

ANTIBACTERIAL EFFECT OF BRAZILIAN BROWN PROPOLIS IN DIFFERENT SOLVENTS AGAINST *Staphylococcus* spp. ISOLATED FROM CAPRINE MASTITIS

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ABSTRACT

The purpose of the present study was to evaluate the antimicrobial activity of propolis extracts diluted in different solvents against bacteria from *Staphylococcus* genus. The study was performed in the Immunology and Microbiology Laboratory from Universidade Federal do Vale do São Francisco. The propolis extracts were prepared using brown propolis diluted in different solvents such as chloroform, methanol, ethyl acetate and grain alcohol. In order to determine the antimicrobial potential of extracts, agar well diffusion method was used, with controls for each diluent. After that, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) methods were used. All tests were performed in triplicate. In the agar well diffusion test, the measurements of the inhibition zone for propolis extract

were as follows: grain alcohol and propolis (2.88mm), methanol and propolis (2.41mm), chloroform and propolis (2.40mm) and ethyl acetate and propolis (0.83mm). The MBC of propolis extracts in different solvents were 93.75 µg/mL for grain alcohol, 375 µg/mL for chloroform and methanol and 3,000 µg/ml for ethyl acetate. Statistically significant differences were achieved comparing the inhibition zones of propolis diluted in grain alcohol and ethyl acetate (2.88 and 0.83 mm, respectively). Considering the low cost of therapy and the activity of the propolis against caprine mastitis pathogens, other studies regarding *in vivo* activity and chemical characterization are necessary, in addition to evaluation of the toxicological aspects of propolis extracts.

KEYWORDS: Antimicrobial therapy; dairy goats; minimal bactericidal concentration; natural products.

AÇÃO ANTIBACTERIANA DA PRÓPOLIS MARROM BRASILEIRA EM DIFERENTES SOLVENTES CONTRA *Staphylococcus* spp. ISOLADOS DE CASOS DE MASTITE CAPRINA

RESUMO

O objetivo deste trabalho foi estudar a ação antimicrobiana do extrato da própolis em diferentes diluentes sobre bactérias do gênero *Staphylococcus*. Os extratos foram preparados utilizando-se diferentes diluentes, sendo eles o álcool de cereais, clorofórmio, acetato de etila e metanol. Foi utilizado o método de difusão em ágar por meio de poço, sendo utilizados

controles para cada diluente. Em seguida, foi empregada a técnica da Concentração Inibitória Mínima (MIC) e da Concentração Bactericida Mínima (CBM). Todos os ensaios foram realizados em triplicata. No método de difusão em ágar, utilizando-se poço, observaram-se as seguintes médias para o halo de inibição: álcool de cereais (2,88 mm), álcool metílico (2,41 mm), clorofórmio (2,40

mm) e acetato de etila (0,83 mm). Foram obtidas as seguintes médias nos testes da CBM: 93,75 µg/mL para o extrato com álcool de cereais, 375 µg/ml para o extrato com clorofórmio e com metanol e 3.000 µg/mL para o extrato com acetato de etila. Foram observadas diferenças estatísticas significativas entre as médias de halo de inibição para a própolis diluída em álcool de cereais e

aquela diluída em acetato de etila (2,88 e 0,83 mm, respectivamente). Considerando o baixo custo dessa terapia e a atividade da propolis contra esses patógenos da mastite, estudos *in vivo* e a caracterização química devem ser realizados, além da determinação dos aspectos toxicológicos do extrato da propolis.

PALAVRAS-CHAVE: Antibiograma; caprina; concentração bactericida mínima; produtos naturais.

INTRODUCTION

Mastitis is considered as one of the most important diseases in dairy goat herds. The most important economic losses associated with this disease are reduction in milk production and the lactation period, time-consuming hygienic procedures and disposal of chronically infected animals (MURICI et al., 2002). Coagulase Negative *Staphylococcus* spp. (CNS) are the most common pathogens in dairy goats mastitis (HARMON, 1994; CONTRERAS et al., 2007). The implementation of early treatment associated with other prophylactic measures is very important for elimination of the source of infection for other animals in the herds (CONTRERAS et al., 2007). In goat and sheep mastitis, the use of any kind of therapeutic action may be guided by *in vitro* antimicrobial tests (COUTINHO et al., 2006). Increased resistance of mastitis associated with bacteria, especially CNS, suggests the importance of adopting the correct therapy, as well as the use of alternative natural products in therapy.

Brown propolis is a resinous substance, collected by *Apis mellifera* bees, from several parts of plants such as buds, floral buds and resinous exudates. Propolis is recognized for its biological properties such as antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, healing, anesthetic, antitumor and antiviral characteristics (PARK et al., 2002). Its composition is complex and its wide variety is related to different geographical origins (GONSALES et al., 2006).

Propolis antimicrobial activity was determined against several gram positive bacteria such as *Bacillus brevis*, *B. cereus*, *B. cereus* var. *mycoides*, *B. megatherium*, *B. polymyxa*, *B. premilus*, *B. sphaericus*, *B. subtilis*, *Cellulomonas fimi*, *Nocardia globerula*, *Leuconostoc mesenteroide*, *Micrococcus lysodeikticus*, *Sarcina lutea*, *Staphylococcus aureus*, and *Streptococcus faecalis*, as well as gram negative bacteria like *Aerobacter aerogenes*, *Alcaligenes* spp., *Bordetella bronchiseptica*, *Escherichia coli*,

Proteus vulgaris, *Pseudomonas aeruginosa*, and *Serratia marcescens* (MARCUCCI, 1996; GARCIA et al., 2004; VARGAS et al., 2004; FERNANDES JÚNIOR et al., 2006).

The present work sought to verify the antimicrobial activity of brown propolis extracts against CNS isolated from dairy goat mastitis in Petrolina, Brazil.

MATERIAL AND METHODS

The propolis was collected in Crato County, State of Ceara, Brazil. The propolis was brown colored, clean and had a pleasant odor. First, the propolis pieces were cut with a scalpel and macerated. Then, 10 g of propolis were mixed with 10 mL of different solvents: chloroform, methanol, ethyl acetate, and grain alcohol. Each solution was submitted to gentle agitation at 36°C. The extracts were diluted to 30% (p/v) according to Brazilian recommendations (BRASIL, 2001) and tested against the isolates.

Twenty (20) coagulase negative *Staphylococcus* (CNS) isolates in subclinical dairy goat mastitis from Pernambuco State were used in our study. The viability of the isolates was previously confirmed by biochemical tests according to QUINN et al. (1994).

The disk diffusion test was performed according to previous descriptions as described (PINTO et al., 2001). In this experiment, we used Muller Hinton Agar. CNS bacterial suspensions were prepared in BHI broth and incubated for 24h at 37°C. Turbidity was adjusted to 0.5 on the Mac Farland scale. Then, wells were filled with 30 µL of propolis extracts. As control, 30% (p/v) of each pure solvent was applied in the wells. The plates were incubated at 37°C for 24h and the inhibition zones were determined.

The minimum bactericidal concentrations (MBC) of propolis extracts with inhibition zones in disk diffusion test were determined by the microdilution technique, as recognized by the Clinical and Laboratory Standards Institute (CLSI, 2007). During the test, Muller-Hinton broth was

mixed with different propolis extracts to obtain the final concentrations of 6,000, 3,000, 1,500, 750, 375, 187.5, 93.75, and 46.87 µg/mL. CNS suspensions were inoculated into propolis extract dilutions and incubated for 24h at 37°C. Aliquots of the mixture were streaked in MH agar to determine the minimum bactericidal concentration of the propolis extracts. The tests were performed in triplicate. An entirely randomized design was used where the mean of the zone of inhibition obtained from each bacterial isolate was considered as the variable response. The results of antimicrobial activity that presented probability of occurrence of the null hypothesis less than 5 % ($p < 0,05$) applying ANOVA were considered statistically different, followed by multiple comparisons of the means of the minimum bactericidal concentration by the Tukey test. The General Linear Models (GLM) procedure of the Statistical Analysis System (SAS, 2003) was used.

RESULTS AND DISCUSSION

The MBC of the different propolis extracts are described in Table 1. In the disk diffusion test the averages of inhibition zones obtained for propolis extracts differed among the solvents. The highest value was found for propolis diluted in grain alcohol (2.88mm). Values lower than that, but still high, were found for propolis and methanol (2.41mm), and for propolis and chloroform (2.40mm). The lowest value was found for propolis and ethyl acetate (0.83mm).

Non-significant statistical differences were observed when comparing diluted propolis extracts and pure diluents on the diffusion agar test. However, statistically significant differences were achieved comparing the inhibition zones of propolis diluted in grain alcohol and ethyl acetate (2.88 and 0.83 mm, respectively).

Table 1. Sensitivity of *Staphylococcus spp.* to propolis extracts with different solvents through well agar diffusion and minimum bactericidal concentration

Propolis + Solvents	Inhibition Zones (mm)	Minimum Bactericidal Concentration	
		Variation	Average \pm sd (µg/mL)
Propolis + grain alcohol	2.88 ^{ab}	93.75 - 6,000	1,579.33 \pm 1,049.45
grain alcohol	1.62 ^{abc}	375.00 - 6,000	1,644.23 \pm 912.65
Propolis + chloroform	2.40 ^{abcd}	375.00 - 6,000	2,708.33 \pm 2,564.99
chloroform	3.62 ^a	375.00 - 6,000	937.50 \pm 795.50
Propolis + methanol	2.41 ^{abcd}	375.00 - 6,000	1,703.13 \pm 1,076.95
Methanol	1.05 ^{bcd}	750.00 - 6,000	2,000.00 \pm 1,154.70
Propolis + ethyl acetate	0.83 ^{cd}	1,500.00 - 6,000	3,000.00 \pm 1,414.21
ethyl acetate	0.62 ^d	750 - 6,000	5,166.67 \pm 1,541.10

The averages followed by the same letters did not differ among themselves (Tukey test, $p < 005$).

Antibiotic therapy is a common procedure for mastitis treatment and control. However, growing resistance in bacteria is a relevant concern in veterinary medicine (PINTO et al., 2001). Propolis is an important therapeutic alternative from the economic and pharmacologic point of view (LONGHINI et al., 2007). Variation in antimicrobial activity of propolis may be explained by the efficiency in extraction of phenolic compounds by solvents (MARCUCCI et al., 2001). Differences in bacterial sensitivity to propolis may also be associated with the species of bacteria. These differences are related to the cell wall chemical composition, with Gram positive bacteria being more sensible than the negative ones (FERNANDES JÚNIOR et al., 1997; VARGAS et al., 2004).

However, according to BANKOVA et al. (1999) and MARCUCCI et al. (2001), the precise antimicrobial mechanisms of propolis action on the bacterial cell wall are still unclear.

Studies regarding resistance of CNS in mastitis showed that these pathogens are more resistant than *S. aureus* (TAPONEN & PYORALA, 2009). *S. aureus* and *Escherichia coli* were sensitive to propolis (PACKER & LUZ, 2007). All CNS tested here were sensitive to propolis extracts and our results are in accordance with the ones described by PINTO et al. (2001) with bovine mastitis pathogens and SANTOS NETO et al. (2009), who evaluated ethanolic and aqueous propolis extracts against *Staphylococcus spp.* isolated from goats. LOGUERCIO et al. (2006) found 94.44% of

propolis sensitivity of coagulase positive *Staphylococcus* spp. (CPS) isolated from cases of bovine mastitis. Similar results were reported by VARGAS et al. (2004). The diverse antimicrobial activity of propolis against *S. aureus* and *S. mutans* was associated with the origin of the extracts (PARK et al., 2002). Differences in the antimicrobial activity of propolis extracts were also described as being associated with collection location and seasonal, temperature and pH (LU et al., 2005; SANTOS NETO et al., 2009).

We found that different solvents used in propolis extracts caused diverse sensitivities in the bacteria. The same result was described by PINTO et al. (2001), comparing the effect of different solvents in propolis extract preparation. Grain alcohol and methanol were more effective as compared to other solvents. As described by PINTO et al. (2001), propolis diluted in chloroform and ethyl acetate presented very weak or no antibacterial effect. These findings are in accordance with the ones reported by SANTOS et al. (2002).

The sensitivity of screening tests of antimicrobial potential for natural products is variable. The agar diffusion test is a very advantageous method due to low costs and practicality, but the potency of different samples may not be compared in opposition to results found with MIC and MBC determinations (COS et al., 2006). Results of the agar diffusion test (< 3 mm zones) were lower than those described by other researchers, who observed inhibition zones of more than 10 mm (PINTO et al., 2001; ESHRAGHI & VALAFAR, 2008; SANTOS NETO et al., 2009). The high MBC of propolis extracts in different solvents against the CNS isolates described here (<1,579.33 µg/mL) were similar to some previous studies (BOVEHI et al., 1994; SANTOS et al., 2002; MIORIN et al., 2003), but were very high when compared to results presented in other studies (PINTO et al., 2001; LU et al., 2005; ESHRAGHI & VALAFAR, 2008; SANTOS NETO et al., 2009). These differences may be due to different pathogens and concentrations used.

The increase in selection of resistant bacteria after antimicrobial therapy in veterinary medicine is a problem that raises concern (COUTINHO et al., 2006). Propolis may be a safe alternative in mastitis therapy due to its natural origin and lack of leaving residues in milk (PINTO, 2001).

CONCLUSIONS

Considering the low cost of therapy and the activity of the propolis against caprine mastitis

pathogens, other studies regarding *in vivo* activity and chemical characterization are necessary, in addition to evaluation of the toxicological aspects of the extracts of propolis.

REFERENCES

- BANKOVA, V.; CHRISTOV, R.; POPOV, S.; MARCUCCI, M.C.; TSVETKOVA, I.; KUJUMGIEV, A. Antibacterial activity of essential oils from Brazilian propolis. **Fitoterapia**, v.70, p.190-193, 1999.
- BRASIL, Ministério da Agricultura, pecuária e Abastecimento. Instrução Normativa n.3, de 19 de janeiro de 2001. Diário Oficial da União 23 jan. seção 1, p.18-23, 2001.
- CLSI (Clinical and Laboratory Standard Institute) 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved standards. Document CLSI M7-A7, CLSI, Wayne, Pennsylvania.
- CONTRERAS, A.; SIERRA, D.; SÁNCHEZ, A.; CORRALES, J.C.; MARCO, J.C.; PAAPE, M.J.; GONZALO, C. Mastitis in small ruminants. **Small Ruminant Research**, v.68, p.145-153, 2007.
- COS, P.; VLIETINCK, A.J.; BERGHE, D.V.; MAES, L. Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept. **Journal of Ethnopharmacology**, v.106, p.290-302, 2006.
- COUTINHO D.A.; COSTA J.N.; RIBEIRO M.G.; TORRES J.A. Etiologia e sensibilidade antimicrobiana in vitro de bactérias isoladas de ovelhas da raça Santa Inês com mastite subclínica. **Revista Brasileira de Saúde e Produção Animal**, v.7, n.2, p.139-151, 2006.
- ESHRAGHI, S.; VALAFAR, S. Evaluation of inhibitory effects of Iranian propolis against filamentous bacteria. **Pakistan Journal of Medical Sciences**, v.24, n.1, p.56-60, 2008.
- FERNANDES JÚNIOR, A.; LOPES, C.A.M.; SFORCIN, J.M.; FUNARI, S.R.C. Population analysis of susceptibility to propolis reference strains of *Staphylococcus aureus* and *Escherichia coli*. **Journal of Venomous Animals and Toxins**, v.3, n.2, p.287-294, 1997.
- FERNANDES JÚNIOR, A.; LOPES, M.M.R.; COLOBARI, V.; MONTEIRO, A.C.M.; VIEIRA, E.P. Atividade antimicrobiana de própolis de *Apis mellifera* obtida três regiões do Brasil. **Ciência Rural**, v.36, n.1, p.294-297, 2006.
- GARCIA, R.C.; PINHEIRO DE SÁ, M.E.; LANGONI, H.; FUNARI, S.R.C. Efeito do extrato alcoólico de própolis sobre *Pasteurella multocida* in vitro e em coelhos. **Acta Scientiarum Animal Sciences**, v.26, n.1, p. 69-77, 2004.
- GONSALES, G.Z.; ORSI, R.O.; FUNARI, S.R.C.; FERNANDES JUNIOR, A. Antibacterial Activity of Propolis collected in different regions of Brazil. **Journal**

- of **Venomous Animals and Toxins**, v.12, p.124-132, 2006.
- HARMON, R.J. Physiology of mastitis and factors affecting somatic cell counts. **Journal of Dairy Science**, v.77, p. 2103-2112, 1994.
- LOGUERCIO, A.P.; MELLO, A.C.M.; PEDROZZO, A.F.; WITT, N.M.; SÁ E SILVA, M.; VARGAS, A.C. Atividade in vitro do extrato de própolis contra agentes bacterianos da mastite bovina. **Pesquisa Agropecuária Brasileira**, v.41, n.2, p.347-349, 2006.
- LONGHINI, R.; RAKSA, S.M.; OLIVEIRA, A.C.P.; SVIDZINSKI, T.I.E.; FRANCO, S.L. Obtenção de extratos de própolis sob diferentes condições e avaliação de sua atividade antifúngica. **Revista Brasileira de Farmacognosia**, v.17, p.388-395, 2007.
- LU, L.C.; CHEN, Y.W.; CHOU, C.C. Antibacterial activity of propolis against *Staphylococcus aureus*. **International Journal of Food Microbiology**, v.102, p.213-220, 2005.
- MARCUCCI, M. C.; DE CAMARGO, F. A.; LOPES, C. M. A. Identification of aminoacids in Brazilian propolis. **Zeitschrift für Naturforschung C**, v.51, n.1-2, p.11-14, 1996.
- MARCUCCI, M.C.; FERRERES, F.; GARCÍA-VIGUERA, C.; BANKOVA, V.S.; DE CASTRO, S.L.; DANTAS, A.P.; VALENTE, P.H.M.; PAULINO, N. Phenolic compounds from Brazilian propolis with pharmacological activities. **Journal of Ethnopharmacology**, v.74, n.2, p.105-112, 2001.
- MIORIN, P.L.; LEVY JUNIOR, N.C.; CUSTODIO, A.R.; BRETZ, W.A.; MARCUCCI, M.C. Antibacterial activity of honey and propolis from *Apis mellifera* and *Tetragonisca angustula* against *Staphylococcus aureus*. **Journal of Applied Microbiology**, v.95, p.913-920, 2003.
- MURICI, R.F.; SELLA, S.; DA SILVA, L.E.; SCHIMIDT, V.; CARDOSO, M.I. Identificação de pontos de contaminação do leite produzido em uma propriedade de caprinos no município de Viamão-RS. **Revista da FZVA**, v.9, n.1, p.111-117, 2002.
- PACKER J.F.; LUZ, M.M.S. Método para avaliação e pesquisa da atividade antimicrobiana de produtos de origem natural. **Revista Brasileira de Farmacognosia**, v.17, p.102-107, 2007.
- PARK, Y.K.; ALENCAR, S.M.; SCAMPARINI, A.R.P.; AGUIAR, C.L. Própolis produzida no sul do Brasil, Argentina e Uruguai: Evidências fitoquímicas de sua origem vegetal. **Ciência Rural**, v.32, n.6, p.997-1003, 2002.
- PINTO, M.S.; FARIA, J.E.; MESSAGE, D.; CASSINI, S.T.A.; PEREIRA, C.S.; GIOSO, M.M. Efeito de extrato de ropolis verde sobre bactérias patogênicas isoladas do leite de vacas com mastite. **Brazilian Journal of Veterinary Research and Animal Science**, v.38, n.6, p. 278-283, 2001.
- QUINN P.J., CARTER, M.E., MARKEY, B., CARTER, G.R. 1994. **Clinical Veterinary Microbiology**. Wolfe, London. 648p.
- SANTOS NETO, T.M.; MOTA, R.M.; SILVA, L.B.G.; VIANA, D.A.; LIMA-FILHO, J.L.; SARUBBO, L.A.; CONVERTI, A.; PORTO, A.L.F. Susceptibility of *Staphylococcus* spp. isolated from milk of goats with mastitis to antibiotics and green propolis extracts. **Letters in Drug Design and Discovery**, v.6, p.63-68, 2009.
- SANTOS, F.A.; BASTOS, E.M.A.; UZEDA, M.; CARVALHO, M.A.R.; FARIAS, L.M.; MOREIRA, E.S.A.; BRAGA, F.C. Antibacterial activity of brazilian própolis and fractions against oral anaerobic bacteria. **Journal of Ethnopharmacology**, v.80, n.1, p.1-7, 2002.
- SAS, Statistical Analysis System. 2003. **Statistical Analysis System User's Guide**, version 6, fifth ed. SAS Institute Inc., Raleigh, NC, USA.
- TAPONEN, S.; PYÖRÄLÄ, S. Coagulase-negative staphylococci as cause of bovine mastitis – Not so different from *Staphylococcus aureus*? **Veterinary Microbiology**, v.134, p.29-36, 2009.
- VARGAS, A.C.; LOGUERCIO, A.P.; WITT, N.M.; COSTA, M.M.; SILVA, M.S.; VIANA, L.R. Atividade antimicrobiana “in vitro” de extrato alcóolico de própolis. **Ciência Rural**, v.34, n.1, p. 159-163, 2004.

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