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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]

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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-oeste 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 clorofórmico-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.

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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. sagitallis* has been used in folk medicine to treat hepatic disorders. Clerodane diterpenes, exhibiting feeding-deterrent activity, have been isolated from this specie (Cifuente *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. schikendanzi*.

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. sckikendanzii* 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 Martínez 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. sckikendanzii*, *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 (yiels 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-amonium (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*-buthanol-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*-buthanol-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 disolved 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. MIC was defined as the lowest concentration of the extracts in the medium in which there was no visible growth. Assays were performed in duplicate and then replicated at least twice.

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 tripticase soya agar plates, in order to evaluate bacterial growth. MBC was defined as the lowest concentration of the extracts that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37° C. Assays were performed in duplicate and then replicated at least twice.

Determination of the combined activity of extracts

Only the acetonic extracts (AE) of *B. ruscifolia* (A), *B. sagittalis* (B), *E. dentata* (C) and *E. schikendanzii* (D) were selected because they presented the higher antibacterial activity. The 1:1 combinations of AE_A + AE_B , AE_A + AE_C , AE_A + AE_D , AE_B + AE_C , AE_B + AE_D and AE_C + AE_D were assayed. In each initial combination, the concentration of each individual extract was 64 mg/mL. Then, serial two-fold dilutions were made in concentration ranges from 64 mg/mL to 0.5 mg/mL. The 96-well plates were prepared by dispensing into each well 95 µL of nutrient broth and 5 µL of the inocula and 100µL of extracts mixture and their serial dilutions initially prepared. 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 FIC_1+FIC_2 where:

$$FIC_1 = \frac{MIC_1 \text{ combination}}{MIC_1 \text{ alone}}$$

$$FIC_2 = \frac{MIC_2 \text{ combination}}{MIC_2 \text{ alone}}$$

where "1" and "2" represent the different extracts in the tested combinations. The results were interpreted as synergy ($\leq$ 0.5), addition (0.5 $\leq$ FIC $\leq$ 1), indifference (1 < FIC $\leq$ 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 gramnegative 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
Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of organic extracts from *B. ruscifolia, B. sagittalis, E. dentata* and *E. schikendanzii*

Plant extracts	Microorganisms							
B. ruscifolia	***S. aureus***		***L. monocytogenes***		***P. aeruginosa***		***E. coli***	
	MIC*	**MBC***	**MIC***	**MBC***	**MIC***	**MBC***	**MIC***	**MBC***
Acetone	2	4	16	16	16	16	16	16
Chloroform:methanol	2	4	ND	ND	ND	ND	ND	ND
Methanol	ND	ND	ND	ND	ND	ND	ND	ND
B. sagittalis								
Acetone	4	4	4	4	16	16	16	16
Chloroform:methanol	8	32	8	16	4	8	8	16
Methanol	ND	ND	ND	ND	ND	ND	ND	ND
E. dentata								
Acetone	4	8	4	8	4	8	8	16
Chloroform:methanol	8	16	8	32	8	16	8	16
Methanol	8	16	8	16	8	16	8	16
E. schikendanzii								
Acetone	4	8	4	8	4	8	4	8
Chloroform:methanol	4	8	4	8	4	8	8	16
Methanol	4	8	8	16	4	8	4	8

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 bacterias. Reports from other *Berberis* and *Baccharis* species found no inhibitory effect of methanol extract on gramnegative 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.*

Extracts	Plants			
	E. dentata	***E. schikendanzii***	***B. sagittalis***	***B. ruscifolia***
Acetone	Flavonoids	Flavonoids	Flavonoids	Flavonoids
Chloroform:methanol	Terpenes	Terpenes	Terpenes	Alkaloids
methanol	Glycosides	Glycosides	Glycosides	Glycosides

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 gramnegative 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 grampositive 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 (Eliopoulus, 1989). Indiference 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.

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Table 3
Minimal inhibitory concentration (MIC) and Fractional inhibitory concentration (FIC) indices of acetonic extracts combinations.

Combination	*S. aureus*					*L. monocytogenes*					*P. aeruginosa*					*E. coli*				
	S	M	S	M	FIC	S	M	S	M	FIC	S	M	S	M	FIC	S	M	S	M	FIC
$AE_A + AE_B$	2	4	4	4	3 (I)	16	2	4	2	0.6 (A)	16	16	16	16	2 (I)	16	16	16	16	2 (I)
$AE_A + AE_C$	2	4	4	4	3 (I)	16	16	4	16	5 (a)	16	16	4	16	5 (a)	16	16	8	16	3 (I)
$AE_A + AE_D$	2	4	4	4	3 (I)	16	16	4	16	5 (a)	16	16	4	16	5 (a)	16	16	4	16	5 (a)
$AE_B + AE_C$	4	4	4	4	2 (I)	4	16	4	16	8 (a)	16	16	4	16	5 (a)	16	16	8	16	3 (I)
$AE_B + AE_D$	4	2	4	2	1 (A)	4	2	4	2	1 (A)	16	16	4	16	5 (a)	16	16	4	16	5 (a)
$AE_C + AE_D$	4	4	4	4	2 (I)	4	16	4	16	8 (a)	4	16	4	16	8 (a)	8	16	4	16	6 (a)

AE_A+AE_B: acetonic extract of *B. ruscifolia* + acetonic extract of *B. sagittalis*, AE_A+AE_C: acetonic extract of *B. ruscifolia* + acetonic extract of *E. dentata*, AE_A+AE_D: acetonic extract of *B. ruscifolia* + acetonic extract of *E. schikendanzii*, AE_B+AE_C: acetonic extract of *B. sagittalis* + acetonic extract of *E. dentata*, AE_B+AE_D: acetonic extract of *B. sagittalis* + acetonic extract of *E. schikendanzii*, AE_C+AE_D: acetonic extract of *E. dentata* + acetonic extract of *E.schikendanzii.* S: MIC (mg/mL) of extract used single. M: MIC (mg/mL) of extract used in mixture. (A): addition, (I): indifference, (a): antagonism

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