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Chemical composition and modulation of antibiotic activity of essential oil of *Lantana caatingensis* M. (Verbenaceae)

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ABSTRACT

This study reports for the first time on the chemical composition, toxicity and antibacterial activity of the of *Lantana caatingensis* essential oil (*LcEO*), a medicinal plant from Northeastern Brazil. The essential oils were analyzed by GC–MS, GC-FID and NMR. The quantitative analysis of the anti-staphylococcal activity was carried out by determining their percentage inhibition degree. From the chemical constituents of the essential oil analyzed, we observed a high proportion of sesquiterpene hydrocarbons, being the β-caryophyllene and bicyclogermacrene its main representatives. The toxicity evaluation was performed on *Artemia salina* with results considered active (LC_{50} < 100 μg/mL). The antimicrobial activity was evaluated separately and in association of aminoglycoside antibiotics with LcEO by microdilution method. The results indicated that LcEO can be an alternative source of natural products with antibacterial action, being capable of inhibiting the bacteria *Staphylococcus aureus* with MIC values of 64 μg/mL. The combination of LcEO with aminoglycoside antibiotics against *S. aureus* showed significant synergism, with 75% reduction in the concentration to the MIC value of amikacin.

1. Introduction

Lantana is a genus of about 150 species. Some of these species are used in folk medicine as antirheumatic and antipyretic, in the treatment of wounds, in relieving intestinal colic and as an ornamental plant. Species of the genus *Lantana* are of great interest for phytochemical, pharmacological and biological studies. Phytochemical investigations established the presence of terpenoids, phenylpropanoids and flavonoids as main class of compounds with significant biological activities (Ghisalberti, 2000). Some species are also known to be immune to herbivory due to the presence of a variety of phytochemical groups (Kohli et al., 2006), some of which are repellents.

The *Lantana caatingensis* Moldenke is native and endemic plant of Brazil. Its geographical distribution lies between Pernambuco, Bahia, Piaui and Minas Gerais. Found in highland forests, *L. caatingensis* (Fig. 1) is represented as a bush, located on the tops of mountain ranges (Rodal and Nascimento, 2002). This species is widely distributed in the municipality of Simões PI, precisely in the Araripe mountain range.

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Fig. 1. Partial photograph of a specimen of *Lantana caatingensis* M. in its natural habitat, highlighting branches with inflorescence, and structures of the major constituents (β-caryophyllene, bicyclogermanecrene, spathulenol and valencene).

Phytochemical studies of *Lantana* genus led to the isolation of triterpenes, steroids and flavonoids, and ethanol extracts of their leaves and roots showed a broad spectrum of biological activities (Costa et al., 2009). In in vitro studies, it was proven the antibacterial action of extracts of several species of the genus *Lantana*, both to strains of gram-positive and gram-negative bacteria (Sousa et al., 2011; Kirimuhuzya et al., 2009; Pereira et al., 2008; Silva et al., 2005).

The chemical composition and antibacterial and/or antifungal activity were also demonstrated to the volatile constituents of various species: *Lantana camara* (Costa et al., 2009; Ghisalberti, 2000; Medeiros et al., 2012), *Lantana montevidensis* (Montanari et al., 2011; Sousa et al., 2011), *Lantana achyrantifolia* (Hernández et al., 2005; Sonibare and Effiong, 2008), *Lantana trifolia*(Montanari et al., 2011). More recently, Sousa and Costa (2012) developed a gender review of *Lantana*, including chemical aspects and biological activities.

Despite geographical differences, the main components observed in some samples obtained from different regions of Brazil were limonene (16.5%), α-phellandrene (16.4%), germacrene-D (13.2%), (*E*)-caryophyllene (10.8%) and sabinene (8.9%) (Silva et al., 1999).

One way to complement the phytochemical studies is to associate them with bioassays. For this reason, many natural products laboratories have entered into their routines simple biological assays in order to select and monitor the research of plant extracts in the search for bioactive substances (Nascimento et al., 2008). This way, the essential oils (EOs) may become an important therapeutic alternative because the low risk of developing bacterial resistance due to the presence of different compounds in the essential oil (Veras et al., 2012).

The antibacterial activity shown by some essential oils can be attributed to the presence of some constituents such as carvacrol, terpineol acetate, cymene, thymol, pinene, cineol, linalool, among others, that have antibacterial activity (Hernández et al., 2005; Oroojalian et al., 2010; Veras et al., 2012), which are also present in several species of the genus *Lantana* (Hernández et al., 2005; Sonibare and Effiong, 2008).

The increased use of antibiotics, along with the overprescription and/or poor adherence to treatment by patients, has led to the development of bacterial resistance to antibiotics. Therefore, there is an increasing interest in the development of new types of effective and non-toxic antimicrobial compounds (Oroojalian et al., 2010). The increasing of bacterial resistance has attracted the attention of the scientific community to search for new drugs and effectiveness of natural origin and led to a combination of antibiotics and natural products to try to reduce this resistance (Botelho et al., 2007).

Considering this fact and the absence of studies from a chemical or biopharrmacological standpoint with *L. caatingensis* M., we decided to analyze the chemical composition of the essential oil of this species, collected in Simões – State of Piaui, in different months of the year, and to determine the antibacterial activity and the modifying antibiotic activity of aminoglycosides against different bacterial strains.

2. Materials and methods

2.1. Plant material

The leaves of *L. caatingensis* M. were collected in the municipality of Simões, in the geographic coordinates of latitude 07°35′S and longitude 040°40′W, in the southeast part of the State of Piaui. Then, a representative sample of the species was deposited in the Herbarium of the Federal University of Piaui, under number 27.183 and identified by Professor Jorge Yoshio Tamashiro (IQ-UNICAMP).

2.2. Extraction of essential oil

The fresh leaves samples were collected at 08:00 am, from different sub bush from the same population of *L. caatingensis* in January (2010 and 2011), June (2011) and March (2012). The essential oil (EO) of fresh leaves (about 300 g) was obtained by hydrodistillation, using a Clevenger type® of device for a period of 3 h. The oil was kept under refrigeration at −5 °C until the execution of the analyses.

2.3. Chemical composition analysis by GC–MS and GC

Analysis method by gas chromatography–mass spectrometry (GC–MS) was performed on a Shimadzu apparatus GC-17A/MS QP5050A with ionization by electron impact at 70 eV. The mass spectra were obtained from 43 to 350 *m*/*z*. The temperature of the injector/detector and the thermal program were maintained at 240 °C. The carrier gas used was helium. The identification was made by comparison with standard spectra from the internal library of data and of the retention times based on the linear retention index (Adams, 2007). The gas chromatography-flame ionization detector (GC-FID) chromatogram was used to determine the relative concentration, using the peak areas, on Agilent system 5975C, of which analysis method was similar to that shown for the GC–MS system above. Phenyl-methylpolysiloxane – 5% capillary column (30 m 0.25 mm di, 0.25 μm film; J & W Scientific, Folsom, CA, USA). Hydrogen was used as carrier gas.

2.4. NMR analysis

The essential oil samples were also analyzed by ^{1}H and ^{13}C NMR to confirm the identification of some components. A Bruker Avance DRX-400 NMR spectrometer was used working at 400 MHz for ^{1}H and 100 MHz for ^{13}C, using $CDCl_3$ as solvent. The chemical shifts (δ) assignments were carried out by comparison with those of pure compound from the literature.

2.5. Toxicity evaluation

The lethality bioassay with *Artemia salina* larvae (Mclaughlin et al., 1991; Medeiros et al., 2013; Araújo et al., 2010Mclaughlin et al., 1991; Medeiros et al., 2013; Araújo et al., 2010 Araújo et al.,

2010), modified from Parra et al. (2001) was conducted to investigate the toxicity of the essential oil of *L. caatingenses*. *A. salina* eggs were hatched in artificial sea water (1.0 L of mineral water and 33.0 g of sea salt) and used after 24 h. The experiments were performed in triplicate and accompanied by control groups. Different concentrations of the oil were used (25, 50, 100, 200, and 400 μg/mL) in a set of three tubes per dose. Survivors were identified after 24 h of incubation and the percentages of deaths in each dose and control were determined. The values of medium lethal concentration (LC_{50}) were obtained by linear regression method (Meyer et al., 1982). The 50% lethal concentration (LC_{50}) was obtained by PROBIT analysis (SPSS 15.0).

2.6. Antibacterial activity and minimum inhibitory concentration (MIC)

The antibacterial activity of the essential oil (collected in January 2011) was investigated in accordance with the methodology recommended by the NCCLS standard M7–A6 (Wikler et al., 2003). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of oil capable of inhibiting the growth of bacteria and the analyses of the results was indicated by the color of resazurin. We used as standards for comparison the antibiotics neomycin, amikacin, and gentamycin. In the assay, two multiresistant strains: *Escherichia coli* (27) from sputum and *Staphylococcus aureus* (358) from surgical wound, obtained from clinical material were used. Brain hear infusion (BHI 3.8%) was used for bacterial growth (24 h, 35 ± 2 °C). The inoculum was an overnight culture of each bacterial species in BHI broth diluted in the same media to a final concentration of approximately 1×10^8 CFU/mL (0.5 nephelometric turbidity units–McFarland scale). After this, the suspension was diluted to 1 × 10 6 CFU/mL in 10% BHI. About 100 μL of each dilution was distributed in 96-well plates plus essential oils, achieving 5 × 10 5 CFU/mL as final concentration of the inoculums. Essential oil was dissolved in distilled water and dimethyl sulfoxide (DMSO) to a concentration of 1024 μg/mL. Further serial dilutions were performed by the addition of BHI broth to reach a final concentration in the range of 8–512 μg/mL. Antibacterial activity was detected using a colorimetric method by adding 25 μL of resazurin staining (0.01%) aqueous solution in each well at the end of the incubation period. Assays were performed in triplicate, following general procedure adopted by Sousa et al. (2011).

2.7. Modulatory activity evaluation

To evaluate the EOLc as a modulator of antibiotic resistance, the MICs of aminoglycosides (neomycin, amikacin and gentamycin) against the analyzed strains (Tables 2 and 3) were determined in the presence or absence of EOLm using the microdilution test. Sub-inhibitory concentrations (MIC 1/8) in 10% BHI were used. The antibiotics solutions (5000 μg/mL) were prepared in distillated water for use the same day. A total of 100 μL of the antibiotic solution, using serial dilutions (1:2), was added to the wells containing 10% BHI and the diluted bacterial suspension (1:10). Microplates were incubed for 24 h at room temperature and the antibacterial activity was determined as described by the literature (Sousa et al., 2011).

3. Results and discussion

The identification of the essential oil components was performed by comparison of their retention indexes in GC–MS. The standard for identification was obtained by Kovats Index. We also used the Kovats Index estimated by a computer program based on the squared linear regression which uses the retention time of small amounts of known components in the chromatogram and compatible with the literature (Alencar et al., 1990).

The yields of the EOs of *L. caatingensis* were similar in all four sampling periods: 0.4% in January 2010, 0.4% in January 2011 ((flowering periods), 0.32% in June 2011 and 0.36% in March 2012, indicating that there is no significant difference associated with the season of the year, despite having been established in other studies that during the dry period there is a bigger loss of essential oil due to increased temperature which may thereby cause further volatilization of volatile constituents of the oil.

In studies of the genus, *Lantana* were observed yields ranging between 0.01 and 0.9 percent (Alencar et al., 1990; Sonibare and Effiong, 2008). Through chromatographic techniques was possible the identification and quantification of a total of 39 constituents present in the oil *L. caatingensis*, being that qualitative and quantitative differences were observed when comparing the different months of sampling (Table 1).

Analyses GC–MS and GC-FID allowed to identify 16 constituents (98% in January 2010, 24 (95%) in January 2011, 22 (89.5%) in June 2011 and 09 (90.46%) in March 2012. The presence of mono and sesquiterpenes as major compounds were also confirmed in several species of the genus *Lantana* by other authors (Dambolena et al., 2010).

According to Chowdhury et al. (2007), the caryophyllene and its isomers are common constituents in essential oils of the aerial parts of the genus *Lantana*. Saleh (1974) verified the presence of α and β-caryophyllene in the essential oil of *L. camara*; and Saxena and Sharma (1999) observed the α-caryophyllene in *L. aculeata*. Sundufu and Shoushab (2004) observed the presence of germacrene D and bicyclogermacrene in the essential oil of *L. camara*.

A significant variation in the presence and content of certain constituents of the essential oil of *L. caatingensis* was detected. This variation of the essential oil of a plant may be due to environmental conditions such as the vegetative cycle or extrinsic factors (temperature, relative humidity, total duration of exposure to the sun and wind regimes) (Medeiros et al., 2012; Simões et al., 2010). The variations in the chemical constituents present in the species are generally plentiful, both in terms of qualitative as well as quantitative, and being that different collection sites, drying and different preparation of plants and their products may hamper the replication of the studies (Farias, 2003). In plants, the oils are presented in mixtures of different concentrations, typically having major constituents (Simões et al., 2010), and being that environmental conditions are capable of causing significant variations.

The β-caryophyllene and bicyclogermanecrene were the main constituents identified in the oil of *L. caatinguensis*, and it was determined that their concentrations vary depending on the season of collection (Fig. 1, Table 1). The spathulenol and β-caryophyllene were the major constituents in the January 2011 sampling, reaching 28% and 21.2%, respectively. The valencene appears as a major component in the sampling of the month of June 2011 (30.3%). In January 2010, the bicyclogermanecrene hit 38.2% in relation to the other constituents and β-caryophyllene reached 37.07% in March 2012. δ-3-carene, β-caryophyllene, cumulene, bicyclogermacrene and spathulenol were found in all periods analyzed at different concentrations (Fig. 2).

The comparison of the ^1H and ^{13}C NMR spectra of the essential oils with literature data (Lima et al., 2012; Silva et al., 2010; Ragasa et al., 2003), allowed the identification and/or confirmation of spathulenol and *E*-caryophyllene, of which spectral data are: the signals at δ_H 0.40–3.69, 4.68 (1H), 4.66 (1H), 4.82 (1H, s), 4.94 (1H, s) and 5.29 (1H). The signals at δ_H 4.68 (1H) and 4.66 (1H) were assigned to H-15α and H-15β of the spathulenol and signals at δ_H 5.29 (1H) were assigned to H-5 and δ_H 4.82 (1H, s) and 4.94 (1H, s) to H-15α and H-15β of the *E*-caryophyllene. The ^{13}C-NMR and DEPT-135 spectra allowed assignment of all carbon signals of

Table 1
Chemical composition and percentages of the constituents of the essential oils of the species of *Lantana caatingensis* obtained in different months of the municipality of Simões-Piaui, Brazil.

Compound	$IK_{experimental}$	January 2010(%, w/w)	January 2011(%, w/w)	June 2011(%, w/w)	March 2012(%, w/w)
Pinene	929	0.40	–	–	–
Sabinene	968	0.60	2.00	–	1.27
δ-3-Carene	1007	1.50	2.10	0.70	2.44
p-Cymene	1019	–	3.10	–	–
Limonene	1024	3.80	–	–	6.06
1,8-Cineol	1024	–	3.80	–	–
β-Phellandrene	1031	–	–	1.20	–
β-*o*-Cymene	1045	1.20	–	0.40	–
γ-Terpinene	1054	2.10	–	0.80	–
α-Terpinolene	1084	0.40	–	–	–
Linalol	1097	–	1.50	0.40	–
Terpinen-4-ol	1171	–	0.60	–	–
Estragol	1196	22.10	–	–	–
α-Copaene	1367	–	0.90	0.50	–
β-Elemene	1384	–	1.70	0.50	–
Itacilene	1405	–	–	0.70	–
β-Caryophyllene	1411	11.10	21.20	21.30	37.07
β-Bergamotene	1428	–	1,00	–	–
α-Humulene	1445	1.50	2.50	2.00	3.16
Aromadendrene	1450	–	1.50	–	–
γ-Muurolene	1468	–	0.70	–	–
Germacrene-D	1471	6.70	2.90	–	2.71
α-Amorfene	1483	–	–	10.30	–
Bicyclogermacrene	1487	38.20	8.40	1.50	15.06
α-Muurolene	1492	–	0.40	–	–
Valencene	1496	–	–	30.30	–
Germacrene A	1497	0.80	–	0.90	–
Pentadecane	1500	–	–	2.20	–
δ-Cadinene	1514	–	0.70	0.70	–
γ-Cadinene	1517	0.40	1.20	1.20	–
Farnesene	1548	–	–	1.50	–
Nerolidol	1561	–	0.70	–	–
Spathulenol	1568	1.10	28.00	2.00	18.88
Caryophyllene oxide	1572	–	6.90	1.50	3.81
Viridiflorol	1581	–	1.50	–	–
Hexadecane	1600	–	–	5.00	–
α-Cedrol	1607	6.10	–	–	–
δ-Cadinol	1637	–	0.70	–	–
Torreiol	1644	–	1.00	3.90	–
Total identified	98.00	95.00	89.50	90.46	
Total sesquiterpenes	66.30	81.90	67.80	80.69	
Total monoterpenes	9.60	13.10	13.80	9.77	
Total – others	22.10	–	7.90	–	

Table 2
Minimal inhibitory concentrations (MICs) of essential oil of *Lantana caatingensis.*

Bacterialstrains	*S. aureus* (ATCC 6538)	*E. coli* (ATCC 25922)	*S. aureus* (358)	*E. coli* (27)	*S. aureus* (ATCC 12692)	*P. aeruginosa* (ATCC 15442)
MIC (μg/mL)	≥1024	512	512	256	64	256

Table 3
MICs of the antibiotic in the absence and presence of essential oil of *L. caatingensis.*

Strains		Neomycin (MIC)	Amikacin (MIC)	Gentamycin (MIC)
S. aureus (12692)	Only antibiotics	64	32	128
	Antibiotics + OE (8 μg/mL)	64	8	512
E. coli (27)	Only antibiotics	16	64	64
	Antibiotics + OE (32 μg/mL)	8	32	64

spathulenol: δ 153.3 (C-10), 106.2 (C-15), 80.4 (C-4), 54.4 (C-5), 53.5 (C-1), 41.7 (C-3), 38.8 (C-9), 29.9 (C-6), 28.6 (C-12), 27.4 (C-7), 26.7 (C-2), 26.0 (C-14), 24.7 (C-8), 20.2 (C-11), 16.3 (C-13) and *E*-caryophyllene: δ151.6 (C-8), 122.3 (C-5), 112.7 (C-15), 53.4 (C-1), 48.7 (C-9), 39.7 (C-10), 39.1 (C-3), 33.9 (C-7), 30.1 (C-13), 29.9 (C-6), 28.6 (C-2), 22.6 (C-12), 16.3 (C-14). These data were used to confirm the structure proposed by MS (ionization by electron impact at 70 e V).

The β-caryophyllene is a sesquiterpene described in several essential oils responsible for strong smell and diverse biological activities such as anti-inflammatory (Fernandes et al., 2007), antiallergic (Ghelardini et al., 2001), local anesthetic (Costa et al., 2009), antifungal (Zheng et al., 1992) and anticarcinogenic (Chinou et al., 1996), besides being a major constituent of the genus *Lantana*.

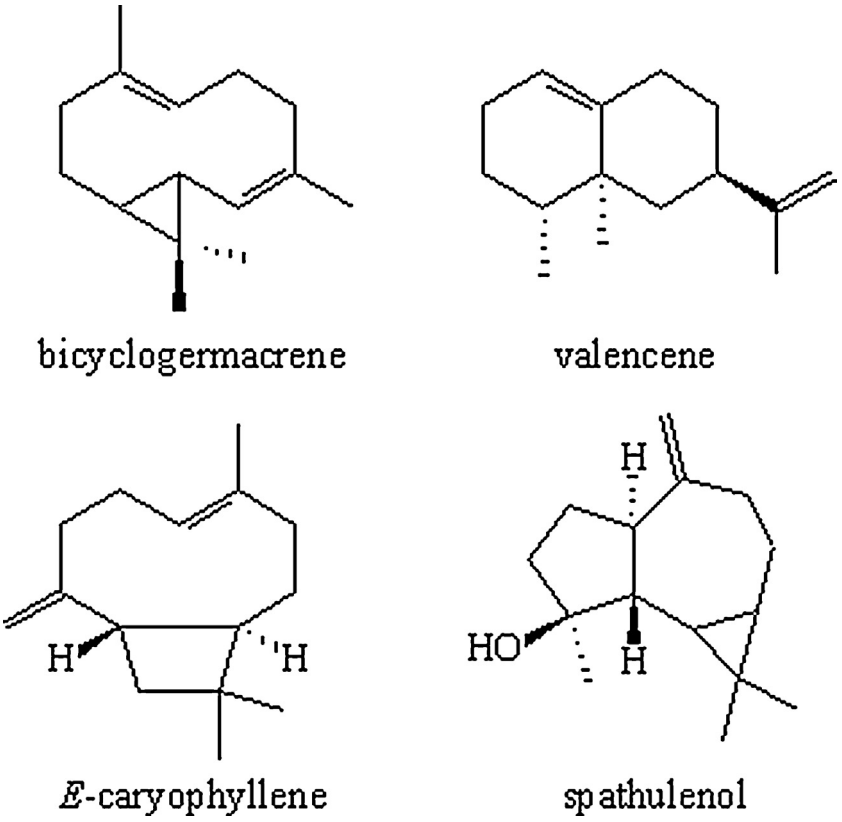

Fig. 2. The major constituents of *L. caatinguensis* oil.

The antimicrobial activity of *L. caatingensis* oil was evaluated by microdilution method separately and in association with aminoglycoside antibiotics*aureu*. The essential oil showed inhibitory activity for six strains analyzed, of which, five showed significant activity. From a clinical point of view, the essential oil of *L. caatingensis*, showed better results for the strain of *S. aureus* (ATCC 12,692) with a MIC of 64 mg/mL, followed by *E.* multiresistant *coli* (EC 27) with a MIC of 256 mg/mL and *P. aeruginosa* (ATCC 15,442) with MIC of 256/mL, as shown in (Table 2).

The results obtained in this study showed that the essential oil has a potential anti-bacterial activity, being capable of inhibiting the bacteria *S. aureus* with MIC values of 64 μg/mL, with activity similar to the antibiotic neomycin (64 μg/mL), inferior to the amikacin (32 μg/mL) and superior to gentamycin (128 μg/mL).

Table 3 shows the EOLc interference on aminoglycosides activities (MIC 1/8). Minimum Inhibitory Concentration reduction for the antibiotics used in this study was observed when LcEO was added to the growth medium.

The results of this trial indicate that the combination of the essential oil with aminoglycoside antibiotics against *S. aureus* (ATCC 12,692) showed significant synergism, with 75% reduction in the concentration to the MIC value of amikacin. In strain of *E. coli* (EC 27) with amikacin, synergism has also been observed with a reduction of 50% of the MIC and with neomycin 50% of the value. The data show the potential of essential oil of *L. caatingensis* antibacterial activities as well as modulatory actions of aminoglycoside antibiotics.

Essential oils have the ability to interfere with the antibacterial effect of antibiotics by means of an antagonistic or synergistic effect. According to Oliveira et al. (2008), the essential oil antimicrobial modifying effect is dependent on the antibiotic, essential oil tested and bacteria strain types analyzed. As evaluated by others authors (Barreto et al., 2014; Sousa et al., 2011; Oliveira et al., 2008) with regard to the modulatory effect, it can be attributed to an interaction of essential oil components in the plasma membrane, leading to an increase in the cell permeability of the aminoglycosides. Furthermore, this interaction could inhibit efflux systems dependent of proton-motive force, as LmrS protein, contributing to the increase in the intracellular levels of antibiotics and leading to an enhancement of the aminoglycoside activity against MRSA.

Mortality rates of larvae in the toxicity bioassay of *L. caatingensis* essential oil (sample of January 2011) on *A. salina* varied between 0 and 100%. The dose required to kill 50% of the larvae (LC_{50}) was calculated to be 62.5 μg/mL, indicating that the oil has activity considered significant by presenting LC_{50} less than 1000 μg/mL (Meyer et al., 1982). Costa et al. (2009) reported that the essential oil of the leaves from *L. camara and L.* sp. showed significant activity with LC_{50} values of 14 μg/mL and 24 μg/mL, respectively.

There is no unanimity on the MIC acceptable for natural products when compared with standard antibiotics, so much so that some authors consider only results similar to antibiotics, while others consider as a good potential even those with higher levels of inhibitions. Aligianis et al. (2001) proposed a classification for plant material on the bases of MIC results: considering as strong inhibition – MIC up to 500 μg/mL, moderate inhibition – MIC of between 600 and 1500 μg/mL and as a weak inhibition – MIC above 1600 μg/mL.

More significant results were observed against *S. aureus* and *E. coli* that are among the most prevalent species of gram-positive and gram-negative bacteria, respectively, that induce clinical mastitis. It is important to mention that *S. aureus* is a bacterium that is a member of the Firmicutes, and is frequently found in the human respiratory tract and on the skin. Although *S. aureus* is not always pathogenic, it is a common cause of skin infections (e.g., boils), respiratory disease (e.g. sinusitis), and food poisoning. The emergence of antibiotic-resistant forms of

pathogenic *S. aureus* (e.g. MRSA) is a worldwide problem in clinical medicine, so the search for alternative forms has been an option in recent years.

The results indicate that LcEO can be an alternative source of natural products with antibacterial action, being observed modulatory effects more promising than the antibiotics amikacin (against *S. aureus*), neomycin and amikacin (against *E. coli*).

4. Conclusion

Among the chemical constituents identified in the LcEO by GC–MS, we observed a high proportion of sesquiterpenes, being β-caryophyllene, spathulenol and bicyclogermanecrene the major compounds. The antimicrobial activity of LcEO was evaluated by microdilution method separately and in association with aminoglycoside antibiotics. The results indicated that LcEO can be an alternative source of natural products with antibacterial action, being observed modulatory effects which increased the activity of the antibiotics amikacin (against *S. aureus*), neomycin and amikacin (against *E. coli*).

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