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RESEARCH ARTICLE

Potential of *Erigeron bonariensis* L. essential oil and its polyacetylene 2-*cis*-lachnophyllum acid methyl ester as neutralizers of bothropic venoms *in vitro*

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Abstract

Snake envenoming causes human organic multisystem failures and local effects (LEs: hemorrhage, edema and necrose). The antivenom serum is not always effective, generating in extreme cases anaphylaxis and being useless against LEs. An alternative under research is the treatment with alexiteric medicinal plants containing metabolites able to interact and inactivate/neutralize the venom 's components. As, in this context, *Erigeron bonariensis* L. is a promising species, we aimed to study the alexiteric potential of its essential oil from Uruguay. As assessed by GC/MS, the main components were: 2-*cis*-lachnophyllum acid methyl ester (*cis*-LAME,32.8%), 4*R*-(+)-limonene (22.6%), germacrene D (8.1%), *trans*-*β*-ocimene (6.9%) and 1*S*-(-)-*β*-pinene (4.8%). *cis*-LAME was isolated (column chromatography) and identified by spectroscopic methods. The oil and *cis*-LAME were incubated with *Bothrops diporus* and *B. alternatus* venoms, indicating alexiteric potential based on a screening performed by 1D-SDS-PAGE. For *B. diporus* venom (which causes more envenoming cases in Argentina) were conducted other *in vitro* tests (inhibition of proteolysis, hemolysis and procoagulant activity). The samples inhibited proteolysis and hemolysis (26.3% and 57.9% for the oil and *cis*-LAME, respectively), whereas only *cis*-LAME inhibited procoagulant activity (60.6%). The results

obtained confirmed the potential of *E. bonariensis* to neutralize bothropic venoms, highlighting *cis*-LAME as a key bioactive component.

Keywords: Asteraceae; snake venom; alexiteric activity; medicinal plants.

Introduction

According to the World Health Organization (WHO), snakebite envenoming (ophidism) is a Neglected Tropical Disease, due to the fang injection of the venom in the body could cause an organic multisystem failure, affecting not only the contact area but also systematically the lungs, kidneys, brain, liver, muscles, and heart[1]. Every year, ophidism causes more than 138.000 mortal victims worldwide in severe cases for sensitive people, frequently when no medical treatments are applied to counteract the deleterious effects because the accidents occurred far away from a public health center[1]. The conventional medical allopathic treatment (antivenom serum composed of protein antibodies obtained from horses after inoculating small amounts of snake venoms) has at the same time many undesirable effects including allergic reactions which may progress to severe anaphylaxis cases, with risk of death[2]. The antivenoms, despite acting systemically and being able to save lives, are not effective against the local effects of the venoms, which include hemorrhage, edema and necrose (leading to amputation of the affected members in some cases)[2]. The severity of ophidism and the incomplete efficacy of antivenom therapy highlight the need for complementary approaches, such as Phytotherapy. In South America, among the most dangerous venomous snakes are those of the genus *Bothrops* (Viperidae), commonly known as “pit vipers”, which include at least 48 species in the region[3,4]. All around the world, and particularly in South America, ethnobotanical specific surveys have demonstrated the potential of medicinal plants to treat the deleterious effects caused by the venomous snakebites, as reported in Brazil[5] and Colombia[6]. Specialized phytometabolites have been proposed for modern pharmacology research as possible neutralizers of the snake venoms and their effects (exerting alexiteric activity), both as pure substances or extracts[7-10].

In South America, several species of the Asteraceae family have been pointed out as relevant for neutralizing snake venoms (in raw state, or to inhibit the main protein components, as the case of phospholipase A_2 and metalloproteinases), as the case of *Baccharis* spp. L.[11-16], *Blainvillea acmella* (L.) Philipson[17,18] (synonym: *Eclipta prostata* Lour.), *Mikania glomerata* Spreng.[19]; *Trixis antimenorrhoea* (Schrank) Kuntze [synonym: *Trixis divaricata* (Kunth) Spreng.][20]; *Ambrosia polystachya* DC. and *Tithonia diversifolia* (Hemsl.) A. Gray[14], among others. In addition, the essential oil of *Erigeron bonariensis* L. (synonym: *Conyza bonariensis* Cronquist) has been highlighted by its capacity to counteract the effect of the snake venoms *in vitro* [14]. The chemical composition of this essential oil has been extensively investigated worldwide ([21] and references therein). However, no studies have characterized the essential oil profile of this species in Uruguay, nor has been explored its potential as a source of isolated compounds capable of neutralizing *Bothrops* venoms.

Thus, the aim of this work was to perform a systematic evaluation of the *in vitro* activity of *E. bonariensis* essential oil obtained from plant material from Uruguay against bothropic venoms (models: *Bothrops diporus* and *B. alternatus*), as well as to assess the biological activity of its major polyacetylene compound, 2-*cis*-lachnophyllum acid methyl ester (hereafter *cis*-LAME). Besides, the chemical composition of the essential oil was evaluated by gas chromatography/mass spectrometry (GC/MS) protocols, including conventional and enantioselective analyses.

Material and Methods

Plant material and snake venoms

Selected healthy aerial parts of *E. bonariensis* were sampled at blooming stage (autumn), in Southern Uruguay, Canelones Department, "Los Cerrillos" area. A voucher sample of the species was deposited in the Faculty of Chemistry, University of the Republic (MVFQ 3424 E. Alonso Paz, *in memoriam*).

Venom pools from captive specimens of *B. alternatus* and *B. diporus* were employed, collected via the milking method by trained personnel at the Interactive Center for Venomous Snakes (CISVA), Faculty of Veterinary Sciences, National University of the Northeast (UNNE), Corrientes, Argentina[16].

Essential oil extraction

The extraction of *E. bonariensis* essential oil was performed by hydro-distillation as previously described[22], employing 200 g of finely powered one-week dried plant material placed in a rounded flask, to which was attached a Clevenger-type glass device. Distillation took place for 90 min, and after recovery of the essential oil, anhydrous sodium sulfate was added (Sigma-Aldrich, St. Louis, MI, USA). Finally, the oil was stored under refrigeration (−4°) until being analyzed and used in biological assays. Yield: 0.65% v/v.

Gas chromatography/mass spectrometry (GC/MS) analyses

The conventional GC/MS analysis was performed as previously described[23] using a Shimadzu GC 2010-QP 2020 instrument (Shimadzu, Kyoto, Japan), equipped with a Rxi-5MS capillary column of 30 m length × 0.25 mm i.d. × 0.25 µm film thickness (5% diphenyl-95%-dimethylpolysiloxane; Restek, Bellefonte, PA, USA). The oven temperature program was as follows: 40°C (4 min), 40 to 180°C at 4°C.min^{-1}, 180°C (2 min), 180 to 280°C at 10°C.min^{-1}, 280°C (10 min) (total run time: 61 min)[22]. Injector, interface and ion source temperatures: 280°C. Carrier gas flow (Helium 99.999% pure; Linde, Woking, UK): 1.0 mL.min^{-1}, kept constant. Injection: 1.0 µL of a 1:100 diluted solution in *n*-hexane (Carlo Erba, Val-de-Reuil, France). The mass spectrometer was operated in electron ionization mode (70 eV), with a m/z scan range of 50-350 a.m.u. Commercial mass spectral libraries were employed for comparison of the fragmentation patterns obtained for each chromatographic peak[24,25]. Linear Retention Indices (LRIs) were determined by injecting a solution of *n*-alkanes (C_8-C_{20}; Sigma-Aldrich) in the same analytical conditions as the samples, and comparing the values obtained with Adams[24], unless other sources are specified. The identified peak raw areas were informed as compounds' abundances (%) without normalization.

GC/MS enantioselective analyses were performed in duplicate for the determination of the enantiomeric distribution of selected monoterpene hydrocarbons of *E. bonariensis* essential oil (*α*-pinene, sabinene, *β*-pinene, and limonene). For this purpose, it was performed the raw area comparison of both enantiomers' peaks obtained after the elution in a capillary column composed of a derivatized *β*-cyclodextrin (chiral selector). The column employed was a CycloSil-B (30% heptakis-2,3-di-*O*-methyl-6-*O*-*tert*-butyl-dimethylsilyl-*β*-cyclodextrin in 70% DB1701 matrix; dimensions: 30 m length × 0.25 mm i.d. × 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA), which was attached to a GC HP6890 coupled to MSD 5973 (Hewlett-Packard; Palo Alto, CA, USA). The oven temperature programming was as follows: 65°C (1 min), 65 to 100°C at 1°C.min^{1}, 100°C (1 min), 100 to 150°C at 2°C.min^{-1}, 150 to 220°C at 10°C.min^{-1}, 220°C (3 min) (total run time: 72 min)[23]. Injector, interface and ion source temperatures: 220°C. The other GC/MS

parameters were the same as above reported for conventional GC/MS analysis. The enantiomers elution order was obtained from the literature employing the same chemically composed stationary phase[26].

Isolation of *cis*-LAME

The polyacetylene *cis*-LAME was isolated from *E. bonariensis* essential oil according to our previous report[27]. Briefly, an open column chromatography was packed with previously activated Silica gel (230-400 mesh; Merck, Darmstadt, Germany) as stationary phase and eluted with a static gradient of *n*-hexane:CH_2Cl_2 (5:1; Cicarelli Lab); flow: 1.8 $mL.min^{-1}$. A total of 24 fractions (around 2.0 mL) were collected. The chromatographic separation was monitored by TLC (Silica Gel 60 mesh F_{254}; mobile phase: *n*-hexane-CH_2Cl_2 2:1; Cicarelli Lab); visualization: UV: 254 nm and *p*-anisaldehyde-sulphuric acid visualization reagent[28]. *cis*-LAME was obtained with 96.4% (GC/MS) purity. The identity of the compound was verified by obtaining of UV-Vis spectra (Beckman DU-610, Fullerton, CA, USA), NMR (Bruker BZH 200/52; Billerica, MA, USA), FT-IR (PerkinElmer GX FTIR provided with a DGTS detector; Shelton, CT, USA), and MS (GC HP6890 and MSD 5973; Hewlett-Packard), by comparing with our previous report[27] and the literature[29-31].

Neutralization of bothropic venoms

The neutralization of the venoms was performed as recently described in detail[16]. In summary, a screening for alexiteric activity was performed for the venoms of both *Bothrops* spp., *B. diporus* and *B. alternatus*, which were incubated with the pure essential oil from *E. bonariensis* and *cis*-LAME, by evaluating the modification of the protein profile using 1D-SDS-PAGE (ratio 1:7). Subsequently, given that the species causing the most of the ophidian accidents is *B. diporus* (90% of accidents caused by *Bothrops* spp. in Argentina; Torres *et al.*[2]), further research was conducted on the *in vitro* inhibition activities against its venom, such as proteolysis inhibition (casein model; ratio 1:120), indirect hemolysis inhibition (agar blood model; ratio 1:40), and inhibition of procoagulant activity (evaluated with a coagulometer; ratio 1:10)[16]. Carquejone (5-methylidene-6-prop-1-en-2-yl-cyclohex-2-en-1-one) and AcOEt extracts of *B. articulata* and *B. genistelloides* var. *crispa* were selected as positive controls for alexiteric activity and for comparison with our previous research[15,16], while the negative control was the solvent in which the samples were diluted (EtOH).

Data availability

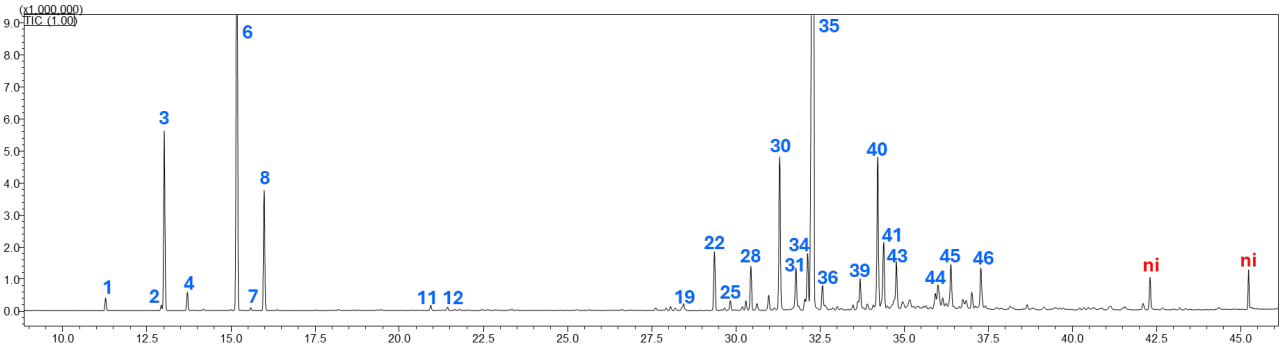
Some data of this article **(FIGURES S1 to S9)** are available at the Figshare online repository[32].

Results and Discussion

Chemical composition of *E. bonariensis* essential oil

FIGURE 1 shows the conventional GC/MS analysis of *E. bonariensis* essential oil from Uruguay in Rxi-5MS capillary column, while in **TABLE 1** is summarized the chemical composition obtained.

FIGURE 1: Chromatogram GC/MS (conventional analysis) of *E. bonariensis* essential oil in Rxi-5MS capillary column. For the analytical conditions see Materials and Methods. The numbers of the peaks are in accordance with the numbers provided in TABLE 1; ni: not identified.



A total of 46 components were identified for *E. bonariensis* essential oil from Uruguay, accounting for a percentage of identification of 91.9% in CycloSil-B column and 93.4% in Rxi-5MS (**TABLE 1**). The main group of identified components were the monoterpene hydrocarbons, followed by C_{10} polyacetylenes, and then sesquiterpene hydrocarbons (**TABLE 1**).

TABLE 1: Chemical composition of *E. bonariensis* essential oil from Uruguay.

Peak #	LRIexp[a]	LRIteo[b]	%A R-5MS[c]	%A Cycl-B[d]	Compound name
1	932	932	0.3	0.4	*α*-pinene
2	972	969	0.1	0.3	sabinene
3	975	974	4.8	4.9	*β*-pinene
4	991	988	0.5	0.7	myrcene
5	1024	1020	0.03	nd	*p*-cymene
6	1028	1024	25.3	23.4	limonene
7	1039	1032	0.08	nd	*cis*-*β*-ocimene
8	1049	1044	3.1	6.9[e]	*trans*-*β*-ocimene
9	1059	1054	0.03	0.2	*γ*-terpinene
10	1105	1106[f]	0.04	nd	6-methyl-3,5-heptadiene-2-one
11	1178	1174	0.2	0.3	terpinen-4-ol
12	1191	1186	0.1	nd	*α*-terpineol
13	1197	1194	0.04	nd	myrtenol
14	1219	1215	0.05	nd	*trans*-carveol
15	1245	1239	0.05	nd	carvone
16	1379	1374	0.07	nd	*α*-copaene
17	1383	1380	0.1	nd	daucene
18	1393	1387	0.07	0.1	*β*-cubebene

Peak #	LRIexp[a]	LRIteo[b]	%A R-5MS[c]	%A Cycl-B[d]	Compound name
19	1395	1389	0.3	0.1	*β*-elemene
20	1396	1390	0.04	nd	sativene
21	1416	1409	0.02	nd	*α*-gurjunene
22	1424	1417	1.7	1.6	*trans*-caryophyllene
23	1431	1434	0.04	nd	*γ*-elemene
24	1433	1430	0.09	nd	*β*-copaene
25	1439	1432	0.3	0.1	*α-trans*-bergamotene
26	1450	na	0.1	nd	lachnophyllum lactone isomer (probably 4-*trans*)[g]
27	1454	1453	0.3	0.2	geranyl acetone
28	1458	1454	1.2	0.8	*trans-β*-farnesene
29	1481	1478	0.08	nd	*γ*-muurolene
30	1486	1484	4.6	8.1	germacrene D
31	1501	1500	1.2	3.7	bicyclogermacrene
32	1504	1500	0.1	nd	*α*-muurolene
33	1510	1508[h]	0.3	nd	2-*trans*-lachnophyllum acid methyl ester
34	1513	1516[h]	1.8	2.3	lachnophyllum lactone isomer (probably 4-*cis*)[g]
35	1519	1516[h]	31.0	32.8	2-*cis*-lachnophyllum acid methyl ester
36	1528	1522	0.8	0.3	*δ*-cadinene
37	1530	1521	0.2	0.1	*β*-sesquiphellandrene
38	1563	1559	0.3	0.2	germacrene B
39	1566	1561	0.9	0.7	*trans*-nerolidol
40	1583	1577	4.8	2.0	spathulenol
41	1589	1582	2.4	0.7	caryophyllene oxide
42	1593	1594	0.07	0.5	salvia-4(14)-en-1-one
43	1602	1594	1.9	0.3	carotol
44	1646	1640	1.1	nd	*α-epi*-muurolol
45	1660	1652	1.5	0.5	*α*-cadinol
46	1692	1685	1.3	nd	germacra-4(15),5,10(14)-trien-1-*α*-ol
Identified (%)			93.4	91.9	
C_{10} hydrocarbons (%)			34.2	36.8	
C_{10} oxygenated (%)			0.7	0.5	

Peak #	LRIexp[a]	LRIteo[b]	%A R-5MS[c]	%A Cycl-B[d]	Compound name
C_{15} hydrocarbons (%)			11.2	15.1	
C_{15} oxygenated (%)			14.0	4.7	
Polyacetylenes (%)			33.2	35.1	
Others (%)			0.1	nd	

Legend: The chromatographic peaks considered were those exhibiting a signal-to-noise ratio higher than 3. References: (a): LRIexp, linear retention indices determined in this work; (b): LRIteo, linear retention indices informed by Adams[24]; (c): abundance percentage in Rxi-5MS analytical column; (d) abundance percentage in CycloSil-B analytical column; (e) co-elution of both ocimenes; (f): LRIteo, linear retention indices informed by Linstrom *et al.*[33]; (g): mass spectra provided in Supplementary Materials (**FIGURES S1** an **S2**)[32]; (h): LRIteo, linear retention indices informed by Minteguiaga *et al.*[34]; (nd): not detected; (na): not available.

As expected from the essential oil composition of *E. bonariensis* reported in the literature[21], the polyacetylene *cis*-LAME [IUPAC name: methyl (*Z*)-deca-2-en-4,6-diynoate] was one of the main components reaching approximately 31.0-32.8% of abundance in the Uruguayan sample (**TABLE 1**, **FIGURE 1**). In fact, hydro-distilled essential oils obtained from the aerial plant materials from other countries also reported it as one of the main ones, i.e. for Brazil (57.2%; Ferreira *et al.*[35]), Greece (10.8-21.2%; Tzakou *et al.*[36]), Italy (14.2%; Benzarti *et al.*[37]), Pakistan (24.9%; Abbas *et al.*[38]), and Togo (9.8%; Adande *et al.*[39]). However, there are also reported *E. bonariensis* essential oils without *cis*-LAME, as for Brazil[40], Venezuela[41] and Tunisia[42], among other countries. Two lachnophyllum lactone isomers were detected by their mass spectra comparison with the literature (see **Supplementary Material**, **FIGURES S1** and **S2**)[32], and despite in our work was not possible to identify the stereochemistry of both, the peak at $LRI_{Rxi\text{-}5MS}$ 1513 (peak 34, **TABLE 1**) probably corresponds to the 4-*cis* isomer because it was the most abundant in our conditions (1.8-2.3%) as was previously informed in the literature for *E. bonariensis* from Spain[43] and Togo[39]. Similarly, the peak at $LRI_{Rxi\text{-}5MS}$= 1450 (peak 26, **TABLE 1**) could be assigned tentatively to the 4-*trans*-lachnophyllum lactone, unreported in the literature for this plant up to our knowledge, and present at 0.1% or lesser in our work (**TABLE 1**). Previously, we identified a lachnophyllum lactone isomer co-eluting with the *cis*-LAME peak in GC/MS, which was then resolved using GCxGC/HRTOFMS[34]. This result matches well with the peak now tentatively identified as the 4-*cis* isomer, because both exhibited closer LRI values in Rxi-5MS: 1513 and 1519 for the lactone and the *cis*-LAME, respectively. Moreover, in our work the matricaria acid methyl esters were not found (these compounds are related to the lachnophyllum acid methyl esters, but having an additional double bond), as reported in the literature[36,42,44]. Other relevant components of *E. bonariensis* essential oil found in this work were limonene (23.4-25.3%), germacrene D (4.6-8.1%), *trans*-*β*-ocimene (3.1-6.9%), *β*-pinene (4.8-4.9%), spathulenol (2.0-4.8%), and bicylogermacrene (1.2-3.7%) (**TABLE 1**). All of them previously reported for essential oils of *E. bonariensis* growing in different geographical regions[21].

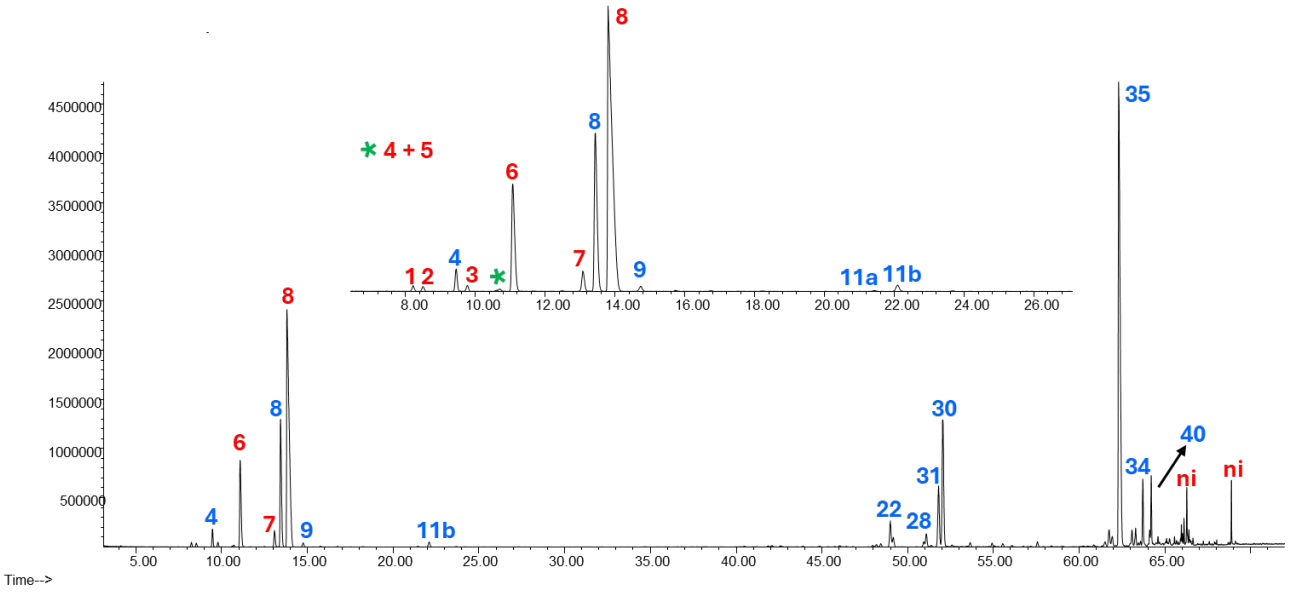
Enantiomeric distribution of selected monoterpene hydrocarbons

FIGURE 2 shows the enantioselective GC/MS analysis of *E. bonariensis* essential oil from Uruguay using a CycloSil-B capillary column, while in **TABLE 2** it is summarized the enantiomeric distribution found for the selected chiral monoterpene hydrocarbons (*α*-pinene, sabinene, *β*-pinene, and limonene). Both *cis*-LAME and lachnophyllum lactone are not chiral (**FIGURE 3**), thus the enantioselective analyses did not apply for such compounds. For other minor or trace-level chiral monoterpene components of *E. bonariensis* essential

oil such as terpinen-4-ol and *α*-terpineol (among others), the enantiomeric distribution could not be determined with confidence, so it was not informed in this report (e.g. peaks 11a and 11b in **FIGURE 2**).

For *E. bonariensis* essential oil, predominated the following enantiomers of the selected chiral hydrocarbons monoterpenes (enantiomeric distribution higher than 95%): 4*R*-(+)-limonene and 1*S*-(-)-*β*-pinene (**TABLE 2**). Whilst the predominance of (1*R*,5*R*)-(+)-sabinene over its counterpart was approximately 2:1, and for *α*-pinene almost a racemic mixture was evidenced (**TABLE 2**). Up to our knowledge, no previous enantioselective analysis has been conducted for this species. Overall, enantioselective studies of major monoterpene hydrocarbons in *Erigeron* spp. and other Asteraceae remain scarce. Thus, for comparison purposes, in **TABLE 2** are included data on the limonene enantiomeric distribution of *E. canadensis*[45], as well as data comparison with another closely related Asteraceae species, *Baccharis tridentata* Vahl., for which the determination of the corresponding enantiomeric distribution values was performed as in the present work[23]. In that way, 4*R*-(+)-limonene appeared to be highly predominant over the 4*S*-(-) counterpart in *Erigeron* genus, a fact not evidenced for *Baccharis* genus. The enantiomeric distributions observed in *E. bonariensis* clearly differ from those of *B. tridentata*, including opposite trends for sabinene and *β*-pinene enantiomers (**TABLE 2**), highlighting their chemotaxonomic significance. Additional studies are required to determine whether these patterns remain consistent across seasons and throughout different ontogenetic stages.

FIGURE 2: Chromatogram GC/MS (enantioselective analysis) of *E. bonariensis* essential oil in CycloSil-B capillary column.



References: For the analytical conditions see Materials and Methods. The numbers of the peaks that are in blue are in accordance to the numbers provided in **TABLE 1**, while the numbers in red are in accordance with the numbers shown in **TABLE 2**; ni: not identified.

TABLE 2: Enantioselective GC/MS analysis results for *E. bonariensis*.

Peak #	Retention time (min)	Compound name	Raw Area	%A Cy-B[a]	ED *Eb* (%)[b]	ED *Ec* (%)[c]	ED *Bt* (%)[d]
1	8.22	1*S*-(-)-*α*-pinene	2005218	0.2	52.8	-	84.3
2	8.50	1*R*-(+)-*α*-pinene	1791232	0.2	47.2	-	15.7
3	9.77	(1*R*,5*R*)-(+)-sabinene	2096669	0.2	68.9	-	24.0

Peak #	Retention time (min)	Compound name	Raw Area	%A Cy-B[a]	ED *Eb* (%)[b]	ED *Ec* (%)[c]	ED *Bt* (%)[d]
4	10.70	(1*S*,5*S*)-(-)-sabinene	947120	0.1	31.1	-	76.0
5	10.63	1*R*-(+)-*β*-pinene	503951	0.1	1.0	-	73.0
6	11.07	1*S*-(-)-*β*-pinene	51337696	4.8	99.0	-	27.0
7	13.07	4*S*-(-)-limonene	8831382	0.8	3.6	1.1	33.8
8	13.80	4*R*-(+)-limonene	235579745	22.6	96.4	98.9	66.2

References: (a): Abundance percentage in CycloSil-B analytical column; (b): enantiomeric distribution (ED) percentage obtained; (c): ED informed by Lis *et al*.[45] for *E. canadensis*; (d): ED informed by Minteguiaga *et al*.[23] for *Baccharis tridentata* (Asteraceae).

Isolation of *cis*-LAME

The main component of *E. bonariensis*, *cis*-LAME, was isolated by column chromatography using silica gel as stationary phase. TLC was employed to assess the separation efficiency (**FIGURE 4**). *p*-Anisaldehyde/sulphuric acid was employed as visualization reagent system; however, the spot of *cis*-LAME was barely observed at such conditions, being its presence detected by using $UV_{\lambda= 365}$ as previously reported by us[34]. From the 24 collected fractions, those numbered 11-16 were mixed (**FIGURE 4**), and after vacuum evaporation of the solvent, about 130 mg of the compound was obtained (94.0% purity by GC/MS). The identity was established by ^{1}H- and ^{13}C-NMR, being compared with literature reports[29-31]. The spectroscopic data, including the chemical shifts in ^{1}H- and ^{13}C-NMR, of this compound was previously published, as well as the mass spectrum at low- and high-resolution and the UV-Vis spectrum[27]. In this contribution we provide the raw ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC spectra of *cis*-LAME in $CDCl_3$ (**FIGURES S3** to **S7**) as well as the UV-Vis and FT-IR spectra of *E. bonariensis* essential oil (**FIGURES S8** and **S9**)[32].

FIGURE 3: Chemical structures of 2-*cis*-lachnophyllum acid methyl ester (*cis*-LAME, I) and of 4-*cis*-lachnophyllum lactone (II), components of the *E. bonariensis* essential oil.

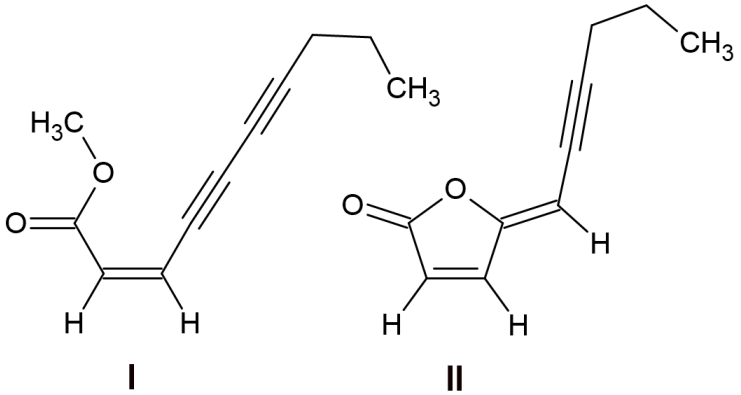
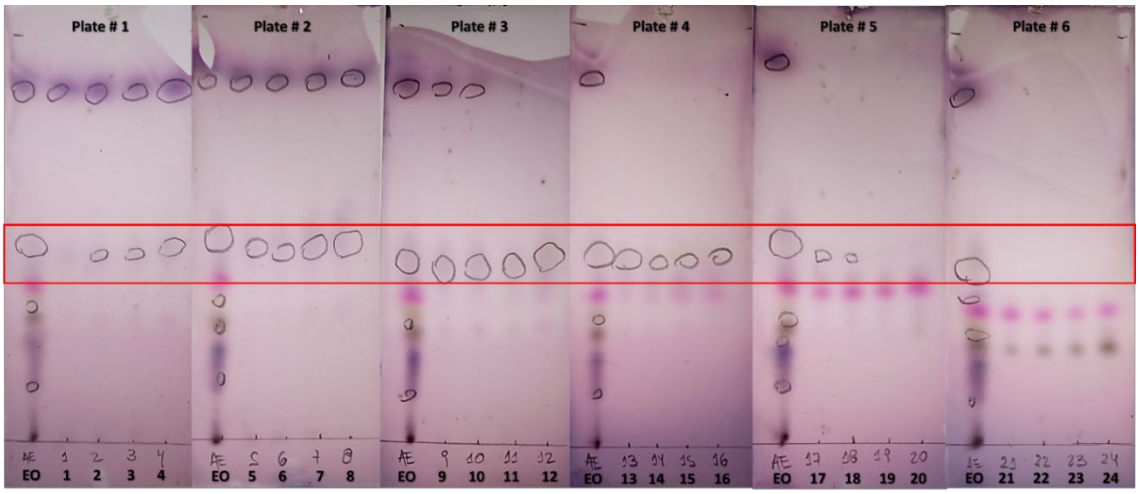


FIGURE 4: TLC results of the column chromatography separation of *cis*-LAME, consisting of 6 consecutive plates and 24 applied fractions (1 to 24).

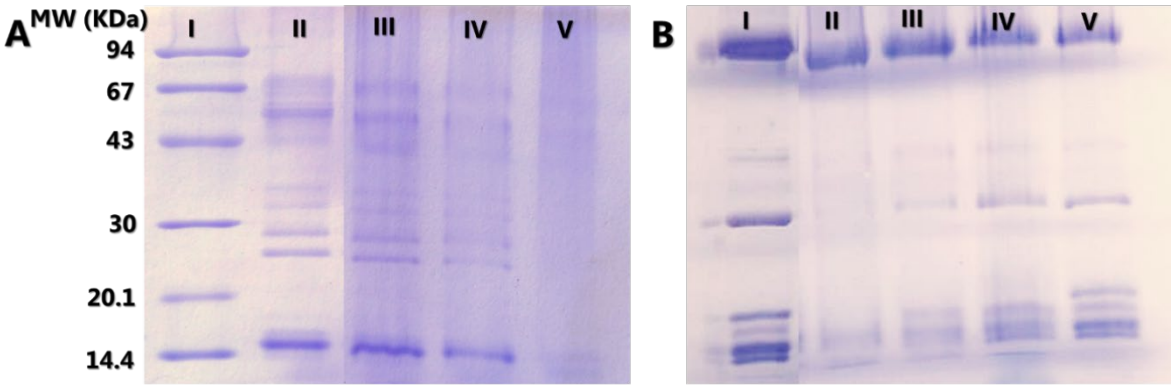


References: EO: *E. bonariensis* essential oil added for comparison purposes of the advancement of the separation. The red rectangle represents the *cis*-LAME band, barely visualized with *p*-anisaldehyde/sulphuric acid reagent, but visible by $UV_{\lambda= 365nm}$ and highlighted with black pen marks. Fractions 11 to 16 were mixed as containing almost pure *cis*-LAME. For experimental conditions see the Materials and Methods section.

Neutralization of bothropic venoms

FIGURE 5 shows examples of the electrophoresis results (1D-SDS-PAGE) following the modification of the protein profile of the *Bothrops* spp. venoms after the incubation with the samples of interest.

FIGURE 5: 1D-SDS-PAGE (venom-sample 1:7).



References: A: Test against *B. diporus* venom: I: molecular weight standard; II. pure venom (V); III. EtOH (negative control); IV. *cis*-LAME (active) + V; V. carquejone (positive control) + V. B: Test against *B. alternatus* venom: I. pure venom (V); II. carquejone (positive control) + V; III. *cis*-LAME + V (active); IV. and V. extracts in AcOEt of *B. articulata* and *B. genistelloides* var. *crispa* (respectively; positive controls) + V.

As demonstrated by our group, the interaction of the venom's components with the phytometabolites *in vitro* by 1D-SDS-PAGE, is a primary indicator of alexiteric activity which in general is corroborated by other neutralizing assays: inhibition of proteolysis, indirect inhibition of hemolysis, and the inhibition of the pro-coagulation effect of the normal plasma[46]. In that approach, both *E. bonariensis* essential oil and its pure component *cis*-LAME interacted with the protein components of *B. diporus* and *B. alternatus* snake venoms as visualized by 1D-SDS-PAGE, which is interpreted as the disappearance (or even the "withening") of most

(or some) of the protein bands (**TABLE 3**, **FIGURE 5**). These results demonstrate the potential of both the essential oil and the polyacetylene compound as neutralizers of *Bothrops* venoms. Further investigations are needed, including a broader range of alexiteric assays, as well as evaluations across additional *Bothrops* spp. and considering the intrinsic variability in venom composition. To further confirm this potential, *in vitro* assays of inhibition of proteolysis (casein model), indirect inhibition of hemolysis (with agar blood medium), and inhibition of the pro-coagulation effect of the normal plasma[15,16] were applied after the incubation of *B. diporus* venom with *E. bonariensis* essential oil and *cis*-LAME (**TABLE 3**).

TABLE 3: Results on the neutralization of *B. diporus* snake venom at *in vitro* conditions by the essential oil of *E. bonariensis* and pure isolated *cis*-LAME.

Sample	Modification of the proteic profile (n=3)	Inhibition of Proteolysis (n=3)	Indirect Inhibition of Hemolysis (n=4)	Inhibition of Pro-coagulation (n=3)
E. bonariensis Ess. Oil	Yes	Yes (+++)	26.3 ± 1.0%	0
cis-LAME	Yes	Yes (+++)	57.9 ± 1.0%	60.6 ± 1.0%
carquejone[a] (C+)	Yes	Yes (+++)	50 ± 10%	20.4 ± 4.4%
B. articulata[b] (C+)	Yes	No	63.2 ± 7.4%	100 ± 0%
B. genistelloides[b] (C+)	Yes	No	10.5 ± 7.4%	57.1 ± 1.0%
EtOH (C-)	No	No	0	0

References: For comparison purposes were employed: (a) carquejone, reported as alexiteric by Minteguiaga *et al.*[15], and (b): *B. articulata* and *B. genistelloides* var. *crispa* extracts in AcOEt, reported as alexiterics by Minteguiaga *et al.*[16].

As is evident from the data presented in **TABLE 3**, both *E. bonariensis* essential oil and *cis*-LAME showed neutralizer properties of *B. diporus* venom activity *in vitro*. Both inhibited the proteolysis of the protein model casein, while *cis*-LAME inhibited the hemolysis by 57.9 ± 1.0% and the pro-coagulation effect by 60.6 ± 1.0%, better than *E. bonariensis* essential oil at the same concentration level (**TABLE 3**). The results obtained are comparable to those obtained for carquejone and other extracts reported by us as highly alexiteric at *in vitro* conditions, as the ones of *B. articulata* and *B. genistelloides* var. *crispa* in AcOEt employed in this work as positive controls[15,16] (**TABLE 3**).

Our results should be evaluated considering the work by Miranda *et al.*[14], which was the first one (to our knowledge) reporting the potential of *E. bonariensis* essential oil as neutralizers of snake venoms. These authors extracted by hydro-distillation the essential oil from plant material collected in Brazil (Lavras, Minas Gerais State). They assessed the inhibition of pro-coagulation (clotting) and fibrinogenolysis (proteolysis of fibrinogen, which forms the core of the clots) induced by the venoms of *B. atrox*, *B. moojeni* (both pit vipers) and *Lachesis muta* (a venomous snake also belonging to the Viperidae family). The oil was predominantly composed by the monoterpene hydrocarbons limonene (56.7%; enantiopurity not studied) and *trans*-*β*-ocimene (26.3%), and the monoterpene alcohol *cis*-verbenol (4.4%); not polyacetylenes were informed in the composition. *E. bonariensis* oil could inhibit the pro-coagulant effect of the three snake venoms at both quantitative relationships of 1:17 and 1:8 (essential oil-venoms). The best results were obtained for *B. moojeni* venom with percentages of inhibition higher than 100% [control clotting time: 108.3 seconds; clotting time after the incubation with the oil at approximately (1:8): 2340 seconds], a fact that the authors associated primarily to the monoterpenes composing the oil[14]. However, the essential oil did not inhibit the fibrinogenolysis in either venom, as followed by 1D-SDS-PAGE. Considering the study of Miranda *et al.*[14]

and ours, it can be found discrepancies: while the essential oil from Brazil was reported as inhibitor of the pro-coagulation and did not affect the proteolysis of fibrinogen, the oil from Uruguay did not displayed inhibition of pro-coagulation but inhibited the proteolysis of casein (**TABLE 3**). However, both works highlight the potential of *E. bonariensis* as a source of neutralizer components (essential oils) against the bothropic venoms. The apparent discrepancies in the level of bioactivity could be related to the different chemical composition found for both essential oils, ours being very rich in polyacetylenes. In fact, *cis*-LAME exhibited positive inhibition of the pro-coagulant effects, also demonstrating inhibition of proteolysis and hemolysis (**TABLE 3**), being a promising scenario for developing a therapeutic alternative for treating *Bothrops* snake envenoming local effects. To our knowledge, no previous mention of the polyacetylenes potential as inhibitors of snake venom effects has been made in the literature, thus stimulating new original research at *in vivo* conditions, and for elucidating the mechanism of action of *cis*-LAME (and eventually the related lactones) against bothropic or non-bothropic venoms.

Previously, we hypothesized by doing a basic structure-activity relationship study, that the *α,β*-unsaturated carbonyl moiety is essential for the alexiteric activity[15]. This is the case of carquejone reported in that work and the same for a *neo*-clerodane diterpenoid reported by other authors[11]. In the current contribution, further evidence is presented, since *cis*-LAME also contains an *α,β*-unsaturated carbonyl motif (carbonyl of an ester group, **FIGURE 3**). It can be hypothesized that the 4-*cis*-lachnophyllum lactone also could be alexiteric due to having the same structural moiety. In fact, such compound has been already reported as bioactive as allelopathic, antileishmanial, antimycobacterial and nematicide[31,43], among others reports.

Considering the serotherapy limitations currently applied to snake envenoming treatment, the developing of alternative or complementary treatments that deals with the local effects (edema, necrose, hemorrhage) of the venoms is priority, and in that circumstance, the use of essential oils eventually formulated to be applied topically could open up new research options[14].

Conclusion

In this contribution we highlighted the potential of *E. bonariensis* essential oil, and its main polyacetylene component (*cis*-LAME), as neutralizer of bothropic venoms (models: *B. diporus* and *B. alternatus*) at *in vitro* conditions, which was demonstrated by interaction of the protein venom components with the phytometabolites by 1D-SDS-PAGE. Further evidence was obtained by confirmation of the inhibition of proteolysis, hemolysis and pro-coagulation effect induced by *B. diporus* venom, particularly in the case of *cis*-LAME. Beyond the bioactivity results, the phytochemical characterization of *E. bonariensis* essential oil from Uruguayan origin, as well as the original report on the enantiomeric distribution of selected chiral monoterpene hydrocarbons (*α*-pinene, sabinene, *β*-pinene, and limonene) were additional findings of this contribution. Future research should focus on evaluating the effects of *E. bonariensis* essential oil and *cis*-LAME *in vivo* (animal models), elucidating their alexiteric mechanisms of action (including those of potential lactone derivatives), and determining the pharmacological and pharmacodynamic conditions required for their topical application.

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Conflicts of Interest

The authors declare no conflict of interest.

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Contributions

Research design and conceptualization: MAMC; AMT; CANC; ED.

Investigation and formal analysis: MAMC; AMT; MNU.

Supervision: CANC; ED.

Writing of the original draft: MAMC.

Writing of the original manuscript: MAMC; AMT; CANC; ED; MNU.

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