Antibacterial activity study of single and combined extracts of *Berberis ruscifolia, Baccharis sagittalis, Euphorbia dentata* and *Euphorbia schikendanzii*, native plants from Argentina

[Estudio de actividad antibacteriana de extractos de *Berberis ruscifolia, Baccharis sagittalis, Euphorbia dentata* and *Euphorbia schikendanzii*, plantas nativas de Argentina y su efecto combinado]

Claudia M. MATTANA¹, Sara E. SATORRES¹, Virginia JUAN², Diego CIFUENTE², Carlos TONN & Analia L. LACIAR¹

¹Área Microbiología
²Área Química Orgánica, INTEQUI CONICET
Universidad Nacional de San Luis, San Luis, Argentina.

Contactos | Contacts: Claudia M. MATTANA - E-mail address: cmmattan@unsl.edu.ar

Abstract

The present work aimed to detect the antibacterial activity of natural species (*Berberis ruscifolia, Baccharis sagittalis, Euphorbia dentata* and *Euphorbia schikendanzii*). Twelve plants organic extracts were tested against *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* and *Pseudomonas aeruginosa*. The combined effect of acetonic extracts was also evaluated. All extracts showed antibacterial activity with MIC varying from 16 to 2 mg/mL. The highest inhibition was observed with acetonic and chloroform-methanolic extracts of *B. ruscifolia* against *S. aureus* (MIC=2 mg/mL). Only, the combinations of *B. ruscifolia* + *B. sagittalis* and *B. sagittalis* + *E. schikendanzii* showed beneficial effect for grampositive bacteria (additive effect).

Keywords: Antibacterial activity, plants from Argentina, single extracts, combined extracts

Resumen

En el presente trabajo, se evaluó la actividad antibacteriana de extractos de 4 plantas nativas de la región centro-este argentina (*Berberis ruscifolia, Baccharis sagittalis, Euphorbia dentata* and *Euphorbia schikendanzii*). Se seleccionaron doce extractos orgánicos para el ensayo frente a *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* y *Pseudomonas aeruginosa*. Asimismo, se determinó el efecto combinado de los extractos acetónicos. Todos los extractos mostraron actividad antibacteriana con valores de CIM comprendidos entre 16 y 2 mg/mL. Los extractos acetónico y clorofómico-metanólico de *B. ruscifolia* exhibieron la mayor inhibición frente a *S. aureus* (CIM= 2 mg/mL). Únicamente las combinaciones de *B. ruscifolia* + *B. sagittalis* y *B. sagittalis* + *E. schikendanzii* presentaron efecto benéfico (aditivo) para las bacterias grampositivas incluidas en este estudio.

Palabras Clave: actividad antibacteriana, plantas de Argentina, combinación de extractos.
INTRODUCTION
The problem of bacterial resistance is growing and the perspective of the use of antimicrobial drugs in the future is highly uncertain (Howden et al., 2010; Rahima et al., 2011; Weyland et al., 2011). This justifies the search of alternative forms for the treatment of infections, such as new compounds with bactericidal properties from natural sources like plants (Vieira et al., 2010; Mattana et al., 2010; Oliveira et al., 2007).

The Argentinean flora presents a great diversity of species which are used with commercial purposes, among these the aromatic and medicinal plants being the most required (Ordoñez et al., 2006; Petenatti et al., 2007). The genus Berberis is represented by 20 species. Some of them are known for their high medicinal value, in particular, B. ruscifolia (“Quebrachillo”) has been reported as antimalaric and for their anti-inflammatory properties. Alkaloids, mainly derived from tyrosine, have been identified as major metabolites (Del Vitto et al., 1997; Imanshahidi et al., 2008). Since 1978, the Argentinean Pharmacopoeia (Farmacopea Nacional Argentina, 1978) describes some species of genus Baccharis called “carquejas” as official drugs. B. sagittalis has been used in folk medicine to treat hepatic disorders. Clerodane diterpenes, exhibiting feeding-deterrent activity, have been isolated from this specie (Cifuentes et al., 2002). Finally, 53 species of Euphorbia (Euphorbiaceae) are recorded in the Argentinean flora. Some of them produce caustic lattices, causing a health hazard to humans and livestock (Prasad et al., 2010). Alkaloids, terpenes, glucosinolates and flavonoids have been reported from extracts of these plants. Nowadays, there is not information of the chemical and biological properties of E. dentata and E. schikendanzii.

On the other hand, it’s also known that many traditional healers rely not only with a single plant extract for therapeutic regimens. Often, they combine various plant parts and even different species in the belief that efficacy may be to enhance effectiveness and synergistic actions and to reduce toxicity (Hawkins and Ehrlich, 2007). Some in vitro antimicrobial combination studies have been undertaken to validate the role of synergism in phytotherapy. (Abu-Hijleh et al., 2009; Eja et al., 2011; Ghaleb and Mohammad, 2008).

The aim of this study was to evaluate the antibacterial properties of four native plants from different provinces of Argentina, separately as well as in combination.

MATERIAL AND METHODS
Plant material
E. dentata and E. schikendanzii were collected in La Pampa province, Argentina. B. sagittalis was collected in Mendoza province and B. ruscifolia in San Luis province, Argentina. B. sagittalis and B. ruscifolia were identified by Ing. Luis Del Vitto, and voucher specimens were deposited at the herbarium of the San Luis University (N° 8841 and 524, respectively). E. dentata and E. schikendanzii were identified by Ing. Oscar Martínez. Voucher specimens were deposited at the herbarium of the La Pampa University (Steibel, Troiani y Martinez N° 12116 and Steibel, Troiani y Prina N° 8136, respectively).

Preparations of organic extracts
Dried and powdered aerial parts (50 g) of E. dentata, E. schikendanzii, B. sagittalis and B. ruscifolia were sequentially extracted at room temperature with solvents (500 mL) of increasing polarity: acetone, chloroform-methanol (1:1) and methanol (three times for each solvent). The extracts were filtered using Whatman N° 4 filter paper, concentrated in vacuo at 40 °C using a rotary evaporator and weighed (yields obtained for acetonic, chloroform:methanolic and methanolic extracts, respectively; E. dentata: 6.3, 12.1, 15.4%; E. schikendanzii: 7.7, 11.4, 13%; B. sagittalis: 8.9; 7.5, 6.4 and B. ruscifolia: 5.3, 6.7, 15%).

Thin layer chromatography (TLC) analysis
A phytochemical screening of extracts was carried out by TLC analysis and visualization reagents. Plates of silica gel 60 GF_{254} (Merck) were used. As mobile phase, mixtures of organic solvents with different polarities, was used. Alkaloids were detected by dragendorff and iodoplatinate reagents, together with the following solvent system: toluene-ethyl acetate-diethylamine (7:2:1), ethyl acetate-methanol-water (11:1.35:1), chloroform-diethylamine (9:1) and acetone-water-ammonium (9:0.7:0.3). Flavonoids were detected by using NP/PEG reagent, aluminum chloride (10%) and UV light. Ethyl acetate-formic acid-acetic acid-water (10:1.1:1:1.2:7), n-butanol-acetic acid-water (4:1:5), ethyl acetate-formic acid-acetic acid-water (5:0.7:0.3:3:1), as solvent system. Detection of terpenes and glycosides was performed with anisaldehyde-sulphuric and vainillin-sulphuric, using...
as solvent system chloroform-methanol-water (6:4:5:1) and n-butanol-acetic acid-water (5:1:4) (Wagner and Bladt, 1996).

**Microorganisms**
The tested microorganisms were: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* CLIP 74910 and *Staphylococcus aureus* ATCC 43300. All organisms were maintained in brain-heart infusion (BHI medium) containing 20% (v/v) glycerol at -20°C (OPS Diagnostics, LLC, 2010). The inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 Mc Farland scale, (10^8 bacterial cells).

**Antibacterial activity: Determination of minimal inhibitory concentration (MIC)**
The MICs of extracts were determined by microplate method (micro-well dilution) according to CLSI method (CLSI, 2009), in tripticase soy broth (Britania, Argentina) pH 7.2 supplemented with 0.01% (W/V) of 2,3,5-triphenyltetrazolium chloride as visual indicator of bacterial growth. The inoculums were diluted 100 times (10^6 CFU/mL). The extracts were dissolved in dimethylsulfoxide (DMSO) to the highest concentration to be tested (64 mg/mL) and, then, serial two-fold dilutions were made in concentration ranges from 64 mg/mL to 0.5 mg/mL. In the assay, the final concentration of DMSO was 1% (v/v). The 96-well plates were prepared by dispensing into each well 95 µL of nutrient broth and 5 µL of the inoculum. One hundred microlitres aliquot from the stock solutions of the extracts and their serial dilutions initially prepared was transferred into eight consecutive wells. The final volume in each well was 200 µL. Controls of nutrient broth, strains, extracts and DMSO were also included in the experiment. The plates were incubated at 37°C for 24 h. Assays were performed in duplicate and then replicated at least twice. MIC values were determined for each of these combinations to establish any interaction effect. The MIC of mixture was compared with MICs of each extract separately to obtain the fractional inhibitory concentration (FIC) index (Schelz et al. 2006). The FIC is expressed as the interaction of two agents where the concentration of each tested agent in the combination is a fraction of the concentration that would produce the same effect when used independently. The FIC indices were calculated as FIC1+FIC2 where:

\[
FIC_1 = \frac{\text{MIC}_1 \text{ combination}}{\text{MIC}_1 \text{ alone}}
\]

\[
FIC_2 = \frac{\text{MIC}_2 \text{ combination}}{\text{MIC}_2 \text{ alone}}
\]

where “1” and “2” represent the different extracts in the tested combinations. The results were interpreted as synergy (≤ 0.5), addition (0.5 ≤ FIC ≤ 1), indifference (1 < FIC ≤ 4) or antagonism (FIC > 4).

**RESULTS AND DISCUSSION**
The extracts of *B. ruscifolia*, *B. sagittalis*, *E. dentata* and *E. schikendanzii* showed antibacterial activity. The
values of MIC of twelve organic extracts tested are showed in Table 1.

Only, the acetonic and chloroform-methanolic extracts of B. ruscifolia inhibited S. aureus with a MIC of 2 mg/mL.

The chloroform-methanolic extract of B. sagittalis, inhibited the development of grampositive and grammegative bacteria with a MIC varying from 4 mg/mL to 8 mg/mL but acetonic extract only inhibited grampositive bacteria (MIC = 4 mg/mL).

### Table 1

<table>
<thead>
<tr>
<th>Plant extracts</th>
<th>S. aureus</th>
<th>L. monocytogenes</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC*</td>
<td>MBC*</td>
<td>MIC*</td>
<td>MBC*</td>
</tr>
<tr>
<td>Acetone</td>
<td>2</td>
<td>4</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Chloroform:methanol</td>
<td>2</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Methanol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>B. sagittalis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Chloroform:methanol</td>
<td>8</td>
<td>32</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Methanol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>E. dentata</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Chloroform:methanol</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Methanol</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>E. schikendanzii</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Chloroform:methanol</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Methanol</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

ND: not detected at maximun concentration tested (64 mg/mL). *mg/mL.

Methanolic extracts B. ruscifolia and B. sagittalis showed no antibacterial activity against all tested bacteria. Reports from other Berberis and Baccharis species found no inhibitory effect of methanol extract on grammegative bacteria (Pasrija et al., 2011, Toribio et al., 2007).

On the other hand, the 3 extracts tested of both species of Euphorbia showed antibacterial activity between 4 and 8 mg/mL against all tested bacteria. These findings are in good accord with studies of other species of Euphorbia, for example, E. macroclada methanolic extracts showed similar MIC values for E. coli (3.12 mg/mL) but higher values for S. aureus (12.5 mg/mL) (Mohammad et al., 2010). In contrast, Chika et al. reported higher MIC values of E. hirta ethanolic extracts for E. coli (58.09 mg/mL) and S. aureus (22.55 mg/mL).

All E. dentata and E. schikendanzii extracts tested were inhibitory to E. coli and S. aureus in lower concentration than the above mentioned extracts. For this reason, our extracts are promising and could be employed in traditional and modern medical domains.

In general, MIC values were bacteriostatic. Higher concentrations (one or two fold higher than the corresponding MICs values) of extract were needed to have bactericidal effect. Only acetonic extracts of B. sagittalis showed the same values of MIC and MBC for L. monocytogenes and S. aureus (Table 1). Hugo and Russell (1984) have reported that the MBC values can either be the same or higher than the MIC values. In this study, the MIC values were either the same or lower than the MBC values, similar to the results of Karou et al. 2006. The MIC and MBC values are predictive of the efficacy of agents in-vivo. However,
the MBC values which are obtained after plating various dilutions of the extracts, is more reliable than the MIC values obtained using turbidity as an index of growth (Junaid et al., 2006).

The antimicrobial potency of plants is believed to be due to flavonoids, terpenes, alkaloids, saponins, phenolic compounds (Aboaba and Efuwape, 2001).

In the acetonic extracts of B. ruscifolia, B. sagittalis and both Euphorbia species, TLC analyses detected flavonoids as major secondary metabolites (Table 2). Antimicrobial activity of flavonoids has been reported against methicillin-resistant Staphylococcus aureus (MRSA) (Li et al., 2002; Tanaka et al., 2007). Here, in accordance with these authors, the extracts containing flavonoids showed inhibitory activity against MRSA. Such results are very interesting, because the control of MRSA infections is very difficult by therapeutic means.

Table 2
Major secondary metabolites detected by TLC analysis and visualization reagents for organic extracts of B. ruscifolia, B. sagittalis, E. dentata and E. schikendanzii.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Plants</th>
<th>E. dentata</th>
<th>E. schikendanzii</th>
<th>B. sagittalis</th>
<th>B. ruscifolia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acetone</strong></td>
<td></td>
<td>Flavonoids</td>
<td>Flavonoids</td>
<td>Flavonoids</td>
<td>Flavonoids</td>
</tr>
<tr>
<td><strong>Chloroform:methanol</strong></td>
<td>Terpenes</td>
<td></td>
<td>Terpenes</td>
<td>Terpenes</td>
<td>Alkaloids</td>
</tr>
<tr>
<td><strong>methanol</strong></td>
<td></td>
<td>Glycosides</td>
<td>Glycosides</td>
<td>Glycosides</td>
<td>Glycosides</td>
</tr>
</tbody>
</table>

On the other hand, terpenes were majoritary detected in chloroform-methanol extracts and their antibacterial activity is generally believed to involved actions on phospholipid membranes bacteria (Laciar et al., 2009). The antimicrobial activity the B. ruscifolia chloroform-methanolic extract could be attributed to the ability of the detected alkaloids to intercalate with DNA (Kumar et al., 2007). Glycosides were majoritary detected in methanolic extracts, their mechanism of action on grampositive and grampnegative bacteria has been demonstrated (Marjorie, 1999).

Synergism between plant extracts is a novel concept and could be beneficial (synergistic or additive interaction) or deleterious (antagonistic or toxic outcome). In our study, the combinations of B. ruscifolia + B. sagittalis, and B. sagittalis + E. schikendanzii showed beneficial effect for gramospositive bacteria. The MIC data of the mixtures of acetonic extracts are presented in Table 3. The FIC for L. monocytogenes (FIC = 0.6) indicated an additive effect when B. ruscifolia and B. sagittalis extracts were combined. When B. sagittalis and E. schikendanzii extracts were combined, the efficacy was enhanced for S. aureus (FIC = 1) and L. monocytogenes (FIC = 1) indicating additive effect, too. Our results showed that additive effect between plant extracts was occurred in grampositive bacteria with reduction in the MICs of the single dilution. The decrease in one or two dilution steps above the MIC values observed in this work, can be considered only as partial synergism (Eliopoulos, 1989). Indifference or antagonism was noted for all combinations against P. aeruginosa and E. coli (Table 3).

The additive effect of B. ruscifolia + B. sagittalis, and B. sagittalis + E. schikendanzii observed herein, probably, suggests the therapeutic applicability of such extracts in combination therapy. However, it is hard to predict combined effects in vivo on the basis of the presented in vitro evidence alone. It must be studied in animal models to determine their efficacy in vivo, and to elucidate their mechanisms of action.

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

REFERENCES
<table>
<thead>
<tr>
<th>Combination</th>
<th>S. aureus</th>
<th>L. monocytogenes</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
<th>FIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>S M S M FIC</td>
<td>S M S M FIC</td>
<td>S M S M FIC</td>
<td>S M S M FIC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEₐ + AEₐ</td>
<td>2 4 4 4 3 (I)</td>
<td>16 2 4 2</td>
<td>0.6 (A)</td>
<td>16 16 16 16 2 (I)</td>
<td></td>
</tr>
<tr>
<td>AEₐ + AEₐ</td>
<td>2 4 4 4 3 (I)</td>
<td>16 16 4 16</td>
<td>5 (a)</td>
<td>16 16 8 16 3 (I)</td>
<td></td>
</tr>
<tr>
<td>AEₐ + AEₐ</td>
<td>2 4 4 4 3 (I)</td>
<td>16 16 4 16</td>
<td>5 (a)</td>
<td>16 16 4 16 5 (a)</td>
<td></td>
</tr>
<tr>
<td>AEₐ + AEₐ</td>
<td>4 4 4 4 2 (I)</td>
<td>4 16 4 16</td>
<td>1 (A)</td>
<td>16 16 16 4 16 5 (a)</td>
<td></td>
</tr>
<tr>
<td>AEₐ + AEₐ</td>
<td>1 2 4 2 1 (A)</td>
<td>4 2 4 2</td>
<td>1 (A)</td>
<td>16 16 16 4 16 5 (a)</td>
<td></td>
</tr>
<tr>
<td>AEₐ + AEₐ</td>
<td>4 4 4 4 2 (I)</td>
<td>4 16 4 16</td>
<td>5 (a)</td>
<td>16 16 8 16 3 (I)</td>
<td></td>
</tr>
<tr>
<td>AEₐ + AEₐ</td>
<td>2 4 4 4 2 (I)</td>
<td>4 16 4 16</td>
<td>8 (a)</td>
<td>16 16 4 16 6 (a)</td>
<td></td>
</tr>
</tbody>
</table>


Junaid SA, Olabode AO, Bonwuliu AEJ, Agina SE. 2006. The antimicrobial properties of Ocimum gratissimum extracts on some...


