Minimum inhibitory concentration of essential oils against *Staphylococcus aureus* isolated from dogs with external otitis

Concentração inibitória mínima de óleos essenciais contra *Staphylococcus aureus* isolados de cães com otite externa

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Highlights

The essential oils showed high antimicrobial action against *Staphylococcus aureus.*
Cinnamomum cassia and *Cymbopogon citratus* showed homogenous MIC for all isolates.
The essential oils tested have potential for therapeutic use in canine otitis.

Abstract

Canine otitis externa is a disease that affects the external acoustic meatus of about 20% of dogs at some point in life, without predilection for race, age, or sex. It is a multifactorial disease whose etiology involves several microorganisms, detaching *Staphylococcus aureus.* Antimicrobials are the basis for treating this illness; however, due to the increase in antimicrobial resistance, conventional drugs have become ineffective, requiring the search for alternative therapies. In this context, essential oils (EOs) have great therapeutic potential due to their broad antimicrobial action. This study aimed to evaluate the minimum inhibitory concentration (MIC) in order to measure the MIC50 and MIC90 of gentamicin and EOs of *Eugenia caryophyllata, Thymus vulgaris, Cymbopogon winterianus, Cymbopogon citratus,* and *Cinnamomum cassia* against 62 *Staphylococcus aureus* strains isolated from the external acoustic meatus of dogs. All EOs showed antibacterial action against the studied microorganisms, and their MIC50 and MIC90 were as follows: Eugenia caryophyllata, 2.42 mg mL^{-1} and 7.25 mg mL^{-1}; *Thymus vulgaris,* 9.51 mg mL^{-1} and 22.94 mg mL^{-1}; *Cymbopogon winterianus,* 26.78 mg mL^{-1} and 157.79 mg mL^{-1}; *Cinnamomum cassia* and *Cymbopogon citratus,* lower than 16.48 and 27.81 mg mL^{-1}, with the same MIC for all isolates. The MIC50 and MIC90 found for gentamicin were 1μg mL^{-1} and 8 μg mL^{-1}. The MIC range found to antibiotic in this assay was 0.5

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Semina: Ciênc. Agrár. Londrina, v. 42, n. 6, suplemento 2, p. 3837-3854, 2021

3837

μg mL⁻¹ to 128 μg mL⁻¹, and the isolates were classified as susceptible [48 strains (77.41%) - MIC range of 0.5-4.0 μg mL⁻¹], intermediate [eight strains (12.90%) - (MIC = 8.0 μg mL⁻¹], or resistant [six strains (9.68%) - MIC ≥ 16 μg mL⁻¹]. The results, according to the *in vitro* assays, showed that resistance to gentamicin, one of the antimicrobials most commonly used to treat canine otitis, is present in the *Staphylococcus aureus* population evaluated. Additionally, the tested EOs have great potential for therapeutic use, however future studies should be carried out to evaluate their in vivo efficacy.

Key words: Phytotherapy. Otopathies. Antibacterials. Natural antimicrobials. MIC.

Resumo

A otite canina externa é uma doença que afeta o meato acústico externo de até 20% dos cães em algum período de suas vidas, sem predileção por raça, idade ou sexo. Trata-se de uma doença multifatorial que tem *Staphylococcus aureus* como um dos principais agentes etiológicos. Antimicrobianos constituem a base para o tratamento desta enfermidade, entretanto, devido ao incremento da resistência antimicrobiana, os fármacos convencionais tem se tornado pouco eficazes, o que requer a busca por terapias alternativas. Neste contexto, os óleos essenciais (OE) apresentam grande potencial terapêutico devido a sua ampla ação antimicrobiana. Este trabalho visou detectar a concentração inibitória mínima (CIM), a partir da qual foram obtidos os índices de MIC50 e MIC90 para a gentamicina e para os OE de *Eugenia caryophyllata, Thymus vulgaris, Cymbopogon winterianus, Cymbopogon citratus* e *Cinnamomum cassi*a contra 62 *Staphylococcus aureus* isolados do meato acústico externo de cães otopatas. Todos os OE mostraram ação antibacteriana contra os microrganismos estudados e foram detectadas as seguintes CIM50 e CIM90: *Eugenia caryophyllata,* 2,42 mg mL⁻¹ e 7,45 mg mL⁻¹; *Thymus vulgaris,* 9,51 mg mL⁻¹ e 22,94 mg mL⁻¹; *Cymbopogon winterianus,* 26,78 mg mL⁻¹ e 157,79 mg mL⁻¹; *Cinnamomum cassia* e *Cymbopogon citratus* com CIM menores que 16,48 e 27,81 mg mL⁻¹, respectivamente já que estes apresentaram CIM única para todos os isolados testados. Para a gentamicina, foram obtidas CIM50 e CIM90 1μg mL⁻¹ e 8 μg/m, respectivamente. Ademais, a faixa de CIM encontrada para o antibiótico variou de 0,5 a 128 μg mL⁻¹, e os isolados foram classificados em susceptíveis [48 isolados (77,41%) - CIM na faixa de 0,5-4 μg mL⁻¹], intermediários [8 isolados (12,90%) - (CIM = 8μg mL⁻¹] e resistentes [6 isolados (9,68%) - CIM ≥16]. Os resultados encontrados mostraram que os micro-organismos testados tem potencial para desenvolver resistência antimicrobiana a terapêutica tradicional e que os OE, de acordo com os resultados *in vitro,* apresentam potencial de uso terapêutico, entretanto futuros estudos devem ser realizados para avaliar a eficácia dos OE *in vivo.*

Palavras-chave: Fitoterapia. Otopatias. Antibacterianos. Antimicrobianos naturais. CIM.

Introduction

Otitis externa is one of the major diseases that affect the canine vestibulocochlear system, with 5 to 27% of animals showing clinical signs at some point in life. However, this frequency varies according to the geographic region (Griffin & Aniya, 2017; Kaimio, Saijonmaa-Koulumies, & Laitinen-Vapaavuori, 2017). The causative factors of otitis have been classified as predisposing, primary, and perpetuating (Sykes, Nagle, & White, 2014). Primary factors are the etiologic or primary cause of disease, while predisposing factors are anatomic or traumas regardless of sex or age, such as dogs with pendulous

3838

Semina: Ciênc. Agrár. Londrina, v. 42, n. 6, suplemento 2, p. 3837-3854, 2021

pinnae (Das, Pradhan, & Behera, 2016; Sykes et al., 2014). Other predisposing factors associated with the disease are anatomic anomalies (auricular hypertrichosis, long or stenotic ear canals), excessive moisture (swimming, humidity), excessive cerumen production, and obstructive formations such as neoplasia or polyps. The factors classified as perpetuating are stenosis, dermal edema, fibrosis, mineralization, osteomyelitis, pH alteration, and secondary infections (Sykes et al., 2014).

Otitis externa is an inflammation of the ear canal. It has multifactorial causes such as trauma, ear canal neoplasia, foreign bodies, parasites, excessive cerumen production, and microorganisms (Penna, Varges, Martins, Martins, & Lilenbaum, 2010; Oliveira et al., 2012; Sykes et al., 2014). Among those, the yeast *Malassezia pachydermatis* and the bacteria *Staphylococcus pseudointermedius, S. aureus, Pseudomonas* spp., *Proteus* spp., *Escherichia coli,* and *Klebsiella* spp. are relevant to the development of this illness. In most cases, the disease is caused by the association of different pathogens (Malayeri, Jamshidi, & Zahraei, 2010; Petrov et al., 2013; Varshney, 2016; Ebani et al., 2017; Gheller, Meirelles, Figueria, & Holsbach, 2017). The major clinical signs shown by dogs are ear pain, scratching, and head shaking, also showing a putrid odor related to exudate and behavioral alterations, irritability, and aggressiveness (Sykes et al., 2014). Erythema, tumefaction, swelling, dermatitis, otorrhea, and alopecia in the ear region are other common symptoms (Hnilica, 2012; Das et al., 2016).

The treatment of otitis externa usually requires antibiotic therapy. In this context, several commercial products used in Brazil have gentamicin in their composition.

However, resistance to conventional drugs and the emergence of multidrug-resistant isolates (MDR) has increased in recent years (Blair, Webber, Baylay, Ogbolu, & Piddock, 2014; Scherer et al., 2018). Therefore, the search for alternative treatments is necessary to fight against resistant microorganisms associated with bacterial diseases. In this circumstance, essential oils (EOs) have great therapeutic potential. They are natural plant compounds yielded as complex secondary metabolites, volatile, and endowed with a strong odor (O'Bryan, Pendleton, Crandall, & Ricke, 2015). Essential oils are usually composed of a mixture of 20 to 60 substances in different amounts and are divided into two groups: terpenes/terpenoids and aromatic and aliphatic compounds (Miguel, 2010; Swamy, Akhtar, & Sinniah, 2016). The main compounds of EOs reflect their physical, chemical, and biological features, and their effects are dependent on the concentration and whether they are used alone or associated with other antimicrobials. In addition, they may also have antimicrobial action against viruses, fungi, and bacteria (Costa et al., 2005; Edris, 2007; Reichling, Schnitzler, Suschke, & Saller, 2009; Nazzaro, Fratianni, Martino, Coppola, & De Feo, 2013; Swamy et al., 2016; Orchard, & Van Vuuren, 2017; Córdoba, Vivot, Szusz, & Albo, 2019).

The strong antimicrobial action of EOs highlighted by several studies suggests the perspective of their use as an alternative to conventional drugs or even in association with them as synergistic agents to treat several infectious diseases, including canine otitis. Therefore, the present study aimed to determine the *in vitro* MIC the EOs of *Eugenia caryophyllata* (clove of India), *Thymus vulgaris* (Thyme), *Cymbopogon winterianus* (Citronella),

Cymbopogon citratus (Lemongrass), and *Cinnamomum cassia* (Cassia cinnamon) and the antibiotic gentamicin against *Staphylococcus aureus* strains isolated from dogs affected by otitis externa.

Material and Methods

Essential oils and antibiotic evaluated

The EOs were produced and traded by FERQUIMA®. They were diluted from 1:20 to 1:640 for the MIC assays, and the concentration in mg mL^{-1} was determined according to the composition specified for each oil by the manufacturer.

The essential oils were evaluated in the following dilution ranges: *Eugenia caryophyllata:* 775 mg mL^{-1} to 1.21 mg mL^{-1}; *Thymus vulgaris*: 460 mg mL^{-1} to 7.18 mg mL^{-1}; *Cymbopogon winterianus*: 442 mg mL^{-1} to 6.91 mg mL^{-1}; *Cymbopogon citratus*: 890 mg mL^{-1} to 8.24 mg mL^{-1}; and *Cinnamomum cassia*: 527.5 mg mL^{-1} to 13.9 mg mL^{-1}. Additionally, the strains were subjected to MIC assays with the aminoglycoside gentamicin ranging from 2 µg mL^{-1} to 16 µg mL^{-1}, as established by the Clinical and Laboratory Standards Institute [CLSI] (2018).

Bacterial strains

Sixty-two (62) *Staphylococcus aureus* strains belonging to the Bacterial Culture Collection of the Laboratory of Veterinary Microbiology of the Federal University of Lavras (UFLA) were used in this study. These strains were isolated from the auricular secretion of dogs with otitis externa and subjected to microbiological analysis in the laboratory from 2010 to 2015. The strains were previously identified by phenotypic tests according to Koneman, Allen, & Janda (2008) and maintained at -70 ºC in brain heart infusion (BHI) (HIMEDIA®) supplemented with 15% glycerol (MERCK®) until their use.

Determination of the Minimum Inhibitory Concentration (MIC)

The MIC of the EOs was evaluated by the microdilution method in microplates containing Muller Hinton Broth (MHB) (HIMEDIA®), using the methodology adapted from the CLSI (2018). For inoculum preparation, the isolates were thawed and incubated in MHB at 37ºC/18-24h. The bacterial concentration was adjusted in sterile saline solution according to the 0.5 McFarland standard (1.5 x 10^8 UFC mL^{-1}). The EOs were diluted to the concentrations mentioned above using dimethyl sulfoxide (DMSO)-MHB and added to the microplate wells in a serial dilution (1:20 -1:640). After dilutions, 10 µL of bacterial inoculum was added to the wells, the plates were incubated at 37 ºC for 18-24h. The quality controls of the medium (wells with MHB and without inoculum) and the strain growth wells (containing MHB and DMSO) (MERCK)® (1:20), with bacteria and without antimicrobial agents, were used in all assays. The MIC results were based on visual evaluation (presence of turbidity and precipitate in each dilution). The MIC was defined as the highest EO dilution without bacterial growth. All assays were performed in duplicate, and the MIC was obtained by their arithmetic mean. The MIC50 and MIC90 values were defined as the lowest extract concentrations at which 50% and 90% of the clinical isolates were inhibited. The microbiological breakpoint of gentamicin was established according to the CLSI (2018).

3840

Semina: Ciênc. Agrár. Londrina, v. 42, n. 6, suplemento 2, p. 3837-3854, 2021

The bactericidal or bacteriostatic activity of the EOs against the isolates was evaluated by randomly choosing ten isolates at the dilution corresponding to the MIC. For this, 5 µL of the medium of the wells corresponding to the MIC values were spread on plates containing Muller Hinton agar (HIMEDIA®), which were incubated at 37 ºC for 18-24h. The absence of growth was interpreted as bactericidal activity, and its presence was interpreted as bacteriostatic activity of the EOs. Fisher's Exact Test was performed using the Epi-Info6.04b (1997) to evaluate the differences between the frequencies of bactericidal and bacteriostatic effects of the EOs, and a value of $p < 0.05$ was considered statistically significant.

Results and Discussion

The MIC50, and MIC90 ranges found for the essentials oils and gentamicin against the tested isolates are summarized in Table 1. Regarding the EOs, only the MIC values were obtained since no standard breakpoints are established. The MIC values of *Eugenia caryophyllata* ranged from 1.21 mg mL^{-1} (1:640) to 19.37 mg mL^{-1} (1:40); the MIC50 and MIC90 were 2.42 mg mL^{-1} and 7.25 mg mL^{-1}, respectively. The MIC values of *Thymus vulgaris* ranged from 7.18 (1:640) mg mL^{-1} to 57.5 mg mL^{-1} (1:80); the MIC50 and MIC90 were established as 9.51 mg mL^{-1} and 22.94 mg mL^{-1}, respectively. *Cymbopogon winterianus* showed an MIC range from 13.82 mg mL^{-1} (1:320) to 22.13 mg mL^{-1} (1:20); the MIC50 and MIC90 were 26.78 mg mL^{-1} and 157.79 mg mL^{-1}, respectively. The EOs of *Cinnamomum cassia* and *Cymbopogon citratus* showed, at their highest dilutions against all isolates (1:640), MIC values corresponding to 16.48

mg mL^{-1} and 27.81 mg mL^{-1}, respectively, while their MIC50 and MIC90 values were lower than these MIC values. One of the isolates classified as gentamicin-resistant showed the highest MIC for *Eugenia caryophyllata* and *Cymbopogon winterianus* (Supplementary Table 1).

The MIC values for gentamicin ranged from 0.5 µg mL^{-1} to 128 µg mL^{-1} and were classified according to the CLSI (2020) as susceptible (77.41% of strains - MIC range 0.5-4 µg mL^{-1}), intermediate (12.90% - MIC = µg mL^{-1}), or resistant (9.68% - MICs ≥16µg mL^{-1}). The MIC results for gentamicin showed four dilution values beyond its breakpoint, while the MIC50 and MIC90 were within the susceptible and intermediate categories, respectively.

All the essential oils showed heterogeneous bacterial activity varying according the isolate assayed, however none showed bactericidal action in the dilution corresponding to the MIC against all tested isolates (Supplementary Table 1).

Canine otitis externa is one of the most frequent illnesses in veterinary clinics, and its treatment is usually based on the administration of topic or systemic antimicrobials. However, therapeutic failures may occur, especially due to multidrug-resistant (MDR) microorganisms (Bourély et al., 2019). Nowadays, the increase in resistance among pathogens that cause diseases in humans and animals is a worldwide concern that demands studies focusing on alternative treatments, and EOs have shown great potential for this purpose. These substances are secondary plant metabolites constituted by a mix of organic compounds, ranging from 20 to 60 components with low molecular weight and

Semina: Ciênc. Agrár. Londrina, v. 42, n. 6, suplemento 2, p. 3837-3854, 2021

3841

at different concentrations. Several EOs have been screened as antimicrobials and are generally regarded as safe (GRAS) as there are no reports of bacterial resistance even when used for a long time (Dal Pozzo et al., 2011; Lang, & Buchbauer, 2012; Akhtar, Degaga, & Azam, 2014; Andrade, Barbosa, Probst, & Fernandes Júnior, 2014; Al-Mariri & Safi, 2014; Lopez-Romero, González-Ríos, Borges, & Simões, 2015; O'Bryan et al., 2015).

Table 1

Minimum Inhibitory Concentration (MIC) of essential oils and gentamicin against 62 *Staphylococcus aureus* strains isolated from dogs with otitis externa

Antimicrobials	MIC (mg mL^{-1})	Number of strains (%)	MIC50 (mg mL^{-1})	MIC90 (mg mL^{-1})	Profile[a]
Gentamicin*	0.5 µg mL^{-1}	29 (46.77%)			Susceptible
	2 µg mL^{-1}	13 (20.96%)			Susceptible
	4 µg mL^{-1}	6 (9.67%)	1 µg mL^{-1}	8 µg mL^{-1}	Susceptible
	8 µg mL^{-1}	8 (12.90%)			Intermediate
	16 µg mL^{-1}	5 (8.06%)			Resistant
	128 µg mL^{-1}	1 (1.61%)			Resistant
Eugenia caryophyllata	1.21	14 (22.58%)			-
	2.42	17 (27.42%)			-
	4.84	19 (30.64%)	2.42	7.25	-
	9.068	10 (16.12%)			-
	19.37	2 (3.22%)			-
Thymus vulgaris	7.18	25 (40.32%)			-
	14.37	19 (30.64%)	9.51	22.94	-
	28.75	14 (22.58%)			-
	57.5	4 (6.45%)			-
Cymbopogon winterianus	13.82	12 (19.35%)			-
	27.65	20 (32.25%)			-
	55.31	14 (22.58%)	22.78	157.79	-
	110.62	7 (11.29%)			-
	221.25	9 (14.51%)			-
Cinnamomum cassia	16.48	62 (100%)	<16.48		-
Cymbopogon citratus	27.81	62 (100%)	<27.81		-

* Gentamicin results are expressed in µg mL^{-1}.
[a] The susceptibility profile was found only to gentamicin according to the CLSI.

3842

Semina: Ciênc. Agrár. Londrina, v. 42, n. 6, suplemento 2, p. 3837-3854, 2021

Although the use of EOs as antimicrobials has been recognized since the dawn of civilization, the scientific interest in these substances as an alternative to antibiotics in the fight against human and animal pathogens has increased due to the MDR phenomenon (Lopez-Romero et al., 2015; O'Bryan et al., 2015). The antibacterial activity of EOs has thus been explored, and several mechanisms have already been reported, such as damage to the cell membrane, causing the loss of selective permeability, the collapse of the proton pump and lysis of the microorganism, and cytoplasmic coagulation (Gustafson et al., 1998; Lopez-Romero et al., 2015; Swamy et al., 2016).

In the present study, the aminoglycoside gentamicin was tested in the MIC assays. This antimicrobial is present in several commercial drugs used in Brazil for the topical therapy of canine otitis externa. The MIC assays showed that 24 (19%) isolates were not susceptible to this antibiotic and were classified as resistant or intermediate strains, being very close to wild-type strains (Bourély et al., 2019). Although the gentamicin concentrations in commercial products used to treat canine otitis are higher than the concentrations evaluated in the MIC assay, our results showed that its use might be a negative factor due to the increase in the selective pressure against resistant strains of *S. aureus* circulating in the canine population. Furthermore, it can become a serious public health concern due to the relationship between dogs and humans and the risks of interspecies infections. Interestingly, it was observed that one isolate classified as gentamicin-resistant showed the highest MIC for two EOs, allowing us to infer that this strain is potentially MDR since the non-susceptibility

to one or more antimicrobials of three or more antimicrobial classes characterizes multidrug resistance (Magiorakos et al., 2012).

In the last decade, a faster resistance propagation among bacterial pathogens has been observed due to the excessive use of antibiotics, limiting the efficacy of treatment of several infectious diseases in animals and humans (Lopez-Romero et al., 2015). Previous studies have suggested a high resistance to gentamicin by *S. aureus* isolated from skin and nosocomial infections in humans (Caraciolo, Maciel, Santos, Rabelo, & Magalhães, 2012; Bortolin, Bidossi, De Vecchi, Avveniente, & Drago, 2017). Moreover, S. aureus has several features related to antimicrobial resistance, including biofilm formation, which impairs the treatment of infected hosts (Swamy et al., 2016). Although biofilm formation was not evaluated in this study, this virulence factor is already known among *S. aureus* strains isolated from canine otitis, increasing the antimicrobial resistance and pathogenicity (Moreira et al., 2012; Lee et al., 2019).

All EOs tested in this study showed MIC variations for the different strains, except *C. cassia* and *C. citratus,* which showed homogeneous results, with all strains showing the same MIC and stopping microbial growth in the last dilution (1:640). The differences in the antimicrobial action of the EOs tested could be related to their different compounds. The composition and antimicrobial effects of EOs can be influenced by the season, geographic region, soil type, plant part used, harvest time, and weather conditions (Castro, Barbosa, Leal, Souza, & Nazareno, 2007; Celiktas et al., 2007; Kamatou et al., 2008; Swamy et al., 2016). Despite the variation in the MIC ranges assessed, the MIC50 values found for all EOs and for the antibiotic

against the isolates corresponded to the lowest concentrations tested, whereas the MIC90 showed greater variability. The MIC50 found showed that irrespective of the OEs of *Eugenia caryophyllata, Thymus vulgaris,* and *Cymbopogon winterianus,* as well as the antimicrobial gentamicin, almost all were able to inhibit 50% of the bacterial strains tested at low concentrations, corresponding to the two highest dilutions tested. Regarding these same EOs, the MIC90 values were approximately three times higher than the MIC50, except for *Cymbopogon winterianus,* which showed a higher ratio between the MIC50 and MIC90 values (6.92). Future assays are necessary to evaluate the MIC50 and MIC90 of *C. cassia* and *C. citratus* as these EOs showed MIC values in the highest dilution tested against all isolates. The MIC90 found for gentamicin was within the intermediate category, highlighting the possible development of antimicrobial resistance by *S. aureus* isolated from otitis to this antibiotic.

Regarding *C. cassia,* several studies have also shown the antimicrobial action of cinnamaldehyde against *Staphylococcus* spp. isolated from bovine mastitis (Baskaran, Kazmer, Hinckley, Andrew, & Venkitanarayanan, 2009; Dal Pozzo et al., 2012). Firmino et al. (2018) found that the major active component of the EO of *C. cassia* with antibacterial activity against *S. aureus* ATCC6538 was cinnamaldehyde, with the MIC ranging from 250 μg mL^{-1} to 500 μg mL^{-1}. In contrast, the MIC of the EO of *C. cassia* observed in our study (<16.48 mg mL^{-1}) showed a higher value. This difference can be explained by the fact that we used crude EO, generally composed of a mixture of 20 to 60 substances (Swamy et al., 2016), resulting in the dilution of its antimicrobial components. Additionally, this difference could be associated with the different bacterial strains used in this study as well as the origin of the EO. Netopilova et al. (2020) demonstrated the synergistic effect of *C. cassia* with 8-hydroxyquinoline against *S. aureus,* highlighting the ability of *C. cassia* to inhibit this bacterial species and showing that the concentration used in this study can be later optimized to evaluate whether the *in vitro* antimicrobial effect will be repeated *in vivo.*

The EO of *C. citratus* has been used as a food preservative agent, as an inhibitor of biofilm formation in different environmental niches, and as an antifungal and antibacterial. This EO acts on membrane permeability, inhibiting essential activities necessary to bacterial survival as well as quorum sensing, preventing biofilm formation in dental caries, and is also present in the food industry (Ekpenyong & Akpan, 2015; Oliveira et al., 2017; Ortega-Cuadros, Tofiño-Rivera, Merini, & Martínez-Pabon, 2018). Its main antimicrobial components are citral (alpha and beta), geranial, and myrcene, which have analgesic action. Its effects are dependent on the concentration and can be improved by the association of its compounds (Aiemsaard, Aiumlamai, Aromdee, Taweechaisupapong, & Khunkitti, 2011; Oliveira et al., 2017; Ortega-Cuadros et al., 2018). In our study, the EO of *C. citratus* inhibited bacterial growth at the concentration of 27.81 mg mL^{-1}. However, different concentration ranges aiming at an optimal dose could be assayed to kill *S. aureus* since its lipophilic activity is increased over higher concentrations (Córdoba et al., 2019). Other studies have demonstrated that the EO of *C. citratus* has a good antimicrobial effect against bacteria isolated from turtle and human newborns (Silva et al., 2018; Oliveira et al., 2019). Sahal et al. (2020) reported this oil

3844

Semina: Ciênc. Agrár. Londrina, v. 42, n. 6, suplemento 2, p. 3837-3854, 2021

as a potential antifungal and antibiofilm agent that can be used to prevent contamination in silicone rubber prostheses and medical devices.

The EO of *Thymus vulgaris* has been reported as an antimicrobial, antiseptic, and antioxidant, and its main action seems to be in the cell membrane, changing the permeability due to its lipolytic action (Lorenzi, & Matos, 2002; Suzuki, Flemming, & Traad, 2008). The main components of *Thymus vulgaris* are thymol, p-cymene, linalool, carvacrol, caryophyllene oxide, and E-caryophyllene. The proportion of its majoritarian compounds varies according to the geographic region, season, and plant part used (Benameur, Gervasi, Pellizzeri, Pľuchtová, Tali-Maama, Assaous, & Ben-Mahdi, 2018).

In our study, the EO of *Thymus vulgaris* showed MIC50 and MIC90 values of 9.51 mg mL^{-1} and 22.94 mg mL^{-1}, respectively. Likewise, Kot, Wierzchowska, Grużewska, & Lohinau (2017) found MIC values ranging from 0.012% to 0.048% against methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from humans and demonstrated that this EO reduced biofilm formation by up to 85%. Nostro et al. (2007) also described the inhibitory action of the EO of *T. vulgaris* on biofilms, highlighting its positive effect against biofilm-producing strains. Another study demonstrated the antimicrobial action of *Thymus vulgaris* against *S. aureus* isolated from canine otitis at the MIC of 17.86 µg mL^{-1}, a concentration below the value obtained in our study, probably due to the different oil composition (Ebani et al., 2017). In another study, Benameur et al. (2018) showed that the components extracted from this EO have a good antimicrobial effect against MDR enterobacteria at MIC values ranging from 2.87 to 11.5 µg mL^{-1}. Additionally, this EO showed good antimicrobial activity against *Staphylococcus* and *Malassezia* isolated from canine dermatitis (Ebani et al., 2020).

The MICs found for the EO of *Cymbopogon winterianus* showed a wide variation, with MIC50 and MIC values of 22.78 mg mL^{-1} and 157.79 mg mL^{-1}, respectively. This EO has been recognized as an effective antibacterial against *S. aureus* isolated from food as well as against resistant bacteria with variable MICs, similar to the results observed in this study (Oussalah, Caillet, Saucier, & Lacroix, 2006.; Brugnera, Liveira, & Piccoli, 2011; Lertsatitthanakorn, Taweechaisupapong, Aarunyanart, Aromdee, & Khunkitti, 2010; Hussain, Anwar, & Nigamet, 2011; Wattanasatcha, Rengpipat, & Wanichwecharungruang, 2012). Imane et al. (2020) found an MIC of 8.07 mg mL^{-1} against MRSA. However, this concentration was unable to kill the bacteria.

The EO of *Eugenia caryophyllata* has eugenyl acetate, B-caryophyllene, and eugenol as its components, with the last being found at higher concentrations. It has bactericidal, fungicidal, and nematicidal activity, acting mainly on the cell membrane, denaturing proteins, reacting with lipids, and changing membrane permeability (Briozzo, Núncez, Chirite, Herszage, & D'aquino, 1989; Cardoso et al., 2007). In our study, *Eugenia caryophyllata* showed different MIC values against the tested strains, with MIC50 and MIC values of 2.42 mg mL^{-1} and 7.25 mg mL^{-1}, respectively. Fu, Zu, Chen, Wang, Sun and Efferth (2007) evaluated the antimicrobial effect of this EO and found an MIC of 0.125% against *S. aureus,* lower than the MIC observed in this study. Al-Shabib, Husain, Ahmad and Baig (2017) demonstrated that eugenol, the main component of the EO

of *Eugenia caryophyllata,* reduces biofilm production and the expression of other virulence genes in MRSA isolated from food handlers. Likewise, Yadav, Chae, Im, Chung, & Song (2015) demonstrated the reduction of *in vitro* and *in vivo* biofilm formation against *S. aureus* isolated from different origins.

The microbicidal action of all EOs in the MIC dilution showed heterogeneity, which can be associated with individual susceptibility characteristics of the isolates and the different compositions of each EO. Regarding the bactericidal action of the EOs, *Cymbopogon winterianus* and *Cinnamomum cassia* seem to be more efficient as they could kill *S. aureus* at the lowest dilutions compared to the other EOs (*p<0.05*). The bacteriostatic effect was found predominantly among the EOs of *Thymus vulgaris, Eugenia caryophyllata,* and *Cymbopogon winterianus,* without significant differences in their frequencies (*p>0.05*). However, the bactericidal effect may occur at EO concentrations higher than the MIC.

Differences in the antimicrobial potency of the EOs were observed in relation to previous studies, which can be explained by several factors, such as the site of isolation, the size of the inoculum, the growth stage of the microorganism, the oil compounds, oil solubility, and the oil diluent used (Hammer, Carson, & Riley, 1999; Opalchenova & Obreshkova, 2003). Regarding the oils diluent, DMSO or Tween 20% can influence bacterial growth or membrane permeability due to the synergistic or antagonistic action of the EOs (Nascimento et al., 2007). Control tests were used in our study, demonstrating that DMSO did not show inhibitory action against the tested microorganisms.

Our study highlighted the potential of EOs in the treatment of canine otitis caused by

S. aureus. However, it should be emphasized that these data refer to an *in vitro* assay and that the inhibitory concentration of an antimicrobial at the site of infection could be different due to intrinsic pharmacological characteristics (Kahlmeter et al., 2003). Also, it should be highlighted that canine otitis has multifactorial causes and involves several mycotic, bacterial, and parasitic pathogens. Thus, additional studies including other agents of importance to its etiology are necessary within a clinical use perspective. Finally, future *in vivo* studies will be necessary to confirm the results obtained *in vitro* as well the perspective of developing new antimicrobial drugs derivate from EOs or their extracts against *S. aureus,* one of the main multidrug-resistant microorganisms (Bourély et al., 2019).

Conclusions

All EOs screened here showed antimicrobial action against *S. aureus.* Future *in vivo* studies will be necessary to confirm the results obtained *in vitro* as well the perspective of developing new antimicrobial drugs based on EOs or their extracts against the pathogens that cause canine otitis and, eventually, their side effects.

Our research addressed a public health concern associated with the possible selective pressure over *S. aureus* isolated from otitis externa in dogs due to gentamicin, highlighting the need for continuous surveillance and studies focusing on new alternative treatments, as shown here.

Acknowledgments

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3851

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3852

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Semina: Ciênc. Agrár. Londrina, v. 42, n. 6, suplemento 2, p. 3837-3854, 2021

3853