DOI: https://doi.org/10.17921/2447-8938.2024v26n3p143-146

Antimicrobial Activity *Banisteriopsis argyrophylla* and *Davilla rugosa* Leaf Extracts Over Human and Veterinary Clinical Isolates of *Staphylococcus aureus*

Atividade Antimicrobiana dos Extratos das Folhas de *Banisteriopsis argyrophylla* e *Davilla rugosa* em Isolados Clínicos de *Staphylococcus aureus* de Origem Humana e Veterinária

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Abstract

The human interactions with companion, production and work animals have changed considerably in the latest years, changing the management of health issues such as bacterial resistance to antimicrobial drugs (BRAD) as well. Natural products represent a valid source of new antimicrobials of interest to treat veterinary and human infectious diseases, looking forward to overcoming BRAD. Here we show the antimicrobial potential of *Banisteriopsis argyrophylla* and *Davilla rugosa* extracts against human and veterinary clinical isolates of *Staphylococcus aureus*. The identity of the isolates was confirmed using VITEK 2 system. The extracts were prepared using dehydrated leaves and different organic solvents. Among them, only the dichloromethane extract was effective, and was tested for cytotoxicity, minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). MIC values ranged from 8 to 64 µg/mL, whilst MBC values ranged from 64 to 512 µg/mL. No cytotoxicity was detected for the extracts. *B. argyrophylla* and *D. rugosa* were active against veterinary and human clinical isolates of *S. aureus*. Further *in vivo* studies with isolated molecules are necessary to confirm their suitability for use in clinical routines.

Keywords: Antimicrobial Activity. *Banisteriopsis argyrophylla. Davilla Rugosa. Staphylococcus aureus.*

Resumo

A interação dos seres humanos com animais de companhia, produção e trabalho tem mudado consideravelmente nos últimos anos, afetando o gerenciamento de questões de saúde como a resistência bacteriana a antimicrobianos (RBAM). Produtos naturais são uma fonte válida de novos antimicrobianos para o tratamento de doenças infecciosas em medicina humana e veterinária, visando superar a RBAM. Neste estudo demonstrou-se o potencial antimicrobiano dos extratos de *Banisteriopsis argyrophylla* e *Davilla rugosa* contra isolados clínicos humanos e veterinários de *Staphylococcus aureus*. A identidade dos isolados foi confirmada com o sistema VITEK 2. Os extratos foram preparados em diferentes solventes orgânicos com folhas desidratadas. Destes, apenas o extrato preparado com diclorometano foi eficaz, e foi testado quanto à citotoxicidade, concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM). Os valores de CIM variaram de 8 a 64 µg/mL e os valores de CBM variaram de 64 a 512 µg/mL, e não foi detectada citotoxicidade. Os extratos de *B. argyrophylla* e *D. rugosa* foram ativos contra isolados clínicos veterinários e humanos de *S. aureus*, tornando necessários estudos *in vivo* com moléculas isoladas para confirmação da viabilidade do uso em rotinas clínicas.

Palavras-chave: Atividade Antimicrobiana. *Banisteriopsis argyrophylla*, *Davilla rugosa. Staphylococcus aureus.*

1 Introduction

Bacterial resistance to antimicrobial drugs (BRAD) is a complex phenomenon that comprise molecular mechanisms that shields bacteria against bactericidal and/or bacteriostatic molecules[1]. BRAD is a critical cause of poor efficiency of antimicrobials and makes the treatment of infectious diseases a very difficult endeavor[1]. The lack of new antimicrobials in the pharmaceutical market makes this situation even more difficult. A recent study described that only 19 new drugs were introduced in the pharmaceutical market – but none of them are from a new pharmacological group[2].

Historically, BRAD has not been investigated in veterinary medicine as in human medicine[3]. The impacts of infectious diseases in animal daily life are quite different from what might happen to humans, and in clinical practice, guidelines recommended euthanasia when animals cannot respond properly to the available treatment options[3-5]. Furthermore, bacterial resistance is considered uncommon in strains isolated from animals[6] - although this is more likely to be due to the lack of studies in the field than due to the bacterial susceptibility to antimicrobials[4].

The human interactions with companion, production and

work animals (CPWA) have changed considerably in the latest years. The growing closeness of animals and humans highlights the "one health" concept, which re-shapes the interpretation of several health issues, including BRAD[4]. A number of events might result in selective pressure on bacterial strains: some of the antimicrobials used in human medicine are shared with different animal species and can be found in sub-inhibitory concentrations in the environment due to contamination by animal and human waste, and also improper disposal[3,4,7]. Antimicrobials might be also present in sub-inhibitory concentrations in animal products consumed by humans (and are often offered by humans to dogs, cats and other species) such as milk and beef[8,9]. The result of such complex scenario is the increasing number of reports of CPWA as sources of pathogenic bacterial strains resistant to antimicrobial drugs[10-14], leading to critical clinical signals due to infectious diseases in humans.

In this context, it is relevant that new antimicrobials are tested on potentially pathogenic species of interest in veterinary and human medicine. Natural products represent a valid source of antimicrobials: the structural and pharmacological diversity of bioactive phytomolecules present in leaves, roots and stembarks, can be effective against different microbial species[15]. Such diversity also explain in parts why bacterial resistance to them is rare. Here we investigated the antimicrobial potential of leaf extracts of *Banisteriopsis argyrophylla* and *Davilla rugosa* (also known as "cipó prata" and "cipó caboclo", respectively) against human and veterinary clinical isolates of *Staphylococcus aureus*, *in vitro*, for the first time. Both plants are used in Brazilian traditional medicine mostly as diuretics or anti-inflammatory agent, but other uses include the treatment of pain and of gastric ulcers[16,17]. We also conducted cytotoxicity tests *in vitro* and classical phytochemical screening. Our data open doors for more studies with isolated molecules from these extracts.

2 Material and Methods

2.1 Preparation of the extracts

Dehydrated leaves of *Banisteriopsis argyrophylla* and *Davilla rugosa* for preparation of hot beverages were purchased from a store at the Brazilian state of São Paulo. It was required from the manufacturer to provide information on the authenticity of the species at the label and in a specific report, as well as an intact and properly sealed package. These are some of the requirements of the Brazilian Ministry for Agriculture, Livestock and Food Supply for registration and commercialization of such products. Furthermore, as required by the Brazilian law, the access to genetic resources was registered in the National System for the management of Genetic Heritage (SisGen, Process N° A826BB6).

The phytomolecules of each plant were extracted by full immersion in different organic solvents (dichloromethane, ethyl acetate, ethanol), in a sealed flask (to avoid evaporation) for 7 days, in home temperature. Following, the solvents were collected avoiding any particulate material, and were dried in a SpeedVac centrifuge (ThermoFisher) at 45 °C. The dried product was then stored at 4 °C until used.

2.1 Phytochemical screening

Classical qualitative phytochemical tests were conducted for the dried extracts prepared in the organic solvent that displayed the best results of antimicrobial activity. Flavonoids were screened using Shinoda test, tannins were screened by observing the formation of precipitates by the addition of $FeCl_3$, and saponins were screened using the foam test[18,19].

2.2Bacterial isolates

Clinical isolates were obtained from the microorganisms collection from Anhanguera College (Ipatinga, MG, Brazil). Human isolates were obtained indwelling catheters of hemodialysis patients. Veterinary isolates were obtained from uterine lavage fluids of mares in preparation for fixed-time artificial insemination. All strains were cultured in Brain Heart Infusion (BHI) broth (Difco) before identity confirmation tests using VITEK 2 system (version R04.02, bioMérieux). Gram-positive identification cards were used with the strains as indicated by the manufacturer. A total of 10 veterinary isolates and 10 human isolates were used in this study.

2.3 Antimicrobial activity

The minimal inhibitory concentration (MIC) of the extracts was determined in untreated sterile 96-well polystyrene microtiter plates as described by the Clinical and Laboratory Standards Institute[20], with slight adaptations described by our group[21]. Bacterial cultures were prepared in Mueller Hinton broth (Difco) in 1 McFarland scale by adjusting the optical density to 1 at 600 nm wavelength, and 100 µL of the suspension was dispensed in the wells. Then, the wells received the extracts serially diluted in final concentrations ranging from 1024 to 8 µg/mL, creating a final concentration of the bacterial inoculum equal to 0.5 McFarland scale. Plates were then incubated at 37 °C overnight. A 0.1% resazurine solution was used for staining procedures. MIC was established as the lowest concentration in which resazurine staining had no color modification from blue to pink in all the strains. The extracts were used as negative control. This assay was performed in triplicate.

Following, we determined the minimum bactericidal concentration (MBC) of the extracts. A total of 10 µL of each well of MIC plates was spotted in Mueller-Hinton agar plates (Difco). The extracts were inoculated in the same volume, as negative controls. The plates were incubated overnight at 35±2 °C and then analyzed for bacterial growth. MBC was

considered the lowest concentration that yielded no growth on the plates, considering all the isolates[20,22]. This assay was performed in triplicate.

2.4 Citotoxicity test

The potential cytotoxicity of the extracts was tested against BGM cells, an immortalized fibroblast-like kidney cell line. The full protocol was described in detail in previous publications of our group[21, 22]. Cell suspensions were prepared in RPMI 1640 media supplemented with glutamine (0.3 mg/L), gentamicin sulfate (40 mg/mL) and heat-inactivated fetal calf serum (10%). Each well of 96-well plates was filled with 3.0 x 10^5 cells diluted in 180 µL of culture medium. RPMI 1640 and glutamine was purchased from Sigma-Aldrich (St Louis, USA), gentamicin from Nova Farma Indústria Farmacêutica (Brazil) and fetal calf serum was obtained from Gibco (Thermo Fisher Scientific, Waltham, USA). All the other reagents were obtained commercially and were of analytical grade.

3 Results and Discussion

3.1 Phytochemical properties and cytotoxicity of the extracts

Flavonoids were the only phytomolecules detected by the classical methods in both extracts. None of them displayed cytotoxicity *in vitro* test when tested at 1000 µg/mL (data not shown).

3.2 Antimicrobial potential of the dichloromethane extract

Different organic solvents were used in the preparation of the extracts. Only the dichloromethane extracts displayed antimicrobial activity, although their MIC values were different for veterinary and human strains (Table 1).

Table 1 - antimicrobial activity of *B. argyrophylla* and *D. rugosa*

Parameter	*B. argyrophylla*	*D. rugosa*
MIC – veterinary strains	64 µg/mL	16 µg/mL
MBC – veterinary strains	X > 1024 µg/mL	512 µg/mL
MIC – human strains	16 µg/mL	8 µg/mL
MBC – human strains	256 µg/mL	64 µg/mL

Results are referent to all veterinary and human strains used in the experiments. MIC: minimal inhibitory concentration. MBC: minimal bactericidal concentration.

Source: research data.

To the best of our knowledge, this is the first study that describes the antimicrobial potential of *B. argyrophylla* and *D. rugosa*. These plants are native from Brazil and are not used in traditional medicine to treat infectious diseases, but as diuretics and anti-inflammatory. One might wonder why exploring plants that are not used as antimicrobials, what could be considered a more interesting pathway. In a previous study of our group, *Passiflora alata* and *Piper methysticum*, which are used to treat insomnia and as anxiolytics, displayed antimicrobial activity against human isolates of *S. aureus* and *Escherichia coli*[23]. This perspective in looking for unconventional plants with antimicrobial potential can provide new data on the search for antimicrobials in the BRAD context. Furthermore, a preliminary study indicated the antiplasmodial potential of *B. argyrophylla*[24].

In general, most phytoextracts are more effective as antimicrobials when prepared with blends of water and solvents like methanol and ethanol, as bioactive polar phytomolecules such as flavonoids, tannins and saponins are better extracted from plants using such blends[21,22]. However, in this study, polar extracts of these plants were not effective against the tested strains, whereas the non-polar dichloromethane extract was effective, and also lacked toxicity. It is possible that non-polar molecules such as alkaloids, sterols, fatty acids and terpene derivatives, eventually present at the extract, along with flavonoids (detected using the Shinoda test), had a role in this pharmacological potential[15, 25]. Due to methodological limitations, we could not detect the mentioned non-polar molecules in the extract.

The effectiveness of the extracts was different for human and veterinary isolates of *S. aureus*. The MIC values for *B. argyrophylla* and *D. rugosa* were four and two times higher for veterinary isolates compared to human isolates, respectively. And, as it would be expected, MBC values for veterinary isolates were also higher than the ones observed for human isolates. The MBC of *B. argyrophylla* could not be detected, as it was superior to the highest concentration tested. Genetic and epigenetic variations can influence the molecular targets of the phytomolecules present at the extract, what partially explains the difference in susceptibility[7,10,15,26]. However, the MIC values remained low for both extracts, and thus, the observed difference does not exclude the possibility of further studies towards a new pharmacological option shared by humans and animals, as it happens for some drugs such as gentamicin and amoxicillin.

4 Conclusion

B. argyrophylla and *D. rugosa* presented antimicrobial activity against veterinary and human clinical isolates of *S. aureus*, and MIC values were higher for veterinary isolates. Our study open doors for more studies with these plant extracts in a "one health" perspective, especially with isolated molecules.

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