Effects of associations of tannins from *Anacardium occidentale* and *Anadenanthera colubrina* with cephalosporin against bovine *Staphylococcus aureus* isolates

**ABSTRACT:** The association of natural compounds isolated from medicinal plants with conventional antibiotics, both with similar mechanisms of action, have become a viable alternative strategy to overcome the problem of drug resistance. This study aimed to evaluate the *in vitro* antimicrobial activity of tannic substances present in the bark of *Anacardium occidentale* and *Anadenanthera colubrina* against samples of *Staphylococcus aureus* when in combination with cephalaxin. These combinations were evaluated by determining the minimum inhibitory concentration (MIC). For this purpose, tannins and cephalaxin were serially dissolved in distilled water at concentrations ranging from 0.976 mg/mL to 500 mg/mL and 2 mg/mL to 512 mg/mL, respectively. When combined, the compounds inhibited *S. aureus* growth forming halos ranging from 0.9 to 46 mm with an MIC of 7.8 mg/mL (tannins) and 4 µg/mL (cephalexin). The resulting effect of the combination of natural and synthetic substances with similar mechanisms of action presented better results than when tested alone. Thus, the conclusion is that both the tannins and cephalaxin had their antimicrobial action enhanced when used in combination, enabling the use of lower concentrations while maintaining their antibacterial effect against strains of *S. aureus*.

**KEYWORDS:** tannins; natural products; antibiotics; microorganisms.

**RESUMO:** A associação de compostos naturais, isolados de plantas medicinais, com antibióticos convencionais, com mecanismos de ação semelhantes, torna‑se uma estratégia alternativa e viável para superar o problema da resistência. Assim, nosso objetivo foi avaliar a atividade antimicrobiana *in vitro* de substâncias tânicas presentes na casca de *Anacardium occidentale* e *Anadenanthera colubrina* associadas à cefalexina, sobre amostras de *Staphylococcus aureus*. Avaliamos essa associação por meio da determinação da concentração mínima inibitória. Dessa forma, taninos e a cefalexina foram dissolvidos de forma seriada em água destilada em concentrações variando de 0,976 mg/mL a 500 mg/mL e 2 µg/mL a 512 µg/mL, respectivamente. Quando associados, inibiram o crescimento de *S. aureus* formando halos que variaram de 0,9 a 46 mm com concentração mínima inibitória de 7,8 mg/mL (taninos)/ 4 µg/mL (cefalexina). O efeito resultante da associação de substâncias, natural e sintética, com mecanismos de ação semelhantes, apresentou resultados superiores aos observados quando testados isoladamente. Podemos concluir que os taninos e a cefalexina tiveram sua ação antimicrobiana potencializada quando utilizados em associação, permitindo o uso de uma menor concentração, mantendo seu efeito antibacteriano sobre cepas de *S. aureus*.

**PALAVRAS‑CHAVE:** taninos; produtos naturais; antibióticos; microorganismos.
INTRODUCTION

The emergence of antibiotic resistant *Staphylococcus aureus* strains occurs due to improper and abusive use of antimicrobial agents, leading to their resistance to drugs in current use. This has become a public health problem (MASURANI; TAVARES, 2007), and the treatment of bacterial infection cases related to this strain is becoming increasingly difficult. This has promoted a growing interest in scientific circles for compounds obtained from medicinal plants, which are effective against microorganisms resistant to conventional medicines. They can minimize side effects and are more affordable. Among the tannins, there is a pool of components, and their synergisms are necessary for bacterial metabolism and growth (JONES et al., 1994; HASLAM, 1996; GUIMARÃES-BEELEN et al., 2006; RODRIGUES et al., 2014). Recent studies indicate that tannins present great potential in binding proteins and adhesins, interfering directly in the substrate availability necessary for bacterial metabolism and growth (JONES et al., 1994; HASLAM, 1996; GUIMARÃES-BEELEN et al., 2006; RODRIGUES et al., 2014).

Among the antibiotics most prescribed to treat infections caused by *S. aureus*, 1st generation cephalosporin (Cephalexin) presents action against many bacteria including: *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli*, *Haemophilus influenzae*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Monsella catarrhalis* (PRESCOTT et al., 2002; RODRIGUES;BERTOLDI, 2010; SAEKI et al., 2011), and constitutes a first option for skin infections, in which *Staphylococcus aureus* is the most probable causative agent (WHITE, 1996; PRESCOTT et al., 2002). Considering the increasing prevalence of multidrug-resistant *S. aureus*, which causes many diseases in humans and in animals (FREITAS et al., 2005; RUEGG, 2007), the use of tannins, associated with conventional antibiotics, can reduce morbidities and combat disease. In this context, tannins isolated from *Anadenanthera colubrina* (Vell) Brenan (Red Angico), and *Anacardium occidentale* Linn. (Cashew) may be good therapeutic candidates. They can be used alone (MENEZES et al., 2014; GONÇALVES et al., 2015; HIGINO et al., 2015; PEREIRA et al., 2015), or as mentioned above, in association with antibiotics. However, studies focusing on their solitary (or associated) biological activities have not been found in the literature. This study aimed to evaluate the *in vitro* antimicrobial activity of tannin substances present in the bark of *A. occidentale* and *A. colubrina* when associated with cephalaxin against multdrug-resistant *S. aureus* samples of bovine origin.

MATERIAL AND METHODS

*Anadenanthera colubrina* (Vell) Brenan and *Anacardium occidentale* L.

Stem husks of “Red Angico” (*A. colubrina*) and “Cashew” (*A. occidentale*) were collected and prepared in NUPEÁRIDO, the Semi-arid Research Nucleus of Universidade Federal de Campina Grande (UFCG). The plants were collected (four samples), and deposited at the Prof. Lauro Pires Xavier Herbarium and the Pharmaceutical Technology Laboratory reference collection, both at Universidade Federal da Paraíba (UFPB). The identification of the samples was carried out in the Virtual Herbarium of Flora and Fungi (INCT) registered with the code CEN 50716 and CEN 6191 respectively.

Extraction of tannin substances for antimicrobial activity evaluation

The husks were packed in plastic bags so as to not lose moisture. In the laboratory, two samples were used; they were cut into smaller fragments, homogenized, weighed and dried in an oven at 103±2°C for 48 h for determination of moisture content (dry basis). The husks were then air dried and milled in a forage harvester. The material was classified, and what passed through a 2 x 2 cm mesh sieve was used. After this process, four samples were collected; two of these were ground in a Willey-type mill to obtain smaller and more homogenous particle sizes aimed to quantify the tannins present in *A. colubrina* and *A. occidentale* in accordance with PAES et al. (2006). The extraction of tannins was performed in water, using a rotary digester, at a temperature of 70±5°C for 2 h. For each 2 kg of husks, 10 L of water were respectively added (1:5). Each sample was submitted to two extractions (GUANGCHENG et al., 1991). The solution obtained was homogenized, strained, and placed in aluminum trays, and then placed in an oven with forced ventilation and kept at 70±3°C until complete moisture evaporation. The dry material, final product of the evaporation process, was crushed in a household multiprocessor and sifted in a 60 mesh sieve; corresponding to pure tannin.

Acquisition of *Staphylococcus aureus* strains

Collection of milk samples and nasal swabs were performed in NUPEÁRIDO, the Semi-arid Research Nucleus of Universidade Federal de Campina Grande (UFCG), in the municipality of Patos, State of Paraíba, Brazil.

Samples were obtained from 30 naturally infected lactating cattle. After washing the udders with soap and water,
Effects of associations of tannins from Anacardium occidentale and Anadenanthera colubrina with cephalosporin against bovine Staphylococcus aureus isolates

Antimicrobial activity

Tannins were dissolved in sterile distilled water and filtered in a 0.22 µm membrane filter (TPP®) at 25 mg of tannin for each mL of distilled water. The initial solution was diluted serially, obtaining concentrations ranging from 500 mg/mL to 0.976 mg/mL; these were added individually to the S. aureus bacterial cultures.

The bacteria were previously grown in BHI medium (Brain Hearth Infusion, Sigma™) at 37°C for 24 h, and the suspensions were adjusted to the Nephelometric 0.5 McFarland standard scale turbidity standard. This corresponded to approximately 1.5x10⁶ colony-forming units (CFU)/mL, which were diluted in BHI broth for a concentration of 1.5x10⁴ CFU/mL.

Aliquots of 50 µL were removed from these tubes and disseminated in Petri plates containing Muller Hinton agar (MHA). Holes were made in the culture mediums with a 6 mm diameter glass tube and filled with 25 µL of tannin and cephalaxin diluted in distilled water. The initial concentration of tannin for serial dilution was 500 mg/mL, and cephalaxin at 512 µg/mL. This concentration was selected in accordance with the results obtained by SULEIMAN et al. (2011). In this study, 11 strains of S. aureus were chosen: one strain of S. aureus (ATCC 25925) and ten of strains of S. aureus from bovine origin obtained from the udders (104U, 120U, 125U, 787U, 250U, 275U, 282U, 301U, 305U and 335U). The strains were tested against β-lactams (ampicillin and penicillin), as well as aminoglycosides (neomycin, streptomycin, and tetracycline).

A qualitative method was used, which allowed classifying the bacterial samples as susceptible or resistant to the association used (CLSI, 2010). Strains were considered sensitive when presenting halos greater than 10 mm, according to the preliminary studies (PEREIRA et al., 2015; MENEZES et al., 2014; CAVALCANTI-DANTAS et al., 2016).

RESULTS

Initially, the resistance profiles of S. aureus strains against β-lactams (ampicillin and penicillin) and aminoglycosides (neomycin, streptomycin, and tetracycline) in this way were verified and considered as S. aureus strains susceptible or resistant to the association used, thus corroborating analyses conducted by BARRETO et al. (2014). The protocol used in this study allowed the determination of the antimicrobial activity (MIC) of stem bark tannins from A. occidentale and A. colubrina when associated with cephalosporin (Cephalexin) against multidrug-resistant S. aureus samples of bovine origin.

The inhibition halos for A. occidentale and A. colubrina associations together with cephalaxin had diameters ranging from 0.9 to 46 mm at all dilutions, thus presenting 100% effectiveness at the 1:64 dilution (Table 1). Inhibition of growth occurred homogeneously and according to the concentration of the tannic substance in associations with cephalaxin.

It was observed that the associations in the higher concentrations of both tannins (mg/mL) and cephalaxin (µg/mL) the diameter of the growth inhibitory halos of S. aureus greater varying between (43.45±2.25); (38.27±1.79); (33.82±2.16) and (25.91±2.16) respectively, without differing between them (p=0.1000). The associations at lower concentrations of both tannins (mg/mL) and cephalaxin (µg/mL) showed the diameter of the smaller growth inhibitory halos of S. aureus varying between (21.09±1.57), (12±1) and (9.9±0.94) respectively, but not differing from each other, differing from the halos values.

STATISTICAL ANALYSIS

Sensitivity testing was carried out in triplicate and evaluated using analysis of variance by the Kruskal-Wallis test, followed by Dunn’s test, comparing tendencies of bacterial growth inhibition (CALEGARI-JACQUES, 2003). Tests were considered significant when the p-value was less than 5%. The data were also recorded in the form of a database in the computer program SPSS (Statistical Package for Social Sciences) for Windows®, version 15.0; and analyzed by means of descriptive and inferential statistics.

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of the highest concentrations (p<0.05). This last concentration (7.8 mg/mL + 8 µg/mL) was considered the MIC (Tables 1 and 2). It is noteworthy that at this last concentration (7.8/8); four strains showed resistance to the Cephalexin – *A. occidentale* association; corresponding to 36.3% of samples (halos less than 9 mm; Table 1).

Another six strains tested showed resistance to the Cephalexin – *A. colubrina* association; corresponding to 54.5% of the samples (halos with less than 9 mm; Table 2). In view of the inhibition halos when Cephalexin and the tannins were used separately, the *A. occidentale* tannin showed an MIC of 62.5 mg/mL, with halos of 11 mm, the *A. colubrina* tannin obtained an MIC of 31.25 mg/mL, with halos that ranged from 10 to 13 mm, and Cephalexin obtained an MIC of 32 µg/mL with halo dimensions that ranged from 29 to 33 mm.

### Table 1. Mean±standard deviation of the minimum inhibitory concentration (MIC); with diameters of inhibition halos in millimeters (mm), on solid medium; association of cephalexin with *Anacardium occidentale* tannin; against *Staphylococcus aureus*.

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**ATCC**

| 104 U       | 42  | 35  | 30  | 25  | 20   | 13   | 10    |
| 120 U       | 46  | 37  | 32  | 27  | 21   | 12   | 09    |
| 125 U       | 44  | 39  | 35  | 29  | 24   | 12   | 10    |
| 156 U       | 42  | 38  | 36  | 28  | 23   | 14   | 12    |
| 250 U       | 45  | 40  | 37  | 29  | 23   | 11   | 09    |
| 275 U       | 43  | 38  | 33  | 25  | 20   | 10   | 09    |
| 282 U       | 40  | 38  | 34  | 26  | 21   | 13   | 10    |
| 301 U       | 46  | 38  | 34  | 25  | 21   | 11   | 09    |
| 305 U       | 40  | 37  | 35  | 22  | 20   | 12   | 10    |
| 335 U       | 44  | 39  | 36  | 25  | 20   | 12   | 10    |
| ATCC        | 46  | 42  | 30  | 24  | 19   | 12   | 11    |

**M±SD**

| 43.45±2.25 | 38.27±1.79 | 33.82±2.16 | 25.91±1.57 | 21.09±1.57 | 12±1.5 | 9.9±0.94 |

Averages followed by different letters present significant difference. The Kruskal-Wallis test (p<0.05); M=Medium; SD=standard deviation; Tan=tannin; U=Udder; Cef=Cephalexin; *MIC=Minimum Inhibitory Concentration at a dilution of 1:64=7.8 mg/mL of tannin and 8 µg/mL of cephalexin.

### Table 2. Mean ± standard deviation of the minimum inhibitory concentration (MIC); with diameters of inhibition halos in millimeters (mm), on solid medium; association of Cephalexin with *Anadenanthera colubrina* extract against *Staphylococcus aureus*.

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**ATCC**

| 40  | 38  | 35  | 29  | 25  | 12   | 00    |

**M±SD**

| 38.8±1.47 | 35.36±1.69 | 31.55±2.42 | 26.73±2.57 | 23±2.28 | 13.36±1.43 | 5.8±5.67 |

Averages followed by different letters show significant difference. The Kruskal-Wallis test (p<0.05); M=Medium; SD=standard deviation; Tan=tannin; U=Udder; Cef=Cephalexin; *MIC=Minimum Inhibitory Concentration at a dilution of 1:64=7.8 mg/mL of tannin and 8 µg/mL of cephalexin.
DISCUSSION

From the results obtained, it can be observed that the tannins presented significant pharmacological effect. In the in vitro assay, the antibacterial activity of tannins isolated from the bark of *Mimosa arenosa* was observed in both Gram-positive and Gram-negative bacteria such as: *S. aureus*, *E. coli*, *P. aeruginosa*, and *B. subtilis* with MICs ranging from 250 to 1000 µg/mL (GONÇALVES et al., 2015). The authors observed that the antibacterial activity exerted by tannins from *M. arenosa* was bacteriostatic against all strains tested.

The results in this study demonstrated synergism between cephalexin and the tested tannins against multidrug-resistant *S. aureus* samples. The tannins of *A. occidentale* and *A. colubrina* presented respective MICs of 62.5 mg/mL and 31.2 mg/mL when used pure. Cephalexin, when used alone, presented an MIC of 32 µg/mL. The associations of tannins with the antibiotic were more expressive. We saw antimicrobial action even at dilutions of 1:64 considering the MICs for tannin contents at 7.8 mg/mL, and at 8 µg/mL for Cephalexin. This demonstrates that the effect of the 抗菌 was potentiated by the tannin addition, which, even in low concentrations, promoted better antimicrobial action. This likely occurred due to their interactions, and to synergism in their mechanisms of action.

Cephalexin interrupts the synthesis of peptidoglycan and inhibits the action of the enzyme involved in cell membrane trans-peptidation, preventing connections between tetra-peptide chains of peptidoglycan, and promoting lysis of bacterial cell wall (SPINOSA, 2011). Tannins are oligomers and polymers formed by poly-condensation of two or more flavanols and flavandiol, which in turn act similarly by forming tannin-protein complexes and/or polysaccharides at the bacterial cell membrane. The formation of these complexes leads to protein solubility reductions, promoting aggregation and precipitation of proteins present in the membrane, and inhibiting the action of microbial enzymes, thus depriving the organism of substrates essential to growth. This causes lysis of the plasma membrane (GUIMARAES-BEELEN et al., 2006; RODRIGUES et al., 2014; CAVALCANTI-DANTAS et al., 2016). In accordance with SPINOSA (2011), substances that act against the plasma membrane of the bacterial cell present mechanisms of action that promote modification of its structure, and interfere in the transport systems of nutrients, ions, and other molecules. Synergism of these two substances promotes an increase of physiological response (SPINOSA, 2011; RANG et al., 2012), and may be responsible for the results obtained in the present study.

In the present study, a potentiating effect was observed in the association of two substances obtained from medicinal plants and a conventional medicine. Both present a similar mechanism of action. The results demonstrated that the combined action between them was significantly greater than the sum of the effects achieved by each one alone. Through this association, it can be observed that the tannins present a significant pharmacological effect when associated with cephalexin, and that both the tannins and cephalexin had their...
in vitro antimicrobial action maximized against the tested multiresistant microorganisms. In vivo studies need to be performed to test dosage safety and to reduce the overuse of the antibiotic, in order to maintain its antibacterial effect against multidrug-resistant *S. aureus* strains. According to SAR et al. (2012), the main advantage in using associated antibiotics that have the same mechanism of action is that the association allows potentiation of their effects, with consequent reductions in the drug dose, while maintaining therapeutic efficacy and promoting its effects.

Currently, there is a growing number of publications concerning the use of medicinal plants and their metabolites in *in vitro* experimental models against multidrug-resistant *S. aureus* (MANSOURI, 1999; HEYMAN et al., 2009; MENEZES et al., 2014; PEREIRA et al., 2015; CAVALCANTI-DANTAS et al., 2016). However, more systematized information is needed concerning possible clinical and pathological aspects resulting from such associations of conventional medicines with tannins.

**CONCLUSION**

When used in association, the tannins and cephalexin presented augmented antimicrobial action. The results of this study suggest that the antibacterial activity demonstrated against multiresistant strains of *S. aureus* was achieved due to a potentiating effect introduced by the tannins. Thus, tannins may be used in association with cephalexin against diseases caused by multidrug-resistant *S. aureus*. These findings, in favor of such economically viable compounds, add to an important list of new alternatives to help in infection control.

**REFERENCES**


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