Antimicrobial activity of *Lippia sidoides* Cham. (Verbenaceae) essential oil against *Staphylococcus aureus* and *Escherichia coli*

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RESUMO: Atividade antimicrobiana do óleo essencial de *Lippia sidoides* Cham. (Verbenaceae) contra *Staphylococcus aureus* e *Escherichia coli*. O efeito antibacteriano do óleo essencial da *Lippia sidoides* (alecrim-pimenta) foi testado contra as bactérias *Staphylococcus aureus* e *Escherichia coli* isoladas de queijo minas artesanal, produzido no Brasil. A Concentração Inibitória Mínima (CIM) determinada por Teste de Diluição foi de 13 μL de óleo mL^{-1} para as duas bactérias, o que caracteriza ação inibitória em caldo durante vinte quatro horas de interação. A Concentração Bactericida Mínima (CBM) determinada através de Teste de Suspensão, com um minuto de contato, foi de 25 μL de óleo mL^{-1} para ambas as bactérias testadas, obtendo-se a esta concentração o efeito bactericida de 99,9% sobre as células bacterianas viáveis em cada uma das amostras. Os resultados demonstraram atividade bactericida do óleo essencial de *Lippia sidoides* contra *S. aureus* e *E. coli*, sugerindo a possibilidade do uso como agente antibacteriano em alimentos.

Palavras-chave: agente antibacteriano, anti-séptico, bactérias contaminantes, *Lippia sidoides*, plantas medicinais

ABSTRACT: The antibacterial effect of *Lippia sidoides* (rosemary pepper) essential oil was tested against the bacteria *Staphylococcus aureus* and *Escherichia coli* isolated from homemade Minas cheese produced in Brazil. The Minimum Inhibitory Concentration (MIC) determined in the Dilution Test was 13 μL oil mL^{-1} for both bacteria, which characterizes inhibitory action in broth for a 24-hour interaction period. The Minimum Bactericidal Concentration (MBC) determined in the Suspension Test, with one minute of contact, was 25 μL oil mL^{-1} for both tested bacteria, obtaining at this concentration a bactericidal effect of 99.9% on the viable bacterial cells from each sample. Results demonstrated the bacterial activity of *Lippia sidoides* essential oil against *S. aureus* and *E. coli*, suggesting its use as an antibacterial agent in foods.

Keywords: antibacterial agent, antiseptics, bacterial contaminants, *Lippia sidoides*, medicinal plants

INTRODUCTION

The industry has tried to reduce the use of chemical preservatives due to the increasing legal demands from law-enforcement agencies and the health risk for consumers. Several plants have been evaluated and presented promising results as preservatives and preventers of pathogenic microorganism growth in food, including *Salmonella enteritidis* and *Staphylococcus aureus* (Tassou et al., 2000).

The utilization of natural antibacterial products is a promising alternative for the control of pathogenic bacteria in food, reducing sanitary risks and economic losses, besides prolonging shelf life. Various studies have already shown that essential oils extracted from medicinal plants may act as antimicrobial agents (Dorman & Deans, 2000).

The antimicrobial activity of essential oils from medicinal plants is performed by a group of terpenoids and phenols. This property is associated with their lipolytic characteristic, causing accumulation on the cell membrane and injuries associated with energy depletion (Oussalah et al., 2006).

Antimicrobial activity may be related to the composition and structural configuration of the plant

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volatile oils, their functional groups and the possibility of synergism among their compounds. Great differences among the results for oils obtained from the same plant have already been reported. This variation is associated with geographical variability, genotype, climate and laboratory methods utilized to extract the oil. These factors may interfere in the chemical composition and proportion of individual compounds of the plant essential oil (Oussalah et al., 2006).

Essential oils from plants have antimicrobial activity against a large number of bacteria, including strains resistant to antibiotics and antifungals (Bertini et al., 2005). Souza et al. (2007) reported that *Lippia sidoides* (Cham.) leaves have up to 4% essential oil content, especially two phenolic terpenoids, with strong antimicrobial activity. The oil has about 60% thymol or a thymol mixture and carvacrol. In addition, the oil content may vary according to the harvest time, location, temperature and other environmental factors.

This study aimed to evaluate the antimicrobial activity and antiseptic potential of *Lippia sidoides* (Cham.) essential oil in foodborne samples of *Staphylococcus aureus* and *Escherichia coli* isolated from homemade Minas cheese.

MATERIAL AND METHOD

Thirty *Staphylococcus aureus* and thirty *Escherichia coli* isolates, obtained from 72 samples of homemade Minas cheese, were studied. The hydrodistillation technique with Clevenger-type apparatus was used to obtain *Lippia sidoides* (Cham.) essential oil. The extracted oil was stored in sterile dark flasks to maintain its phytochemical properties.

To standardize the quantitative and qualitative aspects of the essential oil, the used plants were collected from the Medicinal Garden of the Institute of Agrarian Sciences, Federal University of Minas Gerais (16º40' 50.92''S, 43º50'22.36''W), in the same season. The plants were also in the same phenological stage and had biophysical characteristics as suggested by Simões (2000).

To determine the Minimum Inhibitory Concentration (MIC), macrodillution was employed to verify the sample sensitivity (Brasil, 2003b). *Lippia sidoides* (Cham.) essential oil was diluted in the emulsifying agent DMSO (dimethyl sulfoxide) to obtain the concentrations 150, 75, 37.5, 18.75 and 9.37 μL essential oil mL^{-1} (Oliveira et al., 2006).

S. aureus and *E. coli* isolates were inoculated into brain heart infusion broth (BHI) and incubated for 24 hours at 37ºC. After the growth of microorganisms, inocula were prepared in 0.85% sterile saline solution (0.5 McFarland scale) and standardized to 6 Log10 CFU mL^{-1}. Aliquots of 200 μL from each inoculum were added to tubes containing 3 mL of sterile BHI. The tubes containing the inocula and BHI were added of up to 200 μL essential oil, resulting in the concentrations of 50, 25, 13 and 3 μL essential oil per mL. The tubes were then incubated at 37ºC for 24 hours.

The tests were conducted in triplicate. The results were compared with three controls: negative control with microbial inoculation and addition of crude oil; positive control with microbial inoculation without addition of essential oil; and control of solvent with microbial inoculation without addition of essential oil, yet containing solvent solution composed of DMSO plus sterile distilled water.

The Minimum Inhibitory Concentration (MIC), which is the smallest concentration of essential oil capable of inhibiting the growth of the tested microorganisms, was assessed according to Oussalah et al. (2006). Standard types of *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25753 were employed (Brasil, 2003b). The Minimum Bactericidal Concentration (MBC) was determined according to the agar plating technique for counting all the oil dilutions that did not present growth for MIC. The plates were incubated at 37ºC for 24 hours. Results were expressed as CFU mL^{-1}.

The disk diffusion technique (Oliveira et al., 2006) was used to determine the sensitivity of bacteria tested for MIC and MBC of *Lippia sidoides* essential oil. Sterile filter paper disks measuring 6mm diameter, saturated with 20 μL crude oil and oil in each of the dilutions, were prepared and dried in a greenhouse.

The diffusion test was conducted by scattering on Mueller Hinton plate count agar; inocula were standardized to 0.5 McFarland scale, corresponding to 10^6 CFU mL^{-1} (Brasil, 2003a). The samples were compared to the negative and positive controls and the solvent. After incubation at 37ºC for 18 hours, the size (mm) of the inhibition halo was determined.

Based on MBC results, the antiseptic activity of *Lippia sidoides* essential oil was evaluated by using the methodology described by Bloomfield & Looney (1992). According to this author, to assure a satisfactory antimicrobial activity, the product should present a microbicidal effect (ME) higher than or equal to 5 in suspension tests after a contact period of five minutes. ME was calculated by using the following formula: ME = logNc – logNo, where Nc is the number of CFU mL^{-1} of the control series and No is the number of CFU mL^{-1} of the series tested for antiseptics.

To conduct the tests, BHI received a 200 μL aliquot of the bacterial cultures standardized to 10^6 CFU mL^{-1} in saline solution. Subsequently, 200 μL of the dilution of essential oil corresponding to the concentration obtained in the MBC (25 μL oil mL^{-1}) was added. An aliquot was taken from this mixture and added to 0.85% sterile saline solution, maintaining a proportion of 1:10 in the final solution. Then, the

cultures were diluted in 0.85% sterile solution, at the proportion of 1:10, varying from 10^{-1} to 10^{-6} and incubated at 37ºC for 1 and 5 minutes, using three controls, as described for MIC and MBC.

A 100 μL aliquot of each dilution was inoculated by scattering on plate count agar (PCA). The plates were incubated for 24 hours at 37ºC. After the incubation period, the number of CFU was determined. Bloofmfield & Looney (1992) recommend that the antiseptic activity of the essential oil be analyzed according to the incubation time.

The results were statistically analyzed by randomization, 2 X 5 factorial arrangement, and were subjected to analysis of variance and Tukey's test at 5% significance.

RESULT AND DISCUSSION

MIC was 13 μL essential oil mL^{-1} for all tested bacteria (Table 1). The essential of *Lippia sidoides* presented a bactericidal effect against *S. aureus* and *E. coli* at the concentration of 25 μL oil mL^{-1} (Table 2).

Several studies have determined the MIC of essential oils in order to establish the balance between acceptability and antimicrobial efficiency (Nascimento et al., 2007). Using the microdilution technique, Oliveira et al. (2006) obtained a MIC of 0.4 μL mL^{-1} for *Lippia sidoides* essential oil against *S. aureus*. Dorman & Deans (2000) stated that MIC is difficult to determine due to the use of different methodologies and bacterial isolates from diverse materials, impairing comparisons.

The chemical composition of essential oils may be influenced by climate, season, geographical conditions, harvest time and distillation technique. Studies done by Bertini et al. (2005) demonstrated that *Lippia sidoides* oil has bactericidal effect against *S. aureus* and *E. coli* at concentrations inferior to 5%, as shown in this study.

Gram-positive and gram-negative bacteria present differences in the cell wall structure and cell membrane permeability (Alarcón, 2007). In this study, however, the obtained MIC was the same for both gram-positive and gram-negative bacteria, proving thus the efficacy of *Lippia sidoides* essential oil against both bacterial agents. The results showing the inhibition halo size in the test of microbial sensitivity in disk are presented in Table 3.

According to MIC determination in the disk, the size of inhibition halos differed between *E. coli* (7 mm) and *S. aureus* (8 mm), which was also observed for MBC, with halos of 10 mm for *E. coli* and 11-12 mm for *S. aureus*. The disks impregnated with crude oil formed inhibition halos of 23 mm and 26 mm for *E.*

TABLE 1. Minimum inhibitory concentration (MIC) of *Lippia sidoides* (Cham.) essential oil against *Escherichia coli* and *Staphylococcus aureus,* according to the macrodilution method (tube) in broth.

Bacteria	Essential oil concentrations (μL mL^{-1})							
	50	25	13	6	3	Positive Control	Solvent Control	Negative Control
S. aureus (Pool of 30 samples)	-*	-	-	+**	+	+	+	-
E. coli (Pool of 30 samples)	-	-	-	+	+	+	+	-
S. aureus ATCC 29213	-	-	-	-	-	+	+	-
E. coli ATCC 25753	-	-	-	-	-	+	+	-

Reading in the test tube. * Absence of microbial growth ** Presence of microbial growth.

TABLE 2. Minimum Bactericidal Concentration (MBC) against *Staphylococcus aureus* and *Escherichia coli*.

Bacteria	Microbial Growth					
	50 μL mL^{-1}	25 μL mL^{-1}	13 μL mL^{-1}	Positive Control	Solvent Control	Negative Control
S. aureus	-*	-	+**	+	+	-
E. coli	-	-	+	+	+	-

* Absence of microbial growth ** Presence of microbial growth

TABLE 3. Sensitivity of *Staphylococcus aureus* and *Escherichia coli* in the test with disks containing *Lippia sidoides* Cham. essential oil.

Bacteria	Concentrations		
	Crude Oil	MBC ([log_{10}]25 µL oil mL^{-1})	MIC ([log_{10}]13 µL oil mL^{-1})
S. aureus	0,822 bA	-0,039 bB	-0,303 aC
E. coli	0,935 aA	0,122 aB	-0,298 aC

Averages followed by different uppercase letters in the lines and lowercase letters in the columns differ significantly according to Tukey's test ($p<0.05$).

coli and *S. aureus*, respectively. Gram-negative bacteria have a less rigid and chemically less complex cell wall than gram-positive ones, besides presenting higher lipid content (Vargas et al., 2004). This may be related to the stronger resistance of *E. coli* to *Lippia sidoides* essential oil, forming smaller inhibition halos compared to *S. aureus*.

Although the recommended contact period between the bacterial cell and the antiseptic product is 5 minutes, the experiment showed that one minute of contact with rosemary pepper essential oil at the concentration of 25 µL mL^{-1} was enough to obtain a microbicidal effect for both tested bacteria.

The microbicidal action of *Lippia sidoides* essential oil was efficient against all tested bacteria. There was no significant difference in the microbicidal effect of *Lippia sidoides* oil on the tested bacteria. These results suggest that *Lippia sidoides* oil is efficient against the bacteria *Staphylococcus aureus* and *Escherichia coli* isolated from homemade cheese.

In food, essential oils may migrate to the lipid segment, reducing thus their concentration on the aqueous phase and consequently their contact with microorganisms located in the hydrophilic parts of the food. These interferences require higher concentrations of essential oil for a good antiseptic activity in food. In addition, the functional components of essential oils can also generate undesired flavors and unpleasant smells and modify the food texture (Alarcón, 2007).

Brazilian laws have established that food industries must have products directed exclusively for sanitation/disinfection of surfaces and equipments that enter in contact with food; these products must also be highly efficient to control microorganisms such as *S. aureus* and *E. coli* (Brasil, 2002). The use of *Lippia sidoides* essential oil as a component in sanitizing products for food industry may be an efficient and economical alternative for the cleaning of equipment and utensil surfaces.

An alternative to reduce the loss of *Lippia sidoides* sanitary properties during processing, storage and commercialization would be the use of encapsulation technologies combined with bioactive and biodegradable packing, so that the active components could be released during consumption. In these packs, bioactive components are fixed in a biodegradable matrix which releases the components through the food humidity (Castro et al., 2001).

The results of this study showed that *Lippia sidoides* essential oil presents good antiseptic properties against the tested microorganisms. In addition, other experiments may be conducted to test the effect of lower concentrations in longer contact with microbial cells. Studies may also be proposed to determine better strategies to make the use of *Lippia sidoides* essential oil as antiseptic in the food industry more technologically and economically viable.

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