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Investigation of Argentinean Plant Extracts for Their Antibacterial Activity

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Abstract: Plants of *Baccharis* (Asteraceae) genus are commonly known in Argentina as "carqueja". The antimicrobial activity and minimal inhibitory concentration of *B. articulata*, *B. trimera* and *B. crispa* aqueous and ethanolic extracts were evaluated by using the micro-well dilution method. Previously, the components of extracts were analyzed by spectroscopial means. Gram-positive bacteria were more sensitive to *Baccharis* species extracts than Gram-negative bacteria. Out of 3 plant species, *B. trimera* showed significant antibacterial activity and aqueous and ethanolic extracts were active against *Staphylococcus aureus* (MIC = 2,500 μg/mL and 1,250 μg/mL, respectively) and *Listeria monocytogenes* (MIC = 625 μg/mL and 625 μg/mL, respectively). All ethanolic extracts inhibited the growth of the selected Gram-positive (MIC values ranged between 625 μg/mL and 1,250 μg/mL). Therefore, all Gram-negative bacteria were resistant to the ethanolic and aqueous extracts tested. One flavone, genkawanin, was identified from the three ethanolic extracts as the responsible of antibacterial activity. Two terpenes, hawtriwaic acid and bacrispine, were identified from ethanolic extract of *B. crispa* and *B. trimera* as the responsibles of antibacterial activity. These preliminary studies corroborated the antimicrobial activity of the selected plants used in folklore medicine. Therefore, they could be potential sources of new antimicrobial agents used in treatment of infectious diseases.

Key words: *Baccharis articulata*, *Baccharis crispa*, *Baccharis trimera*, ethanolic extract, aqueous extract.

1. Introduction

Many natural compounds isolated from plants have demonstrated a wide spectrum of biological activities [1].

Among these various kinds of natural substances, medicinal plants have received particular attention as a novel way to reduce the proliferation of microorganisms [2].

The American genus *Baccharis* (Asteraceae) consists of approximately 500 species [3, 4], near 100 of them are present in Argentina.

Plants of the Baccharis genus are very rich in secondary metabolites as sesquiterpenes, clerodan type diterpenes, triterpenes, and phenylpropanoids [5, 6]. It is very frequent the isolation of several types of flavones, usually with a high degree of oxygenation. Some of the aforementioned metabolites are described as interesting chemotaxonomic markers.

Some metabolites such as diterpenoids, flavonoids and volatile terpenoids have been earlier determined by spectroscopical means in *Baccharis* sp. ethanolic extracts tested in the present study (Table 1) [7, 8].

Several *Baccharis* species are commercially used in folk medicine as antiseptics and anti-inflammatory agents, and to treat both gastric ulcers and skin sores [6]. For example, *Baccharis incarum* phytopreparation applied topically could be used to treat skin and soft tissues infection produced by methicillin-resistant *Staphylococcus aureus* [9]. *Baccharis dracunculifolia*

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Table 1 Characteristics of *Baccharis* species used against pathogenic bacteria isolated in San Luis (Argentina).

Specie	Chemical composition	Traditional uses
Baccharis articulate (Lam.) Persoon	Genkwanin 7-4'-di-O-metilapigenin Acacetin Circimaritin Salvigenin Barticulidiol malonate Bacchotricuneatin A	Decoctions from leaves are used for diarrhoea and respiratory and urinary infections
B. trimera (Less) DC.	Genkwanin 7-4'-di-O-metilapigenin Hawtriwaic acid Hawtriwaic acid lactone 1-desoxibacrispine Bacrispine	Infusion, decoctions or tintures of the aerial parts for liver disease, wound, diarrhea, angina, rheumatism, renal disorders, diabetes
B. crispa Sprengel	Genkwanin 7-4'-di-O-methylapigenin Hawtriwaic acid Hawtriwaic acid lactone 1-desoxibacrispine Bacrispine Butenolids	Infusion, decoctions of the aerial parts are used for digestive disorders and ulcers

leaves extracts present antifungal and antibacterial activity [10]. Hexane and dichloromethane extracts of *B. grisebacchii* are active on methicillin-resistant and methicillin sensitive *S. aureus* [11].

Nevertheless, to our knowledge there are few reports available in the literature on the biologically active constituents of *Baccharis articulata, B. trimera* and *B. crispa*, and their biological properties against pathogenic bacteria.

Therefore, our purpose in the present study was evaluated the antibacterial activity of aqueous and organic extracts from these three native plants against Gram positive and Gram negative pathogenic bacteria species. The results should be provided scientific evidences about the efficacy of their use in folklore medicine.

2. Materials and Methods

2.1 Plant Collection and Identification

Baccharis articulata (Lam.) Persoon., *B. trimera* (Less) DC. and *B. crispa* Sprengel, aerial parts were collected in Departamento La Capital, San Luis Province, Argentina, between November 2009 and February 2010. Voucher specimens were identified by Ing. Luis del Vitto and Dr. Elisa Petenatti and lodged in the University of San Luis (Argentina) Herbarium (Nº 7366, 6709, 376, respectively).

2.2 Preparation of Extracts

Ground plant materials (5 g) were extracted with 100 mL of 70% ethanol (EtOH) in a sonication bath at room temperature for 1 h. The extracts were then filtered under vacuum through Whatman Nº1 filter paper. They were concentrated *in vacuo* using a rotary evaporator at 30 ºC. The resultant extracts were air-dried at room temperature [12]. 5 g of the ground material were soaked in 100 mL of sterile water and allowed to stand for 72 h. Then, the crude extracts were obtained by filtration [13]. Extracts were dissolved in small drops of dimethylsulfoxide (DMSO) and toped up with physiological saline and the stock solution kept at 4 ºC.

2.3 Microorganisms

The following bacteria were selected for this study. One strain of *Listeria monocytogenes* was obtained from the Pasteur Institute, France, culture collection: CLIP 74910. It was chosen in order to represent any specie responsible of food-borne disease.

Staphylococcus aureus ATCC 43300 was used as a wound/skin pathogen. *Escherichia coli* (isolated in UNSL laboratory), was used to represent pathogens that cause gastro enteritis while *Pseudomonas aeruginosa* ATCC 27853 was used as an environmental pathogen.

2.4 Antimicrobial Activity

2.4.1 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of *Baccharis articulata*, *B. trimera* and *B. crispa* extracts were determined by microplate method (micro-well dilution) [14], in MH broth (Britania, Argentina) pH 7.2 supplemented with 0.01% (w/v) of 2,3,5-triphenyltetrazolium chloride (TTC) as visual indicator of bacterial growth. The inoculum of each strain was prepared from 18 h broth culture and adjusted to the tube 0.5 of Mc Farland scale (10^8 bacterial cells). Then, they were diluted 10 times. The extracts were disolved in 20% Tween-80 and then diluted with phosphate buffer saline (PBS) to the highest concentration to be tested (5,000 μg/mL), and then serial two-fold dilutions were made in concentration ranges from 5,000 to 78 μg/mL. The 96-well plates were prepared by dispensing into each well 95 μL of nutrient broth and 5 μL of the inoculum. 100 μL aliquot from the stock solutions of the extracts and their serial dilutions initially prepared was transferred into seven consecutive wells. The final volume in each well was 200 μL. The plates were covered with sterile plate sealer and then incubated at 37 °C for 24 h. MIC was defined as the lowest concentration of the extract in the medium in which there was no visible growth after incubation (no red colour signifying live growth). It is established that TTC, a water-insoluble, colorless compound, can be reduced to water-insoluble red formazan by a variety of organisms. TTC reduction is used as a quantitative method in the evaluation of tissue viability. The experiments were replicated at least twice.

2.4.2 Determination of Minimal Bactericidal Concentration (MBC)

Extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture on the surface of the tripticase soya agar plates, in order to evaluate bacterial growth. MBC was determined as the lowest concentration that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37 ºC.

2.5 Thin-Layer Chromatography (TLC)

Merck F_{254} plates, 10 × 10 cm, 1 mm thick were used. *B. articulata*, *B. trimera* and *B. crispa* aqueous and ethanolic extracts were applied and the chromatogram was developed using chloroform-methanol (9:1, v/v) and chloroform-ethanol (7:1, v/v) as solvent systems, respectively. TLC plates were run in duplicate. Spots and bands were visualized using the following spray reagents *p*-anisaldehyde : acetic acid : sulfuric acid (1:97:2; v/v) and sulfuric acid : acetic acid : water (16:80:4; v/v), followed by heating in an oven at 150 °C for 5 min. The diterpenes bacchotricuneatin A, hautriwaic acid, and bacrispine as well as the flavone genkwanin were used as standards. The set of TLC plates for the bioautography assays were dried overnight in a sterile room for complete removal of solvent and were used unrevealed.

2.6 Bioautography

Plates TLC were covered with 1-2 mm layer of soft medium (BHI with 0.6% agar) containing 0.1% (w/v) TTC and an aliquot of an overnight culture of *S. aureus* ATCC 43300 (10^8 CFU/mL) and *L. monocytogenes* CLIP 74910 (10^8 CFU/mL), respectively. The plates were placed in a sterile tray, sealed to prevent the thin agar layer from drying, and incubated at 37 °C for 24 h. Where microbial growth has been inhibited an uncoloured area can be seen on the deep pink-red background. The plates were run in duplicate.

3. Results and Discussion

Extracts of regional *Baccharis* species from semi-arid western region Argentina, were screened for their antibacterial activity against *S. aureus*, *L. monoytogenes*, *E. coli* and *P. aeruginosa*.

Previously, the chemical composition of *B. articulata*, *B. crispa*, and *B. trimera* extracts were investigated by spectroscopical means [7, 8]. A total of 12 components were identified (Table 1).

The extracts of *B. crispa* and *B. trimera* resulted in the identification of the same diterpenoids and flavonoids but butenolids were only present in the extract of *B. crispa*. *B. crispa* and *B. trimera* yielded extracts rich in hautriwaic acid and their lactone as well as the neoclerodane diterpenes 1-desoxibacrispine and bacrispine. From *B. articulata* the neoclerodanes barticulidiol malonate and bacchotricuneatin A were the most important metabolites (Table 1).

There are few data in the literature on the compounds with antibacterial activity from the species included in this study, whereas data have been reported on the antimicrobial activity of terpenoids and flavonoids isolated from other *Baccharis* species such as *B. incarum* [15-18] and *B. boliviensis* [15]. The antibacterial activity of terpenoids is generally believed to involve actions at phospholipids membranes, where partitioning results in destabilisation and disorder culminating in ion leakage in bacteria and disruption of membrane dependent energy generating processes in eukaryotic microorganisms [19].

Traditional healers use primarily water as the solvent but in our studies we found that plant extracts in organic solvent (ethanol) provided more consistent antimicrobial activity compared to those extracted in water. These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay.

In agreement with previous reports, gram-positive bacteria were more sensitive to *Baccharis* species extracts than Gram-negative bacteria [20, 21]. The minor susceptibility of Gram-negative bacteria may be attributed to an outer membrane surrounding the cell wall which restricts diffusion of hydrophobic compounds through the lipopolysaccharide. Moreover, the periplasmic space contains enzymes, which are able of breaking down foreign molecules introduced from outside [22].

Out of 3 plant species, *B. trimera* showed significant antibacterial activity and both the extracts (aqueous and ethanolic) were active against the Gram-positive investigated bacteria.

All aqueous extracts inhibited *S. aureus* but only *B. trimera* aqueous extract was active against *L. monocytogenes* (Table 2).

The TLC plates corresponding to the *B. trímera* aqueous extract showing an intense spot in the polar region of the plate. This result probably is due to the presence of some glicoside type compound (Fig. 1a).

Table 2 Antibacterial activity of aqueous extracts against Gram-positive and Gram-negative bacteria.

	B. articulata		*B. trimera*		*B. crispa*	
	[2]MIC	[3] MBC	MIC	MBC	MIC	MBC
S. aureus ATCC 43300	1,250	2,500	1,250	2,500	625	1,250
L. monocytogenes [1]CLIP 74910	[4]NA	NA	625	1,250	NA	NA
E. coli	NA	NA	NA	NA	NA	NA
P. aeruginosa ATCC 27853	NA	NA	NA	NA	NA	NA

[1]CLIP: *Listeria* Collection of the Pasteur Institute; [2]MIC: Minimum inhibitory concentration (μg/mL); [3]MBC: Minimun bactericidal concentration (μg/mL); [4]NA: no activity.

All ethanolic extracts inhibited the growth of the selected Gram-positive bacteria (Table 3).

Therefore, all Gram-negative bacteria were resistant to the ethanolic and aqueous extracts tested (Tables 2, 3).

The MICs of extracts determined by microplate method (micro-well dilution) ranged from 625 to 2,500 μg/mL. The most sensitive microorganism to extracts from *B. trimera* and *B. crispa* was *L. monocytogenes*, with MIC of 625 μg/mL (Tables 2, 3). Similary, the ethanolic extract of *B. crispa* was active against *S. aureus* with MIC of 625 μg/mL.

S. aureus was inhibited by *B. articulata*, *B. trimera* and *B. crispa* aqueous extracts at the highest MIC (2,500 μg/mL) (Table 2).

MBC values were one or two fold higher than the corresponding MIC values in both extracts (Tables 2, 3).

To obtain some information on the active components, the extracts were analyzed by TLC on silica gel and assayed for bioautography. This assay for qualitative antibacterial activity detection demonstrated well-defined inhibition zones against *S. aureus* (Fig. 1) in correspondence with those flavonoids and sapogenines bands.

Fig. 2 shows the appearance of the chromatogram after treatment with *L. monocytogenes*, indicating the localization of bacterial inhibition zone.

One flavone, genkawanin, was identified from the three ethanolic extracts as the responsible of antibacterial activity (Fig. 1). Two terpenes, hawtriwaic acid and bacrispine, were identified from ethanolic extract of *B.crispa* and *B. trimera* as the responsibles of antibacterial activity (Fig. 2).

4. Conclusions

The results of the present study support the folkloric usage of the studied plants and suggest that

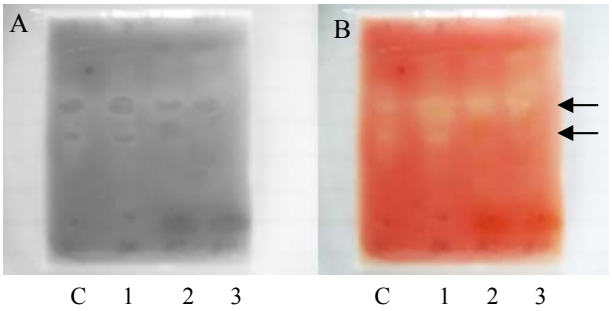

Fig. 1 Thin layer chromatography plate of (1) *B. articulata*, (2) *B. crispa* and (3) *B. trimera* ethanolic extracts. A: visual appearance. B: *S. aureus* bioautography overlay. Arrows indicate regions of inhibition growth visualized wih tetrazolium red. C: Flavones (standard).

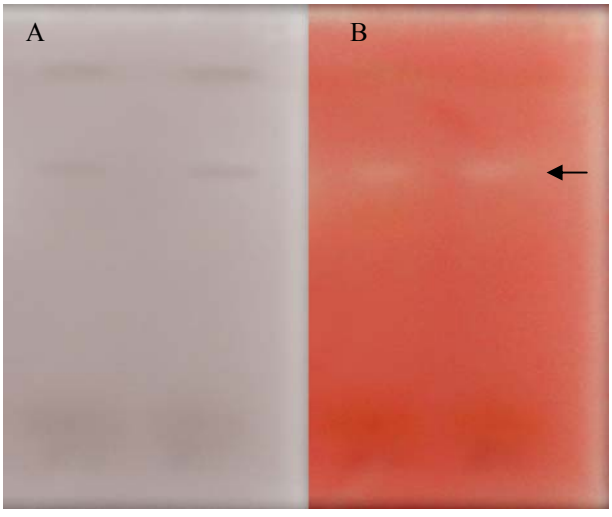

Fig. 2 Thin layer chromatography plate of (1) *B. trimera* aqueous extract. A: visual appearance. B: *L. monocytogenes* bioautography overlay. Arrow indicate regions of inhibition growth visualized wih tetrazolium red. C: Glycoside (standard).

Table 3 Antibacterial activity of ethanolic extracts against Gram-positive and Gram-negative bacteria.

	B. articulata		*B. trimera*		*B. crispa*	
	[2]MIC	[3] MBC	MIC	MBC	MIC	MBC
S. aureus ATCC 43300	1,250	2,500	1,250	2,500	625	1,250
L. monocytogenes [1]CLIP 74910	1,250	2,500	625	1,250	625	1,250
E. coli	[4]NA	NA	NA	NA	NA	NA
P. aeruginosa ATCC 27853	NA	NA	NA	NA	NA	NA

[1] CLIP: *Listeria* Collection of the Pasteur Institute; [2] MIC: Minimum inhibitory concentration (μg/mL); [3] MBC: Minimun bactericidal concentration (μg/mL); [4]NA: no activity.

some of the plant extracts possess compounds with antibacterial properties that can be used in new drugs for the therapy of infectious diseases caused by pathogens.

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