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Antibacterial activity tannin-rich fraction from leaves of *Anacardium humile*

Atividade antibacteriana de frações tânicas de folhas de *Anacardium humile*

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- NOTE -

ABSTRACT

In vitro tests conducted with extracts rich in tannins have identified several biological activities of this class of substance. Thus, this paper intends to evaluate the antibacterial activity of tannin-rich fraction obtained from leaf extracts of *Anacardium humile* A.St.-Hil. Extracts of *A. humile* leaves in 70% acetone were semi-purified with ethyl acetate and butanol. We quantified the total tannins of the semi-purified fractions, of the crude extract and of aqueous residues and then performed tests of the antibacterial activity of the tannins against *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212. All positive extracts underwent tannin isolation using a Sephadex LH-20 column. The tannins isolated from the samples were quantified and tested for the minimal inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC). The tannins from crude extracts, semi-purifications and residues showed inhibition of *S. aureus* growth with MIC=500 µg mL⁻¹. All tannin fractions showed MIC against all strains and MBC, except against *E. faecalis*. The tannin fractions from *Anacardium humile* have antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* and, therefore, may be promising for future synthesis of new antibacterial agents.

Key words: phenolic, cajuí, antibacterial, MIC, MBC, Sephadex LH-20.

RESUMO

Testes in vitro realizados com extratos ricos em taninos têm identificado muitas atividades biológicas dessa classe de substâncias. Nesse contexto, esse artigo propõe a

avaliação da atividade antibacteriana de frações tânicas, obtidas de extratos de folhas de *Anacardium humile* A.St.-Hil. Extratos de *A. humile* em acetona 70% foram particionados com acetato de etila e butanol. Foram quantificados taninos totais das frações particionadas, do extrato bruto e dos resíduos aquosos, e foi realizado teste de atividade antibacteriana dos taninos contra *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853 e *Enterococcus faecalis* ATCC 29212. Todos os extratos positivos foram submetidos ao isolamento de taninos, utilizando-se coluna de Sephadex LH-20. Os taninos isolados das amostras foram quantificados e testados sua concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM). Os taninos do extrato bruto, partições e resíduos apresentaram inibição do crescimento de *S.aureus* com CIM =500 µg mL⁻¹. As frações tânicas apresentaram CIM contra todas as cepas e CBM, exceto contra *E. faecalis*. As frações tânicas de *Anacardium humile* possuem atividade antibacteriana contra *Staphylococcus aureus*, *Pseudomonas aeruginosa* e *Enterococcus faecalis* e, portanto, podem ser promissoras em sínteses futuras de novos agentes antimicrobianos.

Palavras-chave: fenólicos, cajuí, antibacterianos, CIM, CBM, Sephadex LH-20.

Anacardium humile A. St.-Hil. is a species found in the Brazilian Savanna belongs to the Anacardiaceae family. All parts of *A. humile* are widely used in folk medicine. The leaves are used to treat diarrhea and as an expectorant (ALMEIDA et al., 1998). Studies have attributed the anti-ulcer activity of *A.*

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humile to the presence of flavonoids and tannins (LUIZ-FERREIRA et al., 2010), however there are few studies of the constituents of the leaves of *A. humile*.

In vitro tests conducted on tannin-rich extracts have identified various biological activities, such as antimicrobial activity (SILVA et al., 2007). In this context, *A. humile*, a species native to the Brazilian Savanna, may become a possible species to be explored for the presence of bioactive substances. The complexity of the special metabolites that are biosynthesized by plants show that such plants are important potential sources for new drugs (MONTARI & BOLZANI, 2001). Thus, this study involved the isolation of tannin and the analysis of antibacterial activity from crude extracts, residues and tannin-rich fraction obtained from the leaves of *A. humile* A. St.-Hil.

A. humile leaves were collected randomly from ten individual plants in the municipality of Januária (coordinates 15°30'27.9"S and 44°45'15.5"W). The material was identified and deposited at the Montes Claros Herbarium of the University of Montes Claros in Minas Gerais with the voucher number 200. The leaves were dried at room temperature in the shade and crushed in a grinder for seven days. Thirty grams were placed in a suspension of 300mL of acetone at a concentration of 70% v/v and stirred constantly for 24 hours at 160rpm. After centrifugation the insoluble residue was extracted twice more. An evaporation of the acetone from the combined extracts was then performed using a rotary evaporator in a vacuum at 30°C. One hundred milliliters of the extracts were freeze-dried and another 100mL of the non-lyophilized extracts underwent a liquid-liquid partition with ethyl acetate and n-butanol. The remaining aqueous fraction was subjected to lyophilization.

The quantification of the total tannins was performed according to the radial diffusion method (HAGERMAN, 1987). The amount of total tannins was determined from the follow calibration curve of tannic acid at concentrations of 5 to 15mg mL⁻¹: $y=4.4924x+0.3902$ ($r^2=0.9974$). Where y (cm²) is the halo diameter formed by tannin-protein precipitate, x (mg mL⁻¹) is the concentration of tannins.

The samples were diluted in water (type 1 purity level) to the following concentrations: 0.5g mL⁻¹ of crude extract, 0.2g mL⁻¹ of ethyl acetate, 0.3g mL⁻¹ of butanol and 0.1g mL⁻¹ of the residues (aqueous). The samples were diluted to the indicated concentrations in 95% ethanol in a 1:1 ratio and placed (2mL) in a Sephadex LH-20 column (25cm x 3cm). The columns were equilibrated with 95% ethanol (MUELLER-HARVEY, 2001). The column was eluted at a flow rate of 1mL min⁻¹ until the absorbance of 280nm tended

towards zero. The tannins were then eluted from the Sephadex LH-20 gel with 50% acetone and the absorbance at 435nm was determined (WANG & LEE, 1996). Following the tannin precipitation method (COSTA, 2002), the fractions that were positive for tannins were collected. Tannins were quantified from the samples using the Radial Diffusion Method.

For the determination of the MIC, the broth micro-dilution method was used as per NCCLS (2003). The medium used in this test was Mueller-Hinton broth, and the bacterial inoculum for *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterococcus faecalis* (ATCC 29212) were previously normalized to a concentration of 1.5x10⁸ CFU.mL⁻¹ based on the standard of the 0.5 McFarland scale. Each of the 96 wells of the microplate contained 100µL of the tested samples, which were diluted in the medium at concentrations ranging from 1.0(g mL⁻¹ to 8.192(g mL⁻¹. A volume of 5µL of bacterial inoculum was added to each well for each ATCC bacteria tested. The negative control used was the broth with the addition of type 1 sterile water. The positive control was the broth medium with the addition of the bacterial inoculum. The plates were placed in a bacteriological incubator at 35°C for 24 hours. After that time, 30µL of a sterile 0.02% resazurin solution was added. After two hours, the plate was read by visual interpretation of the color in each well. A blue color indicated the absence of bacterial growth and a reddish pink color indicated the presence of bacteria.

The MBC was determined from the MIC. The test was performed by re-inoculating the MIC cultures from the dilutions without bacterial growth (blue color) on plates containing a Mueller-Hinton Agar medium. After incubation (24 h at 37°C), the plates were visually inspected for the presence or absence of bacterial growth. Inhibition in the MIC assay and bacterial growth in the subculture indicated bacteriostatic action, while the absence of growth indicated bactericidal action. The MBC was considered to be the lowest concentration of the extract in which no cell growth was present on the surface of the inoculated agar.

Statistical evaluation of the results was performed using the R statistical system, version 2.8.0 (2009), analysis of variance (ANOVA) and contrast analysis was applied.

The material isolated in the Sephadex LH-20 column from the crude extract showed a total tannin content equal to 0.072±0.002mg, followed by ethyl acetate with 0.107±0.008mg, butanol with 0.044±0.006mg and residues equal to 0.016±0.001mg. Therefore, the material from the semi-purification with

ethyl acetate displayed the highest content of total tannins, and the residues displayed the lowest level (ANOVA, $P < 0.01$).

All of the isolated tannins promoted the inhibition of *S. aureus* (ATCC 6538); no differences between the tannins occurred with regard to either MIC or MBC. In the tests against *P. aeruginosa* (ATCC 27853), the tannins isolated from butanol and the residues showed MIC values (4.1 and $8.2 \mu\text{g L}^{-1}$, respectively) that were higher than those observed for tannins isolated from the crude or the ethyl acetate extracts (both $2 \mu\text{g L}^{-1}$). A similar result was noted in isolates from the residues tested against *E. faecalis* (ATCC 29212), which showed MIC values ($2 \mu\text{g L}^{-1}$) that were greater than those of the other tested tannin isolates ($1 \mu\text{g L}^{-1}$); this result may be explained by the lower levels of tannins found in these isolates (residues).

Based on the MBC values *E. faecalis* was the bacterium for which all of the tested tannin isolates exhibited bacteriostatic activity rather than bactericidal activity. The isolates were most effective against *S. aureus* (ATCC 6538) when lower tannin content was required to determine the MIC and MBC ($0.5 \mu\text{g L}^{-1}$). While the work of PEREIRA et al. (2011) MIC values slightly higher ($1.0 \mu\text{g L}^{-1}$) was necessary in *A. humile* leaf extract for *S. aureus* inhibition. Our results are interesting because low MIC values exhibited by plant extracts represent a possible replacement of the usual antibiotics used to treat infections (TANAVADE et al, 2012).

This result reinforces the idea that tannins can act as natural antibiotics (CHUNG et al., 1998). The complexation of tannins with proteins is the most significant of their toxic effects (HAGERMAN & BUTLER, 1994). Compounds similar to tannins with antibacterial activity have already been synthesized (ROY et al., 2009). So, tannins from extracts from *A. humile* showed antimicrobial activity. These data indicate that this species may be a source of molecules that can serve as natural antibiotics or which may be used in combination with other drugs to increase efficiency.

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