Detection of Slime-Producing *Staphylococcus aureus* Strains Isolated from Food and their Sensitivity against *Mulinum echegarayii* and *Azorella trifurcata* Extracts

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Abstract— *The contamination of food with pathogenic microorganisms producing biofilm, implies a high cost for the food industry and represents a serious risk for the health of consumers. The antibacterial activity of organic extracts of Azorella trifurcata and Mulinum echegarayii was evaluated against 4 Staphylococcus aureus slime-producing strains isolated from bakery foods and against S. aureus ATCC 35556 slime-producing strain and S. aureus ATCC 25923 non slime-producing strain. The plant extracts showed antibacterial effectiveness against all the strains of S. aureus tested with minimum inhibitory concentration (MIC) between 500 and 8000 µg/ml. M. echegarayii 30:70% AcOEt:HEX showed the best activity: five strains of S. aureus showed MIC of 1000 µg/ml and S. aureus ATCC 25923 was inhibited at doses of 500 µg/ml. The values of minimum bactericidal concentration (MBC) of the extracts assayed were one or two times higher than corresponding MIC values. This study showed that extracts of Azorella trifurcata and Mulinum echegarayii are promising for future natural therapy against slime-producing S. aureus. Plant extracts with activity against slime producing S. aureus strains could provide benefits for of food technology and public health.*

Keywords— Antibacterial activity, Slime, Staphylococcus aureus, Organic plant extracts.

I. INTRODUCTION

Poor hygiene practices may result in the contamination of food products with pathogens, which means a serious risk for the health of consumers. The contamination of food during processing, storage and sale causes serious problems in the food industry. *S. aureus* is one of the most commonly identified bacteria that cause food- borne diseases in human (Gutiérrez et al., 2012) Biofilms are the most common bacterial lifestyle in nature. After initial attachment of cells to a surface, they start to multiply and secrete a consistent matrix of extracellular polymeric substances in which cells are wrapped. These biofilms are a common cause of food contamination with undesirable bacteria, and constitute a serious problem in many sectors of the food industry (Brooks and Flint, 2008). Some strains of *S. aureus* produce this biofilm and have the ability to colonize surfaces, which serves as a reservoir for the continuous release of the bacteria to food. Also, there is evidence that adhered bacteria are very difficult to remove because they are extremely resistant to antibiotics and disinfectants (Gunduz and Tuncel, 2006).

Natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have been the basis for the development of new pharmaceutical products. In fact, these plants contain active metabolites with antibacterial principles. *Mulinum* and *Azorella* belong to the family Apiaceae, which includes several species of herbs and some shrubs, traditionally called umbellifers. *Mulinum* is a genus that includes 15-20 species confined to Argentina, Bolivia and Chile (Constance, 1988). *Mulinum echegarayii* Hieron is an Argentinean endemic species known as "Grass in cushion". It is a small and compact shrub widely distributed in the Argentina Patagonian steppe and there are no known references to its use in traditional medicine (Martínez, 1999). *Azorella trifurcata* (Gaertn.) Pers is a native plant known by the vernacular names "Yareta". This species is used in folk medicine as antitussive and expectorant, as well as antiseptic, antiparasitic, antirheumatic and hypoglycaemic (Fuentes et al., 2005).

The purpose of this work was to detect slime-producing *S. aureus* strains isolated from food and evaluate their sensitivity against *M. echegarayii* and *A. trifurcata* extracts.

II. MATERIALS AND METHODS

2.1. Microorganisms

A total of 11 *S. aureus* strains isolated from 41 samples of bakery foods and kept in the ceparium of the Laboratory of Microbiology of the National University of San Luis, were assayed for the production of slime. Then, antibacterial activity

was tested against a total of 6 strains, 4 of those slime-producing strains (a, b, c, d) all isolated from bakery foods and *S. aureus* ATCC 35556 slime-producing strain and *S. aureus* ATCC 25923 non slime-producing strain. Bacterial strains were maintained on trypticase soy broth (TSB) supplemented with 20% glycerol at -80°C until use.

2.2 Slime Production

Slime production was performed by using 2 methods:

2.2.1. Congo Red Agar Method: was performed according to Freeman et al., (Freeman et al., 1989) with the following modifications: the strains were streaked onto Congo red agar (CRA) plates (0.8g of Congo red and 50g of sucrose in 1 liter of brain heart infusion agar), incubated for 24 h at 37ºC and subsequently overnight at room temperature. Plates were inspected for the color of the colonies at 24h. For colonies evaluation, a four-color reference scale was used: black and almost black bordeaux for slime-producing strains, and bordeaux and red for non slime-producing strains.

2.2.2. The spectrophotometric micromethod: was performed according to Pfaller et al., (Pfaller et al., 1988) using tissue culture plates of 96 flat-bottomed wells. Each well was filled with 0.2ml of 10^5 CFU/ml of a bacterial suspension in tryptic soy broth (TSB). After 48h incubation in aerobiosis at 35ºC, the contents were aspirated and the plates were washed twice with saline phosphate-buffered (pH 7.2). The wells were stained with 0.25% safranin for 30seg. The plates were read in an ELISA reader (Benchmark Biorad) at 490nm. Sterile TSB was used as negative control. All the experiments were repeated at least twice and the samples were tested in quintuplicate; the values of optical density (OD) were then averaged. A three-grade scale was used to evaluate the strains slime producing ability: negative, ODs < 0.500; (+) ODs 0.500-1.500; (++) ODs >1.500. *S. aureus* ATCC 35556 was used as positive control for slime production and *S. aureus* ATCC 25923 was used as negative control.

2.3. Plant material

Azorella trifurcata and *Mulinum echegarayii* Hieron were collected in Malargüe, Mendoza, Argentina. Voucher specimens of both species were identified by Ing Luis Del Vitto et al. and lodged in the University of San Luis (Argentina) herbarium (Del Vitto et al., 1997).

2.3.1. Preparation of extracts

A. trifurcata (6.950kg) and *M. echegarayii* (2.100kg) were processed separately. The aerial parts of the plants were previously dried at ambient temperature and finely ground in mill blades, and then macerated with cold acetone for 48h. Acetone extract was separated by filtration. This procedure was repeated three times. The combined extraction liquids were concentrated under reduced pressure yielding 86g and 270g of syrupy material, respectively. The acetone extracts were dissolved in the same solvent and adsorbed on 400g of silica gel 60 G. After evaporation of the solvent a chromatography "flash" column was prepared using n-hexane (HEX) as eluents and mixtures of ethyl acetate (EtOAc)-HEX to increase polarity until 100% EtOAc was reached. The progress of the separation was monitored by thin layer chromatography (TLC), using as mobile phase benzene: dioxane: acetic acid (AcOH) (120:20:4) and as revealing a mixture of H_2SO_4: AcOH: H_2O (4:20:1) or anisaldehyde: H_2SO_4: ethanol: H_2O (1:20:90:90) followed by heating to 120°C.

2.4. Antibacterial activity

2.4.1. Determination of Minimal Inhibitory Concentration (MIC)

The antibacterial activity was assayed *in vitro* using microplate method (microwell dilution) according to the CLSI method (CLSI, 2011) in TSB, 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 24h broth culture and adjusted to concentration of 10^6CFU/ml. Organic extracts were dissolved in dimethylsulfoxide and tested in a concentration ranging from 8000 to 100µg/ml. The 96-well plates were prepared by dispensing into each well 95µl of nutrient broth and 5µl of the inoculum (final concentration of 10^4 CFU/ml). One hundred microlitre aliquots from the serial dilutions of extracts were transferred into four consecutive wells. The final volume in each well was 200µl. Controls of nutrient broth, strains and extracts were included. After 24h incubation at 37ºC, the MIC was defined as the lowest concentration of the extract in the medium in which there was no visible growth. The experiments were replicated at least twice.

2.4.2. Determination of Minimal Bactericidal Concentration (MBC)

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

III. RESULTS AND DISCUSSION

Of the 11 *S. aureus* strains studied, 4 were slime positive by both methods assayed. The slime-production by CRA method is shown in Fig.1: *S. aureus* strains appear as black colonies.

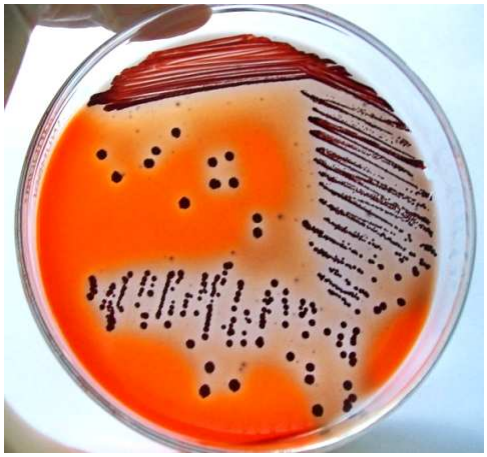

FIGURE 1. CULTURE ON CRA: BLACK COLONIES OF SLIME-PRODUCING S. AUREUS

Also, Table 1. shows the means of the absorbance values obtained from slime producing *S. aureus* strains and *S. aureus* used as positive and negative controls by spectrophotometric micromethod. Three slime producing strains showed values of optical density ODs 0.500-1.500 (+) and one ODs >1.500 (++). *S. aureus* ATCC 35556 showed ODs 0,849 and *S. aureus* ATCC 25923 ODs 0,303.

TABLE 1

MEANS OF THE ABSORBANCE VALUES OBTAINED BY SPECTROPHOTOMETRIC MICROMETHOD FROM STRAINS OF *STAPHYLOCOCCUS AUREUS*

Staphylococcus aureus	OD (490nm)	Scale (Pfaller y col.)
a	0, 818	(+)
b	1,520	(++)
c	1,179	(+)
d	0,868	(+)
Staphylococcus aureus ATCC 35556	0,849	(+)
Staphylococcus aureus ATCC 25923	0,303	(-)

OD: optical density; a, b, c, and d: Staphylococcus aureus isolate from bakery foods

The large numbers of *S. aureus* nasal carriers, between 30 and 50% of the population, could become an important source of food contamination during handling or storage. Since this microorganism can develop in a wide pH range and in environments with high percentage of sodium chloride concentration, a range of products may be responsible for outbreaks of food poisoning by *S. aureus* (Gutiérrez et al., 2012). Moreover, the attachment of the bacteria to the food product leads to serious hygienic problem, and economic losses due to food spoilage. In addition, food borne pathogens such as *S. aureus* biofilm producers are more virulent and resistant to antibiotics and biocides which leads to a serious risk to public health (Gunduz and Tuncel, 2006; Raggi, 2013).

In this study, the plants extracts showed antibacterial effectiveness against all the strains of *S. aureus* tested. Extract of *M. echegarayii* 10:90% AcOEt:HEX showed activity against 3 slime producing *S. aureus* strains isolated from food at doses of 4000 µg/ml and one showed MIC 8000 µg/ml. *S. aureus* ATCC 35556 and *S. aureus* ATCC 25923 were sensitive to this extract with values of MIC of 2000µg/ml and 4000µg/ml respectively. An interesting finding was that the best effect was obtained with *M. echegarayii* 30:70% AcOEt:HEX. At this concentration, all slime-producing *S. aureus* strains showed MIC of 1000µg/ml and *S. aureus* ATCC 25923 was inhibited at doses of 500µg/ml. *A. trifurcata* 40:60/50:50% AcOEt:HEX extract showed less activity against *S. aureus* isolated from food (MIC: 8000µg/ml) and *S. aureus* ATCC 35556 was inhibited at 2000µg/ml. The best activity of this extract was against non slime-producing *S. aureus* ATCC 25923 with MIC of 500µg/ml. MBC values were one or two times higher than the corresponding MIC values (Table 2). Fig.2 shows the microdilution plate used for broth microdilution method with *Mulinum echegarayii* 10:90 ethyl acetate/n-hexane extract and *A. trifurcata* 40:60/50:50% ethyl acetate/n- hexane extract against *S. aureus* ATCC 35556, *S. aureus* ATCC 25923 and three (a, b and c) slime-producing *S. aureus* isolate from food.

TABLE 2
MINIMAL INHIBITORY CONCENTRATION AND MINIMAL BACTERICIDAL CONCENTRATION OF *MULINUM ECHEGARAYII* AND *AZORELLA TRIFURCATA* EXTRACTS.

	Ethyl acetate/n-hexane *Mulinum echegarayii* extracts MIC/MBC (µg/ml)		Ethyl acetate/n-hexane *Azorella trifurcata* extract MIC/MBC (µg/ml)
Staphylococcus aureus	10:90	30:70	40:60/50:50
A	8000/ND	1000/4000	8000/ND
B	4000/8000	1000/4000	8000/ND
C	4000/8000	1000/4000	8000/ND
D	4000/8000	1000/4000	8000/ND
Staphylococcus aureus ATCC 35556	2000/4000	1000/4000	2000/8000
Staphylococcus aureus ATCC 25923	4000/8000	500/2000	500/2000

MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration. ND: not detected at maximum concentration tested (16000µg/ml).

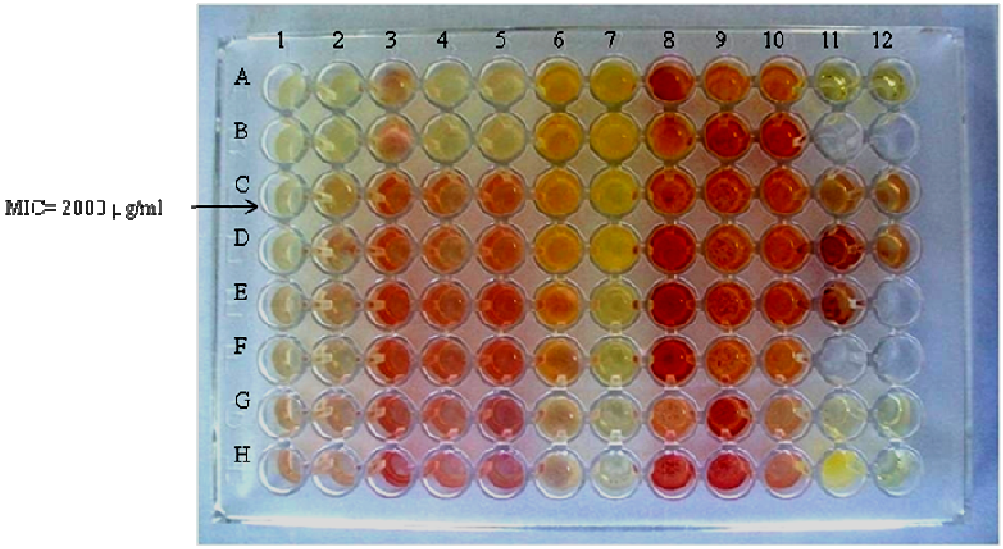

FIG.2. MICRODILUTION PLATE USED FOR BROTH MICRODILUTION METHOD WITH: FILE 1-5 MULINUM ECHEGARAYII 10:90 ETHYL ACETATE/N-HEXANE EXTRACT; FILE 6-10 A. TRIFURCATA 40:60/50:50% ETHYL ACETATE/N- HEXANE EXTRACT; BACTERIAL STRAINS: 1 AND 6: S. AUREUS ATCC 35556; 2 AND 7: S. AUREUS ATCC 25923, 3 AND 8: S. AUREUS A, 4 AND 9: S. AUREUS B, 5 AND 10: S. AUREUS C ; FILE 11-12: A BROTH CONTROLS; C- E: CONTROLS OF STRAINS AND G-H EXTRACT CONTROLS; DILUTIONS OF THE EXTRACTS (µG/ML): FILES A AND B: 4000, C AND D: 2000, E AND F 1000, G AND H: 500.

To our knowledge, there are few reports available in the literature on activity of organic extracts of *M. echegarayii* and *A. trifurcata* against pathogenic bacteria tested in this study (Satorres et al., 2013). Snowden et al. (Snowden et al., 2014), evaluated the antimicrobial activity of several botanical extracts of other plants against *S. aureus*. Some of these extracts, which include *Salvia*, *Eucalyptus*, *Coleus*, *Arctostaphylos*, *Coptis*, *Turnera*, *Anemopsis*, *Allium*, and *Larrea*, showed higher activity than that found in our study (MIC < 200µ/ml) and *Berberis*, *Baptisia* and *Glycyrrhiza* extracts showed MIC > 1000µ/ml. Other extracts tested by Snowden et al. were ineffective.

The ethanol extract of *Rhus javanica* was assayed against methicillin-resistant *S. aureus* (MRSA), inhibiting the growth of this bacterium at concentrations ranging from 0.05 to 0.2mg/ml. This extract also inhibited biofilm formation by MRSA. Yong-Ouk You et al. found in *R. javanica* strong presence of phenolics, moderate presence of glycosides, and weak presence of flavonoids, steroids (terpenoids), and organic acids, suggesting that phenolics may have been responsible for the antibacterial activity observed in our study (Yong-Ouk You et al., 2013).

Andreana et al. (Andreana et al., 2010) evaluated the *in vitro* effect of branch methanol and aqueous extracts of five *Juniperus* species on the growth, adherence and biofilm formation of *S. aureus*. All the extracts affected the biofilm development depending on the biofilm-forming strain capacity and as in our study, inhibited bacterial growth of all assayed strains. Also, the phytochemical screening of the extracts revealed the presence of polyphenols, coumarins, lignans, steroids, alkaloids and terpenes. These authors suggest that all these compounds, alone or in combination, could be responsible for the antimicrobial properties observed.

Some authors have demonstrated that *Rhodomyrtus tomentosa* ethanol extract possesses strong activity against biofilm-forming staphylococci isolated from acne lesions, with MIC values of 32-128µg/ml, somewhat lower than those found in our study (Saising et al., 2011).

Bioactive metabolites, such as triterpenoids and diterpenoids with azorellane and mulinane skeletons have been obtained from Andean species of the genus tested in this study *Azorella* and *Mulinum* (Borquez et al., 2014). Diterpenoids and terpenoids are a large family of natural products exhibiting a wide range of interesting biological activities such as antibacterial (Areche et al., 2010), antiviral (Abdel et al., 1996), trypanosomicidal, trichomonacidal, toxoplasmocidal (Loyola et al., 2001), antitubercular (Molina-Salinas et al., 2010), among others.

Kuzma et al. (Kuzman et al., 2007) demonstrated that derivatives of terpenes such as salvipisone presented a very interesting activity on staphylococcal biofilm cells viability, included this compound in the list of potential anti-biofilm agents, better than most of known antibiotics.

Chiaramelo et al. (Chiaramello et al., 2007) demonstrated the presence of diterpenoid acids in both plant species used in this study**.** Wächer et al. reported that diterpenoid acids isolated from *Azorella compact* showed inhibitory activity against methicillin-resistant *S. aureus* and methicillin-susceptible *S. aureus* (Wachter et al., 1999).

Therefore, the anti-staphylococcal activity of *A. trifurcata* and *M. echegarayii* organic extracts observed in this study could be partly or completely attributed to the presence of these active compounds. Previous research on the mode of action of terpenes has shown that these compounds have multiple cellular targets, rather than one specific site of action (Sikkema et al., 1995). As reported by several authors, the action of these compounds causes destruction of cellular integrity, inhibit respiratory activity, and causes a dissipation of the proton motive force (Sikkema et al., 1994)

IV. CONCLUSION

Natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have been the basis for the development of new chemicals for pharmaceuticals.

S. aureus continues to be a public health problem because of its implication in food-borne diseases in humans. The discovery of plant extracts with antibacterial activity against slime-producing *S. aureus* could contribute to reduce public health hazards and also to inhibit the colonization and persistence of this bacterium in the food environment.

Further studies will be carried out to characterize the active components of *M. echegarayii* and *A. trifurcata* extracts effective against *S. aureus* biofilm producers.

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CONFLICT OF INTEREST

None declared till now

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