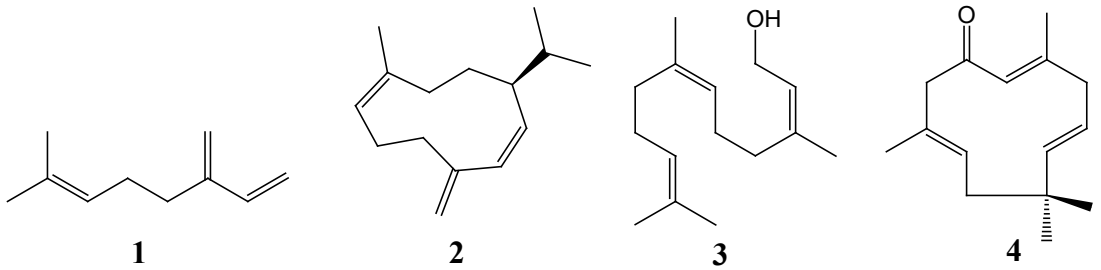

Figure 1. Chemical structures of the four main constituents of the essential oil from leaves of *S. guianensis*: **(1)** β-myrcene, **(2)** germacrene-D, **(3)** *E*,*E*-farnesol and **(4)** siparunone.

Table 1. Chemical constituents of the essential oil from fresh leaves of *S. guianensis*.

Compounds	RI_{lit}	RI_{exp}	% RA
α-Pinene	931	930	1.9
β-Pinene	939	938	0.9
Camphene	953	954	0.1
β-Myrcene	991	992	**16.0**
α-Phellandrene	995	995	0.1
δ-3-Carene	1000	1000	0.1
β-Phellandrene	1006	1005	0.1
Limonene	1031	1033	1.4
p-Cymene	1023	1023	0.8
2-Undecanone	1293	1293	1.8
δ-Elemene	1331	1332	0.5
α-Cubene	1345	1345	0.1
α-Copaene	1374	1375	0.3
β-Bourbonene	1387	1384	0.2
β-Cubebene	1385	1385	0.2
β-Elemene	1392	1396	2.0
E-Caryophyllene	1420	1419	1.2
γ-Elemene	1339	1341	0.1
β-Copaene	1428	1428	0.1
Aromadendrene	1439	1444	0.2
α-Humulene	1456	1458	2.0
Alloaromadendrene	1462	1463	0.1
Germacrene-D	1484	1486	**10.0**
β-Selinene	1476	1476	0.3
Ledol	1562	1562	2.1
E,*E*-Farnesol	1702	1706	**18.0**
α-Muurolene	1500	1497	1.2
γ-Cadinene	1513	1511	2.0
trans-Calamene	1506	1506	0.2
δ-Cadinene	1522	1520	1.0
Germacrene-B	1533	1533	2.0
Spathulenol	1574	1574	0.3
β-Caryophyllene-oxide	1583	1583	0.4
Globulol	1566	1566	0.2
Viridiflorol	1569	1569	1.4
Siparunone	1663	1663	**14.6**
1-Epi-cubenol	1601	1600	0.5
T-Cadinol	1664	1660	3.0
β-Eudesmol	1620	1620	1.0
α-Cadinol	1652	1651	1.8
α-Bisabolol	1685	1682	2.5
Total			**92.7**

RI_{lit}: Retention index found in the literature. **RI_{exp}:** Retention index relative to n-alkanes (C_9-C_{22}) in the DB-5 column. **% RA:** Relative area (peak area relative to the total peak in the GC-MS chromatogram), average of three replicates.

The chemical composition found in this study is similar to that previously reported by a study which quantified and identified the main chemical compounds of the volatile oil extracted from leaves of *S. guianensis* grown in area of Cerrado in Mato Grosso state [21].

A study of the essential oil extracted from *S. guianensis* collected in Tocantins, Minas Gerais, Brazil, showed the predominance of both α-terpinolene and α-bisabolol, which corresponded to about 80 % of the oil all over the year [22]. This result disagrees with the findings of the present study, in which only α-bisabolol was detected, but in a smaller amount.

The variations observed in the concentrations of chemical constituents of essential oils from specimens grown in different regions can be explained by the influence of several factors such as seasonality, circadian rhythm, developmental stage, age, temperature, water availability, UV radiation, soil nutrients, altitude, atmospheric composition, and tissue damage, since they all act in secondary metabolism and influence the total amount of yielded metabolites, as well as their relative proportions [23].

The essential oil under study showed good antibacterial activity against the bacterium *S. mutans* (MIC = 50 µg/mL) and moderate inhibitory activity against the other bacteria under evaluation (Table 2). This result is remarkable because few natural

compounds are known to inhibit this microorganism, which is one of the primary causative agents of tooth decay [24-25]. Additionally, the literature describes samples that had MIC values below 100 µg/mL, with good antibacterial activity. MIC from 100 to 500 µg/mL was considered as moderate, whereas from 500 to 1000 µg/mL was considered as weak. MIC above 1000 µg/mL was considered as inactive [26].

Table 2. Values of minimum inhibitory concentrations (MICs) in µg/mL of essential oil from fresh leaves of *S. guianensis* (SG-EO) against selected cariogenic bacteria.

Microorganisms	MIC SG-EO	MIC CHC
Streptococcus mutans	50	0.922
Streptococcus mitis	100	1.844
Streptococcus sanguinis	400	0.922
Streptococcus sobrinus	200	0.922
Streptococcus salivarius	200	0.737
Lactobacillus casei	400	0.922

MIC: Minimum Inhibitory Concentrations (µg/mL); **CHC:** Chlorhexidine dihydrochloride (positive control); **SG-EO:** essential oil from *S. guianensis*

Several mechanisms have been proposed to explain the antimicrobial activity of essential oils. Inhibition of microbial growth carried out by essential oils is due to direct damage caused to the integrity of the cell membrane by lipophilic components of essential oils, which affects the maintenance of the cell pH and the balance of inorganic ions. Inhibitory effects of essential oils are consistent with the action of monoterpenes and sesquiterpenes on the cell membrane and the damage caused to the membrane leads to different effects in different microorganisms [27]. Therefore, the anticariogenic activity of the essential oil of *S. guianensis* against the selected oral pathogens may be related to the presence of the sesquiterpene (*E*,*E*)-farnesol (18.0 %), one of its main constituents. This compound was considered as active against *S. sobrinus* and *S. mutans* at concentrations of 14 µg/mL and 20 µg/mL, respectively [28]. As previously reported in the literature, the compounds β-myrcene (16.0 %) and p-cymene (0.8 %) identified in the essential oil from fresh leaves of *S. guianensis* have also shown significant antimicrobial activity [29].

Regarding the antimycobacterial activity, the literature reports that essential oils with MIC values of 500 µg/mL and 250 µg/mL are considered moderately active and active, respectively, against mycobacteria under evaluation [9]. In the present study, the essential oil from fresh leaves of *S. guianensis* was evaluated against *Mycobacterium tuberculosis*, *M. kansasii* and *M. avium* (Table 3). It is likely that its moderate activity against *M. tuberculosis* and *M. kansasii* (both MICs = 500 µg/mL), along with the good activity against *M. avium* (MIC = 250 µg/mL) are caused by the presence of the compounds viridiflorol (1.4 %), α-pinene (1.9 %), α-bisabolol (2.5 %), β-myrcene (16.0 %) and limonene (1.4 %). These terpenes have been previously identified in the essential oils of the species *Allophylus edulis* and *Mutellina purpurea* and have already had their biological activity acknowledged against the genus *Mycobacterium* [30-31].

Table 3. *In vitro* antimycobacterial activity of essential oil from *Siparuna guianensis* (MIC = µg/mL).

S. guianensis	*Mycobacterium tuberculosis*	*Mycobacterium kansasii*	*Mycobacterium avium*
Essential Oil	500	500	250
Isoniazid*	0.06	1	> 1

*Positive control

4. CONCLUSION

The chemical composition of the essential oil from fresh leaves of *S. guianensis* collected in Machado, MG state, was similar to that previously reported for the essential oil of specimens of *S. guianensis* occurring in Mato Grosso and Minas Gerais (Tocantins County) states. It showed a mixture of monoterpenes and sesquiterpenes, and its main constituents were *E*,*E*-farnesol, β-myrcene, siparunone and germacrene-D. *S. guianensis* essential oil showed anticariogenic activity against some cariogenic bacteria, especially *S. mutans*, which is one of the main causative agents of the tooth decay. It also had moderate antimycobacterial activity against *Mycobacterium tuberculosis* and *M. kansasii*, as well as good activity against *M. avium*. In sum, the results of this study imply that the essential oil from fresh leaves of *S. guianensis* collected in Machado, MG, might be a promising source of bioactive compounds for the development of new medication. However,

further studies should be carried out on the identification, isolation, and evaluation of the biological properties of the chemical constituents of the *S. guianensis* essential oil.

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