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Antimicrobial activity of the interaction between essential oils against resistant *Staphylococcus aureus* isolates from bovine mastitis

Atividade antimicrobiana da interação entre óleos essenciais contra *Staphylococcus aureus* resistentes isolados de mastite bovina

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Gabriel Cariolato Schlemper
ORCID: https://orcid.org/0000-0003-2520-2226
Instituto de Zootecnia, Brazil
E-mail: gabrielcariolato1@gmail.com
Lívia Castelani
ORCID: https://orcid.org/0000-0001-7801-3256
Instituto de Zootecnia, Brazil
E-mail: liviacastelani@gmail.com
Geovana Menegão de Souza
ORCID: https://orcid.org/0000-0002-2623-250X
Instituto de Zootecnia, Brazil
E-mail: geovanamenegao@gmail.com
Weber Vilas Boas Soares
ORCID: https://orcid.org/ 0000-0003-3617-446X
Instituto de Zootecnia, Brazil
E-mail: weber.soares@sp.gov.br
Luiz Carlos Roma Junior
ORCID: https://orcid.org/ 0000-0002-0019-2538
Instituto de Zootecnia, Brazil
E-mail: lcromajr@gmail.com

ABSTRACT

The control of bovine mastitis is crucial for the sustainability of dairy production. Phytotherapy emerges as a potential alternative for controlling resistant bacteria and potentially replacing conventional therapies. This study aimed to assess the in vitro antimicrobial effect of the *Pelargonium graveolens* (GEO) and *Thymus vulgaris* (TEO) essential oils, as well as the interaction between them against strains of *Staphylococcus aureus* isolated from bovine mastitis. Eight strains of *S. aureus* isolated from bovine mastitis and two standard strains of human origin were used. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined using the microdilution method. The interaction between the oils was assessed using the checkerboard method. It was observed that both essential oils exhibited inhibitory effects when evaluated separately; however, TEO demonstrated superior bactericidal efficacy. The combination of both oils resulted in a bactericidal complex with a 99% reduction in the effective concentration of GEO. In conclusion, the TEO/GEO complex may contribute to improved therapeutic efficacy, reducing adverse effects, costs, and bacterial resistance development.

Keywords: *Pelargonium graveolens*; *Thymus vulgaris;* Synergism; Mammary Gland; Checkerboard Method.

RESUMO

O controle da mastite bovina é fundamental para a sustentabilidade da produção leiteira. A fitoterapia surge como potencial alternativa para o controle de bactérias resistentes e a possível substituição das terapias convencionais. Os objetivos deste estudo foram avaliar o efeito antimicrobiano in vitro dos óleos essenciais de *Pelargonium graveolens* (GEO) e *Thymus vulgaris* (TEO) e a interação entre eles contra cepas de *Staphylococcus aureus* resistentes isoladas de mastite bovina. Foram utilizadas oito cepas de *S. aureus* isoladas de mastite bovina e duas cepas padrão de origem humana. A Concentração Inibitória Mínima e a Concentração Bactericida Mínima foram determinadas pelo método de microdiluição. A interação entre os óleos foi feita pelo método de checkerboard. Observou-se que ambos os óleos essenciais apresentaram efeito inibitório quando avaliados separadamente, porém TEO apresentou melhor eficácia bactericida. A associação de ambos os óleos resultou em um complexo bactericida com redução de 99% da concentração efetiva de GEO. Conclui-se que o complexo TEO/GEO pode contribuir para melhor eficácia terapêutica, reduzindo os efeitos adversos, os custos e o desenvolvimento de resistência bacteriana.

Palavras-chave: *Pelargonium graveolens; Thymus vulgaris*; Sinergismo; Glândula Mamária; Método de Checkerboard.

INTRODUCTION

The bovine mastitis is a mammary gland inflammation that causes countless losses for the dairy industry and for the consumer’s health. This disease results in the stiffening of mammary tissue, directly affecting the production and milk quality. In more severe cases, can commit one or more mammary quarters, potentially even causing the death of the animal (DIAS; ANTES, 2014).

The economic impact of bovine mastitis includes reduction of dairy production due to clinical and subclinical cases, milk disposal, expenses on medications, labor costs, reduced milk selling prices, and animal culling (GUIMARÃES et al., 2017). According to Demeu et al (2015), the average cost of each mastitis treatment is around US$ 150.40.

Bovine mastitis typically has a bacterial origin and can be classified as environmental and contagious, depending on the respective etiological agents (CAPELLARI; ROSSI; MARTINS BONOTTO, 2022). Among contagious microorganisms, *Staphylococcus aureus* is considered one of the main due to its ability to produce various virulence factors and high resistance to the main antimicrobials used in therapy. These microorganisms are part of the microbiota of skin and animal mucous, and transmission primarily occurs from animal to animal during milking (LOCATELLI; JÚNIOR, 2016).

The control and prevention of staphylococcal mastitis are generally achieved through the adoption of good hygiene practices and antimicrobial therapy. However, *S. aureus* exhibits a low response to conventional therapies. Furthermore, the use of antibiotics can lead to milk residues, causing economic losses for the producer and potential health risks for consumers (LANGONI et al., 2017). Therefore, the search for new alternative therapies is relevant, and the use of essential oils may be promising, since they are widely employed in medicine and the food industry. These molecules have antimicrobial, anti-inflammatory, and antioxidant properties (KUROSAWA et al., 2020)

One of the essential oils with significant therapeutic potential is thyme essential oil (*Thymus vulgaris* L.). This plant, belonging to the *Lamiaceae* family, exhibits high antimicrobial activity primarily due to the phenolic compounds thymol and carvacrol present in its composition (ALMASI; RADI; AMIRI, 2020). These compounds can act by interfering with the permeability of the microbial cell membrane, inhibiting protein synthesis, and the activity of specific enzymes, such as those responsible for cellular respiration (VAIČIULIENĖ et al., 2020).

Another oil that stands out for its pharmacological antimicrobial properties is geranium oil (*Pelargonium graveolens*). This plant, belonging to the *Geraniaceae* family, present anti-inflammatory and antioxidant activity (JARADAT et al., 2022). Its antimicrobial activity is associated with phytoconstituents of the monoterpenes class, such as β-citronellol, limonene, α-pinene, and ß-pinene, which induce alterations in the permeability of the microbial cell membrane (GHANNADI A et al., 2012; IOAN COCOȘ et al., 2023).

The association of different medicinal plants is a promising strategy to controlling resistant bacteria. These combinations can affect several biochemical processes in bacteria, acting on different molecular targets or biological pathways (BASSOLÉ; JULIANI, 2012). Furthermore, formulations that combine different molecules may have reduced concentrations, leading to a consequent decrease in side effects and costs (BAG; CHATTOPADHYAY, 2015)..

The present study assumes that there is a synergistic interaction between the of thyme and geranium essential oils, forming a complex with antimicrobial effects against resistant strains of *S. aureus*. Therefore, the objective was to evaluate the in vitro antimicrobial effect of essential oils from geranium (*Pelargonium graveolens*) and thyme (*Thymus vulgaris* L.) and their interaction against resistant strains of *S. aureus* isolated from bovine mastitis and ATCC strains of human origin.

MATERIAL AND METHODS

Ethics statement: All procedures involved in the experiment were approved by the Ethics Committee on Animal Use (Approval CEUA/IZ No. 355-2022, dated 22 June 2022) of the Institute of Animal Science, Nova Odessa, SP, Brazil.

Bacterial isolates: Eight strains of *S. aureus* isolated from bovine mastitis were used in this study. The strains belong to the collection of the Milk Quality Laboratory of the Institute of Animal Science, Dairy Cattle Research and Development Center, Nova Odessa, São Paulo, Brazil. In addition, two standard American Type Culture Collection (ATCC®) strains of human origin were used: *S. aureus* ATCC BAA® 976 and *S. aureus* ATCC® 1026. The identification of bacterial species was done by biochemical tests (MURRAY et al., 2003). The strains were chosen according to the resistance profile determined by the agar disk-diffusion method on Müeller-Hinton Agar (MHA), as recommended by the Brazilian Committee on Antimicrobial Susceptibility Testing, BRCast (BRCAST - BRAZILIAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING, 2022). The resistance profile of the strains is described in Table 1.

Table 1. *Staphylococcus aureus* strains resistant to at least one class of antimicrobial, by the disk diffusion method, used in the present study.

Strain	Antibiotic Classes	Resistance Profile
1005P	aminoglycoside, folate inhibitor, tetracycline	Gen, Tet, Sxt
1020P	aminoglycoside, folate inhibitor, tetracycline	Gen, Tet, Sxt
45T	aminoglycoside, amphenicol, quinolone, oxazolidinone	Ery, Flf, Kan, Lzd, Neo
906T	beta-lactam	Amp, Pen
1808T	aminoglycoside, beta-lactam	Amp, Pen, Str
1849T	aminoglycoside, beta-lactam, lincosamine, macrolide, quinolone	Amp, Cip, Cli, Eno, Ery, Pen, Str
929F	Beta-lactam, folate inhibitor, quinolone, tetracycline	Cip, Eno, Pen, Sxt Tet
2016F	Beta-lactam, folate inhibitor, tetracycline	Pen, Sxt, Tet
ATCC® BAA-976	aminoglycoside, amphenicol, beta-lactam, macrolide	Amp, Flf, Ery, Neo, Oxa, Pen
ATCC® BAA-1026	aminoglycoside, amphenicol, beta-lactam, cephalosporin, folate inhibitor, lincosamine, macrolide, quinolone, tetracycline	Amp, Cf, Flf, Cli, Eno, Ery, Gen, Neo, Oxa, Pen, Sxt

Amp: ampicillin; Cf: cephalothin; Cip: ciprofloxacin; Cli: clindamycin; Eno: enrofloxacin; Ery: erythromycin; Flf: florfenicol; Gen: gentamicin; Kan: kanamycin; Lzd: linezolid; Neo: neomycin; Oxa: oxacillin; Pen: penicillin G; Str: streptomycin; Sxt: sulfamethoxazole/trimethoprim; Tet: tetracycline.

Preparation of the essential oils stock solution: T. vulgaris (TEO) and *P. graveolens* (GEO) essential oils was obtained from "*Farmácia Professor Accorsi Plantas Medicinais*" and "*BioEssência*", respectively. The solubilization of oils was performed in Müeller-Hinton Broth (MHB) supplemented with 0.5% Tween 80, being sterilized by filtration using a 0.45-µm filter.

In vitro determination of the antimicrobial activity of essential oils: An initial inoculum of 1.5 x 10^7 CFU/mL was prepared from each *S. aureus* isolate in MHB. The essential oils were diluted in MHB, followed by two-fold dilutions. The Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC) values of biomolecules were determined by the microbroth dilution technique (ELOFF, 1998). The plates were incubated at 36.5 °C for 24 hours under aerobic conditions.

To determine the MIC, after incubation, 50 µL of 0.5% TTC (Triphenyl-Tetrazolium Chloride) solution was added to the wells, and the plates were reincubated for another 3 hours. The MIC is defined as the lowest concentration capable of preventing bacterial growth, characterized by the appearance of red color, given to the medium when the cells show respiratory activity (KLANČNIK et al., 2010). To determine the CBM, before adding the TTC solution, a 5-µL aliquot from all wells was seeded onto MHA plates and incubated overnight at 36.5 °C. The MBC was defined as the lowest concentration of the antimicrobial agent that kills > 99.9% of the initial bacterial population, indicated by the absence of visible growth of bacteria on the plates (BALOUIRI; SADIKI; IBNSOUDA, 2016).

Checkerboard method: An initial inoculum of 1.5 x 10^7 CFU/mL was prepared from each *S. aureus* isolate in MHB. Essential oils concentrations were prepared in MHB and distributed in the wells of the microdilution plates, as a checkerboard (LINCOPAN; CARMONA-RIBEIRO, 2006; ODDS, 2003). A 50-µL aliquot of TEO was serially diluted along the abscissa, while a 50 µL aliquot of GEO was diluted along the ordinate, for a total of 100 µL in each well. After 1 h of interaction, 5 µL of the inoculum was added and the plates were incubated for 24 h at 36.5 °C under aerobic conditions. The determination of MIC and MBC was made as previously described.

The fractional inhibitory concentration and fractional bactericidal concentration (ΣFIC and ΣFBC) was calculated as follows:

$$\text{FIC or FBC (A or B)} = \frac{\text{MIC/MBC A (or B) in combination}}{\text{MIC/MBC A (or B) alone}}$$

The combination is classified as synergistic when the ΣFIC/FBC is ≤0.5, partially synergistic when the ΣFIC/FBC > 0.5 - ≤ 1.0, indifferent when the ΣFIC/FBC is 1.0 - 4.0, and antagonistic when the ΣFIC/FBC is ≥ 4.0. (FERNÁNDEZ-CUENCA et al., 2003; HALL; MIDDLETON; WESTMACOTT, 1983; ODDS, 2003).

RESULTS

The minimum inhibitory concentration that inhibited 80% and 60% of analyzed *S. aureus* strains was 625 µg/mL and 2500 µg/mL for thyme (TEO) and geranium essential oils (GEO), respectively. The checkerboard test confirmed the in vitro activity of the combination of TEO and GEO, referred to as the TEO/GEO complex, resulting in a reduced MIC of 312/625 µg/mL. The determination of ΣFIC revealed synergistic activity of the complex in 20% of the isolates, partially synergistic in 70% and indifferent in 10% (Table 2). However, it should be noted that the combination of oils resulted in a 50% reduction in the MIC for thyme essential oil and a 75% for geranium essential oil.

Table 2. Minimum Inhibitory Concentration (MIC) for *Thymus vulgaris* essential oil (TEO), *Pelargonium graveolens* essential oil (GEO) and TEO/GEO complexes against *Staphylococcus aureus* isolated from bovine mastitis and *S. aureus* reference strains.

Strain	MIC (µg/mL)			FIC[1]			
	TEO	GEO	TEO/GEO	TEO	GEO	ΣFIC	Interp.[2]
1005P	625	2500	312/312	0,50	0,12	0,62	PS
1020P	625	2500	312/312	0,50	0,12	0,62	PS
45T	1250	1250	312/625	0,25	0,50	0,75	PS
906T	1250	2500	312/625	0,25	0,25	0,50	S
1808T	625	2500	312/625	0,50	0,25	0,75	PS
1849T	625	2500	312/625	0,50	0,25	0,75	PS
929F	625	2500	312/625	0,50	0,25	0,75	PS
2016F	625	625	156/312	0,25	0,50	0,75	PS
ATCC® BAA-976	625	625	156/625	0,25	1,00	1,25	I
ATCC® BAA-1026	625	1250	156/312	0,25	0,25	0,50	S

[1]FIC = fractional bactericidal concentration (FIC = MIC combination/MIC alone); ΣFIC = FIC summation. [2]Interpretation: The combination is classified as synergistic (S) when ΣFIC is ≤0.5, partially synergistic (PS) when ΣFIC is >0.5 to ≤1.0, indifferent (I) when ΣFIC is >1.0 to 4.0, and antagonistic (A) when ΣFIC is ≥4.0 (HALL; MIDDLETON; WESTMACOTT, 1983).

Regarding the minimum bactericidal concentration, MBC values of 625 µg/mL were observed in 40% of the resistant *S. aureus* strains, 40% of the strains had values of 2500 µg/mL for TEO, and 40% exhibited MBC values of 40000 µg/mL for GEO. For the TEO/GEO complex, a reduction in MBC to 1250/312 µg/mL was observed in 40% of the strains. The determination of ΣMBC revealed partially synergistic activity of the complex in 70% of the isolates, indifferent in 10%, and antagonism in 10% (Table 3). It is worth mentioning that the interaction of the oils resulted in a reduction in MBC of 50% for thyme and a 99% for geranium.

Table 3. Minimum Bactericidal Concentration (MBC) for *Thymus vulgaris* essential oil (TEO), *Pelargonium graveolens* essential oil (GEO) and TEO/GEO complexes against *Staphylococcus aureus* isolated from bovine mastitis and *S.* reference strains.

Strain	MBC (μg/mL)			FBC			
	TEO	GEO	TEO/GEO	TEO	GEO	ΣFBC	Interp.[2]
1005P	2500	40000	1250/312	0,50	0,01	0,51	PS
1020P	2500	40000	1250/312	0,50	0,01	0,51	PS
45T	1250	2500	312/1250	0,25	0,50	0,75	PS
906T	1250	5000	312/1250	0,25	0,25	0,50	S
1808T	625	>40000	2500/312	4,00	0,01	4,01	A
1849T	2500	40000	1250/312	0,50	0,01	0,51	PS
929F	2500	40000	1250/312	0,50	0,01	0,51	PS
2016F	625	1250	156/1250	0,25	1,00	1,25	I
ATCC® BAA-976	625	1250	312/625	0,50	0,50	1,00	PS
ATCC® BAA-1026	625	625	156/312	0,25	0,50	0,75	PS

[1]FBC = fractional bactericidal concentration (FBC = MBC combination/MBC alone); ΣFBC = FBC summation. [2]Interpretation: The combination is classified as synergistic (S) when ΣFBC is ≤0.5, partially synergistic (PS) when ΣFBC is >0.5 to ≤1.0, indifferent (I) when ΣFBC is >1.0 to 4.0, and antagonistic (A) when ΣFBC is ≥4.0 (HALL; MIDDLETON; WESTMACOTT, 1983).

DISCUSSION

Mastitis is the main disease that affects dairy herds, often resulting in permanent and irreversible damage to the mammary glands. Antibiotic therapies are generally used for its control and treatment. However, the presence of residues that these molecules generate in milk and bacterial resistance result in significant restrictions on this practice (EL-SAYED; KAMEL, 2021). In addition, *S. aureus*, one of the main etiological agents of mastitis, does not respond satisfactorily to available therapies, and the cure rate is around 10 to 30% (GOMES; HENRIQUES, 2016).

Phytotherapy has been used by humanity since ancient times and is based on the use of active compounds from plants or their derivatives. These active compounds are used for the prevention and treatment of different human and animal pathologies, including mastitis. Currently, the medicinal properties of these plants have received interest from the scientific community as a natural alternative for controlling bacteria resistant to conventional antibiotics (MUSHTAQ et al., 2018).

With the objective of evaluating the in vitro antimicrobial effect of TEO and GEO oils and the interaction between them against resistant strains of *S. aureus* isolated from bovine mastitis, it was observed that the oils, when evaluated individually, showed an inhibitory effect. However, TEO demonstrated superior bactericidal efficacy. Geranium oil showed higher MBC values, and was therefore considered bacteriostatic. Although, when the association of both oils was evaluated, it was observed that the TEO/GEO complex was capable of killing the strains at lower concentrations, with MBC reductions of 50% for TEO and 99% for GEO, with a partially synergistic effect. Individual essential oils contain complex elements that, when combined, can produce synergistic, additive, indifferent or antagonistic effects (BASSOLÉ; JULIANI, 2012).

The TEO primarily contains thymol, a phenolic monoterpene, and p-cymene, which are associated with intense bactericidal, fungicidal, and antiparasitic properties, with relatively low toxicity for humans and animals (WIŃSKA et al., 2019). The antimicrobial activity of GEO is mainly related to citronellol and geraniol, a monoterpenoid alcohol (DŽAMIĆ et al., 2014). According to Bassolé and Juliani (2012), essential oils that have aldehydes or phenols in their composition, such as cinnamaldehyde, citral, carvacrol, eugenol or thymol as main components exhibit greater antibacterial activity, followed by oils that contain terpenic alcohols. Furthermore, the authors mention that studies evaluating the combination of phenolic compounds with monoterpene alcohols have found synergistic effects against different microorganisms.

Gallucci et al., (2009) evaluated the antimicrobial activity of different terpenes, including thymol, geraniol and their combinations, and observed that the bactericidal or bacteriostatic effect is dependent on the evaluated dose. Furthermore, geraniol showed bacteriostatic activity and thymol bactericidal activity against *S. aureus*, corroborating the results of the present study. However, the association of both compounds resulted in a complex with an indifferent effect, which differs from that observed in the present study, in which the complex showed a partially synergistic activity. This fact may be related to differences in the methodology used and the use of thyme and geranium essential oils and not the major compounds thymol and geraniol. Essential oils are composed of major and minor components, and the association of these contributes to more effective antimicrobial activity, when compared to the use of isolated compounds (BASSOLÉ; JULIANI, 2012).

Another important aspect to be highlighted is that despite the bacteriostatic action of GEO being observed, this molecule can act as a potent anti-inflammatory agent. This oil contains antioxidant substances in its composition that can capture free radicals, protecting cells against damage (AL-MIJALLI et al., 2022). Controlling inflammation can contribute to the reduction of somatic cells, which are elevated in cases of mastitis. The increase in these cells contributes significantly to the loss of the industrial quality of milk, generating significant economic impacts on the producer and the dairy industry (ARGAW, 2016).

The use of essential oils for mastitis treatment can be an effective and environmentally sustainable option for pathogens control. As a plant-based product, they can be used in both conventional and organic dairy herds, which can be attractive to producers seeking for more sustainable options (LOPES et al., 2020). Furthermore, the association of different essential oils represents an alternative to avoid mechanisms of bacterial resistance, as these molecules may have distinct cellular targets (CARVALHO et al., 2016). Additionally, this combination may be significant for the control of bacteria that are multi-resistant to conventional antibiotics. Furthermore, the reduction in the antimicrobial concentration of the complex observed in this present study can contribute to reducing the risk of undesirable side effects, as well as a reduction in the costs of antimicrobial therapies for bovine mastitis control.

CONCLUSION

The in vitro association of essential oils from *Thymus vulgaris* L. and *Pelargonium graveolens* L. demonstrated the potential to improve the antimicrobial capacity of the oils against resistant strains of *S. aureus*. This combination can contribute to better therapeutic efficacy, since lower concentrations are used, thus reducing adverse effects and costs, in addition to hindering the development of resistance mechanisms by bacteria. However, it is necessary to carry out additional in vitro and in vivo studies to ensure its efficacy and safety in the context of bovine mastitis.

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