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Full Length Research Paper

Exploring the Antimicrobial Potential and Medicinal Properties of Siparuna guianensis in the Brazilian Cerrado Forest: A Global Hotspot

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Siparuna guianensis **Aubl. (Siparunaceae) is an aromatic medicinal plant native to South America. This study aimed to investigate the biomass and bioactivity of leaf extracts and essential oil of *S. guianensis* from savanna forests in Central Brazil, Tocantins State. Crude leaf extract and leaf essential oil were tested in different concentrations against bacteria and fungi. At concentrations equivalent to crude oil (380 µg ml^{-1}), oil diluted to half (190 µg ml^{-1}) and oil diluted to one quarter (95 µg ml^{-1}), the oil inhibited bacterial growth of *Staphylococcus aureus.* This finding may be related to the major monoterpene and sesquiterpene, especially bisabolol and bisabolene, which have antibacterial properties. The extracts did not exhibit antimicrobial action. Under the natural conditions of the forest legal reserve, the estimated medicinal biomass was 3300 kg ha^{-1}, with the leaf fraction corresponding to 38 kg ha^{-1} of crude extract and 5 L ha^{-1} of essential oil. It is an important reservoir of medicinal biomass foliar for prospective studies and popular use against antimicrobial which are of interest to health or agriculture.**

Keywords: *Siparuna guianensis*, bioactivity, medicinal biomass, gas chromatography, Brazil, agroecology.

INTRODUCTION

The aromatic medicinal species *Siparuna guianensis* Aubl. (Siparunaceae) is native to South America and with greater frequency in Northern Brazil, used as a traditional natural medicine (Renner and Hausner, 2005; Montanari, 2010), including Brazil, but they are poorly known in Tocantins. It´s a shrub that grows in the Cerrado and the aromatic leaves of *S. guianensis* are its largest reservoirs of essential oils and other chemical compounds of biological interest, such as flavonoids, tannins and terpenoids. In addition, the medicinal biomass

(raw material) of the species has not been quantified, and there are few reports in the literature (Montanari, 2010; Valentini et al., 2010; Andrade et al., 2015) related to its potential antimicrobial activity, and no records of this approach in Tocantins State (277,720.520 km²), Brazil. In a recent study by Aguiar et al. (2015), demonstrated highly toxic of essential oil of the *S. guianensis* against *Aedes aegypti* and *Culex quinquefasciatus*, showing potential for use as a natural insecticide against mosquitoes.

In Brazil, *S. guianensis* is threatened with extinction in the Cerrado (savanna) biome because of the degradation of seasonal forests without associations with watercourses, a currently rare ecosystem that is preferred by this species. *S. guianensis* is suggested as a priority species for biodiversity conservation (Vieira and Alves, 2003; Valentini et al., 2011). In the state of Tocantins, the occurrence of *S. guianensis* was recorded in a seasonal semideciduous forest remnant (currently a rare vegetation type in Brazil), suggesting the importance of these Brazilian savanna forests as reservoirs of the medicinal biomass of this species. Tocantins occupies 3.2% (277,720.520 km²) of the Brazilian territory, and it is slightly smaller than the neighboring country of Ecuador and approximately three times larger than Portugal.

The state is located in the center of Brazil, and its transportation routes include highways, railways and, in the future, waterways. Negramina or folha santa are the local names of *S. guianensis*, and there are no records of its antimicrobial potential, which is important because of the growing global search for natural products as an alternative in the control of pathogenic microorganisms. This scenario suggests the vulnerability and impaired biodiversity of *S. guianensis*, and there is strong evidence that popular knowledge of the species is being lost and that the habitats preferred by the species are being anthropogenically degraded.

The Cerrado biome is the most diverse and rich savannas worldwide, and it is one of the last hotspots in the world, harboring 40% of endemic species and more than 7000 plant species (Klink and Machado, 2005). The medicinal species richness of the Cerrado is explained by the morphological characteristics of plants, which includes xylopodia and thick barks that accumulate reserves and frequently have active substances of pharmacological interest (Silva et al., 2010), including antibacterial and antifungal action (Pinhol et al., 2012; Silva et al., 2012; Andrade et al., 2015).

In Brazil, this genus constitutes nearly 40 species that are mainly distributed in the Brazilian Amazon (Montanari, 2010). *S. guianensis* is recommended for conservation because of its great ethnobotanical value and popular uses. Leaf infusions followed by baths are indicated for treating headache, malaria, rheumatic pain, sinusitis and fever, and the plant is used as a postpartum antibiotic and vaginal antiseptic (Grenand et al., 2004; Valentini et al., 2010). *S. guianensis* leaf smears are also used externally to repel insects, and leaves are placed in chicken nests to repel lice (Valentini et al., 2009; Valentini et al., 2010a). The ethanolic leaf extract is used in injuries and as an anti-inflammatory agent (Montanari, 2010). The tea, in turn, is considered an abortifacient, febrifuge and stimulant (Montanari, 2010), and it is used as a flavoring because of its anxiolytic potential (Negri et al., 2012). The leaves of *S. guianensis* are the richest plant part in terms of essential oils. Because the leaves do not undergo significant yield variations related to the seasonality of production under natural conditions (Castellani et al., 2006), they represent interesting biomass reservoirs for prospecting. Barbosa and Ferreira (2004), found que as native species sheets of dry forest in Roraima, north of the Brazilian Amazon, corresponding to 12% of the air component of tree species – shrubs. This reserve under native conditions is significant for obtaining extracts and/or essential oil.

Considering the global scenario motivated by new discoveries of natural products and biodiversity prospecting (Tulp and Bohlin, 2002; Montanari, 2010), *S. guianensis* is a potential species for pharmaceutical production and agroecology, which is an under -studied field. The phytochemistry of the leaf crude extract suggests antimicrobial action, most likely because of the presence of terpenes, tannins and flavonoids (Bessa et al., 2013). Flavonoids of *S. guianensis* have anxiolytic potential and are indicated in complementary therapies to treat anxiety disorders and delay the aging process (Negri et al., 2012). There are also reports of the presence of flavonoids that may have anti-inflammatory action (Facundo et al., 2012). The essential oil, in turn, is mainly composed of the alcohol bisabolol and monoterpene terpinolene (Montanari, 2010). The synthetic bisabolol promoted a wide zone of inhibition for *S. aureus* and *E. coli*, are probably related to the mechanisms antibacterial action of essential oils and antimicrobial activity (Montanari, 2010).

These terpenes are secondary metabolites that are most common in essential oils; therefore, they are pharmacologically active compounds with high biological activity (Duarte, 2006; Miguel, 2010) and may compose close to 85% of the essential oils along with other trace constituents (Miguel, 2010). This characteristic of *S. guianensis* also justifies studies aimed at controlling vector insects of tropical diseases (Porto et al., 2008; Furtado et al., 2005) and phytopathogenic microorganisms such as *Fusarium* sp. (Seixas et al., 2011). Essential or volatile oils are aromatic liquids obtained from plant material such as leaves, and their current uses are primarily as alternative substances for controlling microorganisms resistant to antibiotics (Acosta et al., 2003). The antimicrobial action of the oils most likely occurs because it affects the structure of the microorganism cell wall, interrupting vital cell processes and leading to cell death (Leitão et al., 1999; Dorman and Deans, 2010; Geromini et al., 2012).

Alternatives discovered through studies of natural products are economically relevant in Brazil because of their potential to replace imported synthetic products with high market value and increase the export of essential oils (approximately 1,500,000 kg.year^{-1}, the 3rd largest export) and their production sources, which include citrus (80%), peppermint, flowers, wood and seeds (Knaak and Fiuza, 2010; Souza et al., 2010). Therefore, societal awareness of the importance of natural resources conservation is a valuable strategy. The high yield of *S. guianensis* essential oil (4 to 7%) and high content of terpinolene and bisabolol, its main components and important antimicrobials, increase the potential of this species as a producer of essential oils with a high aggregate market value (Montanari, 2010). Therefore, studies such as the one presented here investigating the characteristics of important species can support public policies regarding the sharing of benefits among family farmers through the sustainable exploitation of medicinal biomass reservoirs in natural ecosystems.

This study aimed to evaluate the bioactivity of leaf essential oils and extracts and the medicinal biomass of *S. guianensis* from savanna forests located in central Brazil, Tocantins State. The antimicrobial activity and inhibitory concentration of crude extracts and leaf essential oils of *S. guianensis* were tested against gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and fungi (*Candida albicans* and *Fusarium oxysporum*).

MATERIALS AND METHODS

Choosing the species and medicinal biomass

The leaf biomass of *S. guianensis* (Siparunaceae), a shrub that grows the Cerrado, was set considering 12% of the air component (Barbosa and Ferreira, 2004) of plants coleted and identified in the inventory of the species, made in permanent plot located in the Legal Reserve area in the Vale Verde rural settlement (S 11°52.582; W 048°58.913), Gurupi, Tocantins, Brazil. The shrubs living woody individuals with diameter at breast height (DBH) greater than 5 cm were inventoried (Felfili et al., 2005). Fresh shoot biomass was obtained using the allometric equation developed by Higuchi et al. (1998) and already used in the Cerrado by Dias and Felfili (2013), as follows: BA= {0,077 + 0,492.DAP ^{2}.Ht} where BA is woody biomass of shoot (ton.ha^{-1}), DAP (diameter at breast height - 1.30 m above the ground) and Ht (total height in m), with r^2 (0.96) and S (0.25). One gets the biomass portion of the air and leaves compartment, leaf yield for oil and extract and the following medicinal booking projection leaf of *S. guianensis* leaf per hectare legal reserve tropical semideciduous forest.

Leaf essential oil crude extract preparation

To prepare the extracts (crude and essential oil), leaves were collected from seasonal semideciduous forest vegetation of the Amazon Legal Reserve area in the Vale Verde rural settlement. After collection, the plant material was preserved as exsiccates, deposited and identificated (register no. 10.218) in a herbarium of UFT, Porto Nacional, Brazil.

The ethanolic leaf extract of *S. guianensis* was prepared in the Laboratory of Natural Products at the UnirG University Center following the methodology described by Matos (2009). Briefly, 100 g (fresh weight) fresh leaves were weighed, placed in an oven at 40°C for 72 h, weighed again to obtain the dry weight, ground in a Wiley mill and and then placed in ethanolic extraction solution 95% for seven days. After filtration, the extract was concentrated in a Fisatom 801 rotary evaporator under reduced pressure at temperatures up to 50°C and weighed to obtain the relationship between the mass (g) of the concentrated extract (m) and dry weight (m), which resulted in a yield (%).

The essential oil from leaves, which were randomly collected from plants in the study area, was prepared in the Laboratory of Weed Science of the Federal University of Tocantins (UFT), using the hydrodistillation method with a Clevenger set according to methodology recommended by Koketsu and Gonçalves (1991), Simões et al. (2003) and Montanari (2010) for *S. Guianensis*. Fresh leaves (250 g) were cut with scissors into small pieces (approximately 1 cm²) and weighed in 5 portions of 50 g, each of which were distributed into one 1000 ml flask, occupying 5 flasks of the Clevenger set. A total of 500 ml of water was added to each flask, and hydrodistillation proceeded for three hours (Montanari (2010). In this essential oil preparation process, the plant material stays in contact with boiling water, and the steam forces the cell walls to open, with the oil between the plant cells evaporating. A mixture of oil and water is contained in the steam, and as the steam passes through a condenser, it is cooled and separated into two liquid phases, allowing the separation of the oil (Silveira, 2012) and collection of the hydrolate.

After collecting the hydrolate in a 5 ml amber flask, the solvent dimethylsulfoxide (DMSO at 1%) was added (Andrade et. al; 2015), and the liquid was concentrated in a rotary evaporator without heating to remove the water, producing the crude oil of *S. guianensis* at a 1.2% yield (v/v). The units (µg.mL^{-1}) of the essential oil concentration were calculated to a simple percentage considering that after inoculation, 1.11 g was used and 1.9 g remained in the flask; thus, the apparent density of the oil was calculated (D=M/V) and which was subsequently converted to µg.mL^{-1}. The extract in crude, diluted to half, and diluted to one quarter concentrations were calculated using the simple rule of three (380 µg.mL^{-1} or 100%; 190 µg.mL^{-1} or 50%; 95 µg.mL^{-1} or 25%).

In vitro microbiological assay

Disk diffusion test

Antimicrobial activity was observed in the Microbiology Laboratory of the UnirG University Center Foundation in Gurupi, Tocantins. The procedures were performed under a laminar flow hood. The disk diffusion sensitivity test was used to evaluate the antimicrobial activity in accordance with recommendations from the ninth edition of “Performance Standards for Antimicrobial Disk Susceptibility Tests," Clinical and Laboratory Standards Institute (CLSI, 2006) and Koneman et al. (1997) and Hartmann and Onofre (2010). The comparison is performed using a standard reference chemotherapeutic agent (positive control) and the diluent or emulsifying agent (negative control), used to determine the different concentrations of extract and oil. The inhibition zones are measured including the disk diameter up to the margin where there is microorganism growth (Ostrosky et al., 2008).

Microorganisms and antimicrobial agents

The antimicrobial agents included the extract and oil of the leaves

of *S. guianensis* at different concentrations. For bacteria, the positive control was gentamicin at 10 µg and the negative control was 1% DMSO, whereas for fungi, the positive control was fluconazole 25 µg and the negative control was 1% DMSO. The microorganisms used in the bioassay were extracted from standard strains (American Type Collection Culture - ATCC) of the gram-positive bacterium *S. aureus* (ATCC 29213), gram-negative bacteria *E. coli* (ATCC 25922 and ATCC 35218) and *P. aeruginosa* (ATCC 10145) and the yeast *Issatchenkia orientalis*, formerly *Candida kruzei* (ATCC 6258), which are recommended for antimicrobial susceptibility testing (CLSI, 2006). These microorganisms were purchased from PlastLabor (Microbiologics®). They were stored in KWIK-STIK™ containers containing a lyophilized pill of a single microorganism strain, a hydrating fluid reservoir and an inoculation swab. For the tests of the fungus of agricultural interest (*F. oxysporum*), the strains were obtained from bean plants provided by the UFT Laboratory of Microbiology, Gurupi, Tocantins, after 72 h of incubation at 25°C of their subculture in Petri dishes and Sabouraud agar. The solid media (BioMérieux Brasil) used for the bacteria trials were Muller-Hinton agar and Sabouraud agar for the fungi (*I. orientalis* and *F. Oxysporum*), which were placed on 150 mm plates, and blood agar, which was placed on 90 mm plates, and the plates were monitored for the growth of subcultures of commercial microorganisms (ATCC).

Inoculation of the test plates

The ATCC strains in KWIK-STIK containers with each microorganism were activated by sowing in 90 mm Petri dishes containing blood agar medium and kept in an oven at ±35°C for 7 days. The fungus *F. oxysporum* was subcultured at UFT prior to use. After this step, all the inoculants were standardized. The blood agar dishes containing the microorganisms were handled with a Drigalski spatula, and each inoculum was transferred to a test tube containing sterile saline solution. The turbidity obtained with this dilution was adjusted to the turbidity of a 0.5 McFarland standard solution to produce approximately 1 to 2 x 10^8 CFU.mL^{-1}. The bacteria were seeded in 150 mm Petri dishes containing Muller-Hinton solid medium, and the fungi were placed in the Sabouraud agar solid medium with a sterile swab dipped in the adjusted inoculum suspension.

The seeding was performed twice, taking into account that the inoculation of antimicrobials was estimated to occur for up to 15 min after inoculation of the dishes. The procedure for the leaf extract was performed first, with 6 mm antimicrobial disks placed on the medium containing the microorganisms. The disks were loaded with 10 µg extract at the three different concentrations as well as the bacterial positive (gentamicin 10 µg) and negative (1% DMSO) controls and fungal positive (fluconazole 25 µg) and negative (1% DMSO) controls. Minimal pressure was applied when placing each disk on the surface of the medium, and they were placed approximately 2.4 cm apart (CLSI, 2006). The microorganism seeding in the dishes was repeated, and the dishes then received the 6 mm antimicrobial disks soaked with essential oil, as well as the positive and negative controls, following the same standardization of plant extracts for both bacteria and fungi. The bioassay was replicated twice for tests on the essential oil and extracts.

The bioassay with the extract consisted of five concentrations (C) (C1=50,000 µg.mL^{-1}/100%; C_2=500 µg.mL^{-1}/1%; C_3=100 µg.mL^{-1}/0.2%; C_4=75 µg.mL^{-1}/0.15%), six antimicrobial agents and two replicates. The bioassay of the essential oil consisted of three concentrations (C_1=380 µg.mL^{-1}/100%; C_2=190 µg.mL^{-1}/50%; C_3=95 µg.mL^{-1}/25%), six antimicrobial agents and two replicates. Inoculation on plates containing the target microorganisms was completed by placing the disks on the plate containing the medium, commercial antibiograms, used as positive controls (25 µg fluconazole for fungi; 10 µg gentamicin for bacteria), and locally prepared antibiograms (negative control, extract and oil). In the latter case, each disk was loaded with 10 µg using a calibrated pipette (Holetz et al., 2002; Mendes et al., 2011). The plates were incubated upside down in an oven at 35°C (±2°C) and subsequently evaluated.

The inhibitory activity was observed when a halo or a zone of inhibition of bacterial or fungal growth was formed around the antimicrobial disks and our diameter was measured in millimeters (mm) using a halometer. Previous studies by Holetz et al. (2002) and Mendes et al. (2011) testing the leaf extracts of Brazilian medicinal plants measured biological activity based on the following concentration ranges: less than 100 µg.mL^{-1} was considered good; from 100 to 500 µg.ml^{-1} was considered moderate; from 500 to 1000 µg.ml^{-1} was considered weak; and greater than 1000 µg.ml^{-1} was considered inactive. Other studies testing leaf extracts have varied the concentrations (%), starting with the crude extract concentration (100%) to dilutions at 50, 25 and 12.5% respectively (Davet et al., 2009).

However, recent recommendations for testing oils were considered, including that of citronella grass (*Cymbopogon nardus*) and rangpur (*Citrus limonia*) at the crude extract concentration (100%), and diluted in DMSO to concentrations ranging from 1.5 to 2.5, 5.0, 10, 15, 25 and 50% respectively (Millezi et al., 2014). Antimicrobial agents were considered inactive when their inhibition zones were larger than 9 mm in diameter, considered partially active with inhibitions zones at 9 to 12 mm, considered active with inhibitions zones at 13 to 18 mm, and considered very active with inhibitions zones larger than 18 mm, acoording to Alves et al. (2000). Readings were performed for bacterial inocula at 18 h after incubation at 35°C (±2°C), with new readings after 24 and 48 h. Readings for fungi inocula were also evaluated at 72 h and 7 days after incubation (CLSI, 2006).

Essential oil components evaluated by gas chromatography

Gas chromatography-mass spectrometry

The GC-MS analyses were performed using a GCMS-QP2010 ULTRA system (Shimadzu) with an Rxi-1MS 30 m x 0.25 mm x 0.25 µm column (Restek) at temperatures ranging from 50°C (2 min), increasing by 3°C.min^{-1} intervals, to 250°C. The injection temperature was 250°C in a split ratio of 1:10, the gas chromatography mass spectrometry (GC/MS) interface temperature was 260°C, and the MS detector operated at an electronic impact of 70 eV at 260°C. Helium was used as a carrier gas at a flow rate of 1.5 ml. min^{-1} and an injection volume of 1 µl. The data acquisition software GCMS solution (Shimadzu) was used to evaluate the components, and the indexes were compared with those from the National Institute of Standards and Technology (NIST) spectral library NIST 11 (.qgd files) based on the Kovats index, a linear retention index used to identify oil components with the calculations including the retention time of a series of n-alkanes.

High resolution gas chromatography

An HP 7820A (Agilent) gas chromatograph was used for gas chromatography-flame ionization detection (GC-FID), which employed HP5 columns measuring 30 m x 0.32 mm x 0.25 µm (Agilent), temperatures ranging from 50°C (2 min), with increases of 3°C.min^{-1}, to 250°C. The injection temperature was 250°C in a split ratio of 1:10, and the FID temperature was 260°C. Hydrogen (H_2) was used as a carrier gas at a flow rate of 3 ml min^{-1} and an

Table 1. Size (mm) of the microorganism growth inhibition zone under various concentrations of leaf essential oil and extracts of *S. guianensis* leaves.

Essential Oil						
Microorganism	Positive control		Negative control	Concentration (µg.mL-1; %)		
	*Gent10µg	*Fluoc 25 µg	DMSO (1%)	380 (100%)	190 (50%)	95 (25%)
E. coli ATCC 25922	30	-	0	0	0	0
E. coli ATCC 35 218	30	-	0	0	0	0
P. aeruginosa ATCC 10145	30	-	0	0	0	0
S. aureus ATCC 29213	30	-	0	12	11	10
I. orientallis ATCC 5258	-	24	0	0	0	0
Fusarium oxysporum	-	24	0	0	0	0

Table 2. Size (mm) of the microorganism growth inhibition zone under various concentrations of leaf essential oil and extracts of *S. guianensis* leaves.

Foliar extract	Positive control		Negative control	Concentration (µg.mL-1; %)			
microorganism	*Gent 10µg	*Fluoc.25 µg	DMSO (1%)	50.000 (100%)	500 (1%)	100 (0.2%)	75 (0.15%)
E. coli ATCC 25922	25	-	0	0	0	0	0
E. coli ATCC 35 218	24	-	0	0	0	0	0
P. aeruginosa ATCC 10145	18	-	0	0	0	0	0
S. aureus ATCC 29213	30	-	0	0	0	0	0
I. orientallis ATCC 5258	-	24	0	0	0	0	0
Fusarium oxysporum	-	24	0	0	0	0	0

*Gent - Gentamicina; Fluoc – Fluoconazol.

injection volume of 1 µl. The data acquisition software was EZChrom Elite Compact (Agilent), which generated.dat files. The analyzes were performed in triplicate and quantitative data obtained by electronic integration of the peak area in relation to the total area of the chromatogram, which resulted in the concentration (%) of each phytocomponent present in the essential oil.

RESULTS

Under the natural conditions of the Forest Legal Reserve, the estimated was one gets the biomass portion of the air and leaves compartment (3300 kg/ha:396Kg/ha), leaf yield for oil (250 g:1.2%; D=0,38) and extract (100 g:9.6%). The potential of the *S. guianensis* essential oils to inhibit the bacterial growth of *S. aureus* (Tables 1 and 2) was observed using the *in vitro* disk diffusion method through the application of concentrations of crude oil (380 µg. ml^{-1}), oil diluted to half (190 $\mu g.mL^{-1}$) and oil diluted to one quarter (95 µg ml^{-1}), and growth inhibition zones measuring 12 mm, 11 mm and 10 mm, respectively, were observed. For the other microorganisms, the essential oil concentrations did not exhibit biological activity, although the positive control for bacteria (gentamicin 10 µg) had an inhibition zone of 30 mm and the control for fungi (fluconazole 25 µg) had an inhibition zone of 24 mm. For the leaf extracts, the result indicated that positive antimicrobial activity was not observed under the concentrations applied except for the positive control.

Table 3. Concentracion (%) of monoterpene component class and Kovats index of the essential oils of leaves of *S. guianensis* from Brazilian savanna forest as measured by GC-MS.

Components	RT (min)	Peack area	Conc (%)	IK calc
α-pinene	4.29	346804	0.61	941
canfene	4.62	36935	0.07	950
β-pinene	5.30	123291	0.22	969
mircene	5.77	16288206	28.74	982
α-phellandrene	6.06	35729	0.06	990
δ-3-carene	6.22	79870	0.14	995
α-terpinene	6.56	103622	0.18	1004
ρ-cimene	6.67	40954	0.07	1007
limonene	6.78	390771	0.69	1010
β-phellandrene	6.86	38583	0.07	1013
cis-β-ocimene	7.15	70677	0.12	1021
trans -β-ocimene	7.48	124920	0.22	1030
γ-terpinene	7.64	35547	0.06	1035
trans-ρ-menthenol	9.29	73032	0.13	1081
cis-ρ-menthenol	10.33	122916	0.22	1110
ρ-menthadienol	15.89	327346	0.58	1266

IKcalc – Kovats Index Calculeted.

This is a novel result for the studied species, which grows in seasonal semideciduous forests in the Brazilian savanna. The antibacterial activity of the essential oil against *S. aureus* was shown by the decrease in size (mm) of the growth inhibition zone with lower concentrations. Effectiveness against the other studied microorganisms was not observed (Figure 1). The bioactivity of *S. guianensis* essential oils against *S. aureus* were dependent on concentration, with the largest inhibition zone observed at the highest concentration (380 µg. mL^{-1}) and smaller inhibitions zones observed in the remaining concentrations. A slight tendency was observed for greater concentrations providing better responses for growth inhibition of this bacterium on the dish. Previous studies using essential oils from other medicinal species reported growth inhibition zones measuring approximately 10 mm, which was considered a positive response to antimicrobial action (Nunes et al., 2006; Packer and Luz, 2007).

The chromatographic evaluation of the essential oil revealed the presence and amounts of 37 components (Figure 2) of the *S. guianensis* leaf essential oil with higher concentration of monoterpene component class and sesquiterpene and acetone (Tables 3 and 4). Under the conditions evaluated, the major secondary metabolites of essential oils in *S. guianensis* leaves are terpenes (71.72%), determined by the predominance of sesquiterpenes (39.54%), such as germacrene, bisabolol, spatulenol, curzerene and atractilone, and monoterpenes (32.18%), and mircene was the major component. Sesquiterpenes and monoterpenes have been identified as major components in the volatile composition of leaves of *S. guianensis* collected from the Brazilian Amazon, Atlantic Forest and Cerrado (Zoghbi et al., 1998; Viana et al., 2002; Fischer et al., 2005; Montanari, 2010, Valentini et al., 2010b), with reports of this predominant composition in plants from neighboring countries as well, such as Panama (Souza and Felfilli, 2005). Another major volatile compound was the aliphatic acetone 2-undecanone, which was identified in previous studies (Fischer et al., 2005; Valentini et al., 2010) and is known as a toxic component of essential oils.

DISCUSSION AND CONCLUSION

Antimicrobial activity of *S. guianensis* from Brazilian savanna forest

Studies using extracts and essential oils are performed to confirm the therapeutic activity or applicability of these components in productive rural systems. In addition, such studies represent the search for new alternative antimicrobials to control epidemiologically important microorganisms, such as gram-positive (*S. aureus*) and gram-negative (*E. coli* and *P. aeruginosa*) bacteria, as well as yeasts, such as *Candida*, which is responsible for severe etiological processes that can lead to death and affect patients with low immunity, such as those in hospital intensive care units (ICUs) (Antunes et al., 2006).

The antibacterial and antifungal activity of essential oils

Table 4. Concentration (%) of acetone and sesquiterpene components class and Kovats index of the essential oils of leaves of *S. guianensis* from Brazilian savanna forest as measured by GC-MS.

Components	RT (min)	Peack area	Conc (%)	IK calc
Acetone class				
2-undecanone	16.96	4745689	8.37	1296
Sesquiterpene class				
γ-elemene	19.88	272232	0.48	1378
β-cubebene	20.61	540302	0.95	1399
β-caryophyllene	21.52	225441	0.40	1424
α-humulene	22.13	333699	0.59	1441
allo-aromadendrene	23.73	844798	1.49	1486
germacrene D	23.95	2030636	3.58	1492
β-selinene	24.32	673123	1.19	1503
bicyclogermacrene	24.55	3451683	6.09	1509
curzerene	24.75	1301144	2.30	1515
γ-cadinene	25.04	1564982	2.76	1523
patchoulene	25.23	491595	0.87	1528
espatulenol	25.68	1887134	3.33	1541
epi-cubenol	28.46	534483	0.94	1619
T-cadinol	28.85	601810	1.06	1630
β-eudesmol	29.60	177984	0.31	1651
α-cadinol	29.94	288517	0.51	1660
α-bisabolol	30.25	945839	1.67	1669
trans-α-bisabolene epoxide	30.95	2757097	4.86	1689
cis-α-bisabolene epoxide	31.98	2164474	3.82	1718
atractilone	34.48	1324550	2.34	1788

IKcalc – Kovats Index Calculeted.

of copaiba, rosemary, melaleuca, andiroba and garlic were evaluated by Packer and Luz (2007) using strains of *S. aureus* (ATCC 6538), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 9027) and *C. albicans* (ATCC 10231) and the agar hole method. Antimicrobial action was only observed for rosemary and melaleuca oils, which formed zones of inhibition smaller than 10 mm and between 10 and 60 mm, respectively. *P. aeruginosa* was tolerant to the oils, and its minimal responses suggested oil action due to the presence of flavonoids. Essential oils are lipophilic, which gives them their antimicrobial properties and causes solubility of the cell membrane in the microorganism, thus affecting its structure and enabling oil entrance (Bakkali et al., 2008; Costa et al., 2011).

In antimicrobial tests involving natural products, inhibitory concentrations vary because of the major components in the natural product and tested microorganisms. However, the essential oils and major components of the plants *Satureja montana* L. (thymol), *Cymbopogon nardus* L. (citronella) and *Citrus limonia* Osbeck (limonene) showed antimicrobial action against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) at concentrations between 1.5 and 5% (Millezi et al., 2014).

Nogueira et al. (2000), performed a chemotaxonomic study in continental Portugal to determine the phytochemical components of essential oils of 13 species of the genus *Hypericum* L. (Guttiferae), which is a potential anti depressive agent. The authors suggest that differences in potency among species were most likely due to the phenological state of the plant and environmental and phytogeographical factors and not due to the major components observed in the species. Therefore, the authors indicate that that differences are more likely caused by phytochemical traits particular to each species, and similar major components are usually found in the extracts and essential oils of plants from the same species.

Therefore, the leaf essential oil of *S. guianensis* has a phytochemical profile formed by monoterpenes and sesquiterpenes. In this case, the presence of bisabolol/

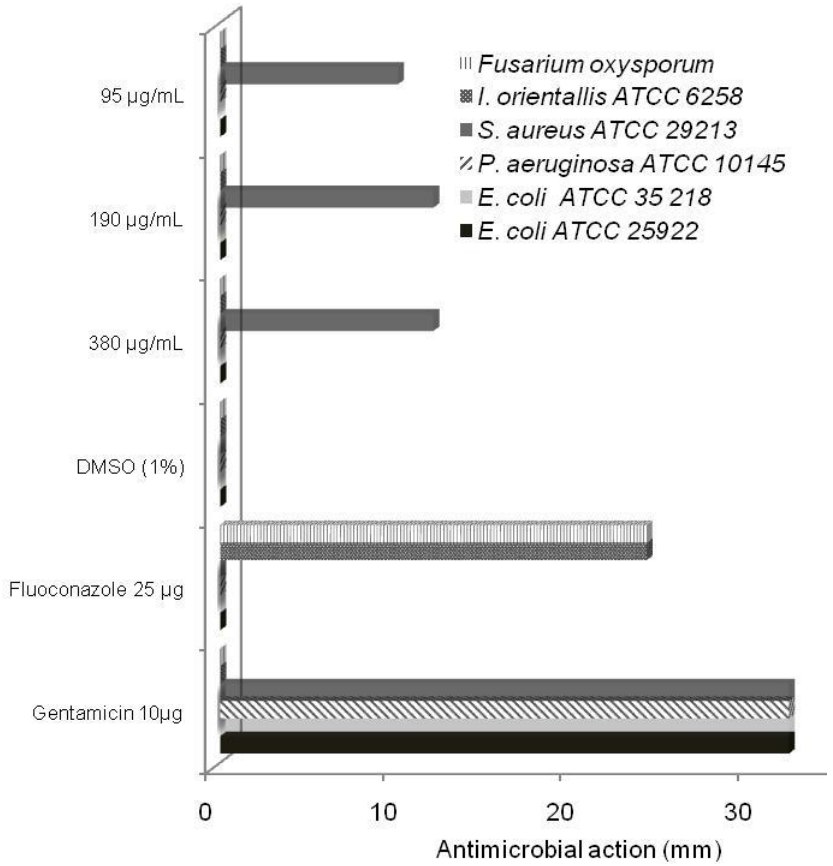
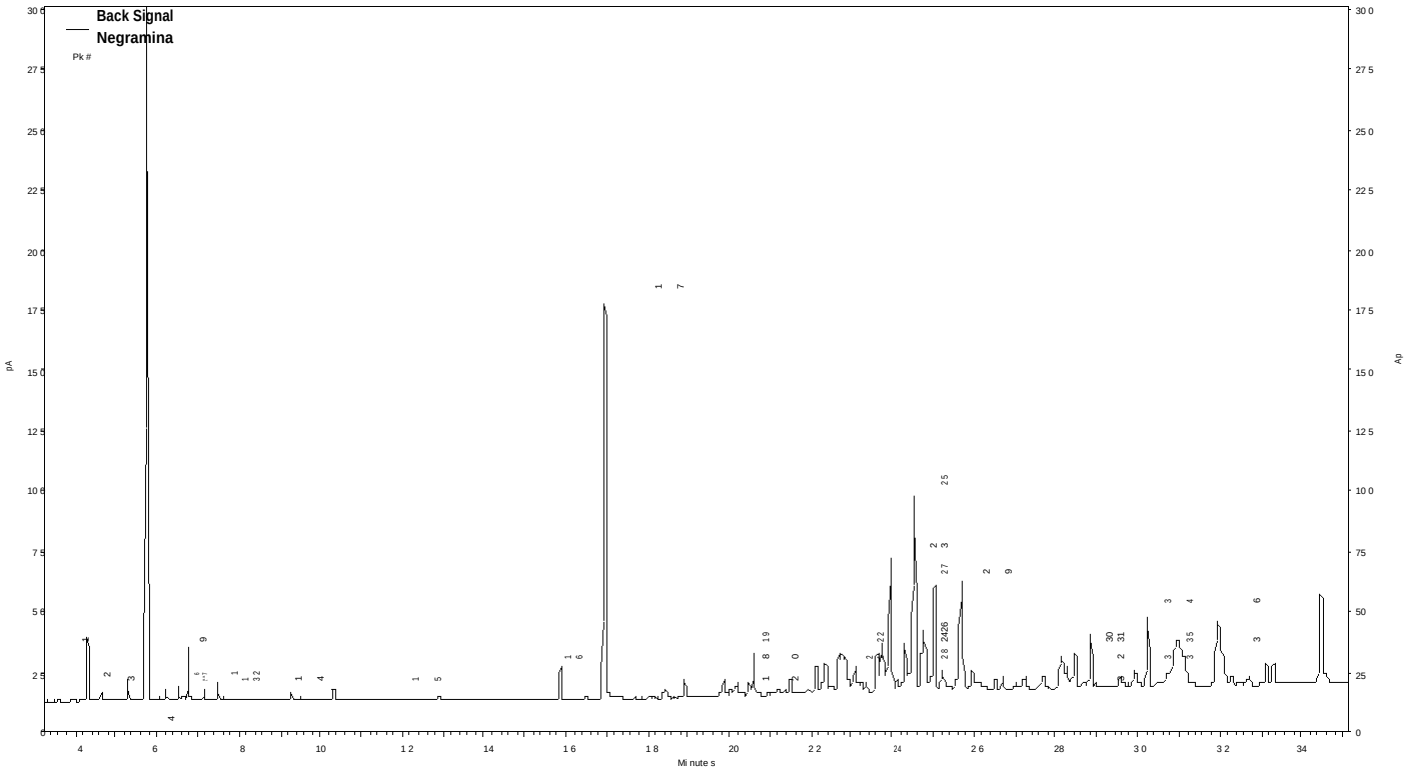


Figure 1. Antimicrobial activity against *S. aureus* by the positive control (gentamicin 10 µg) and three concentrations of essential oil from *S. guianensis* leaves.

bisabolene (10.35%), which are important anti-inflammatory and analgesic agents and appear to be consistent volatile components of the leaves of this species, regardless of geographic location, was observed, as in previous studies. These components are considered essential for the antibacterial activity against *S. aureus*, and this finding highlights the need for additional studies to verify whether such components may be used as chemical markers of the essential oil of this species and be useful for controlling the raw material quality, which would promote the safe and effective use of this medicinal plant and future phytotherapeutic and/or phytochemical applications.

Therefore, additional studies on the chemotaxonomy of this species in the Cerrado biome are important because the native biomass of this species is still abundant in certain Cerrado forest remnants. Chemotypes indicate a portion of the genetic variability of plants, which is closely related to the quality of volatile oils and may be used to map plants from the same species that occur in different localities (Tavares et al., 2005). The presence of monoterpenes raises the possibility of performing studies on the control of mosquito vectors of neglected diseases, such as leishmaniasis and dengue, which is still common in northern Brazil. Popular knowledge prescribes caution and does not recommend drinking teas made with the leaves of *S. guianensis* because of its high abortive power and potential to damage the nervous system. Such consequences of the use of *S. guianensis* may be explained by the high levels (8.37%) of 2-undecanone, a toxic component, in the essential oil.

The bioactivity of the essential oil of *S. guianensis* in the concentrations used against *S. aureus* most likely occurred due to the lower cell wall resistance to the entrance of this natural antimicrobial compared with the other tested strains. Such findings have been explained by studies evaluating the antimicrobial activity of medicinal plants where the action against gram-positive bacterium (*S. aureus*) and not gram-negative bacteria occurs because of the great permeability of the cell wall and its surface properties. Despite being thick walled, the cells of this bacterium only have one type of macromolecule, increasing its sensitivity to specific antimicrobials and enabling the entrance of



Legenda

1 α-pinene	11 cis-β-ocimene	21 α-humulene	31 T -cadinol
2 canfene	12 trans-β-ocimene	22 allo-aromadendrene	32 β-eudesmol
3 β-pinene	13 γ-terpinene	23 germacrene D	33 α-cadinol
4 mircene	14 trans-p-menthenol	24 β-selinene	34 α-bisabolol
5 α-phelllandrene	15 cis-p -menteenol	25 bicyclogermacrene	35 trans-α-bisabolene epoxide
6 δ- 3-carene	16 p-menthadienol	26 curzerene	36 cis-α-bisabolene epoxide
7 α-terpinene	17 2-undecanone	27 γ-cadinene	37 atractilone
8 p-cimene	18 γ-elemene	28 patchoulene	
9 limonene	19 β-cubebene	29 spatulenol	
10 β-phellandrene	20 β-caryophyllene	30 epi-cubenol	

Figure 2. Chemical components of the essential oil of leaves of *S. guianensis* from Brazilian savanna forest. CG-FID chromatogram.

microorganisms through the cell wall (Yokota and Fujii, 2007). Nevertheless, the ability of the phytochemicals of the species to create the necessary conditions for microorganism cell penetration and microorganism growth inhibition must also be considered because under natural conditions, aromatic plant species are capable of resisting fungal and pathogenic bacteria attacks. This behavior may be related to the presence of terpenes, which are components of plant chemical defense mechanisms (Castro et al., 2004). The mechanism of action of monoterpenes from essential oils is known to promote toxic effects to the structure and function of the target vector cell membrane (Oliveira et al., 2011). Therefore, monoterpenes enable oils to enter the microorganism and consequently inhibit its growth. The main components that were extracted from medicinal plants of Brazil and have antimicrobial properties include terpenoids, alkaloids, lectins, polypeptides, flavonoids, tannins and coumarins (Haida et al., 2007; Duarte, 2006). In essential oils, the predominant components are terpenes (Duarte, 2006; Miguel, 2010). For *S. guianensis,* terpenes were previously confirmed as the main chemical

components of the volatile compounds extracted from the leaves of plants native to Minas Gerais State (Montanari, 2010) and savannas of Mato Grosso State (Valentini et al., 2010a). Such findings may be indicative of the presence of these phytochemicals in plants of the studied remnant (i.e., the Brazilian seasonal semideciduous forest) in the southern region of Tocantins State. The presence of terpenes may explain the potential action of the essential oil of *S. guianensis* (Porto, 2008) as well as its typical aroma, which is similar to citric or sweet lemon, and these components are readily obtained by distillation of plant material (Castro et al., 2004).

Among the few Brazilian studies investigating the phytochemistry of *S. guianensis,* certain common points deserve attention, especially those related to the major components of the essential oil of plants from distinct regions of the country. For plants from remnant forests of Minas Gerais in southeast Brazil, essential oils were primarily composed of the alcohol bisabolol and the monoterpene terpinolene (Montanari, 2010). In the Cerrado of Mato Grosso (Valentini et al., 2010a) and Pará State, northern Brazil, (Zoghbi et al., 1998) several terpenoids, including the sesquiterpene bisabolol, are the major components of essential oil. Monoterpenes and sesquiterpenes were also identified in plants from the Amazon (Viana et al., 2002). Overall, terpenes and terpenoids are the most common secondary metabolites in essential oils; thus, they are the major pharmacologically active components (Duarte, 2006; Miguel, 2010), which is consistent with the findings of the present study.

The observed bioactivity of the essential oil against the same microorganism supports the findings of Alves (2007) and Aguiar et al. (2015), who tested the insecticidal action of *S. guianensis* leaves and evaluated the chemical profile of the essential oil. Alves (2007), hypothesized that the major bioactivity was performed by the essential oil, with the oil including a smaller number of different chemical substances in higher concentrations compared with the crude extract, which may contain hundreds of substances in small amounts. The result of his study with plants from the Brazilian Central Plateau Cerrado confirmed the presence of terpenoids (curzerene), insecticide action and the absence of alkaloids.

For *S. brasiliensis* plants from Minas Gerais, low effectiveness of leaf extract was observed at a concentration of 400 $\mu g.mL^{-1}$ for five tested microorganisms, including *S. aureus* and *P. aeruginosa* (Souza et al., 2013). The absence of antimicrobial action was also found in leaf extracts of *S. apiosyce* plants from Southeast Brazil against the microorganisms *E. coli* (ATCC 10531), *S. aureus* (ATCC 6538) and *C. albicans* (ATCC 10231) at a concentration of 50 $\mu L.mL^{-1}$. For the essential oil extracted from *S. guianensis* leaves, only one study of agrochemical interest detected bioactivity against gram-negative (*E. coli*) and gram-positive (*B. cereus* and *S. aureus*) strains; these results were observed under minimal inhibitory concentrations of 63 and 31 $\mu g.mL^{-1}$, respectively, and the response was ten times lower than that of the essential oils of Anacardiaceae and Verbenaceae (Montanari, 2010).

The concentrations used in this study for both the extracts and essential oils are considered the primary reason for the low bioactivity observed because they may not have maximized the response. This finding suggests the need to perform exhaustive basic research with *S. guianensis* to determine the minimum inhibitory concentration (MIC) based on essential oils using scales starting with 8 $\mu g.mL^{-1}$ and increasing by intervals of 50 $\mu g.mL^{-1}$ until 500 $\mu g.mL^{-1}$, thus capturing a range from optimal to moderate activity responses following the methods of Holetz et al. (2002) and Mendes et al. (2011).

The method involves *F. oxysporum*, a fungus of agricultural interest, should be improved. This is because obtaining a natural alternative for the control of agricultural crops family of diseases such as bean crops and is of utmost importance. This study increases the understanding of natural plant products and their potential, and it supports the search for new antimicrobial agents, especially when considering the results obtained for *S. aureus*. The relevance of this result is related to the high virulence of *S. aureus,* its resistance to conventional antimicrobials, and its involvement in several diseases, including potentially fatal systemic diseases, skin infections, opportunist infections and food poisoning (Ardura et al., 2009; Millezi et al., 2014). *S. aureus* is part of the human microbiota and is typically found on the skin and in nasal cavities of healthy individuals (Trabulsi and Altherthum, 2005). This bacterium is capable of causing diseases that range from simple skin infections, such as pimples and boils, to more serious diseases, such as necrotizing pneumonia in previously health children and youth, meningitis, endocarditis, bone infections, septic arthritis and bone prosthesis infections (Santos et al., 2007). *S. aureus* was one of the first bacteria to be controlled after the discovery of antibiotics.

However, because of its adaptability and resistance, *S. aureus* has become one of the most important causes of hospital and community-acquired infections (Trabulsi and Altherthum, 2005). In Brazil, rates of multi-drug resistant strains in hospitals vary between 40 and 80%, especially in ICUs, which has increased the interest in discovering new and more effective antibiotics to combat this infectious agent (Santos et al., 2007). Biomass and chemical components and effective essencial oil from leaves of *S. guianensis* from savanna forest. Determining the biomass of native medicinal flora and of their leaf yield is important for estimating the raw material for biodiversity prospection. Leaves are the most popular plant part of medicinal species, including species that have different characteristics or those originating from different vegetation types.

The aromatic leaves of *S. guianensis* are the main

essential oil reserve of the species, and they do not present significant seasonal variations under the silviculture conditions of the Atlantic Forest biome (Castellani et al., 2006). Castellani et al. (2006), found yields ranging from 0.12 to 0.25%, with better results observed in autumn, which is a period of critical drought and when the plant emits floral buds, and with worse results observed in spring, which is a period when intense fructification and sprouting occurs, likely depleting the oil phytochemicals. In a study with *S. guianensis,* Montanari (2010) found low yield variation (from 4.5 to 7%) in the leaf oil content in plants from the Atlantic Forest of Minas Gerais over 1 year, with the lowest values observed between September and November, a period of critical drought and plant defoliation. In the Cerrado, the monitoring performed by Valentini et al. (2010) in vegetation from Mato Grosso detected 5-fold higher yields, which was most likely related to local edaphoclimatic factors and the harvest season, with lower yields obtained during the vegetative phenological stage, which occurs from February to March.

The leaves of *S. guianensis* may be sources of chemical components of biological interest, such as flavonoids (with anti-inflammatory action), tannins (antimicrobial action) and terpenoids (antimicrobial and insecticidal actions) (Montanari, 2010), and the oil of this species is mainly composed of sesquiterpene (70%) (Valentini et al., 2010b), a substance that was also detected in other studies performed in northern Brazil (Fischer et al., 2005). Such factors are essential to controlling medicinal plant raw material and must be considered when characterizing production at a regional scale, which has not been performed for *S. guianensis*.

In this study, *S. guianensis* plants had a substantial reserve of fresh biomass in the shoot (3300 $kg.ha^{-1}$). However, it is important to estimate the amount of biomass in the leaf compartment because the extract and essential oil extractions were performed on the leaves. Because this information is not available in the literature for this species, estimations were performed according to the results obtained in study with native species developed by Barbosa and Ferreira (2004). The result of this present study suggests a potential prospective use of leaf raw material of this medicinal species, which would produce 23 tons of extract and approximately 3000 L of essential oil from the Amazon Legal Reserve seasonal semideciduous forest (610 ha). This information is new for *S. guianensis* and the Cerrado biome and is relevant for prospection.

However, such reserves would not be readily available at the same time because of the assumptions of uses based on sustainable management of native ecosystems, where destructive methods for plant removal are not supported and rational collection is required.

Concentrations of the natural antimicrobial may affect the size of the inhibition zone, and this behavior was confirmed in this study for the Gram-positive bacteria *S. aureus*, which was inhibited by the *S. guianensis* essential oil. Growth inhibition occurred from the use of the *in vitro* disk diffusion method with the crude leaf essential oil concentration (380 $\mu g.mL^{-1}$) and oil diluted to half (190 $\mu g.mL^{-1}$) and one quarter (95 $\mu g.mL^{-1}$) strength. The largest growth inhibition zone was produced with the crude concentration, and the smallest was produced with the lowest concentration level. The major monoterpene and sesquiterpene components, particularly bisabolol/bisabolene, were most likely responsible for this antibacterial response against *S. aureus*. Montanari (2010), confirmed the antimicrobial activity of the synthetic bisabolol against this microorganism, which confirms the results found in this study of natural oil.

For the leaf extract, the tested conditions were not capable of inhibiting the growth of the bacteria (*S. aureus* - ATCC 29213; *E. coli* - ATCC 25922 and ATCC 35218; and *P. aeruginosa* - ATCC 10145) or fungi (*I. orientalis* - ATCC 6258; and *F. oxysporum*). The concentrations used here were low, which were estimated to provide a heightened response. *S. guianensis* is an evergreen species found in forests of the Cerrado biome, and it is of ecological interest because of its abundance in the Amazon Legal Reserve seasonal semideciduous forest, a reservoir of medicinal biomass with the potential for use as antimicrobial agents and as a supply of raw material. Such information is relevant for planning the prospective uses of the raw material of *S. guianensis* under natural conditions, which will enable future studies on sustainable management of the Legal Reserve in the context of family farming in northern Brazil (rural settlements).

The leaf essential oil of *S. guianensis* is promising and may present biological activity against microorganisms of agricultural or livestock interest as well as organisms considered vectors of neglected diseases in Brazil. Therefore, further studies to map the chemotypes of volatile components extracted from the leaves of *S. guianensis* are recommended.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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