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Antibacterial activity of extracts obtained from *Mulinum spinosum* and *Mulinum echegarayii*

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Abstract

Plants have always been a source of beneficial products to maintain health and increase the quality of human life. *M. spinosum* (Cav.) Pers. and *Mulinum echegarayii* Hieron are plants used in Argentine folk medicine. The antibacterial activity *in vitro* of organic extracts of this native species was evaluated against strains of methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* CLIP 74910, *Escherichia coli* and *Bacillus cereus*. Different organic extracts were prepared using solvents n-hexane, mixtures of *n*-hexane and ethyl acetate of increasing polarity and mixture ethyl acetate and methanol 2% on flash chromatography. Extracts of *M. spinosum* at 10% ethyl acetate/n-hexane and at 2% methanol/ethyl acetate and extracts of *M.echegarayii* n-hexane and at 10% ethyl acetate/n-hexane, showed activity against methicillin-resistant *S. aureus*. *B.cereus* and *L. monocytogenes* were sensitive to extract of *M. spinosum* at 2% methanol/ethyl acetate. The *n*-hexane extract of *M. echegarayii* only showed activity against *S. aureus.* All gram-negative bacteria were resistant to the extracts tested. This study contributes to the discovery of new plants with antibacterial properties and opens a way to isolation and identification of active principles with antibiotic activity.

Key words: Mulinum spinosum, Mulinum echegarayii, antibacterial activity

Introduction

Since remotes times have known the benefits of natural medicine, thus plants have always been a source of beneficial products to maintain health and increase the quality of human life. Despite the number of antimicrobial agents developed by the pharmaceutical industry, traditional indigenous phytotherapy is still practiced in many rural areas of Argentina, and the using treatments handed down from generation to generation (1).

Mulinum is a genus of the Apiaceae family that includes 15–20 species confined to Argentina, Bolivia and Chile (2). *M. spinosum* (Cav.) Pers. is a shrub of the central Andes in Argentina and Chile and Patagonia steppe in Argentina, where it is known as 'neneo' or 'yerba negra'. This specie is used as an analgesic for the treatment of dental neuralgias, in the hepatic and urinary diseases and altitude sickness (3,4,5). Moreover, in the field, is used as medicinal plant against toothache and veterinarians use crushed and boiled roots for purging the mares (6).*Mulinum echegarayii* Hieron is Argentinean endemic specie known as "Hierba en cojín". It is a shrub smaller and more compact than *M. spinosum* widely distributed in the Argentina Patagonian steppe and there are no known references to its use in traditional medicine (7). Also, as far as our knowledge, there are no previous studies of the antibacterial properties of these species. About the chemical composition of *M. spinosum* some authors investigated families of secondary metabolites in leaves, flowers and fruits. Three groups compounds (saponins, flavonoids and terpenoids/sterols) were identified in fruits and flowers but they were absented in leaves (8, 9, 10).

The purpose of the study presented here, was to evaluate *in vitro* the antibacterial activity of organic extracts of two native species, *M. spinosum* and *M. echegarayii* against pathogenic bacteria in order to detect new sources of antimicrobial agents.

Materials and methods

Plant collection and identification

Mulinum spinosum (Cav.) Pers was collected in the Cordillera de Los Andes, Uspallata (Mendoza, Argentina) and *Mulinum echegarayii* Hieron in the valley of Atuel river, Malargue (Mendoza, Argentina) in March 2000. Vouchers specimens were identified by Ing Luis Del Vitto *et al.* and lodged in the University of San Luis (Argentine) herbarium (N° 9092 and 494 respectively).

Preparation of extracts

Previously dried aerial parts at room temperature and finely powdered were macerated with acetone at room temperature for 48h. Acetone extract was separated by filtration. Extraction was replicated three times. Extraction fluids were concentrated under reduced pressure yielding 330 g of dark syrup, then, it was dissolved with acetone and absorbed on silica gel column. Each acetone extract was partitioned by chromatography "Flash" using as elution solvents n-hexane and mixtures of *n*-hexane and ethyl acetate (AcOEt) of increasing polarity and finally was eluted with a mixture ethyl acetate and methanol (MeOH) 2%. The progress of separation was monitored by thin layer chromatography (TLC) using as mobile phase benzene: dioxane: acetic acid (120:20:4) and as revealing a mixture of H_2SO: AcOH: H_2O (2:20:1) followed by heating at 120 ° C

In the present study, we evaluated *in vitro* the antibacterial activity of *n*-hexane, 10% AcOEt/*n*-hexane, 2% MeOH/AcOEt extracts of *M. spinosum*, and *n*-hexane and 10% AcOEt /*n*-hexane of *M. echegarayii* extracts.

Microorganism

A total of five bacteria were selected for this study. Methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* CLIP 74902 (Collection

Listeria Institute Pasteur), *Escherichia coli* and *Bacillus cereus* isolated in UNSL Laboratory.

Antibacterial activity

Determination of Minimal Inhibitory Concentration (MIC)

The antibacterial activity was assayed in vitro using microplate method (microwell dilution) according to the CLSI method (11) in tripticase soya broth (Britania, Argentina) pH7,2 supplemented with 0,01% (w/v) of 2,3,5-triphenyltetrazolium chloride (TTC) used as visual indicator of bacterial growth. The inoculum of each strain was prepared from 24h broth culture and adjusted to concentration of 10^6CFU/ml. Organic extracts were dissolved in dimethylsulfoxide and tested in a concentration ranging from 8 to 0.1 mg/ml. The 96-well plates were prepared by dispensing into each well 95µl of nutrient broth and 5 µl of the inoculum (final concentration of 10^4 CFU/ml). One hundred microlitre aliquot from the serial dilutions of extracts was transferred into four consecutive wells. The final volume in each well was 200 µl. Controls of nutrient broth, strains and extracts were included. After 24-hour incubation at 37°C, the antibacterial activity of the extracts (MIC) was defined as the lowest concentration of the extract in the medium in which there no visible grown. The experiments were replicated at least twice.

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°.

Results and Discussion

Antimicrobial activity

Extracts of *M. spinosum* at 10% AcOEt/*n*-hexane and at 2% MeOH/AcOEt showed significant activities against methicillin- resistant *S. aureus* at doses of 2 and 4 mg/ml respectively. Also *M.echegarayii n*-hexane extract and *M.echegarayii* 10% AcOEt/*n*-hexane extract showed activity against this microorganism a concentration of 2 mg/ ml. It is known that methicillin-resistant *S. aureus* is a microorganism leading to serious epidemiological and therapeutic problems since these microorganisms frequently present associated resistance to other antibiotics (12). (Table 1)

On the other hand, *B.cereus* and *L. monocytogenes* were only sensitive to extract of *M. spinosum* at 2% MeOH /AcOEt with MIC of 1 mg/ml, and 4 mg/ml respectively. *L. monocytogenes* gradually and possibly influenced, among other factors by the exposure of this bacteria to sub-inhibitory concentrations of chemical decontaminants, has changed its pattern of sensitivity and initiated the shift towards the development of resistance to antibiotics commonly used against this organism. Usually these decontaminants, are used to remove surface contamination from poultry carcasses (13). Therefore, all gram-negative bacteria were resistant to the extracts tested. 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 to break down foreign molecules introduced from outside (14).

The *n*- hexane extract of *M. echegarayii* only showed activity against *S. aureus*. The MBC values were one or two fold higher than the corresponding MIC values. (Table 1)

Some studies have demonstrated in plants belonging to the *Azorella*, *Mulinum*, *Laretia* and *Bolax* genus an interesting group of bioactive metabolites, such as azorellane and mulinane

diterpenoids (10, 15, 16, 17). Chiaramello et al. have isolated two new diterpenes from *M. spinosum* acetone extracts: 14-α-hydroximulinolic (C1) and mulin-12-ene-14-one-20-oic acids (C2). Other compounds such as mulinolic acid (C3) and 11,13-dien-20-oico mulin acid (C4), were identified from the fraction 10% AcOEt/*n*-hexane obtained of *M. spinosum*, too (10). Wächter et al. reported the presence of C3, C4 and compounds with antibacterial activity (for example mulin-12-14 dien- 11-one-20-oic acid (C5)) in *Azorella compact* organic extract (16). C5 showed inhibitory activity against methicillin-resistant *S. aureus* and methicillin susceptible *S. aureus, Enterococcus faecium* and *E. coli* (16). C5 has a chemical structure similar to C2. These results suggest that the antibacterial activity of *M. spinosum* 10% *n*-hexane/ AcOEt extract observed in our study could be due, partly or completely, to the presence of C2.

To our knowledge, there are few reports available in the literature on *M. echegarayii*. Chiaramello et al. carried out a phytochemical study of 10% AcOEt /*n*-hexane extract by spectroscopic methods. They have obtained some diterpenes of mulinane nuclei, and azorellane such as 17-acetoximulinic acid (C6), 14-α-hydroximulinolic acid (C1), azorellan-17,13-β olide and sphatulenol (18, 19). Some of these diterpenes C6 and C1 showed a similar phytochemical pattern that those isolates from *M. spinosum*, showing a narrow taxonomic relation and probably a similar antibacterial activity.

This study contributes to the discovery of new plants with antibacterial properties and opens a way to isolation and identification of active principles with antibiotic activity.

see Table 1.

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Bacterial strains	*Mulinum spinosum* extracts MIC/MBC (mg/ml)			*Mulinum echegarayii* extracts MIC/MBC (mg/ml)	
	n-hexane	10% ethyl acetate/*n*-hexane	2% methanol/ethyl acetate	*n*-hexane	10% ethyl acetate/n-hexane
S. aureus ATCC 43300	NA	2/4	4/8	2/8	2/4
P.aeruginosa	NA	NA	NA	NA	NA
L.monocytogenes CLIP 74902	NA	NA	4/8	NA	NA
E.coli	NA	NA	NA	NA	NA
B.cereus	NA	NA	1/2	NA	NA

Table1. Minimal Inhibitory Concentration and Minimal Bactericidal Concentration of *M. spinosum* and *M. echegarayii* extracts.

MIC: Minimal Inhibitory Concentration

MBC: Minimal Bactericidal Concentration

NA: no activity