

The antimicrobial activity of plant extract against *Staphylococcus aureus* isolated from cows milk samples with subclinical mastitis in Brazil

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Abstract: The aim of the present study was to analyse the susceptibility profile in vitro of *Staphylococcus aureus* and hydroalcoholic extract of leaf *Psidium guajava* L. This analysis was against *S. aureus* which were isolated from milk samples of cows with mastitis. The harvest was performed in three rural properties that are located in Brazil. Furthermore, the sampling was not probabilistic and from the evaluated animals, 60 were positive. The milk samples were submitted to a selective medium for verifying the presence of *S. aureus*. The isolated bacteria were identified and they were subjected to tests to check the antimicrobial susceptibility. The extract was appropriately prepared for this research and the antimicrobial tests were performed by the wells technique. The contamination by *S. aureus* was proved in 55% of the samples that were analyzed. After this procedure, it was noted that the most effective antibiotics were clindamycin, erythromycin and rifampicin. In addition, the extract demonstrated antimicrobial activity against the isolated. Thus, microbial growth was inhibited in the dilutions that were tested. Therefore, the use of this plant extract may be an alternative method to reduce the resistance of microorganisms to antimicrobials which are sold for mastitis treatment.

Keywords: Antibiotic, Cattle, Microbial resistance, Microbiology, *Psidium guajava* L.

I. Introduction

The milk is an ideal medium for the development of microorganisms due to its various nutrients. The direct contact with infected animals ceilings can result in contamination. Furthermore, it can be caused by microbial growth due to the poor hygiene of residues in milking utensils. This multiplication provokes physical and chemical changes that limit the product stability. As a result, economic and health problems arise. The quality of raw and pasteurized milk has been influenced by many variables. A prime example is mastitis accompanied by an increase in the somatic cell count. The composition, enzyme activity, the coagulation time, milk derivatives quality are negatively affected due to this increase [1].

Bovine mastitis, which is inflammation of mammary gland, is considered a challenge for dairy industries. *Staphylococcus aureus* are frequently the most isolated bacteria in samples of milk from the carrier cows. These bacteria are not only lead to losses in the dairy farming but also they can be resistant to many antibiotics that are used in the treatment of disease. Researches related to bovine mastitis point out that there is a greater bacterial resistance to antimicrobials of the β -lactam group, for example penicillin G. This is caused due to the microbial production of beta lactamases, which are enzymes that are able to split the beta-lactam ring of the structure of antimicrobial [2]. The increasing prevalence of multi-resistant *S. aureus* that cause bovine mastitis is serious due to the reduction of the effectiveness of antimicrobials and the increase in morbidity and the costs to combat the disease. The main cause is the inadequate and misuse of antibiotics. Means alternatives, for instance natural compounds, have been used as a way to reduce or eliminate these problems. In this context, many researches have proved scientifically the antimicrobial efficacy of some plants, for example guava (*Psidium guajava* L.), that is an alternative therapy for certain pathology [3]. The aim of this study was to evaluate the profile of microbial sensitivity in vitro of *S. aureus*. They were isolated from milk of cows with mastitis. In addition, it was associated with many antimicrobials and hydroalcoholic extract of *P. guajava* L. leaf.

II. Material and methods

Milk harvesting was performed in three rural properties of bovine dairy production. In addition, different management types were used in the region of the city of Alfenas, Southern of Minas Gerais State. The sample of animals was not probabilistic because not all elements of the population had the same probability of

belonging to the sample. Sixty dairy cattle with clinical and subclinical mastitis were used and they were not previously treated with antimicrobials commercials.

The animals were evaluated in different stages of lactation. In cases of clinical mastitis, the result was given by the presence of signs and places with edema, hardening, palpitation and the increase udder temperature. According to Rangel [4], the presence of lumps in milk that was used in the black background mug test was also observed in the results.

The remaining animals performed the CMT test (California Mastitis Test) for the detection of Subclinical Mastitis. After noting the degree of coagulation, the comparison with the standard for the diagnosis was made as described by Schalm et al. [5]. Milk harvesting was performed immediately before milking. In the beginning, the hygiene of milker's hands and animal teats was performed with with soap and water solution. After that, they were dried with disposable paper towels.

Approximately 50mL milk of each animal were gathered in sterile bottles. At the end of the milk harvesting in the three rural properties, 60 samples were made. These samples were put into cool boxes, they were transported under refrigeration and forwarded to the laboratory of Biology and Physiology of Microorganisms, José de Rosário Vellano University, UNIFENAS, Alfenas, Minas Gerais State, Brazil.

The samples were diluted (10^{-1} - 10^{-3}), seeded in BHI agar and incubated at 37°C for a 24 hours. After this procedure, they were sown in Baird Parker agar that was enriched with egg yolk + potassium tellurite. Furthermore, they were incubated at 37 °C for 48 hours. The growth and morphology of colonies were noted after the incubation period. Thus, these have been identified as *Staphylococcus* sp by morphological analysis (colonies of black color and glossy, with precipitation zone surrounded by a clear halo) and bacterioscopy, using the Gram stain method. Subsequently, the blades were observed under a microscope and the bacterial genre was identified as purplish coccus groups. According to Machado et al.[1] samples were subjected to biochemical tests for identification of the species *S. aureus* (evidence of catalase, DNase, mannitol and coagulase) after the morphological analysis.

In the DNA extraction process, followed the protocol suggested by Vannuffel et al. [6]. The spectrophotometry was used to quantify and determine the purity of extracted DNA.

On the basis of the DNA sequences of the femA genes, the following two oligonucleotides were designed to amplify staphylococcal DNA [6]: primers F1 (217-[5']CTTACTTACTGGCTGTACCTG-237) and F2 (902-[5']ATGT CGCTTGTTATGTGC-884), which amplified 686-bp fragment of the femA gene.

Twenty microliters of DNA samples was added to 30 µL of PCR mixture consisting of 25 mM MgCl₂ (6 µL), 200 µM (each) desoxynucleoside triphosphates (1 µL), 25 pmol of each primer and 5 x Green GoTaq Flexi Buffer (10 µL), 5U.µL-1 GoTaq® Flexi DNA polymerase (0,25 µL) (Promega Corporation, Madison WI, USA). After an initial denaturation step (3 min at 92°C), 30 cycles of amplification were performed as follows: denaturation at 92°C for 1 min, annealing at 56°C for 1 min, and DNA extension at 72°C for 1 min with an increment of 2 s per cycle. The reaction was achieved with a final extension at 72°C for 3 min. Amplification was carried out in a Termociclador Perkin Elmer mod. 9700 (Applied Biosystems, Foster City, CA, USA). After amplification, PCR samples was loaded on a 2% (wt/vol) agarose gel and horizontal electrophoresis was performed in 0.1 M TrisHCl (pH 8.6)–80 mM boric acid–1 mM EDTA containing 0.5 mg of ethidium bromide per ml. Amplified, ethidium bromide-stained DNA fragments were then visualized on a UV transilluminator. It was used as positive control ATCC 25923.

Susceptibility tests were performed through diffusion technique [7], using standardized disks with antimicrobials, following the criteria established by Clinical and Laboratory Standards Institute [8]. The colonies, which grown in BHI Agar, were calibrated in the range of 0.5 McFarland turbidity. Moreover, they were sown with the assistance of sterile swabs on plates containing Mueller-Hinton Agar. According to Rangel (2007), they were adequately put on discs with antimicrobials.

Discs with vancomycin (30 µg), cefepime (30 µg), rifampicin (5 µg), penicillin G (10 µg), gentamicin (10 µg), clindamycin (2 µg), ciprofloxacin (5 µg), Chloramphenicol (30 µg), erythromycin (15 µg), tetracycline (30 µg) and oxacillin (1 µg) [8] were employed in this study. Due to the comparative tests, two strains of *S. aureus* resistant and sensitive to oxacillin were used. They were identified as *S. aureus* ATCC 29213 (sensitive to oxacillin) and *S. aureus* ATCC 43300 (oxacillin-resistant). After the incubation for 24 hours at 37°C, the analysis was performed with the aid of a caliper. The inhibition halos diameters (mm) were compared as CLSI table [8]. Thus, the isolates were classified as sensitive or resistant to antimicrobial that was tested.

The minimum inhibitory concentration (MIC), the bacterial inoculum was prepared through direct method technique with turbidity in the range of 0.5 McFarland. Furthermore, it was spread with the aid of sterile swab on the surface of agar Mueller-Hinton which was added of 2% NaCl. The Etest technique (*Epsilon* test) was performed in thin and inert ribbons with antimicrobials oxacillin and vancomycin. Furthermore, they were calibrated with visual scale that indicates the concentration of drugs which were placed on the inoculum. After this procedure, they were incubated at 35 °C for 24 hours. The halos of growth inhibition were carefully

evaluated by the use of light. This light was transmitted for detecting small colonies or scanty growth within the area of inhibition [8].

The leaves of *P.guajava* L. were collected in October/2012 at the University José of theRosárioVellano, UNIFENAS, Alfenas, MinasGerais State, Brazil. After the harvest, the leaves were separated and dried an oven at 35 °C during one week for removing the moisture and for the stabilization of enzymatic content. The material has been reduced to dust in electric and heavy mill. The extracts were obtained in 70% ethanol/water at a rate of 800 mL/150 g of botanical material. Moreover, they suffered maceration for 15 days in glass container with a capacity up to 2000 mL. This glass was capped at room temperature and protected from light, with daily agitation. The extract was filtered and concentrated on rotating evaporator, becoming lyophilized. At the time of use, the same extract was suspended in sterile distilled water and it was sterilized by the filtration in Millipore filter of 0.22 µm [9].

Microorganisms were cultured on BHI agar. Furthermore, they were seeded on Mueller-Hinton agar with the sterile swabs aid. Antimicrobial tests were performed using the technique of wells. The extract of *P.guajava* L. was diluted in sterile water. After that, aliquots of 75,150 and 300 µLwere added on the wells. A negative control that consists of distilled water and positive control was used in this procedure. In addition, it was represented by the antimicrobial clindamycin, one of which presented the highest action in the antibiogram that was performed. The In vitro activity was based on the formation of growth inhibition halos for concentrations which were tested according to the method described by Bauer and Kirby [7]. It also carried out the techniques of the minimum inhibitory concentration (MIC) in 96-well platesand minimum bactericidal concentration in Baird Parker agar to the extract.

III. Results and discussion

Samples of milk from cows with clinical and subclinical mastitis were collected in three farms in the south region of Minas Gerais State from July to October 2012. The presence and the infection in milk by *Staphylococcus aureus* in 55% of the samples (33/60) were proved and identified due to microbiological, biochemical and morphological evidences.The significance of this finding is related also the transmission of opportunistic microorganisms to humans through contaminated milk and cause infections.

In 94% of these positive samples related to microbiological identification of *S. aureus* (31/33), it was found the presence of the gene femA in the molecular analysis. Dias et al. [10] argued that there was amplification of the femA gene in 72.5% of the milk samples which were analysed. Teixeira et al. [11] developed a study that isolated colonies of *S. aureus* were obtained from samples of raw milk. These colonies were identified by phenotypic and microbiological methods. The results also were positive due to the presence of the gene femA.

After the analysis of antibiograms, it was observed the following percentages of antimicrobial resistance: cefepime, 24% (8/33); ciprofloxacin, 3% (1/33); chloramphenicol, 3% (1/33); clindamycin, 0% (0/33); erythromycin, 0% (0/33); gentamicin, 15% (5/33); oxacillin, 73% (24/33); penicillin G, 97% (32/33); rifampin, 0% (0/33); tetracycline, 12% (4/33) and vancomycin, 12% (4/33). In the minimum inhibitory concentration (MIC) there were found the values of 0, 25 µg/mL to vancomycin and 0,125 µg/mL to oxacillin.

Most antibiotics has presented potential in vitro antimicrobial activity against the microorganisms that were isolated. This evidence may indicate that due to the contents of resistance which were found in the investigated properties, the same antimicrobials in treating mammary inflammation were used. Furthermore, others antimicrobials that present good sensitivity can be applied to the control of the disease. In this context, it is evident the importance of application of susceptibility tests to lead to the best treatment.

Some antibiotics, such as clindamycin, erythromycin and rifampin with 100% effectiveness obtained the highest action. In addition, ciprofloxacin, chloramphenicol, tetracycline and vancomycin,also presented significant action, with sensitivity indexes close to or greater than 90%.

The Penicillin G was antimicrobial with the lowest response due to the action of beta lactamases and microbial enzymes that separate the β-lactam ring of this antibiotic. This evidence is similar to the results which were found by Zafalon et al. [2]

The knowledge of the sensitivity and resistance patterns to various antibiotics which has been used in the treatment of bovine mastitis caused by *S. aureus* is necessary and fundamental for the development of preventive methods that are effective, as well as for the construction of treatment strategies [12].

The hydroalcoholic extract of *P.guajava* L. leaf showed antimicrobial activity against all isolates *S. aureus* by inhibiting microbial growth on all aliquots. In addition, it was observed the formation of inhibition halos whose diameters varied from 13 to 36 mm.For minimum inhibitory concentration was found values 37.5µg/mL and 18.75 µg/mL and it was determined by the absence of turbidity in the well. For the minimum microbicidal concentration there was growth in all concentrations tested, this shows that the extract can have bacteriostatic action. Holecz et al. [13]observed antimicrobial activity of hydroalcoholic extract of leaves of *P. guajava* L. In this case, it was against *Candida albicans*, *S. aureus* and *Escherichia coli*. Alves et al. [14] also

detected activity against fungi. In research carried out by Amancio et al. [15] the diameters of zones of inhibition of microbial growth for the guava leaf extracts for *Staphylococcus aureus* were 16 mm and considering the size of the inhibition halos, it was found that Gram positive bacteria are more sensitive and Gram-negative bacteria and yeast are more resistant.

IV. Conclusion

Staphylococcus aureus has showed high prevalence in the infections. In addition, most of the used antibiotics in the evaluation has presented significant antimicrobial action against the isolates that were studied. In the present study, the results are consistent with the history of antimicrobial activity of the species that were analyzed. Moreover, it was considered the demonstration that the isolates were sensitive to the hydroalcoholic extract which was tested. The use of leaf extract of *P. guajava* L. can be an alternative to the increasing resistance of microorganisms to antimicrobial agents that have been used in veterinary clinical practice for the mastitis treatment. The genetic characteristics of this microorganism can be observed through molecular biology that is an important method for the identification. Thus, it is necessary more research about it.

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