

***In vitro* antimicrobial activity of *Spiranthera odoratissima* A. St. Hil. essential oils against foodborne pathogens and food spoilage bacteria**

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Abstract

In Brazilian folk medicine, *Spiranthera odoratissima* has been used to treat rheumatism, infection and abdominal pain. Essential oils (EOs) are technological options that may be employed in natural foods due to their antimicrobial activities. This paper aimed to investigate the chemical composition and *in vitro* antibacterial effects of EOs from *S. odoratissima* leaves and flowers against foodborne and spoilage bacteria *Listeria monocytogenes*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Escherichia coli*, *Clostridium botulinum*, *Bacillus cereus* and *Pseudomonas aeruginosa*. Minimum inhibitory concentration (MIC) values of EOs were calculated by the broth microdilution method on 96-well microplates. Both GC-FID and GC-MS analyses revealed that the major components determined in EOs from *S. odoratissima* leaves were β -caryophyllene (23.8%), bicyclogermacrene (10.8%) and δ -cadinene (7.1%). Major constituents found in EOs from its flowers were β -caryophyllene (14.1%), spathulenol (8.1%) and γ -cadinene (7.2%). EOs from *S. odoratissima* leaves and flowers showed strong antibacterial activity against *Yersinia enterocolitica* (MIC = 0.30 mg/mL), *Staphylococcus aureus* (MIC = 0.12 mg/mL), *Clostridium botulinum* (MIC = 0.30 mg/mL), *Bacillus cereus* (MIC = 0.20 mg/mL) and *Listeria monocytogenes* (MIC = 0.25 mg/mL). These EOs could be important natural alternatives to prevent bacterial growth in food products.

Keywords: *Listeria monocytogenes*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Clostridium botulinum*, *Bacillus cereus*.

Abbreviations: GC-FID_gas chromatography-flame ionization detector; GC-MS_gas chromatography-mass spectrometry; Eos_essential oils; MIC_minimum inhibitory concentration; DMSO_dimethylsulfoxide; NCCLS_National Committee for Clinical Laboratory Standards.

Introduction

Even though biological properties of essential oils (EOs) extracted from aromatic and medicinal plants have been explored for many years, the use of natural antimicrobial compounds has recently gained more attention with the objective of food preservation (Barbosa et al., 2015).

Foodborne pathogens, which are widely distributed in nature, have been the main cause of public health issues in developed and developing countries. Since these agents are responsible for considerable morbidity and mortality, they increase health care costs and loss of both productivity and control in the food industry. *Salmonella* sp., *Listeria monocytogenes* and *Escherichia coli* are among the most dangerous foodborne pathogens because they cause the highest number of diseases and deaths (Valeriano et al., 2012).

It is well-known that the largest challenges faced by the use of antimicrobial agents in food are barriers of additive regularization, even though several components of EOs have

been registered to be used as flavor enhancers in some countries, such as the USA. Since the use of natural food aromatizers is allowed in Brazil, these compounds do not need to be registered at the Ministry of Health, which considers that they do not pose any risk to consumers' health. Carvacrol, carvone, cinnamaldehyde, citral, eugenol, menthol, thymol, p-cymene and limonene are some of them. However, other compounds, such as methyl eugenol and estragole, have been considered toxic and must not be added to food (Santos et al., 2011).

In order to mitigate diseases and economic losses caused by pathogenic microorganisms, the use of natural products, such as antimicrobial compounds, seems to be a relevant form to control pathogenic bacteria and extend shelf life of processed food, mainly when there is prevalence of microorganisms which are resistant to conventional antiseptics and antibiotics, besides increase in popular concepts of food quality and the negative impact of

synthetic additives on health. Thus, consumers' increasing demand for effective and safe natural products has triggered investigations into effects of phytochemicals. As a result, the number of studies of EOs has increased (Valeriano et al., 2012).

EOs are new technological options that may be employed as preservatives. Since they have a broad-spectrum activity against bacteria, viruses, fungi, parasites and insects, they may potentially be applied to pharmaceutical, sanitary, cosmetic, agriculture and food industries. Due to their extraction procedure, generally by steam distillation, they contain a variety of volatile molecules, such as terpenes, terpenoids, aromatic compounds derived from phenol and aliphatic components (Franciscato et al., 2018). Studies of antibacterial activity of EOs against foodborne pathogens have been carried out, mainly *in vitro* ones and by applying bacteria and EOs to food products simultaneously (Valeriano et al., 2012).

It is important to emphasize that food must be pathogen-free and protected against microbial spoilage during shelf life. Since consumers' demand for safe and natural products without any chemical preservatives has increased, research on the evaluation of alternative techniques which aim at preserving their microbiological quality and keeping their nutritional and sensory properties is on the increase (El-Salam and Ibrahim, 2014).

Spiranthera odoratissima (Rutaceae), popularly known as *manacá*, is a shrub found in the savannah and forest in central Brazil and Bolivia. In folk medicine, it has been used in the treatment of rheumatism, gout, kidney infections, urinary retention, abdominal pains and acne. Anti-inflammatory, analgesic, anxiolytic and antiprotozoal activities of EOs from *S. odoratissima* have been reported by the literature (Souza et al., 2018). However, to the best of our knowledge, the antibacterial activity of EOs from *S. odoratissima* flowers has not been documented yet.

In order to continue studies of EOs from *S. odoratissima* (Cabral et al., 2019), this research aimed at carrying out a pioneer investigation into the *in vitro* antimicrobial activity of EOs from *S. odoratissima* leaves and flowers (Figure 1) against the following foodborne and spoilage bacteria: *Listeria monocytogenes*, *Salmonella enteritidis*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Escherichia coli*, *Clostridium botulinum*, *Salmonella typhimurium*, *Bacillus cereus* and *Pseudomonas aeruginosa*.

Results and Discussion

EOs were extracted from *S. odoratissima* leaves and flowers, whose yields were 2.5% and 3.0% (w/w), respectively. Volatile compounds were identified by gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Table 1 shows that 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%, **1**), bicyclogermacrene (10.8%, **2**) and δ -cadinene (7.1%, **3**) (Figure 2), while the ones found in EOs from flowers were β -caryophyllene (14.1%), spathulenol (8.1%, **4**) and γ -cadinene (7.2%, **5**) (Figure 2). Twenty-eight components were identified in EOs from *S. odoratissima* leaves, which exhibited about 93.8% of the oil total composition, whereas twenty-nine components were

identified in EOs from flowers, which showed about 94.4% of the oil total composition (Table 1).

EOs extracted from *S. odoratissima* leaves had high concentrations of sesquiterpene hydrocarbons, mainly: β -caryophyllene (23.8%), bicyclogermacrene (10.8%) and δ -cadinene (7.1%). It represented 41.7% of the oil total composition. They were also found in EOs from other species of the family Rutaceae, as major compounds: *Haplophyllum linifolium* (bicyclogermacrene and β -caryophyllene) and *Pilocarpus spicatus* (δ -cadinene) (Iñigo et al., 2002; Andrade-Neto et al., 2002). β -caryophyllene was also identified as a major component in EOs from *Galeopsis bifida* (22%), *Vernonia remotiflora* (42.2%), *Vernonia brasiliensis* (36.7%) and *Annona foetida* (14.19%) (Olennikov et al., 2010; Maia et al., 2010; Costa et al., 2009). In addition, bicyclogermacrene has already been identified as a major constituent in EOs from *Hortia oreadica* flowers (23.28%), fruits (20.64%) and leaves (from 14.71% to 31.37%) (Santos et al., 2016). *In vitro* antibacterial activity of EOs from *S. odoratissima* against the microorganisms under study and their potential activities were assessed quantitatively by minimum inhibitory concentration (MIC) values. Data obtained from MIC values of EOs were calculated by the broth microdilution method on 96-well microplates. MIC results are shown in Table 2. Data indicated that EOs exhibited varying levels of antibacterial activity against the microorganisms under investigation. Inhibitory properties of EOs were observed within a range of concentrations, from 1.0 to 0.12 mg/mL. Maximum activity was observed against *Listeria monocytogenes* (MIC = 0.25 mg/mL), *Staphylococcus aureus* (MIC = 0.12 mg/mL), *Yersinia enterocolitica* (MIC = 0.30 mg/mL), *Clostridium botulinum* (MIC = 0.30 mg/mL) and *Bacillus cereus* (MIC = 0.20 mg/mL). Higher MIC values were obtained against *Pseudomonas aeruginosa* (MIC = 0.60 mg/mL), *Escherichia coli* (MIC = 1.0 mg/mL), *Salmonella enteritidis* (MIC = 0.70 mg/mL) and *Salmonella typhimurium* (MIC = 0.70 mg/mL). It should be mentioned that the methodology used by this study is the one previously published by Chaibub et al., (2013). However, some MIC values found for the same bacterial strains by this study were very different from the previously described ones.

Aligiannis et al. (2001) used the broth dilution method and reported that samples whose MIC values were \leq 0.5 mg/mL had strong antimicrobial inhibition; the ones whose MIC values ranged from 0.6 mg/mL to 1.5 mg/mL exhibited moderate inhibition and when MIC values were $>$ 1.6 mg/mL, inhibition was weak. Considering these parameters, both EOs extracted from *S. odoratissima* leaves and flowers were found to exhibit strong inhibition against *Yersinia enterocolitica*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium botulinum* and *Bacillus cereus*. Moderate antibacterial activity was shown against the other bacteria under evaluation. *Yersinia enterocolitica* is an enteropathogenic bacterium which is responsible for human gastroenteritis. Enteric yersiniosis is a foodborne disease, which is transmitted through the fecal-oral route. Clinical presentation of yersiniosis includes diarrhea, abdominal pain, fever and, sometimes, vomiting (Rahman et al., 2011). *Listeria monocytogenes* is a bacterial pathogen that causes a potentially potentially severe disease, called listeriosis, both in humans and animals. It has been estimated that the most human cases occur after consumption of contaminated food worldwide. The most frequently implicated foodstuffs, both

Table 1. Chemical composition of EOs from *S. odoratissima* leaves and flowers.

Compounds	RI _{exp}	RI _{lit}	RA%	
			Leaves	Flowers
Limonene	1030	1031	0.5	0.5
Linalool	1101	1098	—	1.0
Terpinen-4-ol	1171	1177	—	0.3
α-Terpineol	1190	1192	—	0.4
Bicycloelemene	1327	1331	1.3	0.3
α-Cubebene	1358	1352	2.3	2.1
α-Copaene	1365	1372	5.5	6.4
β-Elemene	1389	1392	5.3	5.4
β-Caryophyllene	1416	1418	23.8	14.1
α-Bergamotene	1430	1436	0.6	1.6
α-Humulene	1447	1455	4.8	3.9
Alloaromadendrene	1455	1461	2.6	5.2
γ-Muurolene	1472	1477	1.1	1.9
Germacrene D	1478	1480	5.9	3.3
β-Selinene	1482	1485	1.5	0.9
Bicyclogermacrene	1490	1496	10.8	4.2
α-Muurolene	1494	1499	—	2.7
Germacrene A	1501	1503	0.4	0.4
γ-Cadinene	1510	1513	3.0	7.2
δ-Cadinene	1521	1524	7.1	5.8
α-Cadinene	1533	1538	0.3	0.7
Germacrene B	1554	1561	0.5	—
Spathulenol	1581	1578	2.9	8.1
Caryophyllene oxide	1585	1583	1.6	3.0
Viridiflorol	1591	1595	1.2	0.7
Epiglobulol	1601	1598	—	0.4
Carotol	1613	1597	0.3	—
Humulane-1,6-dien-3-ol	1618	1619	0.3	—
Isoaromadendrene epoxide	1623	1622	0.5	0.4
τ-Muurolol	1643	1640	4.3	6.3
δ-Cadinol	1647	1645	0.5	0.6
α-Cadinol	1658	1653	4.7	6.6
Nootkatone	1810	1807	0.2	—
Monoterpene hydrocarbons			0.5	0.5
Oxygenated monoterpenes			—	1.7
Sesquiterpene hydrocarbons			76.6	66.1
Oxygenated sesquiterpenes			16.7	26.1
Total			93.8	94.4

RI_{exp}: Retention index relative to *n*-alkanes (C₈–C₂₀) in the Rtx-5MS column; RI_{lit}: Retention index found in the literature [6]. RA%: relative area. (–) = not detected.

**Fig 1.** *Spiranthera odoratissima* leaves and flowers (Rutaceae).**Table 2.** Determination of MIC (μg/mL) of EOs from *S. odoratissima* leaves and flowers against foodborne pathogens and food spoilage bacteria.

Bacteria	Flowers	Leaves
	MIC	MIC
<i>Salmonella enteritidis</i> (ALI 1132)	0.70	0.70
<i>Salmonella typhimurium</i> (ATCC 14028)	0.70	0.70
<i>Clostridium botulinum</i> (ATCC 3502)	0.30	0.30
<i>Yersinia enterocolitica</i> (ATCC 9610)	0.30	0.30
<i>Bacillus cereus</i> (ATCC 14579)	0.20	0.20
<i>Staphylococcus aureus</i> (ATCC 29213)	0.12	0.12
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	0.60	0.60
<i>Listeria monocytogenes</i> (ATCC 15313)	0.25	0.25
<i>Escherichia coli</i> (ATCC 14948)	1.00	1.00

ALI (Adolfo Lutz Institute); ATCC (American Type Culture Collection).

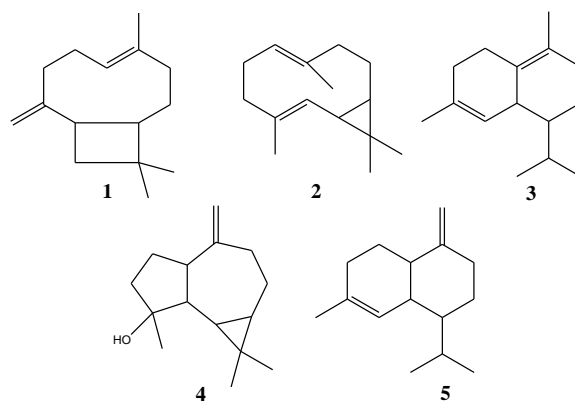


Fig 2. Major chemical constituents of EOs from *S. odoratissima* (Rutaceae) leaves and flowers: β -caryophyllene (1), bicyclogermacrene (2), δ -cadinene (3), spathulenol (4) and γ -cadinene (5).

in outbreaks and sporadic cases, are soft cheese, frankfurters, unpasteurized milk, deli meats, smoked fish, dairy products, salads and refrigerated ready-to-eat products (Buchanan et al., 2017). *Staphylococcus aureus* is the leading cause of diverse infections in humans, including gastroenteritis. Staphylococcal food poisoning occurs due to the ingestion of enterotoxins preformed in food and its symptoms include vomiting, diarrhea and cramps. This illness is one of the most prevalent foodborne disease worldwide (Dittmann et al., 2017). Based on the problems caused by this bacterium, application of EOs from aromatic and medicinal plants and their components has reinforced antimicrobial, antioxidant and food preservative activities against a wide range of microbial pathogens (Pellegrini et al., 2018).

EOs from *S. odoratissima* also showed high inhibitory potential against two bacteria that cause food contamination: *Clostridium botulinum* and *Bacillus cereus*. The latter is a Gram-positive, facultatively anaerobic bacterium, which forms spores and is broadly distributed in the environment due to its capacity to survive in hostile conditions. Researchers worldwide have described it as a food contaminant, since it can be found in different types of raw food, such as rice, meat, vegetables, milk and dairy products, besides cooked food. *B. Cereus* in food is usually associated with deterioration and food intoxication, which lead to emetic and diarrheal syndromes (Organji et al., 2015). Botulism is a disease which results from the activity of a potent protein-derived neurotoxin produced by *Clostridium botulinum*, usually as the result of ingestion of food in which the bacterium produced the toxin. Four types of human diseases are caused by the toxin: foodborne, wound, adult intestinal colonization and infant. Foodborne botulism results from the ingestion of pre-formed toxins, while the other three types occur by infection, multiplication and production of toxins by clostridial microorganisms either in wounds or in the gastrointestinal tract (Peck and Vliet, 2016).

Some chemical constituents identified in EOs from *S. odoratissima* have had their antibacterial activity reported by the literature (Pandey et al., 2017). For example, β -caryophyllene and bicyclogermacrene were found to be highly concentrated in EOs from Verbenaceae species and to exhibit promising antimicrobial activity (Montanari et al., 2011). Besides, antibacterial activity of individual components of EOs, such as α -humulene, spathulenol and β -caryophyllene, has been previously reported (Rahman et al.,

2016). In the same way as EOs from *S. odoratissima*, γ -cadinene was one of the major constituents of the oil. In addition, the compound γ -cadinene was one of the major constituents of EOs from *Xenophyllum poposum*, which also exhibited pronounced antibacterial activity against two strains of *Staphylococcus aureus* (González et al., 2012).

It is usually assumed that non-polar compounds diffuse easily across cell membranes and kill microorganisms by affecting bacterial metabolic pathways or organelles. Additionally, these compounds could interact with bacterium membrane, induce drastic physiological changes and cause loss of membrane permeability, which ultimately leads to cell death (Raut and Karuppaiyil, 2014). Antimicrobial activity of given EOs may depend only on one or two of the major constituents that make up the entire oil. In accordance with the increasing level of evidence, the ratio of the main active constituents may not be the only factor responsible for the inherent activity of EOs, but interactions among them and minor constituents of oils are also important. In short, possible synergistic interactions among components of EOs are beneficial for their activity against foodborne pathogens and food spoilage bacteria (Baldim et al., 2018).

Materials and methods

Plant material and extraction of EOs

Spiranthera odoratissima leaves and flowers (S16°24'11.2''S and 51°06'41.4''W) were collected in November 2017 in Iporá, Goiás state, Brazil. The plant was identified by botanist Erika Amaral, M. Sc., and a voucher specimen (#1039) was deposited in the herbarium in Rio Verde, at the Instituto Federal Goiano (IFGOIANO). EOs were extracted from *S. odoratissima* leaves and flowers by hydrodistillation in a Clevenger apparatus for 2 h. Hydrodistillation was performed in triplicate. The plant material was divided into three 500-g samples and 500 mL distilled water was added to each sample. After manual collection of EOs, traces of water remaining in the oil were removed with anhydrous sodium sulfate; this process was followed by filtration. EOs were stored in an amber bottle and kept in a refrigerator at 4°C until analysis. EOs yield was calculated from the weight of the leaves and flowers and expressed as the average of the triplicate analyses.

Analysis of EOs

EOs were dissolved in ethyl ether and analyzed by GC-FID and GC-MS with the use of both Shimadzu QP5000 Plus and GCMS2010 Plus (Shimadzu Corporation, Kyoto, Japan) systems. Temperature of the column in GC-FID was programmed to rise from 60 to 240°C at 3°C/min and was held at 240°C for 5 min; the carrier gas was H₂ at a flow rate of 1.0 mL/min. The equipment was set to operate in the injection mode; the injection volume was 0.1 µL (split ratio of 1:10), while injector and detector temperatures were 240 and 280°C, respectively. Relative concentrations of components were obtained by normalizing peak areas (%). Relative areas consisted of the average of triplicate GC-FID analyses. GC-MS conditions and identification of EOs have been previously reported (Melo et al., 2015). Identification of volatile components of EOs from *S. odoratissima* was based on their retention indices on an Rtx-5MS (30 m X 0.25 mm; 0.250 µm) capillary column under the same operating conditions used for GC relative to a homologous series of *n*-alkanes (C₈-C₂₀). Structures were computer-matched with Wiley 7, NIST 08, and FFNSC 1.2. Their fragmentation patterns were compared with literature data (Adams 2007).

Antibacterial activity

Bacteria from the American Type Culture Collection (ATCC) and the Adolfo Lutz Institute (ALI) collection were employed (Table 2). The microorganisms were maintained in a freezer at -80°C in 20% glycerol solution.

MIC was determined by using 96-well microplates, as recommended by the NCCLS (2003). In the process of plate preparation, 500 mg EOs were solubilized in 2 mL dimethylsulfoxide (DMSO) and subjected to 1: 2 serial dilutions in 8 tubes. Then, 19 mL Muller-Hinton agar was added to each tube at 50°C. Tubes were vortexed and their contents were poured on Petri dishes so as to reach concentrations which ranged from 12.50 mg/mL to 0.098 mg/mL EOs. Plates with DMSO and the culture medium alone, which had been prepared under the same conditions, were used as MIC controls. Suspensions of microorganisms were prepared in 0.9% sterile saline solution and turbidity was adjusted to a turbidity equivalent to half the 1.0 McFarland scale (NCCLS, 2003). Bacterial inocula were applied to the plates by the Steers inoculator and plates were incubated at 37°C for 24 hours (Steers et al., 1959). MIC was considered to be the lowest concentration of EOs that inhibited the development of bacteria. The experiment was carried out in duplicate.

Conclusions

Our findings showed that EOs from *S. odoratissima*, exhibit high contents and have high *in vitro* antibacterial activities against *Yersinia enterocolitica*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium botulinum* and *Bacillus cereus*. EOs from *S. odoratissima* leaves and flowers showed to be moderately active against *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Escherichia coli*. The highest level of antibacterial activity of EOs may be related to the synergistic interactions among their components. After this screening experiment, further studies are required to analyze the mechanism of

antibacterial activities. *In vivo* studies should also be conducted. Results of this study initially suggest that natural products derived from *S. odoratissima* may have potential use in food, pharmaceutical and/or agroindustrial products, such as preservatives and antimicrobial agents. In sum, further studies of identification, isolation and evaluation of biological properties of chemical constituents of EOs from *S. odoratissima* should be carried out.

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