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ARTICLE

Antimicrobial effect of the essential oil from *Rosmarinus officinalis* L. against *Staphylococcus pseudintermedius* isolated from dogs

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ABSTRACT: (Antimicrobial effect of the essential oil from *Rosmarinus officinalis* L. against *Staphylococcus pseudintermedius* isolated from dogs). This study evaluated the inhibitory effect of *Rosmarinus officinalis* L. (rosemary) essential oil (ROEO) against 18 isolates of *Staphylococcus pseudintermedius* isolated from dogs affected by pyoderma in Brazil, by determining the minimum inhibitory concentrations (MIC) and the effects on the bacterial cell viability over 16 h. MIC values of ROEO against the isolates were 2.5 µL/mL (3/18), 5 µL/mL (12/18) or 10 µL/mL (3/18). At all concentrations tested (¼, ½, 1x, 2x and 4x MIC) the ROEO inhibited bacterial cell viability after 30 min of exposure, and no recovery in viable cell counts was noted in the later intervals. The ROEO at ½, 1x, 2x and 4x MIC established its bactericidal effect within a maximum exposure time of 16 h. These findings reveal an interesting anti-*S. pseudintermedius* effect of ROEO, with a rapid and steady kill rate.
Key words: anti-*S. pseudintermedius* effect, *R. officinalis* L., rosemary.

RESUMO: (Efeito antimicrobiano do óleo essencial de *Rosmarinus officinalis* L. contra *Staphylococcus pseudintermedius* isolados de cães). O objetivo deste trabalho foi avaliar o efeito inibitório do óleo essencial de *Rosmarinus officinalis* L. (alecrim) (OERO) frente a 18 linhagens *Staphylococcus pseudintermedius* isoladas de cães com piodermite no Brasil, determinando a concentração inibitória mínima (CIM) e os efeitos da viabilidade da célula bacteriana, durante 16 horas. Os valores da CIM do OERO, frente aos isolados avaliados, foram 2,5 µL/mL (3/18), 5 µL/mL (12/18) ou 10 µL/mL (3/18). Em todas as concentrações testadas (¼, ½, 1x, 2x e 4x CIM), o OERO causou inibição da viabilidade celular já após 30 minutos de exposição e não foi constatado restabelecimento da viabilidade celular na contagem de células viáveis nos intervalos mais tardios avaliados. O OERO exerceu atividade bactericida a partir da concentração 1/2 CIM. Esses resultados revelam um interessante efeito anti-*S. pseudointermedius* do OERO com uma rápida e constante taxa bactericida.
Palavras-chave: efeito anti-*S. pseudointermedius*, *R. officinalis* L., alecrim.

INTRODUCTION

Staphylococcus pseudintermedius (formerly *Staphylococcus intermedius*; Devriese *et al*. 2009), a coagulase-positive staphylococcus, forms part of the indigenous microflora of cats, minks, pigeons, horses and mainly dogs, where this bacterium colonizes the upper respiratory tract, skin and mucosal surfaces. *Staphylococcus pseudintermedius* may also act as invasive pathogen, causing pyoderma, otitis, cystitis and osteomyelitis (Penna *et al*. 2010, Pereira *et al.* 2009, Oliveira *et al.* 2006, Girard & Higgins 1999). Although rarely isolated from humans, *S. pseudintermedius* is known as a zoonotic pathogen that causes some infections of canine-inflicted wound infections and other invasive and non-invasive infections in humans (Pottumarthy *et al.* 2004, Mahoudeau *et al*. 1997).

Resistance of *S. pseudintermedius* to classical antibiotics used in clinical veterinary therapy is now a factual reality, related to their wide use over time (Futagawa-Saito *et al*. 2007, Lima *et al*. 2012). Regarding the increasing clinical importance of the development of drug resistance in *S. pseudintermedius,* there is a need to discover new and effective antimicrobial agents to control this bacterium.

Currently, there has been increasing interest in studying the biological properties of plants and derivatives in order to find alternative antimicrobial compounds. *Rosmarinus officinalis* L. (Lamiaceae), commonly known as rosemary, is a popular herb in many western countries and is widely used in folk medicine, cosmetics and phytopharmacy (Bozin *et al.* 2007). More than 30 compounds have been identified in the essential oil from *R. officinalis* (ROEO), although one study found that α-pinene, 1,8-cineole, camphor, verbenone and borneol comprised approximately 80 g/100 g of the total essential oil (Santoyo *et al*. 2005).

The aim of this study was to evaluate the inhibitory effect of the ROEO against isolates of *S. pseudintermedius* from dogs affected by pyoderma in Brazil.

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MATERIAL AND METHODS

Essential oil

ROEO was supplied by Ferquima Ltda. (Vargem Grande Paulista, SP, Brazil) and its quality parameters were described in an accompanying technical report (density 0.919; refraction index 1.465; major components: 1,8-cineole, 47 g/100 g; camphor, 16.7 g/100 g; and α-pinene, 13.5 g/100 g). The stock solution of the ROEO was prepared in sterile distilled water using Tween 80 at 4 g/100 mL (Sigma Chemical Co., USA) as a stabilizing agent. Tween 80 at its highest final concentration after dilution in Brain Heart Infusion (BHI) broth (0.5 g/100 mL) caused no inhibition of the bacterial growth.

Bacterial isolates

Eighteen isolates of *S. pseudintermedius* were obtained from superficial lesions of dogs affected by pyoderma in a private veterinary clinic in the city of Natal (Rio Grande do Norte State, Brazil). The isolates were identified as coagulase positive staphylococci by standard microbiological procedures, and were differentiated in particular from *S. aureus* by exhibiting sensitivity to polymyxin B, lack of acid production from maltose aerobically (Devriese *et al*. 2005) and from mannitol aerobically and anaerobically (Roberson *et al.*1992), lack of acetoin production in MR-VP medium (Laboratórios Difco Ltda., Brazil) with added of creatine at 0.06 g/100 mL (Sigma Chemical Co., USA) (Roberson *et al*. 1992, Devriese *et al*. 2005), lack of growth in Baird-Parker Agar (Laboratórios Difco Ltda., Brazil) with added potassium telluride at 0.1 g/100 mL (Sigma Chemical Co., USA) (Raus & Love 1983) and lack of growth in Brain Heart Infusion (BHI) Agar (Laboratórios Difco Ltda., Brazil) with added acriflavine at 7 µg/mL (Sigma Chemical Co., USA) (Roberson *et al.*,1992). The isolates were maintained in Stock Culture Agar (Difco Becton Dicknson, USA), and prior to use, the cells were grown overnight at 37°C in BHI.

Susceptibility testing

The Minimum Inhibitory Concentration (MIC) of the ROEO was determined in BHI (Laboratórios Difco Ltda., Brazil) by the macrodilution assay using a bacterial suspension of *ca.* 10^5 cfu/mL, and an ROEO concentration ranging from 10 - 0.62 µL/mL (twofold serial dilutions) (Barros *et al.* 2009). MIC was defined as the lowest concentration at which no growth was observed.

Time-kill assay

The bacteria were grown in Mueller-Hinton broth (Laboratórios Difco Ltda., Brazil) until they reached the late exponential phase (16-18 h at 37 ºC) and were resuspended in the same medium at a density of *ca*. 10^6 cfu/mL. The ROEO was added to obtain final concentrations of ¼ MIC, ½ MIC, 1x MIC, 2x MIC and 4x MIC, and the cultures were incubated at 37 ºC. Next, 0, ½, 1, 2, 4, 8 and 16 h aliquots (100 µL) were taken, serially diluted in saline solution (0.85 g/100 mL) and spread-plated onto Mueller-Hinton agar (Laboratórios Difco Ltda., Brazil) (Barros *et al*. 2009). After 24 h of incubation at 37 ºC, colonies were counted and the relative bacterial titer calculated (bacterial titer at time t / bacterial titer at time 0). Assays without adding the ROEO to the growth media were tested similarly as positive control.

RESULTS AND DISCUSSION

MIC values of ROEO against the 18 isolates of *S. pseudintermedius* were 2.5 µL/mL (3/18), 5 µL/mL (12/18) or 10 µL/mL (3/18). Previous studies found that the efficacy of ROEO in inhibiting a variety of classical and opportunistic pathogens depends on the plant location and seasonal variations, the method of extraction of the essential oil, the procedure used in the antimicrobial assays, and the target microbial isolate (Gachkar *et al*. 2007, Santoyo *et al*. 2005).

The effect of different concentrations of ROEO on the cell viability of *S. pseudintermedius* (S28; MIC: 5 µL/mL) is shown in Figure 1.

All time-kill assays were repeated three times, with consistent results (standard error ≤ 12.6%). At all concentrations tested, the ROEO caused inhibition of the bacterial cell viability already after 30 min of exposure, and no recovery in viable cell counts was noted in the later evaluated exposure times. The ROEO at ½ MIC, MIC, 2x MC and 4x MIC established their bactericidal effect (≥3 $\log_{10}$ reduction of the initial inocula, *i.e.,* ≥99.9% kill; LaPlante 2007) at a maximum exposure time of 16 h. However, after 16 h of exposure the ROEO at ¼ MIC decreased the viable cell counts in 2 log cycles (*i.e.,*90% kill). ROEO at 4x MIC and 2x MIC revealed the fastest bactericidal effect, noted after 1 and 3 h of exposure, respectively.

The anti-*S. pseudintermedius* activity of ROEO has not been previously reported. Even regarding *S. aureus*, time-kill curves of ROEO had not been reported before the studies of Gachkar *et al*. (2007) and Fu *et al*. (2007). The results reported here revealed a rapid and steady kill rate of the ROEO tested, and a dose- and time-dependent bactericidal effect against *S. pseudintermedius*. Interestingly, Ganière *et al*. (2004) found a steady bactericidal effect of orbifloxacin (at 2 x MIC) toward *S. pseudintermedius* after 10 h of exposure. Orbifloxacin is a fluorquinolone that is exclusively used in veterinary medicine and is frequently applied in the treatment of pyoderma.

Besides the likely safety of ROEO when used topically, some researchers have suggested that the risk of development of bacterial resistance to it is low because of the multicomponent nature of essential oils (Patriginani *et al*. 2008).

The results obtained in this study show an interesting anti-*S. pseudintermedius* effect of ROEO, which may show promise for inclusion in topical formulations used to eradicate *S. pseudintermedius* in animal infections, particularly in pyoderma.

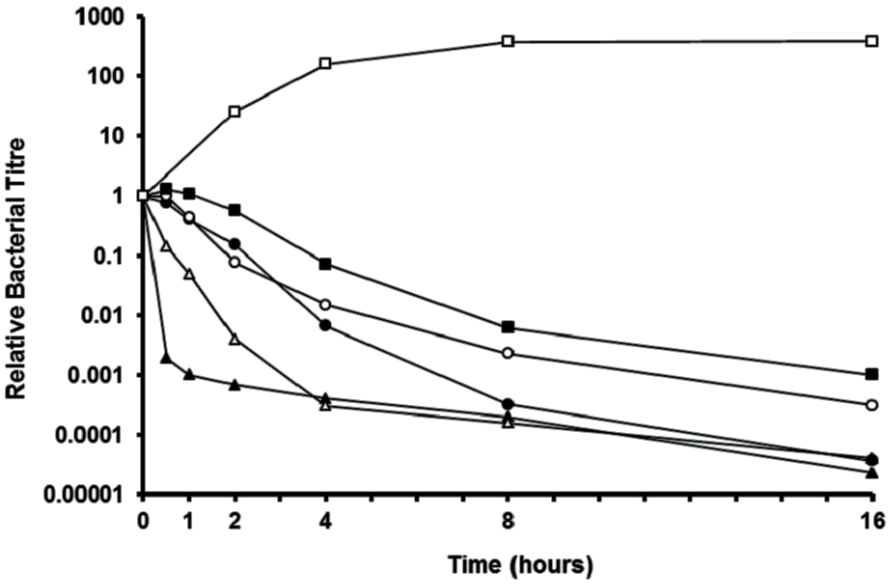

Figure 1. Effect of the essential oil from *R. officinalis* at different concentrations on the cell viability of *Staphylococcus pseudintermedius* S28 over 16 h at 37 °C. (▲): 4x MIC (20 µL/mL); (Δ): 2x MIC (10 µL/mL); (●): 1x MIC (5 µL/mL); (○): ½ MIC (2.5 µL/mL); (■): ¼ MIC (1.25 µL/mL); (□): control (0 µL/mL).

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REFERENCES

BARROS, J.C., CONCEIÇÃO, M.L., GOMES NETO, N.J., COSTA, A.C.V., SIQUEIRA JÚNIOR, J.P., BASÍLIO JÚNIOR, I.D. & SOUZA, E.L. 2009. Interference of *Origanum vulgare* L. essential oil on the growth and some physiological characteristics of *Staphylococcus aureus* strains isolated from foods. *LWT-Food Science and Technology, 42*: 1139-1143.

BOZIN, B., MIMICA-DUKIC, N., SAMOJLIK, I. & JOVIN, E. 2007. Antimicrobial and antioxidant properties of rosemary and sage (*Rosmarinus officinalis* L. and *Salvia officinalis* L., Lamiaceae) essential oils. *Journal of Agricultural and Food Chemistry, 55:* 7879-7885.

DEVRIESE, L.A., HERMANS, K., BAELE, M. & HAESEBROUCK, F. 2009. *Staphylococcus pseudintermedius* versus *Staphylococcus intermedius*. *Veterinary Microbiology, 133:* 206-207.

DEVRIESE, L.A., VANCANNEYT, M., BAELE, M., VANEECHOUTTE, M., DE GRAEF, E., SNAUWAERT, C., CLEENWERCK, I., DAWYNDT, P., SWINGS, J., DECOSTERE A. & HAESEBROUCK, F. 2005. *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. *International Journal of Systematic and Evolutionary Microbiology, 55:* 1569-1573.

FU, Y.J., ZU, Y.G., CHEN, L.Y., SHI, X.G., WANG, Z., SUN, S. & EFFERTH, T. 2007. Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytotherapy Research, 21:* 989-994.

FUTAGAWA-SAITO, K., BA-THEIN, W. & FUKUYASU, T. 2007. High occurrence of multi-antimicrobial resistance in *Staphylococcus intermedius* isolates from healthy and diseased dogs and domesticated pigeons. *Research in Veterinary Science*, *83*: 336-339.

GACHKAR, L., YADEGARI, D., REZAEI, M.B., TAGHIDEH, M., ASTANEH, S.A. & RASOOLI, I. 2007. Chemical and biological characteristics of *Cuminum cyminum* and *Rosmarinus officinalis* essential oils. *Food Chemistry*, *102:* 898-904.

GANIÈRE, J.P., MÉDAILLE, C. & ETORÉ, F. 2004. In vitro antimicrobial activity of orbifloxacin against *Staphylococcus intermedius* isolates from canine skin and ear infections. *Research in Veterinary Science*, *77:* 67-71.

GIRARD, C. & HIGGINS, R. 1999. *Staphylococcus intermedius* cellulitis and toxic shock in a dog. *Canadian Veterinary Journal*, *40:* 501-502.

LAPLANTE, K.L. 2007. In vitro activity of lysostaphin, mupirocin, and tea tree oil against clinical methicillin-resistant *Staphylococcus aureus*. *Diagnostic Microbiology and Infectious Diseases*, *57:* 413-418.

LIMA, L.F.A., LIRA, A.C., COUTINHO, H.D.M., SIQUEIRA-JÚNIOR, J.P. & BARRETO, H.M. 2012. Antimicrobial resistance in staphylococci isolated from canine pyoderma. C*omunicata Scientiae*, *3*: 181-185.

MAHOUDEAU, I., DELABRANCHE, X., PREVOST, G., MONTEIL, H. & PIEMONT, Y. 1997. Frequency of isolation of *Staphylococcus intermedius* from humans. *Journal of Clinical Microbiology*, *35:* 2153-2154.

OLIVEIRA, L.C., LEITE, C.A.L., BRILHANTE, R.S.N. & CARVALHO, C.B.M. 2006. Etiology of canine otitis media and antimicrobial susceptibility of coagulase-positive staphylococci in Fortaleza city, Brazil. *Brazilian Journal of Microbiology, 37*: 144-147.

PATRIGNANI, F., IUCCI, L., BELLETTI, N., GARDINI, F., GUERZONI, M.E. & LANCIOTTI, R. 2008. Effects of sub-lethal concentrations of hexanal and 2-(E)-hexenal on membrane fatty acid composition and volatile compounds of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Escherichia coli*. *International Journal of Food Microbiology*, *123:* 1-8.

PENNA, B., VARGES, R., MARTINS, R., MARTINS, G. & LILENBAUM, W. 2010. In vitro antimicrobial resistance of staphylococci isolated from canine urinary tract infection. *The Canadian Veterinary Journal*, *5*: 738-742.

PEREIRA, I.A., SOARES, L.C., COELHO, S.M.O., BALBINO, F.A., PRIBUL, B.R. & SOUZA, M.M.S. 2009. Susceptibilidade à azitromicina de agentes bacterianos isolados de processos infecciosos em diferentes sítios de animais de companhia. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *61*: 577-584.

POTTUMARTHY, S., SCHAPIRO, J.M., PRENTICE, J.L., HOUZE, Y.B., SWANZY, S.R., FANG, F.C. & COOKSON, B.T. 2004. Clinical

isolates of *Staphylococcus intermedius* masquerading as methicillin-resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology*, *42*: 5881-5884.

RAUS, J. & LOVE, D.N. 1983. Characterization of coagulase-positive *Staphylococcus intermedius* and *Staphylococcus aureus* isolated from veterinary clinical specimens. *Journal of Clinical Microbiology*, *18:* 789-792.

ROBERSON, J.R., FOX, L.K., HANCOCK, D.D. & BESSER, T.E. 1992. Evaluation of methods for differentiation of coagulase-positive staphylococci. *Journal of Clinical Microbiology*, *30*: 3217-3219.

SANTOYO, S., CAVERO, S., JAIME, L., IBAÑEZ, E., SEÑORÁNS, F.J. & REGLERO, G. 2005. Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. essential oil obtained via supercritical fluid extration. *Journal of Food Protection*, *68:* 790-795.