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Antimicrobial activity of essential oils against positive coagulase Staphylococcus isolated from External Canine Otitis cases. Atividade antimicrobiana de óleos essenciais frente à Staphylococcus coagulase positiva isolados de casos de Otite Externa Canina.

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Abstract

This study aimed to evaluate the antimicrobial activity of clove (*Syzygium aromaticum*), citronella (*Cymbopogon winteranius*) and melaleuca (*Melaleuca alternifolia*) essential oils (EOs) on CoPS. Of the 67 cerumen samples collected, 17 CoPS strains were isolated. Using the microdilution method in a 96-well plate, EOs were added at a maximum concentration of 10% and minimum of 0.04%. It is concluded that the EOs of clove and citronella demonstrate antimicrobial activity against strains of CoPS isolated from canine external otitis. At the concentrations used, melaleuca essential oil did not show any bacteriostatic or bactericidal effects.

Keywords: Antimicrobials. Natural products. Auditory canal. Dogs. Veterinary dermatology.

Resumo

Este estudo teve como objetivo avaliar a atividade antimicrobiana dos óleos essenciais (OE) de cravo-da-índia (*Syzygium aromaticum*), citronela (*Cymbopogon winteranius*) e melaleuca (*Melaleuca alternifolia*) em CoPS. Das 67 amostras de cerume coletadas, 17 cepas de CoPS foram isoladas. Usando o método de microdiluição em uma placa de 96 poços, os OE foram adicionados a uma concentração máxima de 10% e mínima de 0,04%. Conclui-se que os OEs de cravo-da-índia e citronela demonstram atividade antimicrobiana contra cepas de CoPS isoladas de otite externa canina. Nas concentrações usadas, o óleo de melaleuca não apresentou nenhum efeito bacteriostático ou bactericida.

Palavras-chave: Antimicrobianos. Produtos naturais. Canal auditivo. Cães. Dermatologia veterinária.

Introduction

Canine external otitis is characterized by acute or chronic inflammation of the epithelium of the external auditory canal of these animals (EBANI et al., 2017, p. 1). Clinical signs include inflammation, pain, discomfort, an unpleasant odor, excessive earwax and intense itching. In chronic cases, patients may present with auditory canal closure, appearance of ear polyps and even rupture of the tympanic membrane, causing discomfort and hearing loss (PERRY et al., 2017, p. 168).

Commonly seen in the clinical routine, otitis is not potentially lethal, but therapeutic intervention can be challenging and frustrating, because several factors can hinder the cure (PIETSCHMANN et al., 2013, p. 439). This difficulty in treatment is reflected by the high prevalence of cases seen in canine dermatology, ranging from 15.9% to 17.5% of canine skin diseases (CARDOSO et al., 2011, p. 69; PERRY et al., 2017, p. 172). Furthermore, the treatment is difficult as it is necessary for a long period of time; in these cases, the ideal scenario would an improvement in the integrity of the dog's tympanic membrane in order to minimize the systemic effects on the animal (RHODES; WERNER, 2014, p. 524).

When part of inadequate treatment regimens, including the provision of inadequate doses and insufficient duration or the wrong choice of treatment type, antibiotics and antifungal treatments can trigger the emergence of resistant strains, limiting therapeutic success. Moreover, in an attempt to eradicate resistant microorganisms, repeated doses of conventional antibiotics increase the occurrence of toxic effects on the animal (EBANI et al., 2017, p. 7).

One of the most etiologic agents prevalent in external otitis in dogs is *Staphylococcus* spp. This type of gram-positive bacteria is commonly found in arrangements similar to "grape clusters" and constitutes a class of opportunistic pathogens that cause infections in animals and humans (PERRY et al., 2017, p. 173).

Staphylococci that exhibit a positive reaction when exposed to rabbit plasma are termed as coagulase-positive. Coagulase-positive *Staphylococci* (CoPS) are a group of bacteria that constitutionally have mechanisms to enable bacteria to become resistant to multiple conventional antibiotics (MA et al., 2019, p. 1; GONZÁLEZ-MARTÍN et al., 2020, p. 118). The CoPS group can produce more virulence factors and is an important and threatening pathogen, given that the elimination of this type of bacteria is very difficult (GONZÁLEZ-MARTÍN et al., 2020, p.120; PENNA et al., 2009, p. 292; DÉGI et al., 2013, p. 2).

Among all species of *Staphylococcus*, the type most frequently found in external otitis cases is *Staphylococcus aureus* (MARKEY, 2013, p. 121). *S. aureus* is a gram-positive, commensal, and coconut-shaped bacterium, coagulase-positive and present in the skin and mucous membranes (QUINN et al., 2005, p. 56). Due to the intense and inadequate use of available conventional treatments, *S. aureus* has shown several mechanisms of resistance to antimicrobials (ENIOUTINA et al., 2017, p. 20; BOURÉLY et al., 2019, p. 3).

In an attempt to find efficient treatments, the latest research has frequently investigated therapy using natural products (ENIOUTINA et al., 2017, p. 20; LACCOURREYE et al., 2017, p. 4). Currently, essential oils have potential to prevent and treat various diseases, including bacterial otitis, when used in combination with conventional therapy. Essential oils have beneficial characteristics that can be exploited, such as antiseptic, antimicrobial, antiviral, anti-inflammatory and immunostimulant properties (ENIOUTINA et al., 2017, p. 4; ALMEIDA et al., 2016, p. 451).

Considering the synergism of essential oil components, continuous exposure to these compounds does not result in the development of resistance (ENIOUTINA et al., 2017, p. 20).

The performance of these essential oils and their components against microorganisms is largely due to the classes of molecules in their constitution. Mostly formed by terpenoids, the compounds present in essential oils have interaction properties with the bacterial cell membrane, modifying its structure. This interaction increases the permeability of the cell membrane, leading to the leakage of molecules and essential ions for structural maintenance, promoting bacterial death (SOLÓRZANO & MIRANDA, 2012, p. 136).

The essential oil extracted from tea tree or melaleuca (*Melaleuca alternifolia*), a plant native to Australia, has a proven antibacterial and antifungal effect. In addition, it has broad antimicrobial and healing effects on wounds, antiviral activity and an acaricidal effect in the form of nanoparticles against *Rhipicephalus microplus* (GAROZZO et al., 2009, p. 807; GE & GE, 2015, p. 316; PAZINATO et al., 2013, p. 82; BOITO et al., 2016, p. 72; LI et al., 2016, p. 8876). *Melaleuca alternifolia*, when administered topically, can be used by most individuals without adverse effects, since the prevalence of allergy in these cases is very low (LARSON & JACOB, 2012, p. 49; HAMMER et al., 2006, p. 618).

The essential oil of clove (*Syzygium aromaticum*) has a strong antioxidant effect (NASSAR et al., 2007, p. 51). Additionally, *S. aromaticum* essential oil has several beneficial characteristics, demonstrating antitumor, antimicrobial, antifungal, antiparasitic, insecticide, analgesic, anesthetic, and even antidiabetic properties (AFFONSO et al., 2012, p. 159). Pinto et al. (2009, p. 1460) observed the antifungal capacity of clove essential oil against several species of Candida, Aspergillus, and dermatophytes, proving effective even for *Candida* species resistant to fluconazole.

The citronella essential oil (*Cymbopogon winterianus*) has antifungal, analgesic, anti-inflammatory, anti-insect, antimicrobial, and antioxidant action. In terms of antimicrobial action, it has activity against *S. aureus*, configuring itself as an option in the treatment of diseases promoted by this bacteria, such as canine otitis (ALMEIDA et al., 2016, p. 451; LEITE et al., 2010, p. 1168; OLIVEIRA et al., 2011, p. 439; SANTOS et al., 2015, p. 19).

Essential oils are not always capable of replacing antibiotics or antimycotics, but they can be constituted as new antimicrobials or even adjuvants in treatment performed with conventional therapy (EBANI et al., 2017, p. 7). In view of the evident antimicrobial activity of essential oils, is possible to evaluate their activity in vitro against the main pathogen involved in canine otitis, *S. aureus*. Thus, melaleuca, citronella and clove could be alternatives to the emergence of resistant bacterial strains when used alone or in combination with conventional drugs.

This study aimed to evaluate the antimicrobial effect of three essential oils from plants (cloves, citronella, and melaleuca) against samples of Coagulase-positive *Staphylococci* (CoPS) isolated from the ear canal of dogs affected by external otitis.

Materials and Method

The study was conducted between June 2018 and October 2019, at the Superintendência Unidade Hospitalar Veterinária Universitária at the Federal University of Fronteira Sul (SUHVU-UFFS, Realeza, PR, Brasil). Cerumen samples were collected, using sterile swabs with *Stuart* transport medium (Olen®), from canine patients of any weight, breed, sex and age. The diagnosis of external otitis was performed according dermatological care, through physical examination of the ears and cytology of the cerumen. To ensure the abundant growth of colonies, samples collected were inoculated on Brain Heart Infusion Broth (BHI, Brain Heart Infusion, Himedia®) agar and kept in an incubator for 24 hours at 37ºC (PENNA et al., 2009, p. 292).

The procedures for identifying the strains were performed using different kinds of agar, colony morphology, Gram staining, catalase test, and subsequent selection of positive isolates in the coagulase test (NewProv®).

External otitis samples were seeded in different culture mediums, using the exhaustion technique of microbiological seeding (CLSI, 2012, p. 12). Microorganisms were identified according to their growth in different culture mediums and classified as differential and/or selective. Firstly, the samples were inoculated in Mueller-Hinton agar to isolate colonies. Samples were seeded in mannitol salt agar (selective for *Staphylococcus* sp. and differential for *Staphylococcus aureus),* MacConkey agar (selective for gram-negative bacteria*)* and cetrimide agar (selective and differential isolation of *Pseudomonas aeruginosa)* and incubated at 37°C for 24/48 h. Samples in Saboureaud agar (selective for fungi) were incubated at 25°C for 7 days (TORTORA, FUNKE & CASE, 2012, p. 168).

After growth in agar, colonies were isolated using a platinum loop and inoculated in microtubes containing 750 µL of BHI broth plus 250 µL of sterile 20% glycerin. After that period, samples were properly stored at -80ºC and constituted the mother inoculum.

From the mother inoculum, a 0.01 ml loop containing isolated CoPS was transferred to a tube containing 5mL BHI broth. This tube was kept in an incubator at 37ºC overnight. After that period, a 0.9% saline solution was added to the broth solution with bacteria, to obtain visually 0.5 on the McFarland scale. The scale was confirmed by spectrophotometry, where an absorbance between 0.08 Å and 0.11Å, at 625nm, represents 1-2 x 10^8 CFU / mL (inoculum).

The minimum inhibitory concentration (MIC) was obtained using the serial microdilution technique in 96-hole microplates (CLSI, 2015, p. 27). BHI broth was added to each well, as well as 10 µL of the standardized inoculum.

The essential oils (EOs) of melaleuca, clove and citronella were obtained commercially (Lazlo Aromatherapy®, Belo Horizonte, MG). Laszlo Aromatherapy analyzes its oils by Gas chromatography–mass spectrometry (GC-MS) and the analysis of the components are available in the package insert.

EOs were mixed with 1% polysorbate 80, to permit oil solubilization in BHI broth. The maximum concentration used was 10% and Eos were serially two-fold diluted until they reached a minimum concentration of 0.04%. One row in the test microplate constituted only wells containing standard inoculums and broth to check if inoculums were growing well in the chosen broth. In addition, a control microplate was prepared the same as the inoculated plates, but without inoculum, in order to confirm that there was no contamination in the process. The microplates were incubated at 37°C for 24 hours. All tests were performed in duplicate.

After incubation, resazurin 0.01% (Sigma-Aldrich® Brasil Ltd, São Paulo, SP, Brazil) was added to each well. The microplate was again kept in an incubator at 37ºC for 1 hour and visually evaluated. A blue color indicated the absence of bacterial growth and a pink / red color indicated cellular metabolism. The first blue well in a row, before a pink well, indicated the minimum oil concentration capable of inhibiting bacterial growth, termed the minimum inhibitory concentration (MIC) (COBAN, 2012, p. 2).

After defining the MIC, the content of each well was inoculated in Petri dishes containing *Mueller Hinton* agar (MHA), to assess the minimum bactericidal concentration (MBC), that is, the minimum amount of oil capable of promoting bacterial death. Then, these plates were kept in an incubator at 37ºC for 24 hours. After incubation, the MBC was obtained by the absence of bacterial growth.

The data obtained were submitted to descriptive statistical analysis and the Shapiro-Wilk normality test. Afterwards, the non-parametric data were submitted to a Kruskal Wallis test, followed by Dunn's post-hoc test. A value of $p < 0.05\%$ was considered statistically significant.

Results

Cerumen samples were collected from 67 different dogs with external otitis between June 2018 and October 2019. About 59.7% (40) of 67 samples were positive for microbial growth on MHA. In 42.5% (17) of these samples we identified CoPS, and in 23 (57.5%) other microorganisms were observed, such as yeasts, negative coagulase *Staphylococcus* sp., Enterobacteria and *Pseudomonas aeruginosa*. An absence of microbial growth was observed in 27 (40.3%) samples.

The results of MICs and MBCs are shown in Table 1. These results demonstrated that melaleuca essential oil was less effective than the other two oils. Comparing the medians obtained, the amount of melaleuca essential oil to inhibit bacterial growth was double that necessary when using citronella, and four times less effective than clove. The antibacterial action of melaleuca essential oil was demonstrated only at the maximum concentration of 10%, in 9 of 17 samples (52.94%). Moreover, the 75^{th} percentile demonstrated that at least 12 of 17 strains were inhibited or killed with 2.5% of citronella or clove essential oils. However, a concentration of melaleuca essential oil of no less than four times higher was required to present the same effect.

Table 1 - Percentages of citronella, clove and melaleuca essential oils necessary to reach Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), in coagulase positive *Staphylococcus* (CoPS) strains isolated from cases of canine external otitis (n=17).

	MIC citronella	MIC clove	MIC melaleuca	MBC citronella	MBC clove	MBC melaleuca
Minimum	0.6	0.1	5	1.25	0.3	5
25% Percentile	1.25	0.925	5	1.875	1.25	7.5
Median	2.5	1.25	5	2.5	2.5	10
75% Percentile	2.5	2.5	10	2.5	2.5	10
Maximum	5	5	10	10	5	10

Note: citronella (*Cymbopogon winterianus*), clove (*Syzygium aromaticum*), melaleuca (*Melaleuca alternifolia*).

MIC and MBC values of clove and citronella oil did not show any significant differences between them. However, they differed statistically from melaleuca essential oil, in MIC and MBC, as demonstrated in Figure 1.

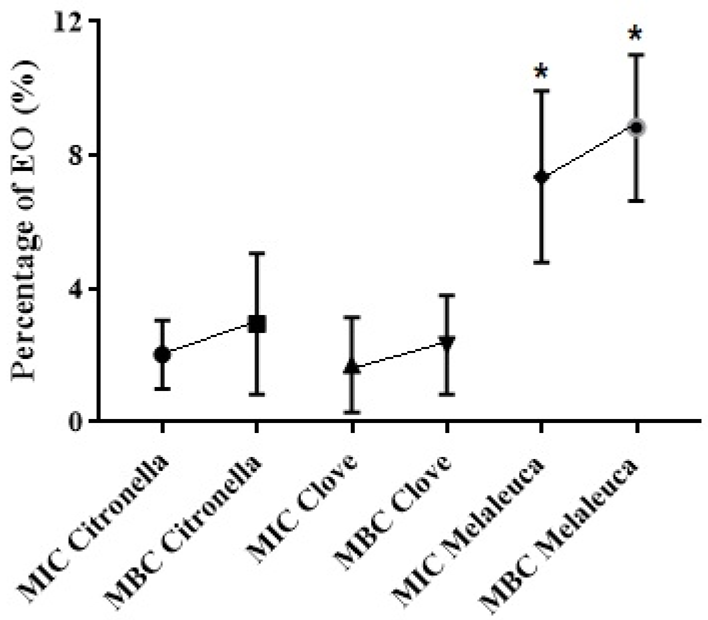

Figure 1 - Statistical differences between Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of citronella, clove and melaleuca essential oils.
Note: The y axis represents the amount, expressed as percentage, of Essential Oil (EO) necessary to produce an inhibitory (in case of MIC) or Bactericidal (in case of MBC) effect. In the x axis, the three EOs and their respective MICs and MBCs are demonstrated. The asterisk indicates statistical difference ($P<0.05$) between the marked groups (melaleuca EO) and the other groups (citronella and clove EO).

Discussion

Despite 59.7% of samples presented positive growth on MHA, in 40.3% (27 samples) no growth was observed. The absence of microbial growth in MHA observed probably occurred due some reasons such as: fungal samples have a preference for Saboreaud (non-tested) agar instead MHA, and because some degree of sample degradation (MARKEY, 2013, p. 23).

Perry and coworkers (2017, p. 171) observed that 28 of 60 samples collected from patients with external otitis showed bacterial growth. The prevalent bacteria isolated was *Staphylococcus* spp., corresponding to 36% of totality. A similar result was observed in the study of Bourély et al. (2019, p. 5), with the prevalence of CoPS in cases of canine external otitis corresponding to 41.2% of isolates. Penna et al. (2009, p. 293) observed that from 151 samples of canine external otitis studied, 91 (60.3%) were *Staphylococcus* sp.

Groot and Schmidt (2016, p. 130) report that several factors that can interfere with the antimicrobial potential of essential oils, modifying their composition. Contact with atmospheric oxygen, exposure to light, humidity, and high temperatures can all degrade essential oils.

It is interesting to explore the synergistic effect of essential oils and their major compounds when associated with conventional low-dose antimicrobials, and in these situations the effects can be even more promising than when conventional drugs are used in isolation, even in the case of resistant microorganisms (SCAZZOCCHIO et al., 2015, p. 2; KHAMENEH et al., 2019, p. 23). In addition, essential oils may have a lower cytotoxic effect than antimicrobials when used at the same concentration (SCAZZOCCHIO et al., 2015, p. 8).

Citronella oil has promising antifungal, antibacterial, antiparasitic, and insect-repellent effects (SHARMA et al., 2019, p. 2). Unlike this study, Scazzocchio et al. (2015, p. 6) observed that citronella had lower antibacterial activity than melaleuca essential oil when tested against standard reference microorganisms. The effect of the oil was different in the present study. This fact may be due to the chemical composition of each oil and bacterial strains and species, since each phytochemical constituent of essential oils affects different microorganisms differently (SCAZZOCCHIO et al., 2015, p. 9).

Silvestri et al (2010, p. 592) tested clove essential oil on seven strains of gram-positive bacteria, including *S. aureus*, and eleven strains of gram-negative bacteria. The higher antibacterial activity of clove oil was demonstrated against *S. aureus*. This fact, observed in Silvestri´s experiment, may suggest that considering the three different essential oils in our experiment, *S. aureus* may be more sensitive to clove essential oil.

In the study of Scherer et al (2009, p. 447), using standard reference bacteria, clove oil showed an antimicrobial action very similar to that observed with citronella essential oil. These results are similar to those obtained in this study, considering that the MIC and MBC values of clove and citronella oil did not show any significant differences between them. However, they differed statistically from melaleuca essential oil, in MIC and MBC.

Melaleuca essential oil has more than 100 components acting synergistically. Each component has a lower activity than in the total oil (HAMMER et al., 2006, p. 623). *Melaleuca alternifolia* has terpinen-4-ol as its major component, which is responsible for a large part of the anti-inflammatory power and antimicrobial properties and can be used to treat dermatological diseases (PAZYAR et al., 2012, p. 1).

In the study of Zhang et al. (2018, p. 3), an antibacterial effect of the essential oil of *Melaleuca alternifolia* was observed, mainly on *S. aureus*. In addition, Buldain et al. (2018, p. 6) found that it is possible to combine and observe a synergism between the antibacterial properties of *M. alternifolia* essential oil and conventional antibiotics against resistant strains of *S. aureus*. Furthermore, Oliva et al. (2018, p. 7) also observed a good action of this oil when associated with conventional antibiotics to treat conditions caused by resistant microorganisms.

However, Saad et al. (2013, p. 276) affirmed that melaleuca is unable to cause a rapid change in the bacterial cell wall and that the lysis of the microorganism, when it occurs, may be due to a change in osmotic pressure. These factors may be responsible for the inefficiency of this oil in our study.

There are reports that essential oils have limited potential as antimicrobial agents due to their high volatility, limiting their action time, becoming less effective than conventional antimicrobials (WINSKA et al., 2019, p. 14). Many factors can affect the effectiveness of the natural product, including tissue penetration, maximum plasma concentration and bioavailability (KHAMENEH et al., 2019, p. 28).

We conclude that essential oils, especially clove and citronella oil, demonstrate potential use as antimicrobials against CoPS, in veterinary medicine. However, further studies are needed to verify the possible use of these oils in cases of canine external otitis, including their interaction with conventional medicines. In addition, at the same time, it will be important to investigate and understand the potential toxic effects caused by these substances.

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