POTENCIAL ANTIBACTERIANO DOS ÓLEOS ESSENCIAIS DE *Melaleuca alternifolia, Mentha piperita E Rosmarinus officinalis* EM ISOLADOS DE *Staphylococcus pseudintermedius*

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RESUMO

Staphylococcus pseudintermedius pertence a microbiota cutânea natural de pequenos animais. Porém, em situações de disbiose ocorre a proliferação desse agente oportunista e, consequentemente, o desenvolvimento de piodermite com sinais clínicos de eritema, pústulas, prurido e inflamação.Relatos de *S. pseudintermedius* resistente à meticilina (MRSP) têm se tornado frequentes na rotina clínica veterinária de animais de companhia. Devido ao aumento mundial de isolados bacterianos resistentes aos antimicrobianos convencionais, os óleos essenciais (OEs) têm sido estudados como alternativa terapêutica. A atividade antibacteriana *in vitro* dos óleos essenciais de *Melaleuca alternifolia, Mentha piperita* e *Rosmarinus officinalis* contra isolados de MRSP e *Staphylococcus pseudintermedius* suscetível à meticilina (MSSP) foi avaliada. Os ensaios foram realizados utilizando o método de microdiluição em caldo com o objetivo de determinar a concentração inibitória mínima (CIM). Diferentes concentrações dos óleos essenciais foram utilizadas (31.25 a 64000 µg/mL). Os valores da CIM dos óleos essenciais de *M. alternifolia, M. piperita* e *R. officinalis* contra o isolado de MRSP foram 10667, 32000 e 42666 µg/mL, respectivamente. *M. piperita* (OEMP) apresentou o menor valor de CIM (5333 µg/mL) e o óleo essencial de *M. alternifolia* (OEMA) apresentou o maior valor de CIM (37333 µg/mL) contra MSSP. Portanto, com a atividade antibacteriana *in vitro,* os OEs podem ser considerados alternativas para tratamento em infecções ocasionadas por *S. pseudintermedius*.

Palavras-chave: Piodermite canina; Resistência bacteriana; MRSP; Concentração inibitória mínima; Óleo de Melaleuca.

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ANTIBACTERIAL POTENTIAL OF ESSENTIAL OILS FROM *Melaleuca alternifolia, Mentha piperita,* AND *Rosmarinus officinalis* AGAINST *Staphylococcus pseudintermedius* ISOLATES

ABSTRACT

Staphylococcus pseudintermedius is an important pathogen in the veterinary routine of companion animals. It is part of the natural skin microbiota of small animals. However, the proliferation of this opportunistic pathogen occurs in situations of dysbiosis, and leads to the development of pyoderma with clinical signs such as erythema, pustules, pruritus, and inflammation. Reports of methicillin-resistant *S. pseudintermedius* (MRSP) have become frequent in the veterinary clinical routine of companion animals. Due to the global increase of multidrug-resistant bacteria, essential oils (EOs) have been studied as a therapeutic alternative. . The *in vitro* antibacterial activity of *Melaleuca alternifolia*, *Mentha piperita,* and *Rosmarinus officinalis* EOs against MRSP isolates and methicillin-susceptible *S. pseudintermedius* (MSSP) was evaluated. Assays were performed using the broth microdilution assay aiming for minimum inhibitory concentrations (MIC). Different concentrations of the EOs were placed (31.25 to 64000 µg/mL). The MIC values of the EOs from *M. alternifolia*, *M. piperita,* and *R. officinalis* against MRSP isolate were 10667, 32000, and 42666 µg/mL, respectively. *M. piperita* (EOMP) showed the lowest MIC value (5333 µg/mL) and EOMA from *M. alternifolia* showed the highest MIC value (37333 µg/mL) against MSSP. Therefore, the antibacterial effectiveness of the EOs can be considered for further therapeutic use.

Keywords: Canine pyoderma; Bacterial resistance; MRSP; Minimum inhibitory concentrations; Tea tree oil.

POTENCIAL ANTIBACTERIANO DE LOS ACEITES ESENCIALES DE *Melaleuca alternifolia, Mentha piperita Y Rosmarinus officinalis* CONTRA AISLADOS DE *Staphylococcus pseudintermedius*

RESUMEN

Staphylococcus pseudintermedius pertenece a la microbiota cutánea natural de los animales pequeños. Sin embargo, en situaciones de disbiosis ocurre la proliferación de este agente oportunista y, en consecuencia, el desarrollo de piodermatitis con signos clínicos de eritema, pústulas, prurito e inflamación. Los informes de *S.pseudintermedius* resistente a meticilina (MRSP) se han vuelto frecuentes en la rutina clínica veterinaria de animales pequeños. Debido al aumento mundial de aislados bacterianos resistentes a los antimicrobianos convencionales, los aceites esenciales (AEs) han sido estudiados y se están convirtiendo en una alternativa terapéutica. Se evaluó la actividad antibacteriana in vitro de los AE de *Melaleuca alternifolia*, *Mentha piperita* y *Rosmarinus officinalis* contra los aislados de MRSP y *Staphylococcus pseudintermedius* susceptible a meticilina (MSSP). Los ensayos se realizaron utilizando el método de microdilución en caldo para determinar las concentraciones inhibitorias mínimas (CIM). Se colocaron diferentes concentraciones de los AE (31.25 a 64000 µg/mL). Los valores de CIM de los AE de *M. alternifolia*, *M. piperita* y *R. officinalis* contra el aislado de MRSP fueron 10667, 32000 y 42666 µg/mL, respectivamente. *M. piperita* (AEMP) mostró el valor más bajo de CIM (5333 µg/mL) y el AE de *M. alternifolia* (AEMA) mostró el valor más alto

de CIM (37333 μg/mL) contra MSSP. Por lo tanto, la efectividad antibacteriana de los AE puede considerarse para su uso terapéutico futuro.

Palabras clave: Pioderma canina; Resistencia bacteriana; MRSP; Concentraciones inhibitorias mínimas; Tea tree.

INTRODUCTION

Canine pyoderma is a bacterial infection usually associated with the opportunistic agent *Staphylococcus pseudintermedius*. It is considered one of the most recurrent dermatological disorders in small animals, especially in dogs, and develops secondary to other dermatopathies. The infections are associated with minor trauma, immunosuppression, parasitic, fungal, or allergic dermatopathies as well as endocrine and metabolic disorders (1).

S. pseudintermedius are Gram-positive cocci and are widely distributed throughout the world. At least 30 species of *Staphylococcus* can be commensal on the skin of animals and humans. In the scope of veterinary medicine, the agents *S. pseudintermedius*, *S. intermedius,* and *S. delphini* comprise the *Staphylococcus intermedius* group (SIG) due to the high degree of genetic similarity. *S. pseudintermedius* is the main pathogenic species isolated from dogs (1,2).

As a result of the increasingly close contact between humans and pets, *S. pseudintermedius* is considered an important pathogen with zoonotic potential. Reports indicate an increase of methicillin-resistant *S. pseudintermedius* (MRSP) isolation in the veterinary routine, creating concerns and occasionally failure of conventional treatments. MRSP isolates have shown multidrug-resistance profiles worldwide, including resistance to several classes of antibiotics such as fluoroquinolones and tetracyclines, but mainly to β-Lactam antibiotics including methicillin, which also implies resistance to other antimicrobials of the same class, such as penicillin, amoxicillin, and oxacillin (3).

EOs have become an alternative for the discovery of new antibacterial agents since they have chemical compounds with antimicrobial properties and prove to be effective in the treatment of infections (4). In addition, these products stand out for having fewer adverse effects, low cost, better biodegradability, and patient tolerance (5,6). The effectiveness of the antimicrobial activity of EOs has been studied, mainly in *in vitro* conditions. Its activity generates irreversible damage to the cell wall of bacteria, inducing the loss of salt and energy substrates and inhibiting the production and action of bacterial toxins that cause infectious and inflammatory processes (7). However, the composition and properties of EOs modify according to the plant species and the influence of environmental factors, and thus their antimicrobial effects. For example, tea tree oil is extracted from the *Melaleuca alternifolia* tree species native to regions of Australia, China, and Kenya (7). *Rosmarinus officinalis* is a species of aromatic herb cultivated worldwide, known for its nutritional values and pharmacological properties that made it famous in traditional medicine and cosmetics production (8) Peppermint EO (*Mentha piperita*) is cultivated in Europe and the United States. It has been widely used as a therapy due to its significant antibacterial and antifungal activity against Gram-positive and Gram-negative bacteria (9,10).

Therefore, this study aimed to evaluate whether commercial EO of tea tree (*M. alternifolia*), rosemary (*R. officinalis*), and peppermint (*M. piperita*) have antibacterial activity against methicillin-resistant *S. pseudintermedius* (MRSP) and methicillin-susceptible *S. pseudintermedius* (MSSP) isolates.

MATERIALS AND METHODS

Essential Oils

The EOs from *M. alternifolia*, *M. piperita,* and *R. officinalis* were obtained by the company By Samia Aromatherapy Ltda. (São Paulo, Brazil) following the Brazilian Norms (NBR) 14725: 2012 and Globally Harmonized System (GHS) standards. The EOs were stored in amber bottles with 10 ml capacity. The chemical composition was obtained by gas chromatography and was provided by the company By Samia Aromaterapia (Table 1).

Table 1. Density and major compounds obtained according to the analysis certificate by the company By Samia Aromatherapy.

Essential oils	Density (g/m)	Major compounds (%)
Melaleuca alternifolia	0.895	terpinen-4-ol (41.1), γ-terpinene (19.5), α-terpinene (9.4), α-terpineol (4.7), p-cimene (3.5), α-pinene (3.4), α-terpinolene (3.2), cineol (2.6), limonene (2.2), aromadendrene (1.9)
Rosmarinus officinalis	0.845	camphor (24.2), α-pinene (21.1), 1,8-cineole (18.8), camphene (9.0), β-pinene (5.2), borneol (3.4), α-terpineol (3.0), limonene (2.7), p-cimene (2.4)
Mentha piperita	0.902	menthol (40.2), menthone (24.4), menthyl acetate (4.7), 1,8-cineole (4.6), menthofuran (4.0), isomenthone (3.9), levomenthol (3.8)

Isolates of *Staphylococcus pseudintermedius* origin and characterization

The samples *S. pseudintermedius* used were stored at the Veterinary Clinic department of the Faculty of Veterinary Medicine and Zootechnics (FMVZ), UNESP, Botucatu/SP, Brazil. They were isolated from dogs with superficial or deep, primary, or recurrent pyoderma, with or without concomitant disease, with no predilection for the breed, sex, or age, routinely treated in different sectors in Botucatu/SP, Brazil, from September 2018 to January 2020.

The technique Matrix Assisted Laser Desorption Ionization-time off Light Mass Spectrometry (MALDI-TOF MS) was used to identify the isolated species. The analysis was conducted at the Milk Quality Research Laboratory at the Department of Animal Nutrition and Production of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo, Pirassununga, SP (11).

The presence of *mec*A resistance gene was performed in each isolate by PCR using the *mec*A1 and *mec*A2 primers. International reference strains were used as positive control (*Staphylococcus aureus* ATCC 33591) and negative (*S. aureus* ATCC 25923). The samples were analyzed in the Molecular Biology Laboratory of the Institute of Biosciences at UNESP (11).

The disk diffusion technique was used to perform the *in vitro* sensibility profile of the isolates. Sensitivity was determined by halo diameters, divided into three categories: sensitive (S), partially sensitive (PS), and resistant (R) (12,13). The isolates were stored in Ligniéres medium after characterization. The samples were transferred into 1.5 ml microtubes containing BHI medium, 30% warmed glycerol, and placed in a storage freezer at -80ºC.

One MRSP isolate and one MSSP isolate were selected according to the *in vitro* microbial sensitivity profile (Table 2), which agreed with the MALDI-TOF MS technique. *S. pseudintermedius* ATCC (49444) strain was used for control.

Table 2. *In vitro* profile performed by disc diffusion of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and methicillin-susceptible *Staphylococcus pseudintermedius* (MSSP) obtained from dogs with pyoderma.

ANTIBIOTICS	MRSP	MSSP
Amicacin	S	S
Amoxicillin + CL[1]	R	S
Ampicillin	R	S
Azithromycin	R	S
Cephalexin	R	S
Cefovecin	R	S
Ceftiofur	R	S
Ceftriaxone	R	S
Ciprofloxacin	R	S
Clarithromycin	R	S
Clindamycin	R	S
Chloramphenicol	R	S
Doxycycline	R	S
Enrofloxacin	R	S
Erythromycin	R	S
Gentamicin	R	S
Levofloxacin	R	S
Neomycin	R	S
Norfloxacin	R	S
Oxacillin	R	S
Penicillin	R	S
Rifampicin	R	S
Tetracycline	R	S
Tigecycline	R	S

[1]CL: Potassium Clavulanate. * Sensitive; ** Resistant

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

Assays were performed using the broth microdilution assay aiming for minimum inhibitory concentrations (MIC). Different concentrations of the essential oils were placed (31.25 to 64000 µg/mL), adjusted by serial dilution in 96-well sterile microtiter plates containing Mueller Hinton Broth (MHB) + 1% Tween 80, obtaining 100 µl of the product tested in each well. Inocula from overnight cultures at 37 ºC were standardized in saline solution according to 0.5 McFarland standard (approximately 1.5×10^8 colony forming units (CFU/mL). 100 µl of the inocula were added to each well, resulting in a final 200 µl and approximately 10^5 CFU/mL per well. Positive controls, consisting of MHB and Amoxicillin + Potassium Clavulanate (250 mg) or Cephalexin (500 mg), were included at 1 to 500 µg/mL concentrations. The microplates were then incubated at 37 °C for 24 h. A solution of resazurin redox revealing compound (0.01%) was used to indicate viable bacteria cells. The MIC was considered the

lowest concentration of EO for which there was no bacterial growth after the incubation period. Subcultures were performed from the microdilution assay in Petri dishes using brain heart infusion (BHI) + agar and were incubated for 24 h at 37 ºC to obtain minimum bactericidal concentration (MBC). MBC values were considered the lowest concentration with no colony growth. The assays followed Clinical & Laboratory Standards Institute (CLSI guidelines) (12).

STATISTICAL ANALYSIS

The assay was performed in a completely randomized design with three replications per treatment. Values were transformed using ln (x) and compared according to Tukey's test ($p < 0.05$) in the statistical program RStudio.

RESULTS

All isolates were sensitive to the tested EOs. It was possible to observe their *in vitro* antibacterial effects and to obtain the MIC. *M. alternifolia* EO had the lowest MIC (10667 µg/mL) against MRSP isolate between the EOs. The EOs from *M. piperita* (32000 µg/mL) and *R. officinalis* (42667 µg/mL) were statistically equal against MRSP isolate (Table 3). Amoxicillin + CL (31.25 µg/mL) was more effective than Cephalexin (666 µg/mL) against MRSP isolate.

Table 3. Minimum inhibitory concentrations (MIC, µg/mL) of the essential oils from *Melaleuca alternifolia, Rosmarinus officinalis, Mentha piperita* and the antibiotics Amoxicillin + Potassium Clavulanate and Cephalexin against *Staphylococcus pseudintermedius.*

	M. alternifolia	***R. officinalis***	***M. piperita***	**Amoxicillin + CL**	**Cephalexin**
Strains	MIC	MIC	MIC	MIC	MIC
MRSP	10667[1,2] Ac	42667 Bd	32000 Bd	31.25 Ca	666 Bb
MSSP	37333 Ad	16000 Acd	5333 Ac	0.33 Aa	1.33 Ab
S. pseudintermedius ATCC (49444)	21333 Ac	64000 Bd	32000 Bcd	1.33 Ba	3.33 Ab

[1]Means within a column followed by the same upper-case letters are not significantly different from each other using the Tukey test ($p < 0.05$).
[2]Means within a row followed by the same lowercase letters are not significantly different from each other using the Tukey test ($p < 0.05$).

The MSSP isolate was more sensitive to the positive control Amoxicillin + CL (0.33 µg/mL). Among the EOs, *M. piperita* EO showed the lowest MIC value, 5333 µg/mL (Figure 1), and the EO from *M. alternifolia* (37333 µg/mL) had the highest MIC against MSSP isolate. There was no statistically significant difference between the EOs of *M. piperita* and *R. officinalis* against MSSP isolate (Table 3).

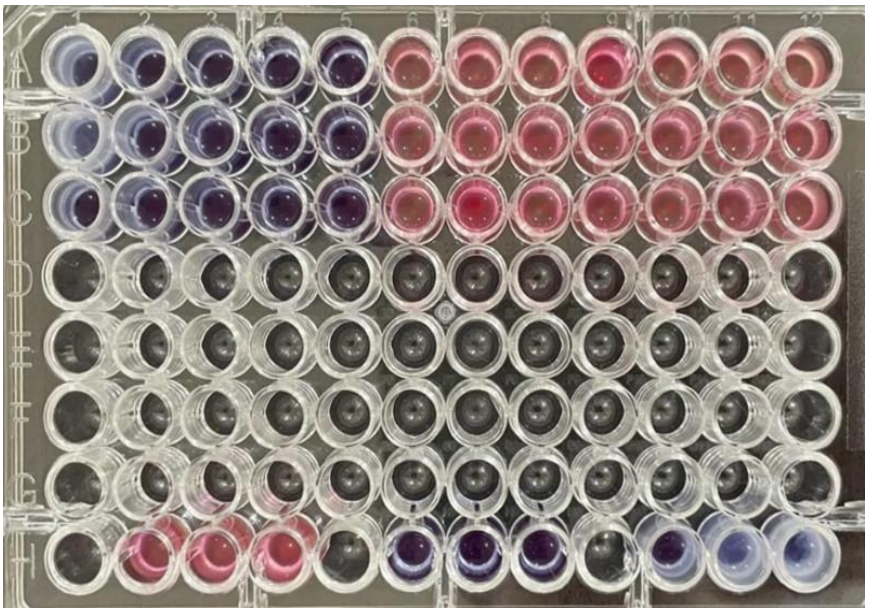

Figure 1. Microtiter plate used in the broth microdilution assay against MSSP isolates demonstrating the MIC value *M. piperita* EO (5333 µg/mL) after adding resazurin (0.01%). Purple wells indicate concentrations without bacterial growth and pink wells with bacterial growth.

In this study, we observed that the EO from *R. officinalis* had the highest MIC value against MRSP isolate and *S. pseudintermedius* ATCC (49444) strain (Table 3). The MBC valuesof the EOs were higher when compared to the MIC. We can see that Amoxicillin + CL showed the lowest MBC value (< 0.25 µg/mL) against MSSP isolate, followed by Cephalexin (2.0 µg/mL) (Table 4). Among the EOs, *M. alternifolia* EO showed the lowest MBC value (16000 µg/mL) against the MRSP isolate, and *M. piperita* EO showed the lowest MBC (8000 µg/mL) against the MSSP isolate. Furthermore, the EO from *R. officinalis* showed a value higher than 64000 µg/mL against the *S. pseudintermedius* ATCC (49444) strain (Table 4).

Table 4. Minimum bactericidal concentration (MBC) in µg/mL of the essential oils from *M. alternifolia*, *R. officinalis*, *M. piperita,* and the antibiotics Amoxicillin + Potassium Clavulanate and Cephalexin.

	M. alternifolia	***R. officinalis***	***M. piperita***	**Amoxicillin + CL**	**Cephalexin**
Strains	MBC	MBC	MBC	MBC	MBC
MRSP	16000	64000	32000	62.50	> 500
MSSP	64000	16000	8000	< 0.25	2.0
S. pseudintermedius ATCC (49444)	64000	> 64000	64000	2.0	4.0

DISCUSSION

Bacterial resistance is a current issue due to the exacerbated and frequent use of antimicrobials that increased the concern of health professionals and triggered the need for alternative treatments in this situation. Since then, the use of essential oils (EOs) has become a therapeutic alternative for dermatopathies due to antibacterial, antifungal, and anti-inflammatory properties (5).

Gram-positive bacteria, such as those of the *Staphylococcus* genus, are more susceptible to EOs than Gram-negative ones (14). One explanation is that Gram-positive bacteria have a cell wall with greater permeability, which facilitates the penetration of the molecule. Otherwise, Gram-negative bacteria have a barrier system consisting of the outer membrane of the bacterial wall formed by phospholipids, lipopolysaccharides, and proteins that provide greater impermeability to antibacterial agents (15). According to this study, we conclude that the EOs tested had *in vitro* antibacterial activity against *S. pseudintermedius* isolates (Table 3).

EOs can easily penetrate bacterial cell membranes due to their lipophilic nature. Therefore, its antimicrobial action primarily destabilizes cellular architecture, disrupting the membrane and increasing permeability, causing the leakage of cellular components and ions (16,17). The disruption of cell membranes by EOs impairs several processes, such as energy conversion, nutrient processing, synthesis of structural macromolecules, and secretion of growth regulators (18).

In this study, we tested the EOs from *M. alternifolia* and *R. officinalis* due to the antibacterial efficacy against *S. pseudintermedius* isolated from canine pyoderma as described by Meroni et al. (19). Our results showed that *M. alternifolia* EO had the lowest MIC (10667 µg/mL) against MRSP isolate between the EOs. However, it showed the highest MIC against MSSP isolate (37333 µg/mL). Andrade et al. (20) described a higher susceptibility of *S. aureus* when compared with the Gram-negative bacteria *Escherichia coli* with *M. alternifolia* EO with MIC values of 210 µg/mL and 4290 µg/mL, respectively.

The *M. piperita* EO is effective in treating bacterial infections due to the activity of its chemical components, mainly menthol, its major compound (21). Desam et al. (9) observed with the disc diffusion method that *S. aureus* and *Staphylococcus pyogenes* species were also more sensitive to *M. piperita* EO with MIC values of 0.75 µg/mL and 2.04 µg/mL, respectively. As observed in our results, *M. piperita* EO showed the lowest MIC value (5333 µg/mL) against MSSP.

The major compounds of *R. officinalis* EO were camphor, α-pinene, and 1,8-cineole (Table 1), considered the main ones with therapeutic activity (22). Gram-positive bacteria have been shown to have greater sensitivity to essential oils (23). Nevertheless, our results show that EO from *R. officinalis* had the highest MIC value against MRSP isolate and *S. pseudintermedius* ATCC (49444) strain.

In literature, the major components are the most studied and generally reflect the biological and biophysical characteristics of EOs. The effect of these isolated compounds is modified according to their concentration when tested alone or synergistically with other compounds (24). In a study conducted by Andrade et al. (25) with the aim of assessing the susceptibilities of tea tree (*M. alternifolia*), geranium (*Pelargonium graveolens*) and palmarosa (*Cymbopogon martinii*) EOs and their major compounds terpinen-4-ol, citronellol and geraniol, respectively, showed that terpinen-4-ol produced a largest inhibitory zone when compared to tea tree EO against *Staphylococcus* spp. Also, suggested that geraniol was probably responsible for the antibacterial activity of *C. martinii* EO.

There is urgency in developing new substances with antibacterial properties against multidrug-resistant bacteria. However, even with the promising *in vitro* results of other studies, there are difficulties to be solved that allow the use of EOs *in vivo* as stability, selectivity, and bioavailability of these natural products. Moreover, ideal proportions and dosages must be optimized for greater effectiveness and decreased toxicity (5).

In recent years, essential oils have acquired importance for their antibacterial potential. The agent's susceptibility and the EOs' activity may vary according to the species or strain of the target bacteria (24). However, more studies evaluating the antibacterial efficacy of EOs against *S. pseudintermedius* isolates still need to be made available.

CONCLUSION

It was observed that the values obtained for MIC and MBC demonstrate that EO had an antibacterial effect against *S. pseudintermedius* isolates. Nevertheless, more studies *in vitro* and *in vivo* conditions evaluating different concentrations, types, and compounds of EOs, must be conducted. Therefore, essential oils can be promising sources of alternative or complementary therapies in the treatment of canine pyoderma.

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ETHICAL STATEMENT

This study was approved by the Ethics Committee for the Use of Animals in Research (CEUA) of the School of Veterinary Medicine and Animal Science of São Paulo State University (UNESP) (no. 0025/2022).

COMPETING INTEREST

The authors have no conflict of interest regarding this publication.

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