Antibacterial Activity of Mulinum spinosum Extracts against Slime-Producing Staphylococcus aureus and Methicillin-Resistant Staphylococcus aureus Isolated from Nasal Carriers

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Nasal carriers of Staphylococcus aureus are important reservoirs with risk of developing endogenous infections or transmitting infections to susceptible individuals. Methicillin-resistant S. aureus (MRSA) are associated with higher rates of treatment failure. Some strains of S. aureus produce slime which is believed to make the microorganisms more resistant to antibiotics and host defenses. The antibacterial activity of ethyl acetate : n-hexane (EtOAc : HEX) extracts of Mulinum spinosum (5 : 95% EtOAc : HEX, 50 : 50% EtOAc : HEX, 70 : 30% EtOAc : HEX, and mix 20 : 80/30 : 70% EtOAc : HEX, 50 : 50/70 : 30/100 : 0% EtOAc : HEX) were assayed against 3 slime-producing S. aureus strains and 2 MRSA strains isolated from nasal carriers. S. aureus ATCC 35556 slime-producing strain and MRSA ATCC 43300 strain were used as controls. The extracts were prepared using flash chromatography. M. spinosum 5 : 95% AcOEt : HEX showed antibacterial effect against all slime-producing strains (MIC: 500 to 1000 μg/mL) and the highest activity against MRSA strains (MIC: 500 to 1000 μg/mL). All M. spinosum extracts assayed were active against slime-producing S. aureus and MRSA at doses between 500 and 4000 μg/mL. Both, slime-producing S. aureus and MRSA are highly contagious and hardly eradicated by antibiotic therapies. So, there is an increasing need to find new substances with the ability to inhibit these strains.

1. Introduction

S. aureus is widely distributed in nature and is part of the bacterial human flora of the skin, armpits, groin, perineum, upper respiratory tract, and anterior nares [1]. In healthy adults the 20% are persistent nasal carriers of this bacterium [2]. So, carriers of S. aureus are important reservoirs with risk of developing endogenous infections or of transmitting infections to susceptible individuals. Certain strains of S. aureus produce slime and this exopolysaccharide is a mucoid material, which is firmly attached to the bacterial cell wall and is released to the environment [3]. Slime production has been implicated as a virulence factor and is postulated to be a mechanism by which bacteria attach to and colonize indwelling medical devices [4]. The importance of the role played by slime is further increased by its frequent association to reduced antibiotic susceptibility [5]. The presence of these microbial communities is often associated with various chronic diseases including cystic fibrosis, periodontitis, chronic prostatitis, otitis media, endocarditis, and recurrent urinary tract infections [6]. Methicillin-resistant S. aureus (MRSA) are associated with higher rates of treatment failure by the limited availability of antibiotics showing activity in vivo. The main impact of this microorganism is that MRSA strains were traditionally limited to the hospital environment and today have become important pathogens of the community [7, 8]. Due to increased resistance to antibiotics there
is an imminent need to search for new therapeutic options [9, 10]. Ethnobotany is the main source for development and research of natural drugs and has received considerable interest in recent years. Latin American countries have a rich tradition in the use of medicinal plants in folk medicine [11]. *Mulinum* is a genus of herbaceous plants belonging to the Apiaceae family. It comprises 37 described species. The type species of this genus is *Mulinum spinosum* Pers. *M. spinosum* (neneo, hierba negra) is a plant endemic of the mountains of Chile and western of Argentina Patagonia region. It is a thorny and perennial shrub [12]. This species is used as an analgesic for the treatment of dental neuralgias, in the hepatic species of this genus is Apiaceae family. It comprises 37 described species. The type tradition in the use of medicinal plants in folk medicine [11].

The aim of this work was to study the inhibitory activity of *M. spinosum* extracts against slime-producing *S. aureus* and MRSA isolated from nasal carriers.

2. Materials and Methods

2.1. Plant Material. *M. spinosum* (Cav.) Pers was collected in the Cordillera de Los Andes, Usquallata, Mendoza, Argentina. Voucher specimen was identified by Ing. Del Vitto et al. and lodged in the University of San Luis, Argentina, herbarium (N’ 9092) [16].

2.2. Preparation of Extracts. Previously dried aerial parts at room temperature and finely powdered were macerated with acetone at room temperature for 48 h. Acetone extract was separated by filtration. Extraction was replicated 3 times. Extraction fluids were concentrated under reduced pressure yielding 330 g of dark syrup and then it was dissolved with acetone and absorbed on silica gel column. Each acetone extract was partitioned by chromatography "flash" using us elution solvents mixtures of ethyl acetate and n-hexane (EtOAc/HEX) of increasing polarity. 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 sulfuric acid : acetic acid : H₂O (4 : 20 : 1) followed by heating at 120 °C [17].

In this study, we evaluated in vitro the antibacterial activity of 5 : 95% EtOAc : HEX, 50 : 50% EtOAc : HEX, 70 : 30% EtOAc : HEX and mix 20 : 80/30 : 70%, EtOAc : HEX 50 : 50/70 : 30/100 : 0% EtOAc : HEX extracts of *M. spinosum.*

2.3. Microorganisms. A total of 24 *S. aureus* strains isolated from nasal carriers, kept in the cebarium (maintained in the culture collection) of the Laboratory of Microbiology of the National University of San Luis, were assayed for slime production and oxacillin resistance. Then, the antibacterial activity was assayed against a total of 5 of those strains: three slime-producing *S. aureus* and 2 MRSA isolated from nasal carriers. *S. aureus* ATCC 35556 slime-producing strain and MRSA ATCC 43300 strain were used as controls.

2.4. Slime Production. Slime production was performed by using 2 methods as follows.

2.4.1. Congo Red Agar Method. It was performed according to Freeman et al. [18] with the following modifications: the strains were streaked onto Congo red agar (CRA) plates (0.8 g of Congo red and 50 g of sucrose in 1 liter of brain heart infusion agar), incubated for 24 h at 37 °C and subsequently overnight at room temperature. Plates were inspected for the color of the colonies at 24 h. For colonies color evaluation, a four-color reference scale was used: black and bordeaux almost black as slime-producing strains and bordeaux and red as nonslime-producing strains.

2.4.2. PCR Method for the Amplification of the icaA and icaD. PCR reactions were performed using the method described by Arciola et al. [19]. In brief, 2 pairs of primers were designed for the detection of icaA. The following primers were used: 5'-ACAGTCGCTACGAAAAGAAA as the forward primer and 5'-GGAAATGCCATATAAGC as the reverse primer, yielding a PCR product of 103 bp. For the detection of icaD the following primers were used: 5'-ATGGTACGGCC-AGACAGAAG and 5'-GCTTTTCACTATTATGCAA as forward and reverse primers, respectively, yielding a PCR product of 198 bp. DNA amplification was carried out with the following thermal cycling profile: initial denaturation at 94 °C for 5 min, followed by 50 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s) with a final extension at 72 °C for 1 min. After the first 30 cycles, a further 1 U of Taq DNA polymerase was added. PCR products were analyzed by electrophoresis in 2% agarose gel for 50 min at 80 V. The bands were stained with GelRed and observed under UV light.

2.5. Oxacillin Resistance. *S. aureus* isolates were screened for oxacillin resistance using disk diffusion method [20].

2.6. Antibacterial Activity

2.6.1. Determination of Minimal Inhibitory Concentration (MIC). The antibacterial activity was assayed in vitro using microplate method (microwell dilution) according to the CLSI method [21] in triplicate soya broth (Britania, Argentina) pH 7.2 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 24 h broth culture and adjusted to concentration of 10⁶ CFU/mL. Organic extracts were dissolved in dimethylsulfoxide (DMSO) and tested in a concentration ranging from 8 to 0.1 mg/mL. The 96-well plates were prepared by dispensing into each well 95 μL of nutrient broth and 5 μL of the inoculum (final concentration of 10⁴ CFU/mL). One hundred microliter aliquots from the serial dilutions of extracts were transferred into 4 consecutive wells. The final volume in each well was 200 μL. Controls of nutrient broth, strains, DMSO, and extracts were included. After 24 h 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 is no visible growth. The experiments were replicated at least twice.
Table 1: Minimal inhibitory concentration and minimal bactericidal concentration of *M. spinosum* extracts against slime-producing *S. aureus*.

<table>
<thead>
<tr>
<th>Slime-producing strains</th>
<th>MIC/MBC (µg/mL)</th>
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<tr>
<td><em>S. aureus</em> NC1</td>
<td>500/1000</td>
<td>500/1000</td>
<td>2000/4000</td>
<td>500/1000</td>
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<td>500/1000</td>
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<tr>
<td><em>S. aureus</em> NC2</td>
<td>500/1000</td>
<td>500/1000</td>
<td>4000/8000</td>
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<tr>
<td><em>S. aureus</em> NC3</td>
<td>500/1000</td>
<td>1000/2000</td>
<td>4000/8000</td>
<td>2000/1000</td>
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<td>500/1000</td>
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<td>500/1000</td>
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<tr>
<td><em>S. aureus</em> ATCC 35556</td>
<td>1000/2000</td>
<td>1000/2000</td>
<td>4000/8000</td>
<td>1000/1000</td>
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NC: nasal carrier.

2.6.2. 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 bactericidal effect. The presence or absence of bacterial growth was determined by visual inspection. MBC was defined as the lowest concentration that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37°C.

3. Results and Discussion

Of the 24 *S. aureus* strains studied, 3 were slime positive by both methods assayed and 2 showed oxacillin resistance. The slime-production by cultures on CRA is observed in Figure 1: *S. aureus* appear as black colonies. Figure 2 shows PCR method: 103-bp band for icaA gen and 198-bp band icaD gen obtained with DNA from slime-producing *S. aureus*.

Extract of *M. spinosum* 5:95% AcOEt:HEX showed antibacterial effect against all slime-producing strains of *S. aureus* isolated from nasal carriers (MIC: 500 µg/mL). *M. spinosum* 50:50% AcOEt:HEX and mix *M. spinosum* 20:80/30:70% AcOEt:HEX extracts showed inhibitory activity against slime-producing strains isolated from nasal carriers with MIC between 500 µg/mL and 2000 µg/mL.

*S. aureus* ATCC 35556 was sensitive to these 3 extracts at doses 1000 µg/mL. The *M. spinosum* 70:30% extract and 50:50/70:30:0% EtOAc:HEX mix showed the lowest antibacterial activity, MICs between 2000 µg/mL and 4000 µg/mL. *S. aureus* ATCC 35556 was inhibited with MIC = 4000 µg/mL. Higher concentrations (one to three times higher than the corresponding MICs values) of extracts were needed to obtain bactericidal effect. Only the extracts *M. spinosum* 70:30% AcOEt:HEX and *M. spinosum* 20:80/30:70% AcOEt:HEX showed the same MIC and MBC values for *S. aureus* ATCC 35556 (Table 1).

In addition, our study on the activity of the extracts against MRSA strains isolated from nasal carriers showed that *M. spinosum* 5:95% AcOEt:HEX extract presented the highest activity (MIC: 500 and 1000 µg/mL). *M. spinosum* 50:50% AcOEt:HEX and 20:80/30:70% AcOEt:HEX extracts showed activity against MRSA (MIC: 2000 µg/mL). Extracts of *M. spinosum* 70:30% and 50:50/70:30/0% AcOEt:HEX inhibited the growth of MRSA at doses of 4000 µg/mL. *S. aureus* ATCC 43300 was inhibited by all extracts (MIC between 500 µg/mL and 4000 µg/mL). The MBC values were one- or twofold higher than the corresponding MIC values, except for extracts *M. spinosum* 5:95% AcOEt:HEX and...
Table 2: Minimal inhibitory concentration and minimal bactericidal concentration of *M. spinosum* extracts against methicillin-resistant *S. aureus*.

<table>
<thead>
<tr>
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<th>MIC/MBC (μg/mL)</th>
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<tr>
<td></td>
<td>5:95%</td>
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<tr>
<td>S. aureus NC4</td>
<td>500/1000</td>
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<tr>
<td>S. aureus NC5</td>
<td>1000/2000</td>
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<tr>
<td>S. aureus ATCC 43300</td>
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NC: nasal carrier.

**Figure 3:** Microwell dilution in broth. Extract: *M. spinosum* 5:95% AcOEt:HEX. 1: *S. aureus* ATCC 43300; 2 and 3: MRSA strains isolated from nasal carriers; 4 and 5 (A): extract control; 4 and 5 (B): broth controls; 4 and 5 (C–E): DMSO controls; and 4 and 5 (F–H): strains controls.

In contrast, our results show that all the tested extracts showed good activity against MRSA.

About the chemical composition of *M. spinosum*, some authors investigated families of secondary metabolites in leaves, flowers, and fruits. Three group compounds (saponins, flavonoids, and terpenoids/sterols) were identified in fruits and flowers but they were absent in leaves [25–27]. Terpenoids, glycosylated flavonoids, and polyphenols are small molecules naturally produced by plants that can inhibit many bacterial species, particularly gram-positive organisms. These compounds are receiving sustained attention regarding their potential use since there has been strong evidence that they possess, in addition to antimicrobial activity, anti-inflammatory and antitumour properties [22, 28–30].

Schito et al. [28] showed that diterpenoid compounds obtained from the exudate produced by the aerial parts of *Salvia corrugata* inhibited the synthesis of biofilm in vitro produced by multiresistant *S. aureus*, *S. epidermidis*, and *Enterococcus faecalis*. These compounds presented MICs of 3200–6400 μg/mL when they were assayed against *S. aureus*. Also, these investigators indicate that such diterpenoids were active against all strains of methicillin-resistant *S. aureus* tested.

Moreover, Saising et al. [23] demonstrated that *Rhodomyrtus tomentosa* ethanol extract and its pure compound rhodomyrtone both possessed strong activity against biofilm-forming staphylococci isolated from acne lesions with MICs of 32–128 μg/mL and 0.5–1 μg/mL, respectively. However, the MIC of this extract was lower than the MIC of all extracts assayed in our study.

Moreover, Quave et al. [24] studied extracts of wild plants grown in southern Italy for the inhibition of growth and biofilm formation in MRSA. These authors demonstrated that ethanolic and water extracts had limited bacteriostatic activity, and, in fact, many promoted planktonic growth.
presented these virulence factors at doses between 500 μg/mL and 4000 μg/mL.

4. Conclusion

Both, slime-producing S. aureus and MRSA present a significant dilemma to medicine today, are highly contagious, and hardly eradicated by antibiotic therapies. So, there is an increasing need to find new compounds with the ability to inhibit these strains. Extracts of M. spinosum were active against all strains of slime-producing S. aureus and MRSA assayed, so extracts of this plant could represent interesting sources of natural antibiotics and justify the realization of further studies about the antimicrobial activity and characterization of new active compounds.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References


