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Communication

[*Comunicação*]

Activity of essential oils from spices against *Staphylococcus* spp. isolated from bovine mastitis

[*Atividade de óleos essenciais de plantas condimentares frente* Staphylococcus *spp. isolados de mastite bovina*]

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Worldwide, economic losses due to mastitis have been estimated at $35 billion (Wellenberg *et al.*, 2002). *Staphylococcus* spp. is the main causative agent of bovine mastitis, with higher prevalence in cases of clinical and subclinical manifestations (Fagundes *et al.*, 2010). The most common treatment is based on intramammary infusion of antibacterial agents. However, cure rates obtained with such drugs are not always effective, because it may determine the emergence of resistant bacteria (Zafalon *et al.*, 2007) as well increase amounts of antibiotic residues in milk (Fagundes *et al.*, 2010). Nevertheless, the treatment of bovine subclinical mastitis caused by *S. aureus* in the lactation can be economically unviable (Zafalon *et al.*, 2007).

Alternative treatments to bovine mastitis with bacteriocins (Pieterse *et al.*, 2010) and plant derived compounds (Baskaran *et al.*, 2009; Mubarack *et al.*, 2011) have been described. Essential oils (EO) are classified as GRAS (generally regarded as safe), show antibacterial proprieties and resistance has not been reported after prolonged exposure. Then, the investigation of their antimicrobial activity against bacterial agents of mastitis is justifiable. In this context, this study aimed to: a) evaluate the *in vitro* antimicrobial activity of EO from spices against *Staphylococcus* spp.; b) compare the activity of EO against *Staphylococcus* spp. isolates with multiple profiles of susceptibility and resistance to penicillin, erithromicin and tetracycline antibiotics.

Thirty two isolates from bovine mastitis, from the Laboratório de Bacteriologia Veterinária, Universidade Federal de Santa Maria, Brazil, were studied. The isolates were identified as coagulase positive *Staphylococcus* spp. The standard strain *S. aureus* ATCC 29213 was employed as reference strain. The antimicrobial susceptibility tests were performed according to CLSI M31-A3 agar diffusion method (CLSI, 2008). Based on these tests, the isolates were divided into subgroups according to the following resistance profiles: 1) susceptible; 2) penicillin-resistant; 3) tetracycline and penicillin-resistant; 4) erythromycin and tetracycline-resistant, and 5) erythromycin, penicillin and tetracycline-resistant.

The EO were purchased from Essential7.com Roewell, NM, USA. The majority constituents were previously determined by Pozzatti *et al.* (2010).

The essential oil of *Origanum vulgare* (oregano) contains 92% of carvacrol; *Thymus vulgaris* (thyme), 64% of γ-terpinene; *Lippia graveolens* (Mexican oregano), 56.8% of carvacrol and 32.2% of *o*-cymene; *Rosmarinus officinalis* L. (rosemary), 28.6% of 1,8-cineole and 26.31% of camphor; *Salvia officinalis* L. (sage), 40.6% of cis-thujone; *Ocimum basilicum* L. (Basil), 32.2% of linalool and 23.6% of 1,8-cineole; *Zingiber officinale* (ginger), 20.8% of zingiberene. The majority constituents were purchased from Acros Organics, Fair Lawn, NJ, USA.

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Carvacrol (99.5% carvacrol), thymol (thymol 99.0%) and cineole (cineole 97.0%) EO were first diluted with methanol to achieve the concentration of 640mg/mL (Solution I) and then diluted to 1:100 in Mueller-Hinton broth, resulting in the concentration of 6,400µg mL^{-1} (Solution II).

The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined based on the document M31-A3 (CLSI, 2008). Briefly, the inoculum was prepared from cultures grown in Muller-Hinton agar, resulting in a bacterial suspension in saline equivalent to the 0.5 McFarland standard ($1x10^8$ CFU mL^{-1}). The solution II was distributed into wells of a microtiter plate and serial dilutions were prepared to obtain final concentrations from 6,400 to 100µg mL^{-1}. Inoculum volumes of 10µL were distributed in each well containing EO incorporated into Mueller-Hinton broth. The MICs were determined after incubation at 35°C/24h under aerobic conditions. These tests were performed in triplicate. To determine the MBC, volumes of 10µL from wells without visible bacterial growth after 24h incubation were transferred to the surface of Mueller-Hinton agar, which was incubated at 35°C/24h. Then, bacterial growth was observed, registering the lowest concentration of EO or majority compounds that did not show bacterial growth. These tests were performed in triplicate.

The Mann Whitney test was used to compare two independent samples in order to observe whether the different study groups had similar patterns of susceptibility or not.

The geometric means (GM) of the MICs and MBCs of EO against *Staphylococcus* spp. were [GM-MIC/GM-MBC]: *Origanum vulgare* [1600/2288µg mL^{-1}], *Lippia graveolens* [1562/2707µgmL^{-1}], *Thymus vulgaris* [1564/2370µg mL^{-1}], carvacrol [584/732µg mL^{-1}] and thymol [427/856µg mL^{-1}]. In this study, ginger, basil, rosemary and sage EO, and the major compound cineole showed no antibacterial activity at the tested concentrations against the microorganisms evaluated. The GM of MICs and MBCs indicated that oregano, thyme and Mexican oregano EO were equally active ($P>0.05$) but less active than carvacrol and thymol ($P<0.001$) (Table 1). Based on the antimicrobial susceptibility, significant differences were not observed between susceptibilities profile of subgroups (Table 2).

Table 1. Antimicrobial activity of essential oils (EO) from spices and from majority constituents carvacrol and thymol against *Staphylococcus* spp. isolated from bovine mastitis

EO and FMs	MIC (µg mL^{-1})			MBC (µg mL^{-1})	
	Range	GM*	MIC_{50}	Range	GM*
oregano	800 – 3,200	1,600A	1,600	1,600 - 6,400	2,288A
lippia	800 – 3,200	1,562A	1,600	1,600 - 6,400	2,707A
thyme	800 – 3,200	1,564A	1,600	1,600 - 3,200	2,370A
carvacrol	200 – 1,600	584B	800	200 - 3,200	732B
thymol	200 – 800	427B	400	400 - 6,400	856B

MIC = minimal inhibitory concentration; MBC = minimal bactericidal concentration; MIC_{50} = minimal inhibitory concentration able to inhibit 50% of isolates *GM = geometric mean. Distinct letters indicate statistical difference by Mann Whitney test, P<0,05.

Table 2. Susceptibility of *Staphylococcus* spp., with distinct profiles of resistance to antibiotics, compared to essential oils and majority constituents

Subgroups (n)	Susceptibility	GM MICs (µg/mL)				
		Ov	Lg	Tv	carv.	thymol
1 (2)	Sensible*	1,200.0	1,200.0	1,600.0	800.0	400.0
2 (5)	PEN resistant	1,600.0	1,837.9	1,600.0	527.8	303.2
3 (9)	PEN-TET resistant	1,568.2	1,600.0	1,600.0	800.0	503.9
4 (4)	ERI-TET resistant	1,600.0	1,600.0	1,345.4	1,345.4	475.7
5 (12)	PEN-ERI-TET resistant	1,795.9	1,600.0	1,600.0	475.7	400.0

TET= tetracycline; PEN= penicillin; ERI= erythromycin; GM MICs= geometric mean of minimum inhibitory concentrations; (n) number of isolates; Ov = *Oreganum vulgare*; Lg = *Lippia graveolens*; Tv = *Thymus vulgaris*; carv. = carvacrol.
*Sensible to tetracycline, penicillin, erythromycin, ceftiofur, ampicillin, cephalotin and oxacilin.

The remarkable antimicrobial activity of *O. vulgare, L. graveolens and T. vulgaris* against *Staphylococcus* spp. from multiple sources has been previously confirmed (Smith-Palmer *et al.*, 1998; Hoferl *et al.*, 2009). These studies reported significant antimicrobial activity of oregano EO against Gram positive and Gram negative bacteria, including *Staphylococcus aureus*. Arana-Sanchez *et al.* (2010) showed antimicrobial activity of *L. graveolens* against *E. coli* ATCC 11229 and *Staphylococcus* species. The phenolic compounds carvacrol and thymol as major constituents ensure such activities. This activity had also been reported against sensible and resistant yeasts to fluconazole (Pozzatti *et al.*, 2010).

Rosemary, sage, basil and ginger EO, and the major constituent cineole did not show antimicrobial activity at the studied concentrations. However, Viuda-Martos *et al*. (2008) and Delamare *et al*. (2007) reported antimicrobial activity of sage and rosemary EO against *Staphylococcus* spp., *Enterobacter gergoviae*, *E. amnigenus*, *Lactobacillus sakei*, and *Lactobacillus curvatus*. The disparity between the results of the present study and those reported by these authors can be attributed to the composition of the EO or the techniques employed. The lack of an internationally standardized technique to evaluate antimicrobial activity of EO and plant extracts invalidate the comparison of some studies.

In this study, the antimicrobial activities of EO against the subgroups of *Staphylococcus* species with different antibiotic susceptibility profiles were partially independent of the pattern of antibacterial resistance which is in agreement with other authors. Mahboubi *et al*. (2010) reported that the *Zataria multiflora* EO – Iranian condiment similar to thyme) consisting of thymol (38%), carvacrol (15.3%) and *p*-cymene (10.2%), showed similar antimicrobial activity against methicillin resistant *Staphylococcus aureus* as well to sensitive susceptible stains. Against fluconazole-sensible and resistant *Candida* spp., Pozzatti *et al*. (2010) reported significant antifungal activities of EO regardless of the previous susceptibility of those yeasts to antifungal agents.

The finding that the activities of oregano, thyme and Mexican oregano EO, and majority constituents carvacrol and thymol are independent of resistance to antibacterials tested, reinforces the status of EO as alternative candidates to be considered in the therapy of bovine mastitis.

In conclusion, this study demonstrated that essential oils of oregano, thyme, Mexican oregano, as well as the major constituents thymol and carvacrol showed antimicrobial activity against *Staphylococcus* spp. and that the EO of oregano, thyme and Mexican oregano exhibited similar antimicrobial activities (MICs and MBCs), but lower than major constituents thymol and carvacrol. The activities of EO were similar against subgroups of isolates, independently of the resistance to antimicrobials used for treatment of mastitis.

Keywords: medicinal plants, phytotherapy, antimicrobials

RESUMO

Avaliou-se a atividade antimicrobiana dos óleos essenciais (OE) de Origanum vulgare (orégano), Thymus vulgaris *(tomilho),* Lippia graveolens *(lipia),* Zingiber officinale *(gengibre),* Salvia officinalis *(sálvia),* Rosmarinus officinalis *(alecrim) e* Ocimum basilicum *(manjericão), e de suas frações majoritárias, carvacrol e timol, frente a 32 isolados de* Staphylococcus *spp, oriundos de rebanhos leiteiros bovinos. A concentração inibitória mínima (CIM) e a concentração bactericida mínima foram determinadas por meio da técnica de microdiluição em caldo. Orégano, tomilho e lípia (Orégano Mexicano) apresentaram atividade antimicrobiana similar, médias geométrica de CIM de 1600μg mL^{-1}; 1564μg mL^{-1}; 1562μg mL^{-1}, respectivamente, no entanto menos ativos que carvacrol, 584μg mL^{-1} e thymol, 427μg mL^{-1}. Isolados com diferentes perfis de susceptibilidade aos antimicrobianos usados no tratamento de mastite bovina, quando subagrupados, foram inibidos por concentrações semelhantes de OE. Estes resultados confirmam a atividade antimicrobiana de OE e algumas frações majoritárias.*

Palavras-chave: plantas medicinais, fitoterapia, antimicrobianos

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