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In vitro activity of propolis: Synergism in combination with antibiotics against *Staphylococcus* spp.

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Mastitis causes inflammation of the mammary gland and physical, chemical and bacteriological alterations in milk. The control of this disease deserves special attention within health programs of the dairy cattle industry. The aim of the present study was to assess the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of a commercial product (gentamicin + amoxicillin) indicated for the treatment of mastitis, propolis,and their association against *Staphylococcus* spp. In total, 70 samples of *Staphylococcus* spp. isolated from cases of subclinical mastitis, were used in the present study. The bacterial isolates were tested against the commercially available association of gentamicin and amoxicillin, as well as commercially available propolis. Muller Hinton (MH) micro-broth dilution was used in the present study. The presence of phenolic compounds and flavonoids was confirmed in the propolis used. The MIC values found for gentamicin and amoxicillin were 0.2258 g/ml and 0.8469 mg/ml, respectively. The MBC values found for gentamicin and amoxicillin were 1.7793 and 6.6722 mg/ml, respectively. The mean MBC values of amoxicillin and gentamicin in synergism with propolis were 0.1384 g/ml and 0.5235 mg/ml, respectively. Analysis of propolis showed the presence of derivatives of the following acids: caffeic; cinnamic; *p*-coumaric; ferulic and 3,4-dihydroxybenzoic. The results of the present study showed that gentamicin and amoxicillin, associated with propolis, exhibit synergistic activity that could reduce the selection of resistant microorganisms. The synergistic effect of propolis is probably due to the presence of cinnamic acid derivatives.

Key words: Antimicrobial, bovine mastitis, propolis, *Staphylococcus* spp.

INTRODUCTION

Mastitis is one of the most common diseases in dairy herds. The etiology is wide-ranging and the disease is primarily caused by microorganisms. Bacteria belonging to the genus *Staphylococcus* are among the main micro-organisms that cause mastitis. These micro-organisms are the etiologic agents that have most frequently been

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isolated from the mammary gland of cows with mastitis. Species of this genus can cause long duration infections and tend to become chronic, resulting in a low cure rate and great losses in milk production (Godden et al., 2002).

Antibiotic therapy is one of the main forms of mastitis control and it has been extensively studied over the years (Sol et al., 1997; Friton et al., 1998; Langoni et al., 2000). However, the use of antimicrobials results in economic losses, either from their cost or by the prohibition of commercial milk containing residues of antibiotics (Crisan et al., 1995). Moreover, mastitis in dairy herds represents a serious risk for the health of the general public.

Natural alternatives, such as propolis, are currently being studied in order to reduce the use of antibiotics to treat mastitis and alleviate the concern for food quality and milk safety (Vargas et al., 2004). Propolis is a complex mixture containing resin material and balsamic. Its chemical composition is associated with the flora of different regions visited by bees and the periodical collection of resin (Lustosa et al., 2008). The mechanism of antimicrobial activity of propolis is complex and can be attributed to the synergistic activity between phenolic compounds and other substances present (Krol et al., 1993), as well as a mechanism of action based on the inhibition of bacterial RNA polymerase (Uzel et al., 2005).

Besides the antimicrobial potential of propolis, studies have demonstrated a synergistic effect between propolis and antimicrobial drugs (Detoma and Ozino, 1991; Scheller et al., 1999; Stepanovic et al., 2003; Junior Fernandez et al., 2005). Given the importance of reducing the use and the concentration of antibiotics in the treatment of production animals, the aim of the present study was to assess the inhibitory concentrations of a commercial product in association with propolis extract.

MATERIALS AND METHODS

Bacterial isolates

We tested 70 strains of *Staphylococcus* spp., which were provided by the microbiology and animal immunology laboratory of the Federal University of Vale do São Francisco. The microorganisms isolated were identified based on morphological (color, size, presence or absence of hemolysis), dyeing (Gram stain) and biochemical characteristics, as described by Quinn et al. (1994). The samples were isolated from cattle with subclinical mastitis in herds from the cities of Petrolina and Arcoverde in the state of Pernambuco (Brazil). The herds contained animals of various breeds and ages, and they were also at different stages of lactation.

Analysis of propolis

The quantification of phenolic compounds was performed by the Folin-Ciocalteau method, which involves the reduction of phenolic compounds based on the reaction of the samples with the concomitant formation of a blue complex, the intensity of which(760 nm) increases linearly, as described by Swain and Hills (1959).

The total flavonoid content was determined by adapting the Dowd method: 500 µg of aluminum sulphate (Al2 (SO4) 3); 5% methanol, mixed with 0.4 mL of the sample. Absorbance readings were made at 300 nm after 30 min of rest in the absence of light, using methanol as blank. The total flavonoid content was determined using a standard curve of quercetin at five concen-trations (1, 5, 10, 20 and 40 mg/mL). Y = 0.0198x + 0.3552, where "y" is the absorbance and "x" is the concentration, R2 = 0.9807. The total flavonoid content was expressed as the mg equivalent of quercetin per gram of propolis, whereas the dry matter content thereof was described as per Lee et al. (2003).

Chromatographic analysis was carried out using a Shimadzu Prominence LC2OAT high performance liquid chromatography (HPLC) device equipped with a photodiode array detector (SPDM2O) with a reversed-phase column (Shimpack CLC-ODS, 4.6 mm x 250 mm x 5 µm). For benzoic and cinnamic acid derivatives, the mobile phase consisted of a mixture of 5% aqueous formic acid (A) and methanol (B) at a flow rate of 1 mL/min. A gradient elution was used, starting with 20% B up to 15 min, 30% B for 20 min, 40% B for 30 min and isocratic at 40% B up to 45 min. The injection volume was 20 µL. Chromatograms were recorded at 290 nm. Phenolics were identified based on retention times and UV-spectra with authentic markers.

The caffeic, p-coumaric, ferulic, cinnamic and 3,4-dihydroxy-benzoic acid phenolics identified in propolis were quantified using the external standard method based on peak area. Analyses were made by plotting a calibration curve. To make the calibration curve of each phenolic compound, appropriate volumes from each stock solution were diluted with methanol to obtain working solutions in the concentration range of 0.5 - 40 mg/mL, which were then correlated with the measured area. The area of these peaks was plotted and the corresponding concentration of phenolics was calculated based on the calibration curve. The quantitative analyses were performed in triplicate at 290 nm.

Commercial antimicrobial

The commercial antimicrobial product (*Gentamox®/Laboratório Hipra Saúde Animal Ltda*, Brazil) used in each ml of solution contained 40 mg of gentamicin, amoxicillin and 150 mg vehicle qs 1 mL, as indicated for the treatment of mastitis caused by bacteria.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The bacterial isolates were tested against commercial propolis from the southeast region of Brazil, specifically São Paulo. The propolis was diluted in alcohol, forming the ethanol extract of propolis (EEP).

The *in vitro* antimicrobial activity of the commercial product indicated for the treatment of mastitis and the ethanolic extract of propolis were determined based on the descriptions of the CLSI (2008). The antimicrobials were diluted in 200 µL (1:2) of Miller Hilton broth (MH). During the preparation of inocula, MH agar colonies were used to obtain a bacterial suspension with a turbidity equivalent to the 0.5 tube of the McFarland scale. From this suspension, 10 µL were inoculated (1 x 10^4 UFC) in each microtube containing a dilution of antibiotics or propolis. The material was incubated at 37°C/24 h, under aerobic conditions. After 24 h of cultivation, a reading was performed to identify the MIC. In the case of dilutions with no signs of bacterial growth, one aliquot of 10 µL was withdrawn and then spread on the surface of MH agar and incubated for 24h at 37°C. Subsequently, the MBC was determined as the lowest concentration capable of causing the death of inocula. The assays were performed in triplicate.

The determination of the minimal inhibitory concentration followed the recommendations of CLSI (2008), using a 200 µL distribution of Mueller-Hinton broth in microtiter plates. Subsequently, 200 µL of the commercial product (amoxicillin and gentamicin) was added

Table 1. Minimal inhibitory concentration and minimal bactericidal concentration of commercial antimicrobial (Amoxicillin + Gentamicin), ethanol extract of propolis (EEP) and the associated commercial antimicrobial (34.35 mg) of EEP against *Staphylococcus* spp. isolates from cattle with subclinical mastitis.

Activity	Commercial antimicrobial		ethanol extract of propolis (EEP)*	EEP + Commercial antimicrobial	
	Gentamicin	Amoxicillin		Gentamicin	Amoxicillin
MIC (µg/ml)	0.2258	0.8469		< 0.00695	< 0.02606
MBC (µg/ml)	1.7793	6.6722	68.7	0.1384	0.5235

*The MICs of antimicrobials associated with EEB were lower than the lowest concentrations studied.

to the first well and, after homogenization, transferred to the second and so on, until the following final concentrations were obtained: gentamicin - 0.889; 0.445; 0.222; 0.111, 0.556; 0.028, 0.014 and 0.007 µg/mL; amoxicillin – 3.336, 1.668, 0.834; 0.417, 0.208, 0.104, 0.051 and 0.026 µg/mL. The concentration of ethanol extract of propolis was common in all wells, with ½ MBC (34.35 µg/mL). Finally, the minimal bactericidal concentration of antimicrobials associated with propolis was determined.

RESULTS

After the morphological, dyeing and biochemical tests, the following distribution of isolates was noted: *Staphylococcus aureus* (n=27), coagulase-positive staphylococci (n=26) and coagulase-negative staphylococci (n=17).

The total phenolic and total flavonoid levels found for commercial propolis were 126.22 mg (12.62%) of gallic acid equivalent per gram of propolis extract, and 51.06 mg (5.10%) equivalent of quercetin per gram of propolis extract. These results are in accordance with the limits set by the Ministry of Agriculture, Livestock and Supply (MAPA), with a minimal percentage of total phenolics of 5% and a minimal percentage of total flavonoids of 0.5% (Brasil, 2001). The HPLC-DAD analysis of propolis revealed the presence of phenolic compounds. Ferulic (7.04 µg), caffeic (106.87 µg), cinnamic (222.55 µg), coumaric (226.55 µg) and 3,4-dihydroxybenzoic (2.20 µg) acids were detected in 5 mg of dry extract of propolis.

Table 1 displays the minimal inhibitory concentrations and minimal bactericidal concentrations for the antibiotics used in the commercial product formulation, as well as the association of ethanol extract of propolis (EEP) with the commercial antimicrobial (gentamicin + amoxicillin).

The minimal bactericidal concentration of gentamicin and amoxicillin, after association with ½ MBC of the ethanol extract of propolis (34.35 mg), provided mean values of 0.1384 and 0.5235 µg/mL, respectively.

DISCUSSION

The results of the present study corroborate the findings of Ashraf and Bassuony (2009), who also found phenolic compounds and flavonoids in propolis samples from Egypt. The concentrations of total phenolics and flavonoids differ due to several factors, including the ecology of the flora, the period of the collection of the resin, the genetics of the queen bee, local flora and the collection region, among others (Park et al., 2002; Bankova, 2005; Sousa et al., 2007). The HPLC-DAD analysis of propolis revealed the presence of cinnamic, ferulic, caffeic, coumaric and 3,4-dihydroxybenzoic acids. These compounds were identified according to their retention time and UV spectral characteristics and then compared with standards. Derivatives of cinnamic acids are commonly found in propolis (Akao et al., 2003).

With regards to the mean MBC, Cos et al. (2006) noted that the ideal antimicrobial concentration of propolis would be between 100-150 µg/ml. Therefore, the propolis tested in the present study was effective at lower concentrations. Trusheva et al. (2010) studied the consti-tuents of propolis and highlighted that the exploitation of propolis is a promising source of biologically active compounds. For Marcucci et al. (2001), the antibacterial activities of propolis are higher against Gram-positive bacteria due to the flavonoids, aromatic esters and acids present in the resin, which affect the cell wall structure of these microorganisms.

The combination of amoxicillin and gentamicin, which has been indicated for the veterinary treatment of mastitis, was effective against all the *Staphylococcus* spp. studied. Very few studies have assessed an asso-ciation between the antibiotics gentamicin and amoxi-cillin. Brito et al. (2001) assessed the MIC of ten anti-biotics against *Staphylococcus* spp. isolated from bovine mastitis and found an MIC_{90} 0.5 mg/ml of gentamicin. Other studies have reported efficacy rates of greater than 96% for gentamicin against microorganisms isolated from milk (Cunha et al., 2006). On the other hand, *Staphylococcus* spp. have shown resistance to amoxi-cillin, which is more effective when used in combination with other antibiotics (Langoni et al., 2000).

In the present study, the combination of amoxicillin and gentamicin with the third antimicrobial, propolis, reduced the mean value of the two MBC antimicrobials by more than twelve times. Associations were found between the antimicrobial aim to potentiate its action, decrease side effects and increase the spectrum of the action on microorganisms. It is believed that a combination of propolis extracts and antimicrobial agents could lead to a reduction in the doses of the drugs used, resulting in less pressure for the emergence of resistant strains. It could also reduce side effects and the waste antimicrobial

substances in industrial products of animal origin, particularly in milk and in the environment (Mirzoeva et al., 1997). It is also well known that cinnamic acid derivatives, including those identified in propolis, exhibit antimicrobial activity (Sova, 2012).

The results of the antimicrobial activity associated with the EEP showed that the MIC values of gentamicin and amoxicillin were lower than the lowest concentrations studied (0.00695 µg/mL for gentamicin and 0.026063 µg/mL for amoxicillin), when compared with 100% of isolates of *Staphylococcus* spp. These results confirm the high synergism of propolis associated with antibacterial agents. Fernandes Júnior et al. (2005) assessed the synergistic activity of propolis with antibiotics against *Staphylococcus* spp. and noted that propolis exhibits synergistic activity with drugs that inhibit protein synthesis (gentamicin, chloramphenicol, tetracycline and netilmicin). The same author also noted the absence of antagonistic activity of ethanolic extract of propolis associated with antimicrobial. Naher et al. (2011) also assessed the antimicrobial activity of propolis in combination with several drugs, including amoxicillin, and high activity was observed for *Staphylococcus aureus* from human samples. The similarity in these results reinforces the antimicrobial activity of these combinations, although the isolates tested were often obtained from different animal species and the methods used sometimes differed.

According to Benhanifia et al. (2012), natural products are still a major source of innovative therapeutic agents for infectious diseases, although further studies are required to elucidate and optimize the effective combination of propolis and antibiotics in clinical practice.

Based on the results obtained in the present study, it is possible to conclude that the chemical composition of propolis is within the standards established, and phenolic compounds and flavonoids are directly associated with the antimicrobial activity of propolis.

Furthermore, it was possible to demonstrate the synergistic effect when combined with greater propolis in combination with amoxi-cillin and gentamicin. The synergism reduces the concen-trations of antimicrobials used and could contribute to a reduction in the selection of resistant bacteria. Therefore, the importance of *in vivo* studies that could lead to the inclusion of these compounds in the treatment of bovine mastitis is worth emphasizing.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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