

Full Length Research Paper

Antioxidant and antibacterial activities of essential oil of *Lippia sidoides* against drug-resistant *Staphylococcus aureus* from food

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Chemical composition, *in vitro* antioxidant and antibacterial activities of essential oil of *Lippia sidoides* (EOLS) and its major constituent was investigated. Chemical composition was analyzed in a gas chromatograph coupled to a mass spectrometer (GC/MS). Antioxidant activity (AA) was obtained using 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging assay. Antimicrobial activity was tested using disk-diffusion test against *Staphylococcus aureus* ATCC 25923 and nine *S. aureus* strains resistant to beta-lactams, tetracycline and quinolone. EOLS chemical composition revealed the presence of 15 components identified as monoterpenes hydrocarbon (1.56%), oxygenated monoterpenes (94.31%) and sesquiterpenes hydrocarbon (4.11%), and the major components were thymol (79.70%). All OELS concentrations analyzed showed AA varying from $19.09 \pm 2.088\%$ (100 $\mu\text{g/mL}$) to $74.32 \pm 1.61\%$ (3.200 $\mu\text{g/mL}$). For thymol, the most efficient AA ($70.11 \pm 3.43\%$) was found with higher concentration (6.400 $\mu\text{g/mL}$). All *S. aureus* strains ($n = 10$) were sensitive to OELS and thymol. When OELS was used, the halo size of the ATCC strain was 74.4 mm and for the resistant-strains, it ranged from 29 to 60.4 mm. Thymol bioactive was lower when compared with OELS: the ATCC strain showed a halo of 24.5 mm and a variation of 17.5 to 45 mm was observed between the halo sizes in resistant strains. The results suggest that OELS as compared to thymol has better potential for use as an antimicrobial and antioxidant agent.

Key words: Antimicrobials, *Lippia*, thymol, essential oil, 2,2-diphenylpicrylhydrazyl (DPPH), *Staphylococcus aureus*, oxygenated monoterpenes, antioxidant.

INTRODUCTION

The *Lippia sidoides*, a northeastern Brazil native plant, is utilized in popular medicine as a local antiseptic on the skin and mucosal tissues (Veras et al., 2012). Furthermore, other activities have been described:

antifungal (Morais et al., 2016), amoebic (Santos et al., 2016), antimalarial (Mota et al., 2012), leishmanicidal (Farias-Junior et al., 2012), larvicidal (Silva et al., 2014) and acaricidal (Gomes et al., 2014), characterizing this

phanerogam as a source of bioactive compounds.

The search for new phytochemicals in plants including *L. sidoides* has also been directed to the worldwide problem related to emergency drug-resistant bacteria. Reports on antibiotic resistant bacteria point to the *Staphylococcus aureus* species recognized as foodborne pathogen (Rasooly et al., 2017) and one of the most frequently isolated (Li et al., 2016; Ge et al., 2017). In this context, the search for new active substances to combat the drug-resistant *S. aureus* is a current need.

This study aimed to evaluate the chemical composition, *in vitro* antioxidant and antibacterial activities of essential oil of *L. sidoides* and its major constituent, against *S. aureus* ATCC 25923 and nine drug-resistant *S. aureus* strains isolated from food.

MATERIALS AND METHODS

The leaves of *L. sidoides* Cham. (Verbanaceae) were collected in May 2014, from the Garden of Medicinal Plants, Farm of Faculdades INTA, City of Cariré, Ceará, Brazil (3°49'51 82"S and 40°24' 37 85"W). The plant was classified and a voucher specimen was deposited in the Francisco José de Abreu Matos Herbarium of the State University of Acaraú Valley under the number: HUVA 17480.

EOLS extraction

The leaves (1 kg) were placed in a 5 L round bottom beaker with 2 L of distilled water and subjected to hydrodistillation in a Clevenger apparatus for 2 h. The oil obtained was dried with anhydrous sodium sulphate, filtered, and then maintained in glass bottles under refrigeration until analysis.

EOLS analysis (gas chromatography/mass spectrometry)

The chemical composition of the essential oil was analyzed in a gas chromatograph coupled to a mass spectrometer (GC/MS-Shimadzu QP-2010 Plus) equipped with a Factor Four/VF 5 ms fused-silica capillary column (30 m × 0.25 mm × 0.25 µm film thickness), using helium as carrier gas at 1 mL/min. The initial oven temperature was 35°C, which after being held constant for 2 min, was increased at a rate of 4°C/min to 180°C, followed by 10°C/min to 250°C, with a final isotherm (250°C) for 20 min. The sample injection volume was 1 µL (1:50 split mode). The injector and detector temperatures were both 250°C. The mass spectra were obtained in a range of 10 to 300 m/z by the electron impact technique at 70 eV.

Gas chromatography/flame ionization detector

Quantitative analysis of the essential oil and chemical composition was carried out in a gas chromatograph coupled to an HP 5890 Series II flame ionization detector (FID), using the same type of column as in the GC/MS analysis. The injector and detector

temperatures were 240 and 300°C, respectively. The percentage of each constituent was calculated by the integral area under its respective peak in relation to the total area of all the sample constituents.

Identification of EOLS constituents

The various chemical constituents of the essential oil were identified by visual comparisons of their mass spectra with those in the literature and spectra supplied by the equipment database (NIST08), as well as by comparison of the retention indices with those in the literature (Adams, 2007). A standard solution of n-alkanes (C8-C20) was injected under the same chromatographic conditions as the sample and used to obtain the retention indices as described by Van Den Dool and Kratz (1963).

2,2-Diphenylpicrylhydrazyl (DPPH) radical scavenging assay

The measurement of the DPPH radical scavenging activity was performed according to methodology described by Brand-Williams et al. (1995) with some modifications. DPPH is a commercially available stable free radical, which is purple in colour. The antioxidant molecules derived from medicinal plants, when incubated, react with DPPH and convert it to di-phenyl hydrazine which is yellow in colour. The discoloration degree of purple to yellow was measured at 517 nm using *Spectramax paradigm*® (Molecular Devices). Sample stock solutions of essential oil of *L. sidoides* (8.0 mg/mL) and thymol (8.0 mg/mL) were diluted to final concentrations of 6.400, 3.200, 1.600, 800, 400, 200 and 100 µg/mL. Eugenol at the same final concentrations was used as a positive control, distilled water in 3% Tween 80 was used as a negative control and an ethanol and methanol solution in a ratio of 1:1 was prepared and used as a blank. For experimental procedure, 150 µL of a 0.27 mM DPPH ethanol : methanol (1:1) solution was added to 50 µL of sample solutions of different concentrations in 96-well plates and were allowed to react for 30 min at room temperature. After 30 min, the absorbance values were measured at 517 nm and converted into the percentage antioxidant activity (AA) using the following formula: $AA (\%) = [(AB - AS) / AB] \times 100$, where AB means absorption of DPPH sample and AS means absorption of sample solution.

Origin of strains

S. aureus ATCC 25923 and nine drug-resistant *S. aureus* strains isolated from shrimp *Litopenaeus vannamei* were used: S14 (ciprofloxacin-resistant), S46 (penicillin-resistant), S82 (oxacilin-resistant), S86 (oxacilin-resistant), S16 (oxacilin and cefepime-resistant), S27 (oxacilin and cefepime-resistant), S83 (oxacilin and cefepime-resistant), S102 (oxacilin, tetracycline and ampicillin-resistant), S26 (oxacilin, penicillin, and ampicillin-resistant) and S81 (cefepime, ceftriaxone, oxacilin and ampicillin-resistant).

Agar disc-diffusion assay

The antimicrobial susceptibility test by disk diffusion was done according to standard of Clinical and Laboratory Standards

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Table 1. Percentage concentrations of the constituents of essential oil of *Lippia sidoides* (EOLS).

Component	CRR	LRR	RT _{CG/FID}	%
<i>p</i> -Cymene	1029	1024	11.745	0.36
Limonene	1033	1029	11.978	0.73
1,8-Cineole	1037	1031	12.165	0.83
γ -Terpinene	1062	1059	13.582	0.47
E-4-Thujanol	1075	1070	14.320	0.45
6,7-Epoxymyrcene	1093	1092	15.528	0.42
Linalool	1102	1096	16.075	0.96
Ipsdienol	1148	1145	18.788	1.30
Citronellal	1157	1153	19.403	0.36
3-Thujen-2-one	1177	1171	20.747	0.11
Terpinen-4-ol	1184	1177	21.269	0.98
Methyletherthymol	1234	1235	24.512	0.43
Carvone	1253	1243	25.780	8.77
Thymol	1302	1290	29.196	79.70
E-Caryophyllene	1422	1419	35.827	4.11
Total				99.98

CRR= Calculated retention rate; LRR = literature retention rate; RT = retention time in the GC-FID.

Institute (CLSI 2012), with modifications. These strains were activated in two bacterial samplings in BHI broth (Difco®) grown for 24 and 18 h, respectively, in an oven at 37°C and adjusted to a 10^6 to 10^8 CFU/mL concentration in 0.85% saline solution equivalent to 0.5 McFarland and then inoculated on Mueller-Hinton agar (Difco®). Sterile 6 mm diameter white discs (Laborclin) were soaked with 20 μ L of the tested substances and, after drying, were placed on plates containing Mueller-Hinton agar (Difco®) where bacteria were inoculated. Meropenem (30 μ g) was used as positive control and sterile distilled water was used as negative control. After 24 to 48 h of plate's incubation at $35 \pm 2^\circ\text{C}$ under aerobic conditions, the inhibition halos were measured.

Statistical analysis

All analyses were performed in triplicates and the results were presented as mean (%) $\pm$ standard error mean (S.E.M). Data were analyzed by the software, GraphPad Prism 5.0, ANOVA followed by Student-Newman Keuls as *post hoc* test. The values of $p < 0.05$ were considered statistically significant.

RESULTS

Table 1 shows EOLS chemical composition. Fifteen components representing 99.98% of the essential oil were determined. Three monoterpenes hydrocarbon (1.56%), 11 oxygenated monoterpenes (94.31%) and one sesquiterpene hydrocarbon (4.11%) were identified. The major components were thymol (79.70%), carvone (8.77%) and E-caryophyllene (4.11%), totalizing 92.58%.

All EOLS concentrations analyzed showed antioxidant activity (AA) varying from 19.09 ± 2.088 (100 $\mu\text{g/mL}$) to $74.32 \pm 1.61\%$ (3.200 $\mu\text{g/mL}$) as compared to the

negative control (0%), as shown in Figure 1A. No statistical difference was observed between concentrations of 3.200 ($74.32 \pm 1.61\%$) and 6.400 $\mu\text{g/mL}$ ($68.88 \pm 6.27\%$) meaning that the best antioxidant activity was visualized at the concentration of 3.200 $\mu\text{g/mL}$. Antioxidant activity decreased with decrease in concentrations to 200 $\mu\text{g/mL}$ ($26.84 \pm 1.028\%$), and there was no difference in the low concentration of 100 $\mu\text{g/mL}$ ($19.09 \pm 2.088\%$).

Figure 1B shows antioxidant activity of different concentrations of thymol. The best activity ($70.11 \pm 3.43\%$) was found with higher concentration (6.400 $\mu\text{g/mL}$) as compared to the negative control (0%). Despite the fact that other concentrations exhibit antioxidant activity, their action decreased considerably from 3.200 ($29.65 \pm 1.53\%$) to 400 $\mu\text{g/mL}$ ($15.65 \pm 3.88\%$). Concentrations of 200 and 100 $\mu\text{g/mL}$ did not present antioxidant effect as compared to the negative control.

Antioxidant activity for eugenol, as a positive control is shown in Figure 1C. As expected, eugenol showed antioxidant activity in all concentrations analyzed and there was no statistical difference between them, with maximal value of $100 \pm 0.46\%$ at concentration of 6.400 $\mu\text{g/mL}$.

All *S. aureus* strains ($n = 10$) were sensitive to EOLS and thymol. Figure 2A shows that there was a significant difference ($p < 0.05$) between the halo size of the ATCC strain (74.4 mm) and the halo size of the resistant strains (29 to 60.4 mm).

Thymol bioactive was lower when compared with EOLS (Figure 2B). The ATCC standard strain showed a halo of

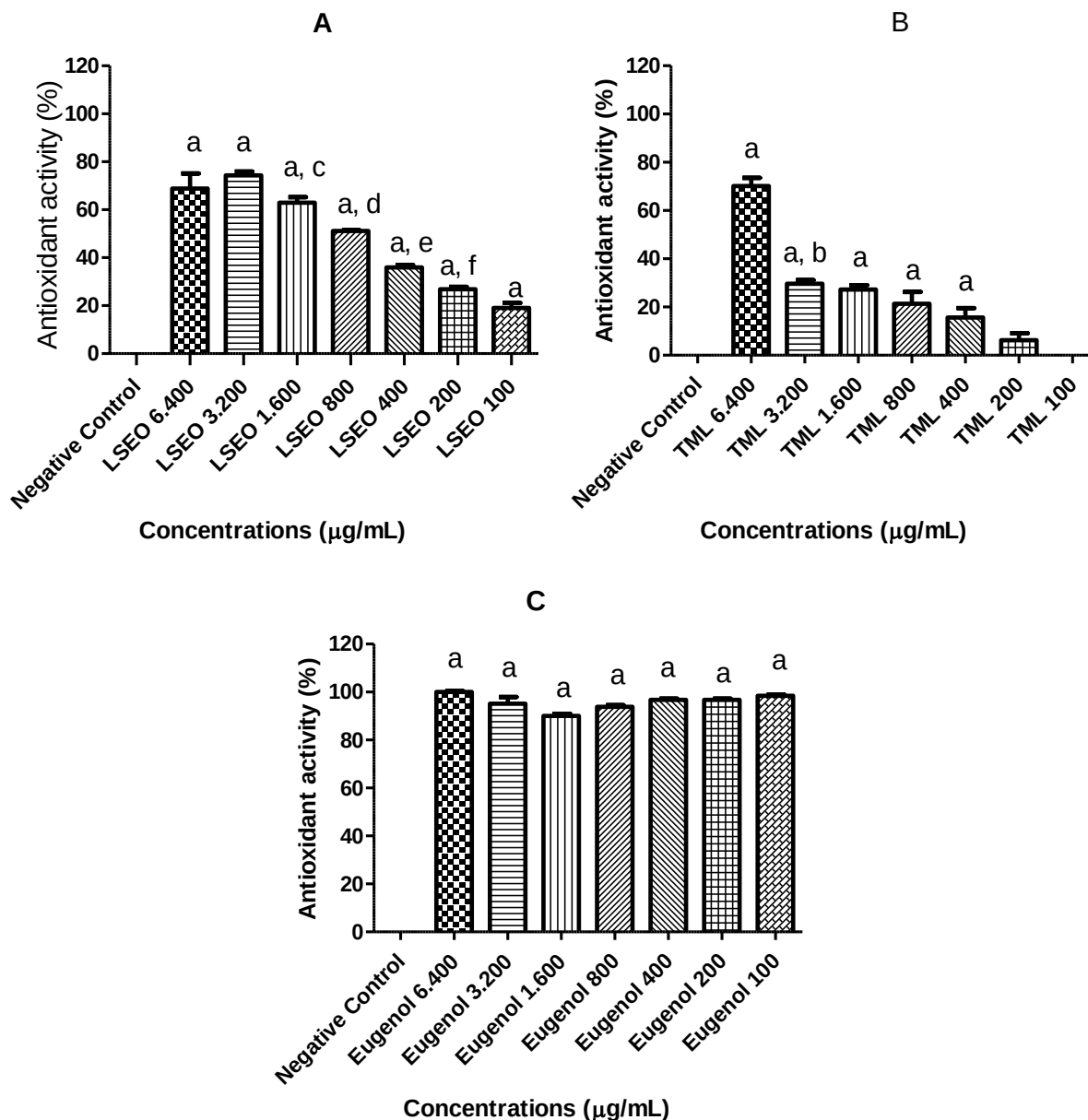


Figure 1. Percentage of antioxidant activity of essential oil of *Lippia sidoides* (EOLS), Thymol (TML) and Eugenol using DPPH radical scavenging assay. (a) Percentage of antioxidant activity of different concentrations of EOLS (10, 100, 200, 400, 800, 1.600, 3.200 and 6.400 µg/mL); (b) Percentage of antioxidant activity of different concentrations of TML (10, 100, 200, 400, 800, 1.600, 3.200 and 6.400 µg/mL); (c) Percentage of antioxidant activity of different concentrations of Eugenol (10, 100, 200, 400, 800, 1.600, 3.200 and 6.400 µg/mL). ANOVA followed by Student-Newman-Keuls as the *post hoc* test. ^ap<0.05 vs. negative control; ^bp<0.05 vs. 6.400; ^cp<0.05 vs. 3.200; ^dp<0.05 vs. 1.600 and ^ep<0.05 vs. 800

24.5 mm and a variation of 17.5 (S16) to 45 mm (S102) was observed between the halo sizes in resistant strains. Significant variation ($p < 0.05$) was observed between the halo size of the standard strain and the halos of eight of the resistant strains.

Antibacterial activity of meropenem, as a positive control, is shown in Figure 2C and all strains were sensitive to this carbapenem. 39.3 mm of halo was observed when ATCC strain was used and halos

ranging from 24.9 (S82) to 48.3 (S102) when tested resistant strains were used. Significant variation ($p < 0.05$) was observed between the halo size of the standard strain and the halos of all strains.

DISCUSSION

The occurrence of thymol (79.7%) as the major

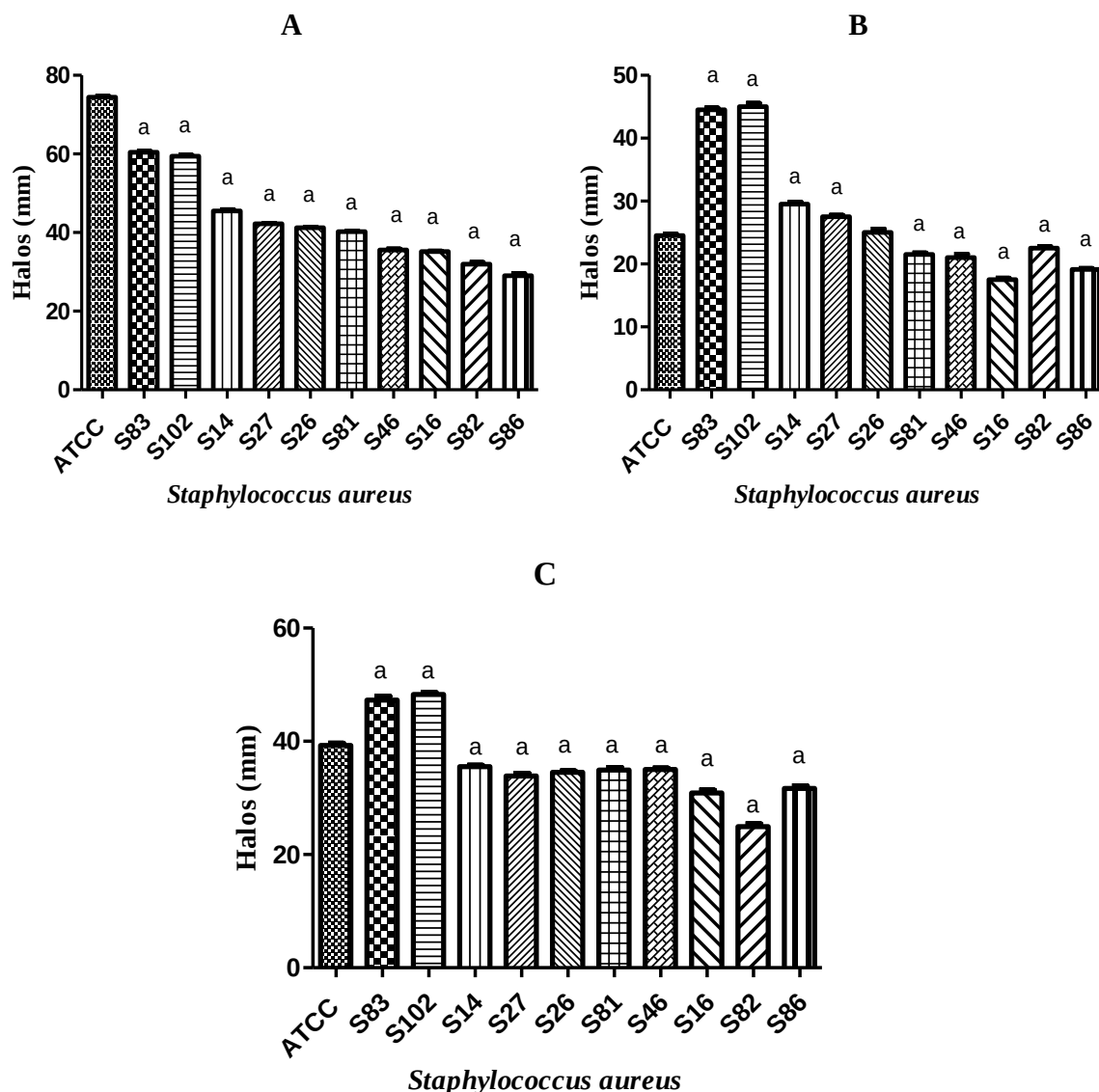


Figure 2. Antibacterial activity of essential oil *Lippia sidoides* (EOLS), Thymol (TML) and Meropenem using agar disc-diffusion assay. (a) Antibacterial activity of essential oil *L. sidoides* (EOLS) against *S. aureus* ATCC 25923 and *S. aureus* drug-resistant strains; (b) Antibacterial activity of Thymol against *S. aureus* ATCC 25923 and *S. aureus* drug-resistant strains; (c) Antibacterial activity of Meropenem against *S. aureus* ATCC 25923 and *S. aureus* drug-resistant strains. ANOVA followed by Student-Newman-Keuls as the *post hoc* test. ^a $p < 0.05$ vs. *S. aureus* ATCC 25923.

constituent of EOLS observed in the present study (Table 1) is in accordance with literature reports (Guimarães et al., 2015). In this context, thymol (2-isopropyl-5-methylphenol) is considered the main monoterpene phenol in essential oils isolated from plants belonging to the Verbenaceae family, including *L. sidoides* (Marchese et al., 2016).

Veras et al. (2012) evaluated the chemical composition of EOLS by the GC-MS analysis and revealed the following main constituents: thymol (84.9%) and p-cymene (5.33%). In addition, 12 compounds were characterized by Botelho et al. (2007) in EOLS, having

the major constituents: thymol (56.7%) and carvacrol (16.7%). Monoterpenes and sesquiterpenes were well represented in the chemical composition of EOLS characterized by Mota et al. (2012).

On the other hand, isoborneol, bornyl acetate, α -humulene, α -fenchene and 1,8-cineole have already been reported as the major components of the EOLS. This fact suggests the existence of two chemotypes of this species (Morais et al., 2012, 2016).

Difference in the chemical composition of EOLS (Table 1) with the essential oils described above may be related to the culture conditions of *L. sidoides* specimens.

For Castro et al. (2011), the chemical composition of essential oils may be influenced by climate, season, geographical conditions, harvest time and distillation technique. The antioxidant activity of species of the genus *Lippia* has been reported (Fabri et al., 2011). These authors reported that the AA of methanolic extracts of the *L. sidoides* leaves is related to the presence of flavonoids and coumarins.

In the present study, AA EOLS is not related only to the presence of thymol, since AA above 70% at concentrations of 6.400 and 3.200 of LSOE was verified (Figure 1A), and there was a drastic reduction of thymol AA in the concentration of 3.200 (29.65%) (Figure 1B). Thus, EOLS AA can be attributed to the presence of thymol and other polar phenolic compounds (Damasceno et al., 2011). Due to the antioxidant activity of naturally occurring substances in plants, attention on the protective activity of these natural antioxidants against chronic disorders caused by oxidative process has increased (Garcia et al., 2012).

The results of the present study also indicated bacterial activity (Figure 2). It is important to note that inhibition halos for EOLS were higher than those formed by thymol for all tested strains (Figure 2A and B), showing that the EOLS (mixture of 15 substances) may present different mechanisms of action with a substance potentiating the action of another. The consequence of this possible event is a greater activity of the essential oil as compared to the activity of its component individually tested. In accordance with the current data, the microbicidal action of essential oil of *L. sidoides* against *S. aureus* isolated from food (homemade cheese) was reported (Castro et al., 2011). This fact highlights the potential of this essential oil against foodborne pathogens.

In the present study, EOLS was able to inhibit the growth of all drug-resistant strains (Figure 1A). Oliveira et al. (2006) observed the action of EOLS against *S. aureus* from clinical material resistant to beta-lactams, aminoglycosides, macrolides, lincosamides and quinolones, with inhibition halos varying from 15 to 21 mm. EOLS was more efficient than meropenem (positive control) when the size of the inhibition halos was observed (Figure 2A and C). This data is important and reveals the potential of EOLS, since meropenem is considered as a carbapenem, which is a drug commonly used and relatively resistant to hydrolysis by most β -lactamases, target penicillin-binding proteins, and generally have broad-spectrum antibacterial effect (El-Gamal et al., 2017).

The major constituent of EOLS, thymol, also had an effect on the ATCC and drug-resistant strains (Figure 2B). The effect of safe plant metabolites, thymol, on regulation of human pathogenic growth has been reported (Gutierrez et al., 2017). As in the current study, thymol was bioactive against *S. aureus* isolated from dairy and meat products, which would be of interest for use in the food industry (Rúa et al., 2011).

Thymol is a natural ingredient used as flavor or preservative agent in food products. This monoterpene, in higher concentrations, disrupted *S. aureus* cell membrane integrity and reduced cell viability. Furthermore, it induced a mild destabilization in the DNA secondary structure. Thus, antibacterial effect of thymol is related to destruction of bacterial cell membrane and binding directly to genomic DNA (Wang et al., 2017).

The data show a promising effect of thymol on drug-resistant strains (Figure 2B). Miladi et al. (2016) reported that thymol may serve as potential source of efflux pump inhibitor in food-borne pathogens (Miladi et al., 2016). Plant-derived antibacterial compounds may be of value as a novel means for controlling antibiotic-resistant bacteria in food animals and their products. It has been reported that thymol is effective against *S. aureus* blaZ and is able to reduce the minimum inhibitory concentration of ampicillin, penicillin and bacitracin (Palaniappan and Holley, 2010).

Conclusion

The results of this study suggest that EOLS, as compared to thymol, presents better potential for use as an antimicrobial and antioxidant agent. In addition, this essential oil has a promising antimicrobial effect against beta-lactam, tetracycline and ciprofloxacin-resistant *S. aureus*, making it of interest for application as a preservative in the food industry.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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