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# SCREENING PHYTOCHEMICAL AND ANTIBACTERIAL ACTIVITY OF THREE SAN LUIS NATIVE SPECIES BELONGING AT THE *FABACEAE* FAMILY

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## Abstract

Ethanolic and aqueous extracts obtained from aerial parts of three San Luis native species belonging at the *Fabaceae* family, "*Acacia caven* (Molina) Molina var. caven, *Acacia furcatispina* Burkart and *Prosopis torquata* (Cav. ex Lag.) DC." were tested for the phytochemical components and to validate their in vitro antibacterial activity against strains of *Staphylococcus aureus* by agar well diffusion, micro-well dilution and agar overlay bioautography assays. Phytochemical study revealed the presence of carbohydrates, tannins, flavonoids, saponins and alkaloids. Thin layer chromatography analysis detected flavonoids (quercetine) and sapogenines (oleanolic acid). Antibacterial activity against *S. aureus* in all the extracts tested was detected. The aqueous extract of *P. torquata* was the most active showing inhibition diameters of 15-20 mm and the lowest MIC values according to the diffusion and microdilution methods respectively. Under both methods ethanol extracts of the three plants were less active than the aqueous extracts against the tested strains. The bioautography assay demonstrated well-defined inhibition zones against *S aureus* in correspondence with those flavonoids and sapogenines bands. These compounds could be considered the main responsible of their popular use for treating of microbial infections.

Key words: antibacterial activity; screening phytochemical; *Prosopis torquata; Acacia cavens; Acacia furcatispina*.

## Introduction

Diverses bacterial infections, especially those produced by *Staphylococcus aureus*, are very difficult to manage. *S. aureus* is well-evidenced as a major human pathogen and is commonly involves in skin and soft tissue infections. In addition, it can cause some serious infections including bacteremia, pneumonia, acute endocarditis, meningitis, osteomyelitis, toxic shock syndrome, and fatal invasive diseases (1, 2, 3, 4). Many factors contribute to the pathogenesis of *S. aureus* such as the presence of capsule, the expression of adhesins, the secretion of various toxins and also immunomodulators (5). Also, many strains are biofilm producers and show a better ability to attach themselves to mucosal surfaces and cause infection than non-biofilm producer strains (6). The emergence and spread of methicillin-resistant *S. aureus* (MRSA) originated in the 1960s remains a major clinical and epidemiological problem in hospital environments (7, 8). The introduction of glycopeptide antibiotics, a last resort to treat such infections, was followed by the isolation of either vancomycin-intermediate *S. aureus* (VISA) or vancomycin-resistant *S. aureus* (VRSA) (9, 10). Bacterial resistance to antimicrobial agents has become a widespread medical problem, especially in hospitals. Therapy with several types of antibiotics is frequently accompanied by side effects and microbial resistance (10). At present, the pharmaceutical arsenal available to control antibiotic-resistant bacteria is limited.

The increase in bacterial resistance has created the necessity of studies directed towards the development of new antimicrobials. Many researchers have focused on the investigation of natural products as source of new bioactive molecules (11, 12, 13). Various plants worldwide have been used in traditional medicine as alternative treatments of bacterial infections, and represent good sources of therapeutic agents (14, 15, 16, 17).

In the San Luis Province of Argentine, the region is characterized by a relatively large biodiversity and the flora consists of about 2.700 species (18). The dominant plant families include *Asteraceae, Poaceae, Fabaceae, Solanaceae, Rosaceae* and *Verbenaceae* among other and any of them are used in domestic medicine for the treatment of most diseases and the alleviation of symptoms and signs (19).

The use of medicinal plants in the treatment of diseases is an ancient tradition in San Luis, Argentina. From the pharmacognostic point of view the *Fabaceae* family is very important because many of its species are used in folk medicine. Three native species: *Acacia caven* (Molina) Molina var. caven "espinillo", "aromo" or "tusca", *Acacia furcatispina* Burkart (= A. gilliesii Steud) "garabato", and *Prosopis torquata* (Cav. ex Lag.) DC. "tintitaco" are used in folk argentine medicine as astringent and antiseptic agent (19).

To our knowledge, there are few reports related to the antimicrobial activity and chemical composition of the species tested in the present study.

The objective of the present study was to identify major biologically active phytoconstituents and to test the antibacterial activity of ethanolic and aqueous extracts obtained from *Prosopis torquata, Acacia cavens* and *Acacia furcatispina* against methicillin resistant *Staphylococcus aureus* strains by agar well diffusion, micro-well dilution and agar overlay bioautography assay.

## Materials and methods

*Plant material*

Plant material were collected in the Northern region of the province of San Luis, Argentina. Aerial parts (leaves) of *Prosopis torquata, Acacia cavens* and *Acacia furcatispina* were used for the study. Voucher specimens were deposited in the herbarium of the San Luis National University (UNSL). Plants material were dried in air flow oven at not more than 50° C and pulverized using a grinder to blade. The extractions were performed by infusion, cooking and alcoholic extraction techniques (20).

*Preparation of extracts*

Aqueous extracts:

• Infusion: It was prepared according to Farmacopea Argentina 6th ed. 1978 (20) applying constant action of hot water during 20 min.

• Decoction: Dried and powdered plant material (5g) was boiled with 70 ml of water for 20 min. After cooling to 40-45 ºC, the liquid was filtered and the volume adjusted to 100 ml with distilled water (20).

Crude ethanol extracts (EE)

The leaf powder was macerated in ethanol 95% (V/V) in a 1:3 proportion at room temperature,

*Microorganisms*

The microorganisms used in this study were as followed: I.- Reference strains: *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 25923 and II.- methicillin resistant *Staphylococcus aureus* (n=.3.), isolated from skin diseases. The written informed consent was obtained from all subjects prior to the study. All organisms were maintained in brain-heart infusion (BHI medium) containing 20% (v/v) glycerol at −80°C. The inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 Mc Farland.

*Phytochemical analysis*

Aqueous extracts were subjected to phytochemical analysis to ascertain the presence of metabolites such as glycosides, tannins, flavonoids, saponins and alkaloids (23).

*Thin layer chromatography*

Phytocompounds of the ethanolic extracts were analyzed using thin layer chromatography (TLC) using aluminium-backed TLC plates (Merck, silica gel 60 F 254 plates) (24). Briefly, the plant extracts were reconstituted in the extracting solvent. Five microlitres of each extract was spotted on the TLC plates and developed in different mobile systems: ethyl acetate: formic acid: methyl ethyl acetone: water (5:1:3:1) as eluent for flavonoids and chloroform: methanol: water (70:30:4) for sapogenines.

Development of the chromatograms was done in a closed tank in which the atmosphere had been saturated with the eluent vapour. The plates were then dried overnight under a stream of air to remove excess solvent. The TLC plates were run in triplicate. In plate A, the reference chromatogram was used to determine the spots as visualized by UV light to see if the separated spots were UV active after which it was sprayed with vanillin sulphuric acid (2%) spray reagent, plate B was used for bioautography. Individual Retention factor (Rf) for each spot was measured and plate C, to assess the chemical profile of the samples visualized with oleum = sulphuric acid: acetic acid: water (80:10.10). Rutine, quercetin, oleanolic acid were used as standards.

*Antibacterial activity assay*

Antibacterial activity of the crude ethanolic extracts and aqueous extracts were determined by the agar well diffusion method (CLSI) (21), modified by Perez et al. (22). The different organic extracts were dissolved in dimethylsulfoxide (DMSO) and the aqueous extracts were dissolved in water.

The extracts were sterilized by filtration through a 0.2 membrane filter.

- Agar well diffusion assay

The bacterial inoculum ($1x10^6$ ufc/ml) was uniformly spread on Mueller Hinton agar and then, 7 mm diameter holes were cut in the agar gel. Each well was filled with 50 µL (20 mg/ml) of each plant extract. Simultaneously, wells containing the same volume de DMSO, ethanol and distilled water served as negative controls while gentamicin sulfate (1µg per well) was used as positive control. After 24h at 37ºC, the diameters of the growth inhibition zones were measured (mm). All tests were performed in duplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced.

-Determination of Minimum Inhibitory Concentration (MIC)

Based on the preliminary screening, ethanol and aqueous extracts that revealed antimicrobial activity were further tested to determine the minimum inhibitory concentration (MIC) for each bacterial sample. The MICs of *Prosopis torquata, Acacia cavens* and *Acacia furcatispina* aqueous and ethanolics extracts were determined by micro-well dilution in tripticase soy broth supplemented with 0.01% (W/V) of 2,3,5,-triphenyltetrazolium chloride as visual indicator of bacterial growth (CLSI). The inoculum used was a suspension of microorganisms of $10^7$ cfu / ml. The ethanolic extracts were dissolved in dimethylsulfoxide (DMSO) and the aqueous extracts were dissolved in distilled water to the highest concentration to be tested (8000 µg/ml), and then serial two-fold dilutions were made in concentrations ranges from 8000 to 62.25 µg/ml. The 96-well microplates were prepared by dispensing into each well 95 µL of culture medium, 5 µl of the inoculum and 100 µl of each dilution of different extracts. The final volume in each well was 200 µl. The plates were incubated at 37°C for 24 h and read visually. MIC was defined as the lowest concentration of the extracts in the medium in which there was no visible growth after incubation. Media, extract and strains controls were included. The test was performed in duplicate.

- Minimum bactericidal concentration (MBC)

Extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture on the surface of the trypticase soya agar plates, in order to evaluate bacterial growth.

MBC was defined as the lowest concentration that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37°C.

-Bioautography agar overlay

The developed TLC plates were thinly overlaid with soft medium (BHI agar 0.6%) containing 0.1 % (w/v) 2,3,5 triphenyltetrazolium chloride and inoculated with an overnight cultures of *S. aureus* at a final concentration of 108 CFU/ml. The plates were placed in a sterile tray, sealed to prevent drying the thin agar layer and incubated at 37°C for 24 h. Microbial growth inhibition appeared as clear zones against a pink background. The Rf values of the spots showing inhibition were determined.

## Results and Discussion

The biological activities of any medicinal plant reflects the effect and nature of its phytochemical compounds. Phytochemical study of the three plants revealed the presence of carbohydrates, tannins, flavonoids, saponins and alkaloids either in both aqueous and ethanol extracts or in any of them. TLC analysis revealed the presence of flavonoids: quercetin (Rf of 0.91) and sapogenines: oleanolic acid (Rf of 0.87) (Fig 1).

The antibacterial activity of plants extracts was assayed in vitro conditions by agar well diffusion and broth microdilution methods against clinical isolates and references strains of *S. aureus*. The results showed antibacterial activity of all extracts assayed against *S. aureus*. Inhibition of bacterial growth by the action of the extracts according to the agar well diffusion method is summarized in Table 1. Maximum inhibition was observed with the *P. torquata* aqueous extracts (inhibition diameters of 15-20 mm) although diameters of inhibition were similar between the different strains of S. aureus tested. No differences in the diameters of inhibition zones between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* strains are observed. This clearly indicates that antibiotic resistance does not interfere with the antimicrobial activity of plant extracts and extracts could have different modes of action on test organisms.

Table 2 shows the results of the MIC of aqueous and ethanolic extracts of the three plants against *S. aureus* using the broth microdilution method. Similar to those results observed by the agar diffusion method the aqueous extract of *P. torquata* was the most active showing the lowest MIC values against *S. aureus*. Under both methods, the ethanol extracts of the three plants were less 8 and 9 mm and MIC values between 1000 and 4000 µg/mL respectively (Tables 1 and 2). This lower microbial activity may be due to low concentration of active compounds in these extracts.

Table 3 shows MBCs values against *S. aureus*. Respect to *P. torquata*, the MBC values of aqueous extracts against *S. aureus* were twice the MIC values, however the MIC and MBC values of ethanol extracts were equal (1000 µg/mL) .

The MBC and MIC values of ethanolic and aqueous extracts of A. cavens were the same (2000 µg/mL) except

the ethanolic extract against *S. aureus* ATCC 43300 (8000µg/mL and 4000µg/mL respectively), while for the *A. furcatispina* extracts, the MBC values were one or two fold higher than the corresponding MIC values.

In order to elucidate whether the observed antibacterial effects were bactericide or bacteriostatic, MBC/MIC ratios were calculated. Extracts with ratios greater than 1 were considered as bacteriostatic, while extracts with ratio less than or equal to 1 were bactericide. These data allow concluding that MIC values of aqueous extracts of *P. torquata* (250 µg/mL) were bacteriostatic and higher concentrations of ethanol extract were required to have bactericidal effect (1000 µg/mL).

*A. cavens* and *P. torquata* aqueous extracts exhibited a higher degree of antibacterial activity, compared with the ethanol extracts, in contrast to *A. furcatispina* whose ethanolic extracts were more active than their water extracts. Previous study have reported that alcohol is a better solvent for extraction of antimicrobial substances from medicinal plants than water (11). Our results partially confirm this observation because the most active *A. furcatispina* extracts against *S. aureus* were ethanolic, however the most active *A. cavens* and *P. torquata* extracts were aqueous.

The bioautography assay for qualitative antibacterial activity detection demonstrated well-defined inhibition zones against the growth *S. aureus* in correspondence with those flavonoids and sapogenines bands. It was observed that flavonoids and sapogenines were positive in both extracts of all three plants.

These results suggest that anti-staphylococcal activity detected in the extracts tested may be due to the presence of these compounds in the plant species studied. In addition to the compounds with antimicrobial activity several compounds on the

reference chromatogram were visible in UV light and other that were visible by using vanillin/sulfuric acid reagent, many of these compounds did not coincide with the antimicrobial components.

This could be attributed to evaporation of the active components or insufficient amount of the active component. Synergism could play an important role in the active extracts when the MIC of the mixture was determined, because a minor antimicrobial activity of individual compounds was observed.

In our laboratory, previous studies have also been reported antimicrobial activity of flavonoides and sapogenines present in regional plants against *S. aureus* (15, 25).

To our knowledge, there is no scientific information concerning the antibacterial activity of the plants used in the present study, therefore, our results support the popular use of these plants for the treatment of infectious skin diseases.

The separation and identification of the main compounds responsible for the bioactivity could help to know more about the properties of the studied plants.

From the present study, it can be concluded that the selected medicinal plants have potential as antimicrobial agents against *S. aureus* strains. Hence, this study would lead to the development of some biologically active compounds which can be employed in the formulation of antimicrobial agents

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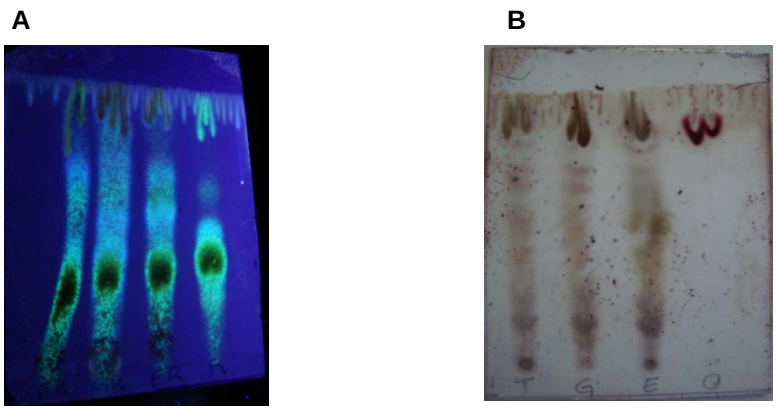


**Fig 1**. TLC with alcoholic extracts. a) TLC (revealed) for flavonoids. UV light. From left to right: Tintitaco, scribble, espinillo, routine control. b) TLC (revealed) for saponins. Natural light. From left to right: Tintitaco, scribble. espinillo, oleanolic acid control.

**Table 1.** Antibacterial activity of ethanolic and aqueous extracts of *A. cavens*, *A. furcatispina* and *P. torquata* by agar well diffusion assay.

| **Microorganisms** | **Inhibition zone diameter (mm)** | | | | | |
|---|---|---|---|---|---|---|
| | ***A. cavens*** | | ***A. Furcatispina*** | | ***P. torquata*** | |
| | **AE** | **EE** | **AE** | **EE** | **AE** | **EE** |
| ***S. aureus* (380)*[1]** | 9 | 8 | 10 | 8 | 15 | 9 |
| ***S. aureus* (383)*[2]** | 10 | 8 | 10 | 8 | 16 | 9 |
| ***S aureus* (386)*[3]** | 9 | 8 | 10 | 8 | 22 | 10 |
| ***S. aureus* ATCC 43300** | 10 | 8 | 9 | 8 | 20 | 9 |
| ***S. aureus* ATCC 25923** | 10 | 8 | 9 | 8 | 18 | 9 |

*: Methicillin resistant *S. aureus* : [1](isolated from wound), [2](isolated from abscess), [3]( isolated from abscess) , AE: aqueous extracts; EE: ethanolic extracts

**Table 2.** MIC (ug/ml) of ethanolic and aqueous extracts of *A. cavens*, *A. furcatispina* and *P. torquata* by micro-well dilution method

| **Microorganisms** | **MIC (ug/ml)** | | | | | |
|---|---|---|---|---|---|---|
| | ***A. cavens*** | | ***A. Furcatispina*** | | ***P. torquata*** | |
| | **AE** | **EE** | **AE** | **EE** | **AE** | **EE** |
| ***S aureus* (386)*** | 2000 | 4000 | 4000 | 1000 | 250 | 1000 |
| ***S. aureus* ATCC 43300** | 2000 | 4000 | 4000 | 1000 | 250 | 1000 |
| ***S. aureus* ATCC 25923** | 2000 | 4000 | 4000 | 1000 | 250 | 1000 |

*: Methicillin resistant *S. aureus*

**Table 3.** MBC (ug/ml) of ethanolic and aqueous extracts of *A. cavens*, *A. furcatispina* and *P. torquata*.

| **Microorganisms** | **MBC (ug/ml)** | | | | | |
|---|---|---|---|---|---|---|
| | ***A. cavens*** | | ***A. Furcatispina*** | | ***P. torquata*** | |
| | **AE** | **EE** | **AE** | **EE** | **AE** | **EE** |
| ***S aureus* (386)*** | 2000 | 4000 | 8000 | 4000 | 500 | 1000 |
| ***S. aureus* ATCC 43300** | 2000 | 8000 | 8000 | 8000 | 500 | 1000 |
| ***S. aureus* ATCC 25923** | 2000 | 4000 | 8000 | 4000 | 500 | 1000 |

*: Methicillin resistant *S. aureus*