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Antibacterial activity of extracts and compounds isolated from the Andean medicinal plant *Azorella cryptantha* (Clos) Reiche, Apiaceae

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ABSTRACT

Azorella cryptantha (Clos) Reiche, Apiaceae, vernacular name "yerba del soldado or cuerno de cabra" is a medicinal herb that grows in the Andean mountains (Argentina). An infusion or decoction of the leaves is employed as cholagogue and digestive, usually to treat food-borne illnesses associated with enterobacteria. Extracts and compounds from two Argentinean populations were subjected to antibacterial assays against pathogenic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Yersinia enterocolitica*) and Gram (+) *Staphylococcus aureus* methicillin-sensitive and methicillin-resistant microorganisms. The antibacterial activity-guided fractionation against a panel of ATCC and clinically isolated bacteria was done according to CLSI protocols. The petroleum ether extracts from both populations showed strong antibacterial activity against *S. enteritidis* with MIC values from 125 to 250 μg/ml, and also towards methicillin-sensitive *Staphylococcus aureus*, and Gram negative strains *E. coli*, *P. aeruginosa*, *Salmonella* sp. and *Yersinia enterocolítica*-PI, (MICs between 400 and 1000 μg/ml). Fractions from the petroleum extracts showed strong antimicrobial activity, against *Escherichia coli* ATCC 25922, *E. coli*-LM$_1$, *E. coli*-LM$_2$, and *Salmonella enteritidis*-MI, with MICs values between 31.2 and 125 μg/ml. The bioassay-guided fractionation of the petroleum ether extracts led to the isolation of nine terpenes: azorellolide (**1**), mulinol (**2**), stachytriol (**3**),1α,10β,4β,5α-diepoxy-7β-germacran-6β-ol (**4**), 1β,10α,4β,5α-diepoxy-7β-germacran-6β-ol (**5**), 1,2,3,3α,4,5,6,7,8,8α-decahydro-7-(1-hydroxy-1-methylethyl)-1,4-dimethylazulene-3α,8α-diol (**6**), madreporanone (**7**), yaretol (**8**) and chrysotol or 6β,10β-epoxy-4α-hydroxyguaiane (**9**). Compounds **3**, **5**, **6** and **9** are reported here for the first time from *A. cryptantha*. Their structures and relative configurations have been determinated by means 1D and 2D NMR techniques. Chrysothol (**9**), madreporanone (**7**), and stachytriol (**3**) showed strong antimicrobial activities (MICs = 50–100 μg/ml) against enterobacteria *E. coli* and *S. enteritidis*. The antibacterial activity found for some of the isolated compounds supports least in part, the commercial exploited of this species to treat food-borne illnesses associated with Gram negative pathogenic bacteria.

1. Introduction

In the Andes Mountain, the flora's development depends on their ability to adapt to a wide variety of factors such as extreme changes of temperature, high radiation, water stress and herbivore exposure. As a result of this situation, plants produce metabolites to protect themselves, which can be an interest source of bioactive compounds.

Over the length of the Andes Mountains in South America (Peru, Ecuador, Chile, Bolivia and Argentina) numerous wild type plants are used as an alternative medical treatment. Because of that, these species are currently in high demand and its marketing in herbalists and popular markets is increasing.

Most of the species are used in local traditional medicine and employed to treat digestive and hepatic disorders, fever, coughs and colds. These include species belonging to the following genera: *Azorella*, *Baccharis* and *Senecio* (Bustos et al., 1996). Around 30 species of the *Azorella* genus develop Andean Mountains, from them

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Table 1
Botanical and vernacular name of two collections of *A. cryptantha*, voucher species and extracts yield.

Species	Vernacular name	Collection site	Vouchers species	Altitude (m.a.s.l.)	Yield (%, w/w)		
					PEE	DCME	MeOHE
Azorella cryptantha (Clos) Reiche	"yerba del soldado"	Bauchaceta, Iglesia district	CORD 1193	2700	8.90	3.30	17.40
	"cuerno de cabra"	Agua Negra, Iglesia district	CORD 1188	4000	4.51	2.02	17.00

PEE, petroleum ether extract; DCME, dichloromethane extract; MeOHE, methanol extract.

15 grow in Argentina (Martinez, 1989). The literature report the chemical composition to *A. madreporica* and *A. yareta* (Loyola et al., 1997a,b,c, 1998a,b, 2002), as well as *A. compacta* (Wächter et al., 1999) collected in Chilean Andes. Biological activities of azorellane and mulinane diterpenoids isolated from *Azorella* species, such as antiplasmodial, trichonomicidal, antituberculosis and antibacterial have been reported (Wächter et al., 1998; Loyola et al., 2001b, 2004; Areche et al., 2010; Molina-Salinas et al., 2010). The aerial parts of the *Azorella cryptantha* (Clos) Reiche, collected in the Central Andes (Argentina) is recommended and sold in herbalists to treat hepatic and intestinal disorders, as well as cleanser and genital infections.

Frequently, this species is used in traditional medicine to treat digestive problems associated with excessive food intake or contamination by bacteria. Here, we report the antibacterial properties of the extracts and isolated bioactive terpenes of the *A. cryptantha*, which were assayed against strains ATCC and isolated from patients with gastrointestinal tract infections (associated to Enterobacteriaceae).

2. Materials and methods

2.1. General reagents

Solvents analytical grade Chloroform (Fisher, US) and methanol (J.T. Baker, US), methanol HPLC grade, acetic and sulphuric acids (Merk), Silica gel F_{254} plates (Merck), *p*-anisaldehyde (Aldrich Chemical Co), Silica gel (Merck Kieselgel 60), Sephadex LH-20 (Pharmacia Inc.) were used. Dry column flash chromatography was done on silica gel (Aldrich Chemical Co).

2.2. Structural identification of the compounds and HPLC conditions

Nuclear magnetic resonance (NMR) spectra of the compounds in $CDCl_3$ were obtained in a Bruker Avance 2 (500 MHz) spectrometer, operating at 500.13 MHz for ^1H and 125.13 MHz for ^{13}C. TMS was employed as internal standard. Two-dimensional experiments (COSY, HSQC, HMBC, and ROESY) were applied using standard sequences. ESI-HRMS spectra were recorded on a Bruker micrOTOFQ2 mass spectrometer. HPLC separations were carrier out using a Thermo Separations Spectra Series P100 pump, a Thermo Separations Refracto monitor IV RI detector and a Thermo Separations Spectra Series UV100 detector, with simultaneous UV (220 nm) and RI detection. An YMC RP-18 (5 mm, 20 mm × 250 mm) column working at a flow rate of 5 ml/min was used for separations.

2.3. Plant material

A. cryptantha (Clos) Reiche (Apiaceae) collected in two locations belong to the Iglesia district in the Central Andes area, San Juan province, Argentina: Bauchaceta (2700 m.a.s.l., n.v. "yerba del soldado", *A. cryptantha*-BAU, voucher number CORD 1193) and Agua Negra (4500 m.a.s.l., n.v. "cuerno de cabra", *A. cryptantha*-AN, voucher number, CORD 1188) during flowering period (April 2010), were identified by Dr. L. Ariza Espinar in Instituto Multidisciplinario de Biología Vegetal (IMBIV-CONICET-Universidad Nacional de Córdoba). The vouchers have been saved into Museo Botánico de Córdoba (Argentina).

2.4. Preparation of extracts from A. cryptantha

Extracts were obtained from the air-dried leaves and stems (250 g finely grinded of each collection), by successive extractions with petroleum ether (PE), dichloromethane (DCM) and methanol (MeOH) under reflux. to afford PEE, DCME, and MeOHE extracts. Table 1, show percentual yield of crude extracts (%, w/w, of dry starting material), voucher number, botanical, and vernacular name of each sample.

2.5. Bioassay-guided fractionation of PEE

According to the results of antimicrobial activity, the petroleum ether extract (PEE, 20 g) was successively permeated through a Sephadex LH-20 column (column length 35 cm, diameter 4 cm) previously equilibrated with petroleum ether–methanol–chloroform (2:1:1). Some eleven fractions of 50 ml each were obtained. After TLC comparison using ethyl acetate–petroleum ether, 2:8 as mobile phase (detection under UV light and after spraying with *p*-anisaldehyde), fractions with similar TLC patterns were combined and tested in antibacterial activity (Table 2): 1 (220 mg); 2 (1467 mg); 3 (1400 mg); 4 (1052 mg); 5 (2410 mg); 6 (4813 mg); 7 (5608 mg); 8 (1992 mg); 9 (750 mg); 10 (176 mg).

Fractions **6** and **7** (9600 mg) were applied to a silica column chromatography (column length 70 cm, diameter 5 cm, containing 300 g silica gel, 0.063–0.2 mesh, Merck 60). Ten fractions were obtained (A–J) based on TLC profiles (mobile phase PE:EtOAc, 8:2). Fraction B showed the highest antibacterial effect and fractions C–E was moderate active.

2.6. Antimicrobial activity

2.6.1. Microorganisms

The following strains were used: *Staphylococcus aureus* methicillin-sensitive ATCC 29213, *Staphylococcus aureus* methicillin-resistant ATCC 43300, *Escherichia coli* ATCC 25922, *Escherichia coli*-LM_1 (LM: Laboratorio de Microbiología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina), *Escherichia coli*-LM_2, *Pseudomonas aeruginosa* ATCC 27853, *Yersinia enterocolitica*-PI (PI: Pasteur Institute), *Salmonella enteritidis*-MI (MI: Malbrán Institute), and *Salmonella sp.*-LM.

2.6.2. Antibacterial susceptibility test

The antibacterial susceptibility was conducted applied the microbroth dilution method according to CLSI (2008). Assay was executed in Mueller–Hinton broth (MHB), using strains 12 h old. The inoculum employed was $1–5 \times 10^5$ CFU/ml. Stock solutions of extracts and compounds in DMSO were prepared to give serial two-fold dilutions to obtain final concentrations between 10 and 2000 μg/ml. The plates were incubated for 24 h at 37 °C. The final concentration of DMSO was ≤1%. A positive control of antibiotic Cefotaxime (Argentia Pharmaceutica) was included, tests were done in triplicate. The plates were incubated for 24 h at 37 °C. The

Table 2
Invitro antibacterial activity of different extracts from *A. cryptantha*-BAU (Bauchaceta) and *A. cryptantha*-AN (Agua Negra) using the broth microdilution methods document M100-S18 recommended by CLSI.

	A. cryptantha-BAU			*A. cryptantha*-AN			Cf
	PEE MIC (μg/ml)	DCME	MeOHE	PEE	DCME	MeOHE	
Staphylococcus aureus methicillin-sensitive ATCC 29213	1000	1000	[a]	400	1000	[a]	0.5
Staphylococcus aureus methicillin-resistant ATCC 43300	1000	1000	[a]	1000	[a]	[a]	0.5
Escherichia coli ATCC 25922	1000	1000	[a]	1000	1000	[a]	0.5
Escherichia coli-LM_1	500	1000	[a]	500	1000	[a]	5
Escherichia coli-LM_2	500	1000	[a]	500	1000	[a]	0.5
Pseudomonas aeruginosa ATCC 27853	1000	1000	[a]	400	1000	[a]	7.5
Yersinia enterocolítica-PI	1000	1000	[a]	400	1000	[a]	0.5
Salmonella enteritidis-MI	125	500	[a]	250	1000	[a]	12.5
Salmonella sp.-LM	1000	1000	[a]	400	1000	[a]	0.5

Cf: cefotaxime,
[a] No active (MIC > 1000 μg/ml).

MIC was defined as the lowest extracts and/or compounds concentration showing no visible bacterial growth after incubation time. Tests were done in triplicate.

3. Results and discussion

3.1. Identification of bioactive compounds in fractions B–D

Bioassay-guided isolation of *A. cryptantha* PE extract showed that fractions B–D were more effective inhibiting bacterial growth. The fraction **B** (2612.4 mg) was purified by medium pressure column chromatography MPCC (column length: 70 cm, diameter: 5 cm, 100 g of silica gel) and eluted with a PE–EtOAc gradient affording 10 fractions of 200 ml each. Compound **1** (azorellolide, 3.7 mg) was obtained from fraction 4 (32.1 mg) by RP-HPLC using water–methanol 2:8 as mobile phase. The NMR data of compound **1** are in agreement with literature (Colloca et al., 2004).

The fraction **C** (721.8 mg) was permeated through a Sephadex LH-20 column (column length 35 cm, diameter 4 cm) in methanol. Fifteen fractions were obtained based on TLC profiles (mobile phase EtOAc-PE 3:7 and sprayed with *p*-anisaldehide).

Fraction C-6 (302.5 mg) was successively purified by preparative RP-HPLC using water-methanol 2:8 as mobile phase, followed by preparative TLC purification using ciclohexane–EtOAc (7:3) as eluent, to yield compounds **2** (mulinol, 11.6 mg), and **3** (stachytriol, 4.6 mg). The NMR data of these compounds are in good agreement with literature (Loyola et al., 1997b; Soliman et al., 2007).

On the other hand, the MeOH–soluble portion (1355 mg) of fraction **D** was permeated through a Sephadex LH-20 column (column length 35 cm, diameter 4 cm) in methanol. The fractions obtained were displayed similar TLC profiles were combined.

Fraction **D**-(5–6) (289 mg) was purified by RP-HPLC using water-methanol (4:6) as eluent to yield compound **4**: 1α,10β, 4β,5α-diepoxy-7β-germacran-6β-ol (23 mg), and its isomer **5**: 1β,10α,4β,5α-diepoxy-7β-germacran-6β-ol (20.6 mg). Additionally, a complex mixture (88.6 mg) was further purified by RP-HPLC using water–methanol (3:7) as mobile phase to yield 0.5 mg of compound **6**:1,2,3,3a,4,5,6,7,8,8a-decahydro-7-(1-hydroxy-1-methylethyl)-1,4-dimethylazulene-3a,8a-diol, and 2.7 mg of compound **7**: madreporanone. All spectroscopic data of the isolated compounds are in agreement with literature (Sanz and Marco, 1991; Anh et al., 1996; Loyola et al., 2002; Colloca et al., 2004).

Fraction **D**-(7–8) (131 mg) was chromatographed on silica gel (column length 47 cm, diameter 2.5 cm, 50) with a PE–EtOAc gradient affording 210 fractions of 25 ml each. After TLC comparison (silica gel, PE–EtOAc 7:3), the fractions with similar TLC patterns were pooled into eight fractions. From fraction 4, was purified by RP–HPLC using water–methanol (3:7) as mobile phase to yield compound **8** (yaretol, 17 mg). The NMR data of compound **8** are in agreement with literature (Loyola et al., 2002).

Fraction **E** (1444 mg) was permeated through a Sephadex LH-20 column (column length 35 cm, diameter 4 cm) in methanol. Eight fractions were obtained based on TLC profiles. The fraction **E**-8 (294.6 mg) was chromatographed on silica gel (column length 45 cm, diameter 2 cm, 100 g) with an EP–EtOAc and then for successive purifications by RP–HPLC to yield compound **9**: chrysotol or 6β,10β-epoxy-4α-hydroxyguaiane (5 mg) being coincident NMR data with literature (Ahmed et al., 2006). The fractionation is summarized in Fig. 1.

3.2. Antibacterial activity

Food-borne illnesses associated with Gram (+) and Gram (−) bacteria including *Staphylococcus aureus*, *E. coli* and *S. enteritidis* present a major world-wide public health concern (Abad et al., 2013).

Table 2 shows the antibacterial activity of the *A. cryptantha* extracts (collected in Bauchaceta and Agua Negra localities). Both, PEE showed activity against Gram (+) and Gram (−) bacteria with MICs values between 125 and 1000 μg/ml. The Gram (−) bacteria *S. enteritidis*-MI was the most sensitive strain with MIC values of the125 μg/ml and 250 μg/ml for PEE of A. *cryptantha*-BAU and *A. cryptantha*-AN, respectively. Also the PEE extract of *A. cryptantha*-AN displayed interesting activities against *P. aeruginosa*, *Yersinia enterocolítica*-PI, and *Salmonella* sp.-LM, presented MIC values of 400 μg/ml, as well as to *S. aureus* methicillin-sensitive.

Both PEE of *A. cryptantha*-BAU and *A. cryptantha*-AN showed similar activities (MIC = 500 μg/ml) against clinical isolated *E. coli* (*E. coli*-LM_1 and *E. coli*-LM_2).

In order to identify the bioactive compounds, a bioassay-guided fractionation was performed of the PEE *Azorella cryptanta*-BAU extract. In Table 3, the antimicrobial activity of fractions, subfractions and pure isolated compounds of the PEE from *Azorella cryptanta*-BAU, the MICs values are expressed as μg/ml are shown.

Fractions **6** and **7**, which displayed an important antibacterial activity against *E. coli*-LM_1, *E. coli*-LM_2, and *S. enteritidis*-MI, with MIC values between 125 and 250 μg/ml were further chromatographed on sílica gel affording 12 fractions (A–J). One of them, subfraction B–E, showed a strong antibacterial activity towards *E. coli*-LM_1, and E. *coli*-LM_2 and *S. enteritidis*-MI (MIC range = 31.2–100 μg/ml) and which were sucessively purified by Sephadex LH 20, silica gel column and preparative HPLC affording: azorellolide (**1**), mulinol (**2**), stachytriol (**3**),1α,10β,4β,5α-diepoxy-7β-germacran-6β-ol (**4**), 1β,10α,4β,5α-diepoxy-7β-germacran-6β-ol (**5**),

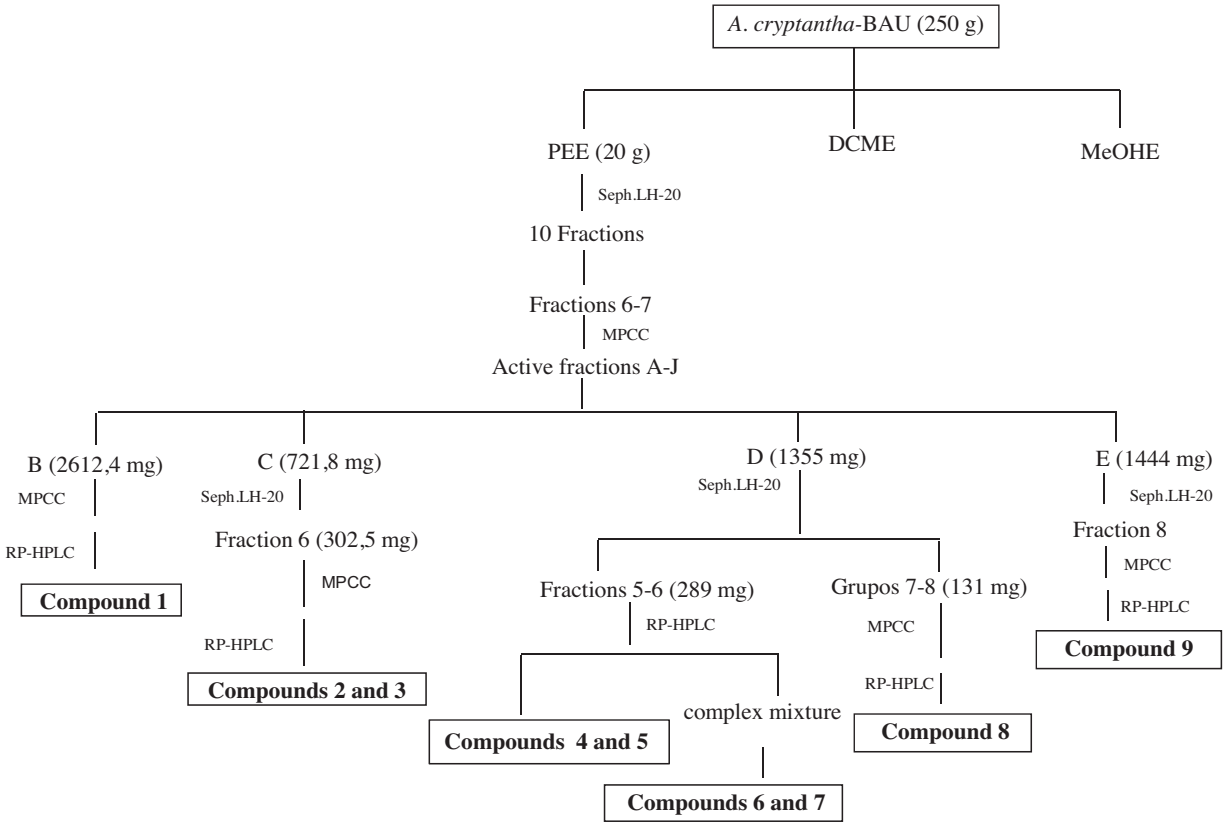


Fig. 1. Bio-guided fractionation of antibacterial compounds from petroleum ether (PEE) of *A. cryptantha* aerial parts.

1,2,3,3a,4,5,6,7,8,8a-decahydro-7-(1-hydroxy-1-methylethyl)-1,4-dimethylazulene-3a,8a-diol (**6**), madreporanone (**7**), yaretol (**8**) and chrysotol o 6β,10β-epoxy-4α-hydroxyguaiane (**9**) (Fig. 2).

Yaretol (**8**) and chrysothol (**9**), showed the stronger antimicrobial activity (MICs = 50–100 μg/ml) against enterobacteria *E. coli*-LM$_1$, and *E. coli*-LM$_2$ and *S. enteritidis*-MI clinical isolated. While stachytriol (**3**) displayed a strong activity against *S. enteritidis* with value of MIC = 100 μg/ml.

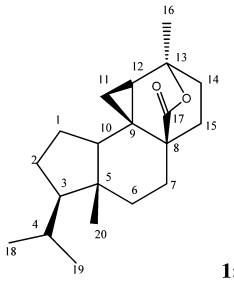
Recently, the antibacterial activity of *A. cryptantha* essential oil against Gram (+) and Gram (−) bacteria was reported (López et al., 2012). Some compounds isolated from *A. cryptantha*-BAU were previously identified in other species of the *Azorella* genus, such as mulinol (**2**) isolated from *A. compacta* (Loyola et al., 1997b), and madreporanone (**7**) identified from *A. madreporica* (Loyola et al., 2002). Previously Colloca et al. (2004), reported the isolation of azorellolide, dihydroazorellolide, yaretol and 1a,10b,4b,5a-diepoxy-7a-germacran-6b-ol from *A. cryptantha.*

While, stachytriol (**3**) was reported as a constituent of *Stachys yemenensis* (Soliman et al., 2007), and the 1α, 10β, 4β, 5α-diepoxy-7β-germacran-6β-ol (**4**) was isolated from *Pallenis spinosa* (Sanz and Marco, 1991). The sesquiterpene1,2,3,3a,4,5,6,7,8,8a-decahydro-7-(1-hydroxy-1-methylethyl)-1,4-dimethylazulene-3a,8a-diol (**6**) was obtained from *Caryodaphnosis tonkinensis* (Anh et al., 1996), and with respect to chrysotol **(9)** was isolated from *Chrysothamnus viscidiflorus* (Ahmed et al., 2006).

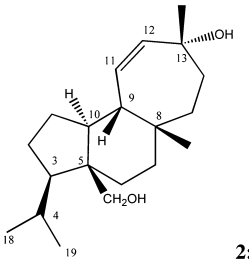
Table 3
Antibacterial activity (fractions, subfractions and compounds) from *Azorella cryptanta*-BAU petroleum ether extract (MIC in μg/ml).

Bacteria											Cf
	Sephadex LH20 fractions from *A. cryptantha*-BAU petroleum ether extract										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
E. coli-LM$_1$	>500	>500	>500	>500	250	125	250	250	500	500	5
E. coli-LM$_2$	>500	>500	>500	500	500	125	250	500	500	500	0.5
S. enteritidis-MI	>500	>500	>500	500	250	125	125	125	200	250	12.5
	Fraccions from Sephadex LH20 of **6–7**										
	A	B	C	D	E	F	G	H	I	J	
E. coli-LM$_1$	>150	62.5	100	100	100	>150	>150	>150	>150	>150	5
E. coli-LM$_2$	>150	125	100	100	100	>150	>150	>150	>150	>150	0.5
S. enteritidis-MI	>150	50	50	100	100	>150	>150	125	>150	>150	12.5
	Compounds										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	–	
E. coli-LM$_1$	>100	>100	>100	>100	>100	>100	>100	100	50	–	5
E. coli-LM$_2$	>100	>100	>100	>100	>100	>100	>100	100	50	–	0.5
S. enteritidis-MI	>100	>100	100	>100	>100	>100	>100	100	50	–	12.5

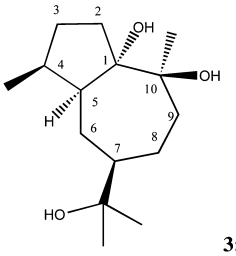
(**1**), azorelloride; (**2**), mulinol; (**3**), stachytriol; (**4**), 1α, 10β, 4β, 5α-diepoxy-7β-germacran-6β-ol; (**5**), 1β, 10α, 4β, 5α-diepoxy-7β-germacran-6β-ol; (**6**), 1,2,3,3a,4,5,6,7,8,8a-decahidro-7-(1-hydroxy-1-methylethyl)-1,4-dimetilazulene-3a,8a-diol; (**7**), madreporanone; (**8**), yaretol; (**9**), chrysotol, Cf: cefotaxima.



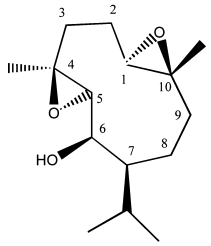
1: azorellolide



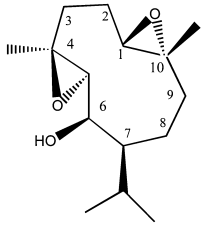
2: mulinol



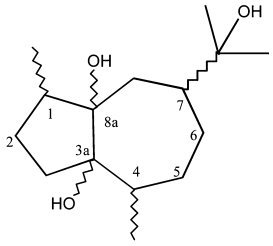
3: stachytriol



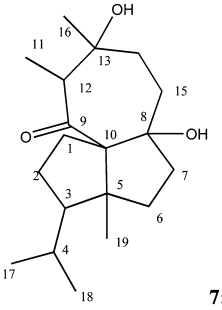
4: 1α, 10β, 4β, 5α-diepoxy-7β-germacran-6β-ol



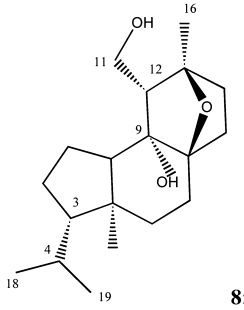
5: 1β, 10α, 4β, 5α-diepoxy-7β-germacran-6β-ol



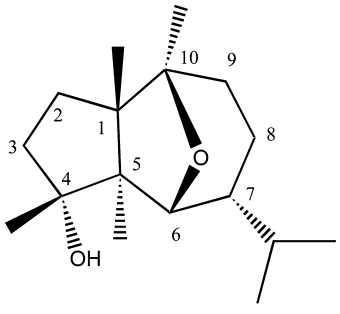
6: 1,2,3,3a,4,5,6,7,8,8a-decahidro-7-(1-hydroxy-1-methylethyl)-1,4-dimetilazulene-3a,8a-diol



7: madreporanone



8: yaretol



9:chrysotol

Fig. 2. Compounds **1–9** from *A. cryptantha*-BAU petroleum ether extract.

Areche et al. (2010) reported antimicrobial effect of the diterpenes isolated from *Azorella madreporica*.

Stachytriol (**3**), 1β,10α,4β,5α-diepoxi-7β-germacran-6β-ol (**5**), 1,2,3,3a,4,5,6,7,8,8a-decahydro-7-(1-hydroxy-1-methylethyl)-1,4-dimethylazulene-3a,8a-diol (**6**) and chrysotol or 6β,10β-epoxy-4α-hydroxyguaiane (**9**) are reported here for first time for *A. cryptantha*. These results are in accordance with previous report on argentinian medicinal plants to treat to bacterial infections (Zampini et al., 2009).

4. Conclusions

The results of the present study showed that the *A. cryptantha* extract present potential antimicrobial activity. The PE extract, fractions and compounds of *A. cryptantha* show a stronger antibacterial activity against enterobacteria *E. coli*-LM_1, *E. coli*-LM_2, and *S. enteritidis*-MI. Of the nine compounds isolated an identified, chrysothol (9) was the most active. The strong antibacterial effect of *A. cryptantha* supports at least in part, the ethnopharmacological use, and its reputed properties for commercialization of this plant. These results could be useful for developing pharmaceuticals preparations to treat food-borne illnesses associated with enterobacteria.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2014.10.065.

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