Emir. J. Food Agric. 2013. 25 (6): 457-462
doi: 10.9755/ejfa.v25i6.15554
http://www.ejfa.info/

PLANT SCIENCE

Azorella trifurcata and *Mulinum echegarayii* obtained from central region of Argentina: antibacterial activity of their organic extracts

S. E. Satorres[1*], A. Chiaramello[2], C. M. Mattana[1], L. E. Alcaráz[1], P. C. Rossomando[2], C. Tonn[2] and A. L. Laciar[1]

[1]Área de Microbiología, INTEQUI-CONICET, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Ejército de los Andes 950 San Luis, Argentina
[2]Área de Química Orgánica, INTEQUI-CONICET, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Ejército de los Andes 950 San Luis, Argentina

Abstract

The natural products derived from medicinal plants have proven to be an abundant source of compounds with antibacterial activity. The antibacterial activity of extracts of *Azorella trifurcata* and *M. echegarayii* was evaluated against strains of Staphylococcus aureus ATCC 43300, Pseudomonas aeruginosa ATCC 27853, *Listeria monocytogenes* CLIP 74902 and *Escherichia coli* ATCC 35218. Organic extracts were prepared using n-hexane, mixtures of n-hexane and ethyl acetate of increasing polarity and a mixture of ethyl acetate and methanol on flash chromatography. All the extracts of *A. trifurcata* showed antibacterial effects against gram-positive bacteria (CIM between 0.5 and 2 mg/ml). Four extracts (100% n-hexane, 40:60/50:50 acetate: n-hexane, 70:30 ethyl acetate: n-hexane and 2:98 methanol: ethyl acetate) of *A. trifurcata* showed antibacterial activity against gram- negative bacteria. *M. echegarayii* 2:98 methanol: ethyl acetate was active against all gram-negative and gram-positive bacteria (CIM between 1 and 2 mg/ml). The values of MBC of the extracts assayed were one or two times higher than corresponding MIC values. The discovery of organics extracts with antibacterial properties could contribute to the treatment of bacterial infections.

Key words: Antibacterial activity, *A. trifurcata*, *M. echegarayii*, Organic extracts

Introduction

In recent decades has increased significantly the development of microbial resistance to conventional antimicrobials and the study of antibacterial properties of plant extracts and natural phytochemicals continues offering a large number of therapeutic options. Latin American countries have a rich tradition in the use of medicinal plants in folk medicine. The use of these plants is very important in primary health care in rural and semi-rural areas. They are used in many circumstances in which the disease is considered as a loss of physical, emotional and spiritual conditions (Estomba et al., 2006; Ladio et al., 2008; Lozada et al., 2006; Eyssartier et al., 2009). *Azorella trifurcata* (Gaertn.) Pers (Apiaceae) is a plant known by the vernacular names "Yareta". This species is used in folk medicine as antitussive and expectorant, to treat asthma, colds and bronchitis, as well as antiseptic, antiparasitic, antirheumatic and hypoglycaemic (Padín,1999; Delporte Vergara et al., 2003; Fuentes et al., 2005).

Mulinum echegarayii (Hieron) is endemic specie to the high Andes of Mendoza and San Juan, western Argentina, where he lived between 2,500 and 3,000 m above sea and has no trivial names. There are no previous data in the literature on this species and are not known references in the literature on its use in folk medicine. Both species belongs to the family Apiaceae or also Umbeliferae called. Species of this family synthesize secondary metabolites as natural diterpenes, which have carbon skeletons and azorellane, mulinane, madreporane and yaretane types (Martinez, 1999; Loyola et al., 2002; Chiaramello et al., 2003a). The purpose of the study presented here, was to evaluate *in vitro* the antibacterial activity of organic extracts of *Azorella trifurcata* and *Mulinum echegarayii.*

Received 08 December 2012; Revised 11 February 2013; Accepted 02 March 2013; Published Online 02 April 2013

*Corresponding Author

S. E. Satorres
Área de Microbiología, INTEQUI-CONICET, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Ejército de los Andes 950 San Luis, Argentina

Email: sasato@unsl.edu.ar

Materials and Methods

Plant material

Azorella trifurcata and *Mulinum echegarayii* Hieron were collected in Malargüe, Department, Mendoza, Argentina. A specimen of both species was available in the Herbarium of the National University of San Luis, Voucher N° 9247 (Figure 1) and N° 494 (Figure 2) respectively.

Figure 1. Aerial parts of Azorella *trifurcata* specimen collected in Mendoza, Argentina.

Figure 2. Aerial parts of *Mulinum echegarayii* specimen collected in Mendoza, Argentina.

Preparation of extracts

A. trifurcata (6.950 kg) and *M. echegarayii* (2.100 kg) were processed separately. The aerial parts of plant previously dried at ambient temperature and finely ground in mill blades, were macerated with cold acetone for 48 hours. 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 respectively, of syrupy material. Those acetone extracts was dissolved in the same solvent and adsorbed on 400g of silica gel 60 G. after evaporation of the solvent proceeded to prepare chromatography "flash" column using n-hexane (HEX) as eluents and mixtures of ethyl acetate (EtOAc) - HEX increasing polarity until reaching 100% EtOAc. The progress of the separation was monitored by thin layer chromatography (TLC), using as mobile phase benzene: dioxane: acetic acid (AcOH) (120:20:4) as developer and a mixture of H_2SO4: AcOH: H_2O (4:20:1) or anisaldehyde: H_2SO_4: ethanol: H_2O (1:20:90:90) followed by heating to 120°C.

Microorganism

A total of four bacteria were selected for this study, methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* CLIP 74902 (Collection *Listeria* Institute Pasteur) and *Escherichia coli* ATCC 35218.

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 in tripticase 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 (CLSI, 2011). 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 0.25 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 microlitres 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 h incubation at 37ºC, 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 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.

Results and Discussion

In the present study, all the extracts of *A. trifurcata* showed antibacterial effects against Gram-positive pathogenic bacteria tested. Extracts

of *A. trifurcata* 100% HEX, 10:90 AcOEt:HEX, 20:80/30:70 AcOEt:HEX, 70:30 AcOEt:HEX and 100% AcOEt showed a MIC of 2 mg/ml against methicillin-resistant *S. aureus.* *A. trifurcata* 40:60/50:50 AcOEt:HEX and 2:98 MeOH:AcOEt showed activities against this bacterium at doses of 0.5 mg/ml and 1 mg/ml respectively. *L. monocytogenes,* whose isolates have been increasing in number and severity over the last years, was inhibited by all these extracts showing MIC values between 1 and 2 mg/ml. Four extracts of *A. trifurcata* tested (100% HEX, 40:60/50:50 AcOEt:HEX, 70:30 AcOEt:HEX and 2:98 MeOH:AcOEt) showed antibacterial activity against Gram- negative bacteria (CIM between 2 and 4 mg/ml) (Table 1). *A. trifurcata* 10:90 AcOEt:HEX, 20:80/30:70 AcOEt:HEX and 100% AcOEt extracts, were no active against *P. aeruginosa* and *E. coli* (Table 1).

Table 1. Minimal Inhibitory Concentration and Minimal Bactericidal Concentration of *Azorella trifurcata* extracts.

Azorella trifurcata extracts MIC/MBC (mg/ml)							
	HEX	AcOEt: HEX				AcOEt	MeOH:AcOEt
Bacterial strains	100%	10:90	20:80/30:70	40:60/50:50	70:30	100%	2:98
S. aureus ATCC 43300	2/4	2/4	2/4	0.5/1	2/4	2/4	1/2
P. aeruginosa ATCC 27853	4/ND	NA	NA	2/4	2/4	NA	2/4
L. monocytogenes CLIP 74902	1/2	1/2	1/4	1/2	2/4	2/4	1/4
E. coli ATCC 35218	4/ND	NA	NA	2/4	2/4	NA	2/4

MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration; HEX: n-hexano; AcOEt: ethyl acetate; MeOH: methanol; ND: no detected; NA: no activity.

Table 2. Minimal Inhibitory Concentration and Minimal Bactericidal Concentration of Mullinum echegarayii extracts.

	Mullinum echegarayii extracts MIC/MBC (mg/ml)				
	HEX	AcOEt: HEX		AcOEt	MeOH:AcOEt
Bacterial strains	100%	10:90	30:70	100%	2:98
S. aureus ATCC 43300	1/4	1/2	0.5/1	4/ND	1/2
P. aeruginosa ATCC 27853	NA	NA	NA	NA	2/4
L. monocytogenes CLIP 74902	NA	NA	4/ND	4/ND	1/4
E. coli ATCC 35218	NA	NA	NA	NA	2/4

MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration; HEX: n-hexano; AcOEt: ethyl acetate; MeOH: methanol; ND: no detected; NA: no activity.

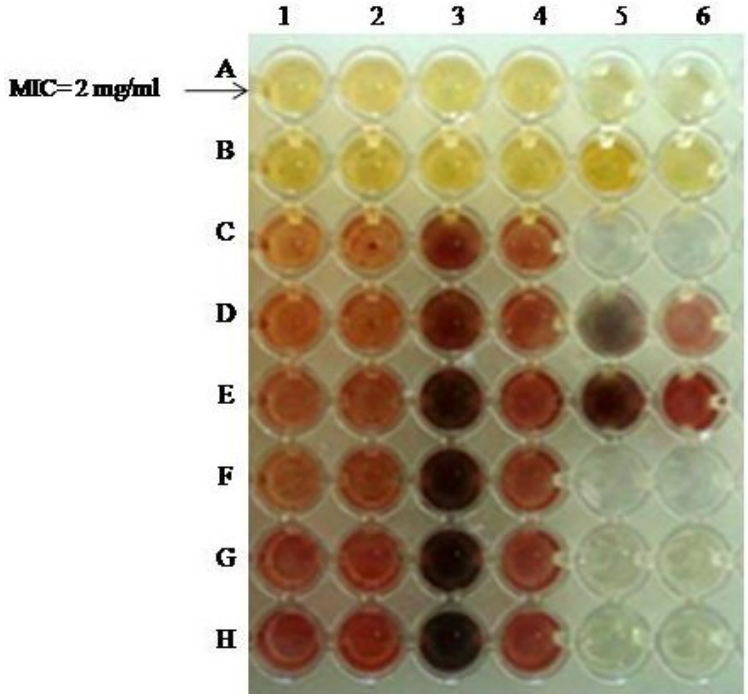

Figure 3. Microdilution plate used for broth microdilution method with *A. trifurcata* 70:30 Acetato de etilo: n-hexane extract against *S. aureus* ATCC 43300 (file 1); *L. monocytogenes* CLIP 74902 (file 2), *P. aeruginosa* ATCC 27853 (file 3); *E. coli* ATCC 35218 (file 4). File 5-6 (A-B): extracts controls; File 5-6 (D-E): controls strains.

An interesting finding was that both extracts of *A. trifurcata* and *M. echegarayii* 2:98 MeOH:AcOEt were active against all Gram-negative and Gram-positive bacteria tested. On the other hand, *S. aureus* was inhibited by all extracts of *M. echegarayii* with MICs between 0.5 and 4 mg/ml (Table 2). Methicillin-resistant *S. aureus* was sensitive to all *A. trifurcata* and *M. echegarayii* extracts. It is important to remember that infections caused by these bacteria have reached epidemic proportions, and therapeutic options are limited because these strains are often multidrug resistant (Chua et al., 2011). All extracts studied showed better antibacterial activity against Gram-positive bacteria, in comparison with Gram-negative bacteria. Several authors have shown that Gram-positive bacteria are more susceptible to the plant extracts that Gram- negative bacteria (Bele A.A. et al., 2009). Therefore it is theorized that Gram-positive bacteria are more susceptible than Gram-negative bacteria due to the differences in their cell wall structure. Gram-negative organisms are considered to be more resistant due to their outer membrane acting as a barrier to many environmental substances, including antibiotics (Lewis, K., 2001).

The Figure 3 shows the MIC obtained from *A. trifurcata* 70:30 AcOEt/HEX extract against the tested bacteria. The values of MBC of the extracts assayed were one or two times higher than corresponding MIC values. The MBC was not active at the highest concentration tested for extracts of both species whose MIC was 4 mg/ml. Table 1 and 2. An interesting group of bioactive metabolites, such as triterpenoids and diterpenoids with azorellane and mulinane skeletons have been obtained from *Azorella*, and *Mulinum* genera (Borquez et al.,2011; Chiaramello et al., 2003a; Colloca et al.,2004; Molina-Salinas et al.,2010; Morales et al., 2003; Areche et al., 2009). So, some authors demonstrated that these metabolites display a wide variety of biological activities, including antimycobacterial activity (Molina-Salinas et al., 2010), trichomonicidal activity (Loyola et al., 2001), trypanocidal activity (Araya et al., 2003) and antiplasmodial activity (Loyola et al., 2004). To our knowledge, there are few reports available in the literature on activity of organic extracts of *M. echegarayii* and *Azorella trifurcata* against pathogenic bacteria tested in this study. Chiaramello et al. (2003b) isolated from both plant species, secondary metabolites mulinane and azorellane-type such as azorellolide and mulinénico acid from *A. trifurcata* and 17-acetoximulinic acid, 14-α-hidroximulinolic acid, azorellolide and sphatulenol from *M. echegarayii* (Chiaramelo et al., 2003b; Chiaramello et al., 2007a; Chiaramello et al., 2007b). Some authors reported that diterpenoid acids isolated from *Azorella compact* showed inhibitory activity against methicillin-resistant *S.aureus,* methicillin-susceptible *S.aureus*, *Enterococcus faecium,* and *E.coli* (Wächer et al., 1999). Therefore, it could be possible to attribute the antibacterial activity of *A. trifurcata* and *M. echegarayii* organic extracts, partly or completely to the presence of diterpenoid acids as previously demonstrated in both plant species used in this study (Chiaramelo et al., 2003b; Chiaramello et al., 2007a).

Conclusions

There is considerable evidence that the organic extracts of the plants tested could be used in therapies preventative or treatment of bacterial infections.

Acknowledgement

This work is part of the Projects 8802 and 27301 financed by University National of San Luis, Argentina.

References

Araya, J. E., I. Neira, S. Silva, R. A. Mortara, P. Manque, E. Cordero, H. Sagua, A. Loyola, J. Bórquez, G. Morales and J. González. 2003. Diterpenoids from *Azorella compacta* (Umbelliferae) active on *Trypanosoma cruzi*. Mem. Inst. Oswaldo Cruz. 98:413-418.

Areche, C., P. Cejas, P. Thomas, I. A. San-Martín, L. Astudillo, M. Gutiérrez and L. A. Loyola. 2009. Triterpenoids from *Azorella trifurcata* (Gaertn.) Pers and their effect against the enzyme acetylcholinesterase. Quim. Nova. 32:2023-2025.

Bele, A. A, V. M. Jadhav, S. R. Nikam and V. J. Kadam. 2009. Antibacterial potential of herbal formulation. Res. J. Microbiol. 4:164-167.

Borquez, J., G. M. Molina-Salinas, L. A. Loyola, A. San-Martın, L. M. Peña-Rodríguez and S. Said-Fernández. 2011. A new azorellane diterpenoid from *Azorella madrepórica*. Nat. Prod. Res. 25:653-657.

Chiaramello, A. I., C. E. Ardanaz, E. E. García and P. C. Rossomando. 2003a. Mulinane-type Diterpenoids from *Mulinum spinosum*. Phytochemistry 63:883-886.

Chiaramello, A. I. and P. C. Rossomando. 2003b. New Azorellane-type Lactone in *Azorella trifurcata* (Apiaceae) "Presented at the XIII National Symposium of Organic Chemistry. XIII SINAQO. Rosario, Argentina. pp. 19.

Chiaramello, A. I. 2007a. Doctoral Thesis in Biochemistry. The Tribe Mulinae "Phytochemical study of species of the genus *Azorella*, *Mulinum* (Apiace-Hydrocotyloidaeae). Bioactivity of Secondary Metabolite. Departament of Chemistry, Biochemistry and Pharmacy. UNSL. San Luis. Argentine.

Chiaramello, A. I. and P. C. Rossomando. 2007b. Study phytochemical of *Mulinum echegarayii* (Apiaceae). XVI National Symposium of Organic Chemistry. SINAQO XVI. Argentina Society for Research in Organic Chemistry. Mar del Plata. Bs. As. Argentine pp.20.

Chua, K., F. Laurent, G. Coombs, M. L. Grayson and B. P. Howden. 2011. Not Community-Associated Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA). A Clinician's Guide to Community MRSA–Its Evolving Antimicrobial Resistance and Implications for Therapy. Antimicrobial Resistance. Clin. Infec. Dis. 52:9-114.

CLSI. 2011. Clinical and Laboratory Standard Institute. Performance Standards for antimicrobial susceptibility testing: M100 - S21. Vol.31, nº1. Wayne, Pensylvannia. USA.

Colloca, C. B., D. B. Pappan, D. A. Bustos, V.E. Sosa, R. F. Baggio, M. T. Garland and R. R. Gil. 2004. Azorellane diterpenes from *Azorella cryptantha*. Phytochemistry 65:2085-2089.

Delporte Vergara, C., N. Backhouse Erazo, P. Salinas, A. San Martín, J. Bórquez and A. Loyola. 2003. Pharmaco-toxicological study of diterpenoids. Bioorg. Med. Chem. 11:1187-1190.

Estomba, D., A. H. Ladio and M. Lozada. 2006. Medicinal wild plant knowledge and gathering patterns in a Mapuche community of North-western Patagonia. J. Ethnopharmacol. 103:109-119.

Eyssartier, C. A., H. Ladio and M. Lozada. 2009. Use of medicinal plants cultivated in a semi-rural community of the Patagonian steppe. BLACPMA. 8:77-85.

Fuentes, N. L., H. Sagua, G. Morales, J. Borquez, A. San-Martín, J. Soto and L. A. Loyola. 2005. Experimental antihyperglycemic effect of diterpenoids of llareta *Azorella compacta* (Umbelliferae) Phil. in rats. Phytother. Res.19:713-716.

Ladio, A. H. and M. Lozada. 2008. Medicinal plant knowledge in rural communities of North-western Patagonia, Argentina. A resilient practice beyond acculturation. In Albuquerque Ulysses Paulino de and Alves Ramos Marcelo (Ed). Curr. Top. Ethnobot. pp. 39-53.

Lewis, K. 2001. In search of natural substrates and inhibitors of MDR pumps. J. Mol. Microbiol. Biotechnol. 3:247-254.

Loyola, L. A., J. Bórquez, G. Morales, J. Araya, J. González, I. Neira, H. Saqua and A. San-Martin. 2001. Diterpenoids from *Azorella yareta* and their trichomonicidal activities. Phytochemistry 56:177-180.

Loyola, L. A., J. Bórquez, G. Morales, A. San-Martín, and J. Darias. 2002. Madreporanone, a unique diterpene with a novel skeleton from *Azorella madreporica*. Tetrahedron Lett. 43:6359-6362.

Loyola, L. A., J. Borquez, G. Morales, A. San-Martın, J. Darias, N. Flores and A. Gimenez. 2004. Mulinane-type diterpenoids from *Azorella compacta* display antiplasmodial activity. Phytochemistry 65:1931-1935.

Lozada M, A. H. Ladio and M. Weigandt. 2006. Cultural transmission of ethnobotanical knowledge in a rural community of northwestern Patagonia. Econ. Bot. 60:374-385.

Martínez, S. 1999. Apiaceae. In: F. Zuloaga and O. Morrone (Eds.), p.74. Catálogo de las Plantas Vasculares de la República Argentina II. Dicotyledoneae. Monog. Syst. Bot. Gard.

Molina-Salinas G. M., J. Bórquez, A. Ardiles, S. Said-Fernández, L. A. Loyola and A. San-Martín, I. González-Collado and L. M. Peña-Rodríguez. 2010. Antituberculosis activity of natural and semisynthetic azorellane and mulinane diterpenoids. Fitoterapia 81:50-54.

Morales, P., M. Kong, E. Pizarro, C. Pasten, G. Morales, J. Borquez and L. A. Loyola. 2003. Effect of Azorellanone, a diterpene from *Azorella yareta* Hauman, on human sperm. Physiol. J. Androl. 24:364-370.

Padín, O. H. 1999. Caracterización de la biodiversidad de la franja costera norte de Tierra del Fuego. Edit. Totalfina Argentina. Pp. 1-80 www.ecopuerto.com/cae/total/pub-biodiversidad.html.

Wächter G. A., S. G. Franzblau, G. Montenegro, E. Suarez, R. H. Fortunato, E. Saavedra and N. Timmermann. 1998. A new antitubercular mulinane diterpenoid from *Azorella madreporica*. Clos. J. Nat. Prod. 61:965-968.

Wächter G. A., G. Matooq , J. J. Hoffmann, W. M. Maiese, M. P., Singh, G. Montenegro and N. Timmermann. 1999. Antibacterial diterpenoid acids from *Azorella compacta*. J. Nat. Prod. 62:1319-1321.