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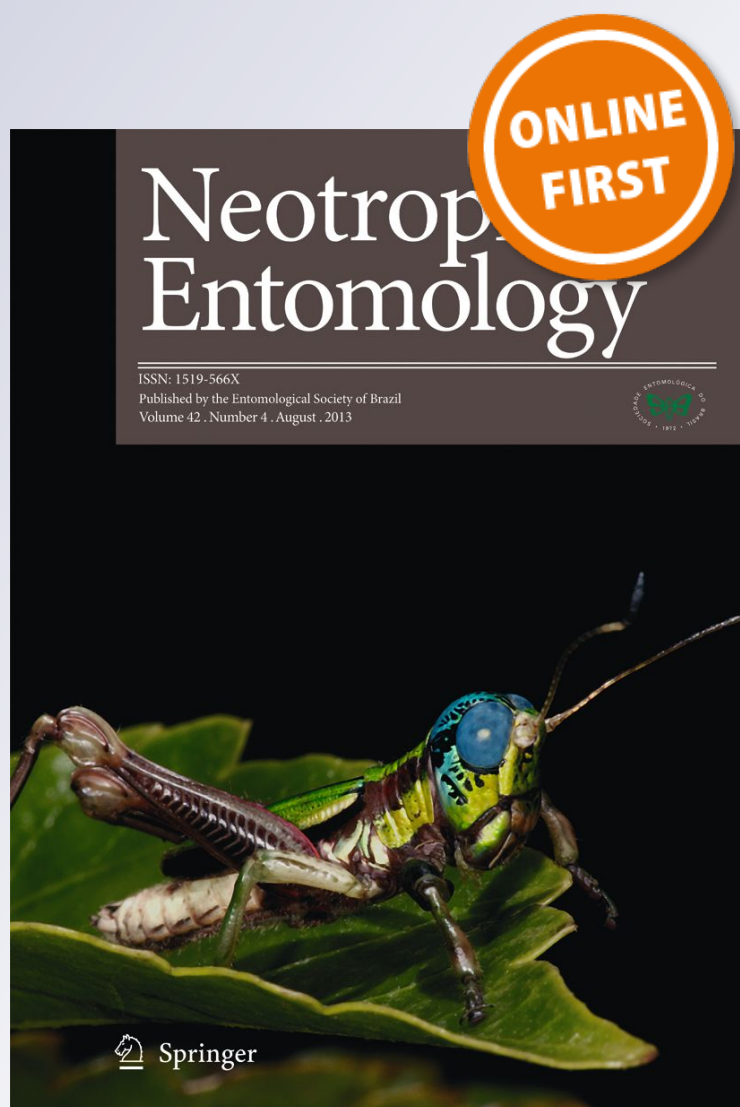
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# Assessment of the Insecticidal Potential of the *Eupatorium buniifolium* Essential Oil Against *Triatoma infestans* (Hemiptera: Reduviidae). A Chiral Recognition Approach

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## Keywords

Ovicidal, activity, Repellent, Chagas disease, Plant extracts, Plant metabolites, Monoterpene

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## Abstract

In this research, bioactivities toward the Chagas' disease vector *Triatoma infestans* (Klug) (Hemiptera: Reduviidae) by the essential oil (EO) of *Eupatorium buniifolium* H. et A. (Asteraceae) are reported. The tests were designed in order to determine ovicidal activity as well as the response to vapor exposure (fumigant) and to topical application (contact toxicity) and as repellent. In the last three bioassays, nymphs from the 3rd and 4th instar were used. The assayed materials were obtained from aerial parts of plants collected during the months of March and December, throughout 4 years, in two locations. The EO samples were subjected to a qualitative analysis by GC-MS and the relative area of each component was reported by GC-FID. The main monoterpene detected was  $\alpha$ -pinene and by using a chiral column through GC-MS experiments and having both stereoisomers as standards, we were able to determine that the enantiomer present was *S,S*-(-)- $\alpha$ -pinene. Although usually in studies of EOs changes in chemical composition are often observed due to the time of collection and the environment where the plant develops, in our case the differences were, with some exception, only at the level of the minor components. The best results were obtained in the experiments to determine ovicidal activity, fumigant action, and repellency. No worthy response was found as insecticide in the trials designed for contact toxicity. The results of the studied bioactivities were independent of the location, month, and year of collection of the plant material. This behavior provides an interesting scope in relation to the potential use of this natural blend for the control of this insect at the nymph stage as repellent as well as for decreasing the population by ovicidal effect. Notably, in the course of the two-choice repellency test, it was possible to demonstrate recognition of one of the enantiomers of the  $\alpha$ -pinene, giving rise to a non-common chirality/response effect. In this assay, the levorotatory isomer was the most active as repellent. Considering the abundance of the wild plant under study and the fact that its EO is easy to obtain, it is suggested that it could be an adequate natural resource to control this vector in a sustainable way as a complementary approach to conventional methods.

## Introduction

In South America, *Triatoma infestans* (Klug) (Hemiptera: Reduviidae) is the main vector of Chagas disease. Pyrethroid insecticides currently constitute the most successful chemical tools for its control and are almost exclusively used by government campaigns. Within this family of chemical compounds, those of greater effectiveness are called third generation pyrethroids and are characterized by being enriched in stereoisomers with higher insecticidal potency. Among pyrethroids, Deltamethrin has proven to be the best control of *T. infestans* (Arias et al 2003, Alzogaray & Zerba 2001). Nevertheless, resistance has been reported for this vector, probably related with the extensive and intensive use of these chemical insecticides. Recently, a possible evolutionary mechanism associated with the resistance to insecticides observed in this insect has been reported (Mougabure-Cueto & Picollo 2015). In addition, insects resistant and not resistant to Deltamethrin have been reported in different houses in the same neighborhood, which indicates the possibility of microgeographic behavior toward this insecticide (Germano et al 2013).

Bearing in mind that about 6 million to 7 million people are estimated to be infected worldwide, mostly in Latin America where Chagas disease is endemic, these results account for the complexity of the control of this insect in connection with the associated parasites, for which the treatment is very challenging in the chronic phase of the disease (Lozano et al 2015).

The environmental impact of Deltamethrin is reported as notable for its low selectivity, which is particularly damaging to all useful auxiliary insects in crops. On the other hand, a recent report on its action in experimental animals shows that this pyrethroid decreases fertility in rats causing histological changes in the ovary, even at sub-lethal doses (Marettova et al 2017). Consequently, it is necessary to investigate alternative insect management strategies and, ideally, additional technologies that generate new ways of regulating triatomine populations and thus the risks of insecticide use.

The province of San Luis is located in the Central-Western region of Argentina where Chagas disease continues to have an important impact, as in the northern region of the country. For this reason, secondary metabolites of the *Salvia* genus, especially diterpenes, have been also studied as potential trypanocidal molecules in the search for new leading structures (Sánchez et al. 2006, Lozano et al 2015, 2016).

Other authors have made efforts to find EO, and organic extracts from plants that can be an alternative for the control of *T. infestans* (Kurdelas et al 2012, Carvajal et al 2012, Valladarres et al. 1999; Schmeda-Hirschmann & Arias 1992). Considering that locals inhabiting the countryside of San Luis province have informed (personal communications) about

the repellent property that *E. buniifolium* presents against some insects, the aim of the present work is to study the chemical composition of the EOs of several collections and evaluate their activity toward *T. infestans*. The experiments here reported were designed with the idea of addressing several possible aspects for the control of the vector. The fumigant, repellent (both by vapor) activities, and contact toxicity were evaluated toward nymphs. Additionally, the eggs were treated in the form of a spray to evaluate the ovicidal activity.

Sometimes the reported chemical composition of other EOs shows high dispersion in their composition. The variability in the qualitative and quantitative content of terpenes can be attributed to different conditions in its habitats as soil, altitude, rainfall, humidity, and temperature, which can affect the expression of some biosynthetic pathways (Sosa et al 2012). Therefore, plant specimens collected over the 4 years, twice every year, in two different places in terms of their environment were used in this study. The months chosen (December and March) correspond to the start and end of flowering time, when higher production of volatile compounds is expected.

If we consider the positive results obtained in our studies, it could be necessary having good information on the chemical composition of the EO in order to provide security in relation to its potential use. For this reason, we have been careful to collect all the analytical data for each of the samples, which assure a reasonable constancy in the composition of the EOs.

## Material and Methods

### Extraction and analysis of EOs

Aerial parts of *Eupatorium buniifolium* Hook et Arn. were collected in Potrero de los Funes (33°12'12"S and 66°15'43" W) (elevation 1225 m) (location I) and Suyuque Nuevo (33°08'02"S and 66°16'45"W) (elevation: 981 m) (location II) in the months of December and March of the years 2010 to 2013. Voucher specimens were deposited at the Herbarium of the Universidad Nacional de San Luis (UNSL no. 495-Del Vitto). Fresh aerial parts of *E. buniifolium* were cut into small pieces and subjected to steam distillation at 96°C for 3 h (2.5 kg/batch) by a FIGMAY hydrodistillator ([www.figmay.com.ar](http://www.figmay.com.ar)). Until the analysis and bioassays were run, the recovered EOs were dried over anhydrous sodium sulfate and stored in cold (−15°C).

Characterizations of the EO components were performed by GC-MS (gas chromatography-mass spectroscopy) using a Thermo Trace 1300 gas chromatograph coupled to an ITQ 900 ion trap mass spectrometer (GC/MS-ITD, Thermo Scientific). Studies were performed using a DB-5 fused silica

capillary column Ohio Valley ([www.ovsc.com](http://www.ovsc.com)) (5% phenyl, 95% dimethylpolysiloxane, 30 m × 0.25 i.d., film thickness 0.25 μm). Mass transfer line and injector temperature were set at 240 and 220°C, respectively. Oven temperature setting: 60°C; 3°C min<sup>-1</sup> to reach 246°C; 15°C min<sup>-1</sup> to reach 300°C. Carrier gas was He (10 psi). Samples (0.1 μL of the EO) in split mode (1:50) were injected. Ionization voltage of mass spectrometer was operating in the EI-mode at 70 eV, and ionization source temperature was 250°C. All the spectra were acquired in a full-scan positive mode in a range between 50 to 450 *m/z*. The EO components were identified by comparison of their linear retention indices (LRIs) in relation to the homologous series of *n*-alkanes (C9–C26) according to Adams (2007), Davies (1990), and Kovats (1958). The fragmentation patterns were compared with those stored in the NIST 2.0 spectra library (Adams 2007, McLafferty & Stauffer 1989, D'Acampora Zellner *et al* 2010).

The quantification of the EO components were performed using a GC-FID Perkin Elmer Clarus 500 ([www.perkinelmer.com](http://www.perkinelmer.com)), equipped with the same DB-5 fused silica capillary column that was used in GC-MS studies. N<sub>2</sub> (31.9 cm s<sup>-1</sup>) was used as carrier gas and FID detector was set at 300°C. The oven temperature program used for the GC-FID analysis was the same already described for GC-MS. Peak identification was carried out by comparing the GC-FID retention indices with those from GC-MS. Relative amounts of each individual component were expressed as percentages of each peak area relative to the total chromatogram peak area (Tables 1, 2, 3, 4).

In order to determine the correct enantiomer of α-pinene present in the essential oil as a major component, a Restek Rt<sup>®</sup>-βDEXsm column (2,3-di-O-methyl-6-O-tert-butylidimethylsilyl-β-cyclodextrin added into 14% cyanopropylphenyl/86% dimethyl polysiloxane; 30 m × 0.25 i.d., film thickness 0.25 μm) ([www.restek.com](http://www.restek.com)) was used for chiral chromatography. The initial oven temperature was 60°C with a ramp of 5°C min<sup>-1</sup> to 200°C and this temperature was kept for 10 min. In this experiment, with the exception of the column, the same equipment, volume of injection, and all the operational conditions that were applied for the GC-MS studies were used. In this study, a sample of *E. buniifolium* EO doped with optically pure standards of each enantiomer were analyzed as controls (see Electronic Supplementary Material). Chiral standard reagents were purchased from Sigma-Aldrich ([www.sigmaaldrich.com/argentina](http://www.sigmaaldrich.com/argentina)): *S,S*-(-)-α-pinene, purity ≥ 99%, ee ≥ 86%, [α]<sub>D</sub> = -50 to -46°. *R,R*-(+)-α-pinene, purity ≥ 99%, ee ≥ 97%, [α]<sub>D</sub> = +50.7°.

The <sup>1</sup>H NMR and <sup>13</sup>C NMR were run in a Bruker Avance II, 200 MHz spectrometer ([www.bruker.com/bruker/biospin](http://www.bruker.com/bruker/biospin)). Spectral data of the whole EOs (see Electronic Supplementary Material) were indicative that the principal monoterpene was α-pinene. A reference sample of this

Table 1 Composition of EOs from Potrero de los Funes (location I), March 2010 to 2013.

	RT (min)	Values in percentages <sup>a</sup>			
		2010	2011	2012	2013
α-Thujene	6.33	1.71	2.04	1.37	2.69
(-)-α-Pinene	6.67	47.21	51.81	45.03	41.48
Camphene	7.00	0.54	0.57	0.53	1.02
Sabinene	7.85	5.37	6.28	8.44	6.79
β-Pinene	7.96	3.98	2.75	3.33	3.54
Myrcene	8.42	0.24	1.23	2.19	2.67
δ-2-Carene	8.80	6.95	5.14	6.96	4.91
α-Phellandrene	8.89	1.39	1.87	1.76	0.36
α-Terpinene	9.34	0.50	0.31	0.49	0.39
<i>p</i> -Cymene	9.63	0.42	0.58	0.19	0.73
<i>R</i> -Limonene	9.87	6.95	7.79	9.53	8.47
Z-β-Ocimene	10.18	1.39	0.49	0.42	2.36
E-β-Ocimene	10.65	8.54	8.09	9.53	11.09
γ-Terpinene	11.00	0.75	0.47	0.02	0.59
Terpinolene	12.19	0.10	0.31	0.02	0.66
Linalool	12.66	0.52	0.14	0.12	0.27
Terpinen-4-ol	16.02	2.90	0.52	0.19	0.80
β-Elemenene	25.33	1.27	0.58	0.10	0.31
E-Caryophyllene	26.45	3.09	2.20	3.31	2.31
Germacrene D	28.99	1.52	1.70	1.08	2.19
Valencene	29.62	0.86	0.92	0.75	2.92
δ-Cadinene	30.66	0.84	0.73	0.36	0.63
Spathulenol	32.73	0.50	0.60	1.10	0.89
Non-identified		2.44	1.00	3.18	1.91
Total (%)		100.00	100.00	100.00	99.98

<sup>a</sup> From the areas of the corresponding peaks.

monoterpene was subjected to this technique to ensure its presence as main component.

#### Insects and bioassays

Eggs, and nymphs of the 3rd and 4th instar from susceptible strains, were provided by the Reference Vector Centre, National Service for Chagas Disease, Santa María de Punilla, Córdoba, Argentina.

#### Ovicidal activity

The methodology described by Valladares *et al* (1999) was used. In each evaluation, three batches of ten eggs per each sample of EO were sprayed with 2 μL of solutions of the EO at concentrations of 5, 10, 25, and 50% v/v in acetone as solvent. Two simultaneous controls were run in triplicate; the first corresponded to the solvent (acetone) and the second to K-Othrina<sup>®</sup> (Deltamethrin 7.5 g/L, Bayer Environmental

Table 2 Composition of EOs from Potrero de los Funes (location I), December 2010 to 2013.

	RT (min)	Values in percentages <sup>a</sup>			
		2010	2011	2012	2013
α-Thujene	6.33	1.69	1.78	1.84	1.33
(-)-α-Pinene	6.67	53.46	52.82	38.02	49.52
Camphene	7.00	1.16	1.01	0.99	1.77
Sabinene	7.85	4.98	5.59	6.93	7.49
β-Pinene	7.96	1.40	5.13	3.13	0.33
Myrcene	8.42	4.08	3.43	2.04	0.19
δ-2-Carene	8.80	7.66	6.06	6.78	6.43
α-Phellandrene	8.89	0.26	1.48	0.48	1.37
α-Terpinene	9.34	0.29	0.43	0.63	0.33
p-Cymene	9.63	0.30	0.22	0.37	0.19
R-Limonene	9.87	7.54	6.56	7.84	6.94
Z-β-Ocimene	10.18	0.48	0.02	1.64	0.47
E-β-Ocimene	10.65	7.54	7.56	8.62	5.16
γ-Terpinene	11.00	0.37	0.52	0.94	0.47
Terpinolene	12.19	0.01	0.04	0.91	0.16
Linalool	12.66	0.02	0.12	0.30	0.04
Terpinen-4-ol	16.02	0.47	0.06	1.64	0.23
β-Elemenene	25.33	1.03	1.81	1.36	4.19
E-Caryophyllene	26.45	1.03	0.25	2.57	0.32
Germacrene D	28.99	1.30	2.84	3.59	5.45
Valencene	29.62	0.11	0.21	3.54	1.12
δ-Cadinene	30.66	1.84	0.05	1.08	0.51
Spathulenol	32.73	0.27	0.01	1.48	0.13
Non-identified		2.71	2.00	3.27	5.68
Total (%)		100.00	100.00	99.99	99.82

<sup>a</sup> From the areas of the corresponding peaks.

Table 3 Composition of EOs from Suyuque Nuevo (location II), March 2010 to 2013.

	RT (min)	Values in percentages <sup>a</sup>			
		2010	2011	2012	2013
α-Thujene	6.33	0.76	0.41	0.15	1.38
(-)-α-Pinene	6.67	42.75	41.98	75.77	36.99
Camphene	7.00	0.89	0.74	0.09	0.58
Sabinene	7.85	8.79	8.75	6.19	8.97
β-Pinene	7.96	3.18	3.86	4.31	2.50
Myrcene	8.42	2.23	1.99	1.49	2.08
δ-2-Carene	8.80	5.18	5.25	5.65	6.63
α-Phellandrene	8.89	0.25	0.55	0.20	0.55
α-Terpinene	9.34	0.55	0.58	0.20	0.42
p-Cymene	9.63	0.12	0.42	0.22	0.55
R-Limonene	9.87	9.39	10.20	0.20	6.69
Z-β-Ocimene	10.18	1.48	1.40	0.06	4.24
E-β-Ocimene	10.65	11.84	11.68	0.01	8.99
γ-Terpinene	11.00	0.85	0.96	0.02	0.62
Terpinolene	12.19	0.68	0.48	0.02	0.64
Linalool	12.66	0.21	0.24	0.28	0.37
Terpinen-4-ol	16.02	0.69	0.29	0.02	1.27
β-Elemenene	25.33	0.29	0.18	0.74	0.64
E-Caryophyllene	26.45	2.13	2.30	0.88	2.45
Germacrene D	28.99	1.81	1.14	0.03	2.33
Valencene	29.62	2.72	2.32	0.01	3.58
δ-Cadinene	30.66	0.39	0.40	0.30	0.67
Spathulenol	32.73	0.43	1.39	0.05	1.20
Non-identified		2.39	2.28	3.11	5.65
Total (%)		100.00	99.79	100.00	99.99

<sup>a</sup> From the areas of the corresponding peaks.

Science) as commercial dilution. After treatment, the eggs were deposited in capsules conditioned in their upper part with a piece of gauze to obtain a normal aeration. Capsules were kept in the breeding chamber at 25°C, with 80% relative humidity in darkness (SEMEDIC model I-291P chamber) ([www.semedix.com.ar/](http://www.semedix.com.ar/)). The hatching of the eggs was monitored for 60 days, and the percentage of hatching was recorded. Results are shown in Fig 1 (for the device, and numerical data, see [Electronic Supplementary Material](#)).

#### Fumigant activity

To determine the behavior by exposure to EO vapor, a series of plastic Falcon type bottles (Becton Dickinson, 250 mL, 75.0 cm<sup>2</sup> of area at the lateral side) were used in this experiment. A piece of filter paper (2.0 cm<sup>2</sup>) tied with a thread to the lid was placed in each bottle. A predetermined volume of the acetone dilution of the EOs (1:1) were applied over the filter paper in the following volumes: 5, 15, 25, 50, and 75 μL,

respectively, in order to reach concentrations of 10, 30, 50, 100, and 150 μL of EO/L of air, respectively. Each trial was performed in triplicate. Solvent control (50 μL of acetone) was carried out. A positive control with K-Othrina® (Deltamethrin 7.5 g/L, Bayer Environmental Science, 150 μL/L of air) as commercial dilution was carried out. Before starting the experiment, the solvent was left to evaporate for 15 min at r.t. in experimental and control arenas. Then, 20 selected nymphs (3rd and 4th instar) were placed in each bottle. Insects remained in the environment containing EOs as vapors for 8 h. Then, the papers were removed and the top of the flasks were slightly loosened to permit air exchange. After 24 h, the number of dead insects was recorded as Mortality Rate (Abbott, 1925; García *et al* 2005, 2007). Abbott's Mortality Rate =  $[(E - C)/(100 - C)] \times 100$ , where  $E$  = number of insects killed by treatment and  $C$  = number of dead insects in control. Results are shown in Fig 2 (for the device and numerical data, see [Electronic Supplementary Material](#)).

Table 4 Composition of EOs from Suyuque Nuevo (location II), December 2010 to 2013.

	RT (min)	Values in percentages <sup>a</sup>			
		2010	2011	2012	2013
α-Thujene	6.33	1.99	2.65	4.44	1.34
(-)-α-Pinene	6.67	55.68	48.62	38.87	46.89
Camphene	7.00	0.55	0.58	0.96	1.35
Sabinene	7.85	6.63	9.66	6.09	5.74
β-Pinene	7.96	3.93	5.37	3.26	6.72
Myrcene	8.42	0.38	0.31	2.21	1.50
δ-2-Carene	8.80	4.95	4.37	4.28	8.39
α-Phellandrene	8.89	0.41	0.31	0.27	0.47
α-Terpinene	9.34	0.38	0.87	0.55	0.26
p-Cymene	9.63	1.89	1.87	0.52	0.49
R-Limonene	9.87	6.77	1.14	8.37	8.72
Z-β-Ocimene	10.18	0.41	0.73	1.51	1.35
E-β-Ocimene	10.65	6.75	6.21	7.77	1.50
γ-Terpinene	11.00	0.15	0.22	0.90	0.47
Terpinolene	12.19	0.41	0.75	1.04	0.35
Linalool	12.66	0.15	0.22	0.26	1.42
Terpinen-4-ol	16.02	0.53	2.02	1.01	1.27
β-Elementene	25.33	0.16	0.60	0.63	0.17
E-Caryophyllene	26.45	1.02	3.15	2.40	2.03
Germacrene D	28.99	1.07	2.56	2.82	2.13
Valencene	29.62	1.67	2.56	3.19	2.03
δ-Cadinene	30.66	0.31	1.27	0.91	0.44
Spathulenol	32.73	1.67	0.11	1.11	1.17
Non-identified		2.14	3.85	6.62	3.80
Total (%)		100.00	100.00	99.99	100.00

**Repellent activity.** A binary choice bioassay was prepared for these tests. Test arenas were two 250-mL Falcon flasks which were linked with a transparent acetate tube of 7.5 cm in length and 2.0 cm in diameter with 0.5-cm<sup>2</sup> holes in the center. Acetone dilutions at 10, 25, and 50% were applied (1.0 μL) in one of the flasks (treated side). The same volume of acetone was added to the second flask (control side). The solvent was allowed to evaporate for 15 min at r.t. Twenty triatomine nymphs were incorporated into the device through the hole and then covered with adhesive tape. The test arenas were placed in the dark at r.t. and the number of insects on the treated side (T) and on the control side (C) was recorded at 120 min. Following the useful *Response Index* (RI) (Phillips *et al* 1993, García *et al* 2005, 2007), the results of the experiments were quantified as  $RI = (T - C/Tot) \times 100$ , where T = treatment, C = control, and Tot = total number of insects. Positive values indicate attraction, and negative ones correspond to repellent effect (Fig 3). Three replicates were performed for each batch of EO within the same day to avoid phenological variations (Messchendorp *et al* 1996) (for the device and numerical data, see [Electronic Supplementary Material](#)).

**Contact toxicity.** Thirty nymphs of the 3rd and 4th instar of *T. infestans* were randomly selected. Acetone dilutions of EOs (10, 25, and 50%) were topically applied (2 μL/nymph) to the ventral surface of the thoracic segments with a Distriman® pipette. A control was treated with the solvent alone, and a positive control was carried out using 2 μL of deltamethrin (7.5 g/L). After treatment, insects were placed into plastic vials (diameter 10 cm, height 7 cm) in a chamber at 25 ± 1°C with a

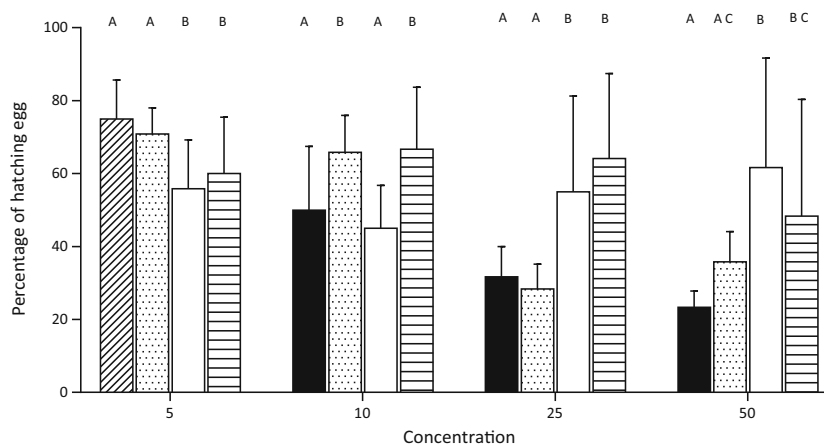


Fig 1 Percentage of hatching eggs. The data reported correspond to an average of three repetitions ± SD, of 4 years. ANOVA test ( $P < 0.0001$ ), followed by Tukey's multiple comparison test ( $P < 0.05$ ), the equal letters indicate that there is no significant difference when comparing. Concentration: percentage of the EO (v/v) in acetone. ■ Potrero de los Funes, March; ▨ Potrero de los Funes, December; □ Suyuque Nuevo, March; and ▤ Suyuque Nuevo, December. Percentage of hatching eggs for solvent control ranged from 83 to 90% and for Deltamethrin control from 83 to 92%.

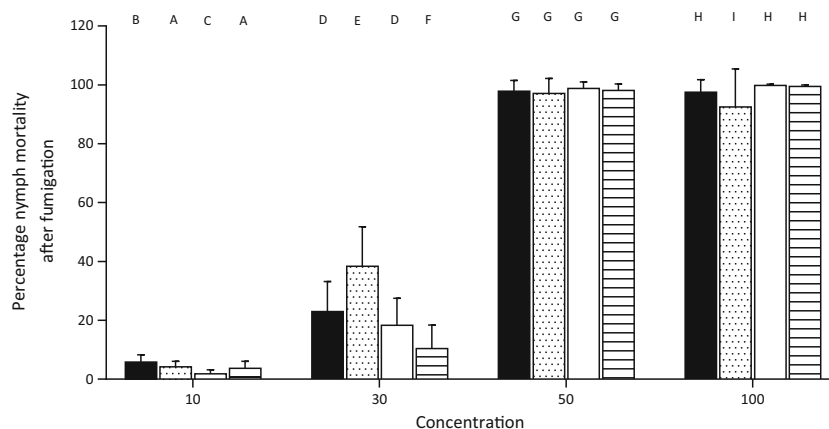


Fig 2 Percentage of nymph mortality after fumigation. The data reported correspond to an average of three repetitions  $\pm$  SD, of 4 years. ANOVA test ( $P < 0.0001$ ), followed by Tukey's multiple comparison test ( $P < 0.05$ ), the equal letters indicate that there is no significant difference when comparing. EOs in microliters per liter of air. ■ Potrero de los Funes, March; ▨ Potrero de los Funes, December; □ Suyuque Nuevo, March; and ▤ Suyuque Nuevo, December. Data from 150  $\mu\text{L/L}$  of air where not included since mortality was 100% in all the tests. Percentage of nymph mortality after fumigation for solvent ranged from 2.0 to 2.4%.

16:8 (L:D) photoperiod. The mortality was recorded every 24 h for 3 days. The experiments were carried out by triplicate. Results are shown in Fig 4 (for the numerical data, see Electronic Supplementary Material).

*Evaluation of chiral recognition to the enantiomers of  $\alpha$ -pinene.* Using the system described above to determine the repellent activity of EOs, the response of *T. infestans* nymphs to each enantiomer of  $\alpha$ -pinene was studied. In this experiment, 4.0  $\mu\text{L}$  of pure commercial isomer was used in each

treatment bottle (16  $\mu\text{L/L}$  of air) without placing acetone in the control. The insect response was evaluated as described above and the results are shown in and Fig 5 (for the numerical data, see Electronic Supplementary Material).

*Statistical analysis*

The lethal dose ( $\text{LD}_{50}$ ) in the ovicidal activity was calculated by the method of Probit (Sakuma 1998). The statistical calculation was made by the software POLO PLUS (LeOra

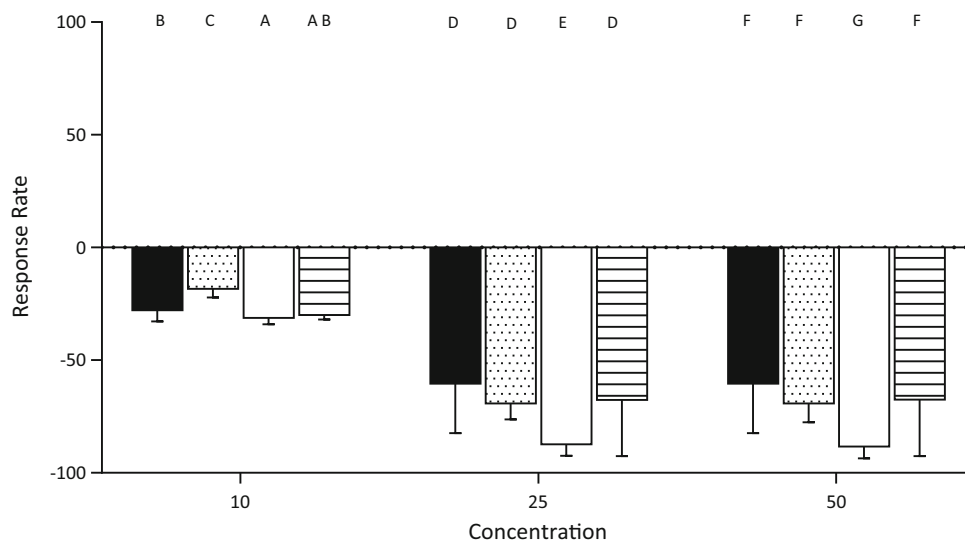


Fig 3 Repellency evaluation by a binary choice test (response rate). The data reported correspond to an average of three repetitions  $\pm$  SD, of 4 years (Phillips *et al* 1993). ANOVA test ( $P < 0.0001$ ), followed by Tukey's multiple comparison test ( $P < 0.05$ ), the equal letters indicate that there is no significant difference when comparing. Concentration: percentage of the EO ( $v/v$ ) in acetone. ■ Potrero de los Funes, March; ▨ Potrero de los Funes, December; □ Suyuque Nuevo, March; and ▤ Suyuque Nuevo, December. Values of solvent control ranged from 21 to 26.



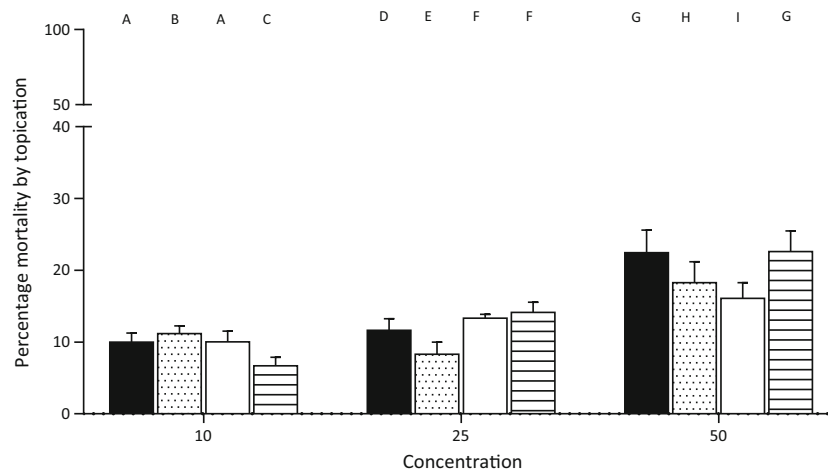


Fig 4 Toxic activity by topication. The data reported correspond to an average of three repetitions  $\pm$  SD, of 4 years. ANOVA test ( $P < 0.0001$ ), followed by Tukey's multiple comparison test ( $P < 0.05$ ), the equal letters indicate that there is no significant difference when comparing. Concentration: percentage 10, 25, and 50 of the EO (v/v) in acetone. ■ Potrero de los Funes, March; ▨ Potrero de los Funes, December; □ Suyuque Nuevo, March; and ▤ Suyuque Nuevo, December. Toxic activity by topication for solvent was 0.0 and for Deltamethrin 100%.

software). Abbott's formula was used to assess mortality by fumigation, and contact toxicity tests. All the data were analyzed by Anova-Tukey (Graph Pad Prism 7.03).

## Results

### Chemical constituents of the EOs

The average yield of EOs was  $3.62 \pm 1.79$  mL/kg for location I, and  $2.92 \pm 1.21$  mL/kg for location II. In order to obtain their chemical composition, each of the 16 EO samples were analyzed as described in "Material and Methods" and the results are presented in Tables 1, 2, 3, 4. As can be observed, in all

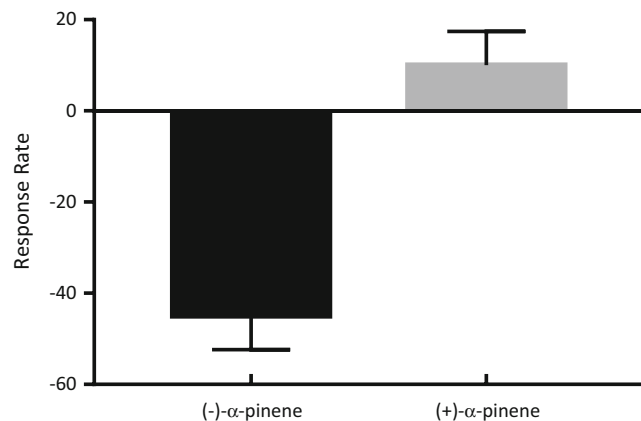


Fig 5 Chiral recognition of the enantiomers of  $\alpha$ -pinene by *Triatoma infestans*. Repellency evaluation toward the enantiomers of  $\alpha$ -pinene. Sixteen microliters per liter of air of *S,S*-(-)- $\alpha$ -pinene, and *R,R*-(+)- $\alpha$ -pinene. The data reported correspond to an average of three repetitions  $\pm$  SD.

cases, the predominant compound was the monoterpene *S,S*-(-)- $\alpha$ -pinene. This compound showed concentrations ranging from  $49.37 \pm 15.4\%$  (location II, March) to  $46.38 \pm 3.74\%$  (location I, March). The most significant data was the increase in its concentration in March 2012 in location II (75.77%), which was accompanied by a striking decrease of the monoterpenes *R*-Limonene, *Z*- $\beta$ -Ocimene, and *E*- $\beta$ -Ocimene at a trace level. A second group of monoterpenes in importance was constituted by *E*- $\beta$ -Ocimene, *R*-limonene, and  $\delta$ -2-carene, which except for the aforementioned results (location II, March 2012) maintain a relatively constant concentration for both locations in both months of collection.

Although it is not possible to determine with certainty the type of enantiomer of  $\alpha$ -pinene as the major monoterpene in the EOs by means of the non-chiral GC-MS methodology, this was achieved by using of a chiral column during the separation process (see Electronic Supplementary Material). Throughout this study, it was possible to unequivocally verify that the stereoisomer of the monoterpene  $\alpha$ -pinene present in all the EOs under studies was the levorotatory isomer, known by the common name of *S,S*-(-)- $\alpha$ -pinene being its systematic name (1*S*,5*S*)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene.

### Ovicidal activity

Figure 1 shows the results of the ovicidal activity on eggs of *T. infestans* of EO samples from the two locations collected throughout the 4 years during the aforementioned months. Results were analyzed considering the percentage of hatching eggs. The  $LD_{50}$  data from location I ( $LD_{50} = 17.09$  March, and 22.67 December) was possible to calculate, whereas the

results from location II were not suitable to reach the LD<sub>50</sub> values.

#### Fumigant activity

The *E. buniifolium* essential oil showed an important fumigant power. Mortality values reaching 92 to 100% were obtained when concentrations of 50, 100, and 150 µL of EOs/L air were assayed (Fig 2). There is no doubt that this toxicity is associated with the volatile components of the essential oil. In the abovementioned concentrations, the fumigant activity is independent of the location and the month of harvest. At low concentrations, the oil from location I showed a slightly higher activity.

#### Repellent activity

The results of repellent activity of EOs against *T. infestans* nymphs are shown in Fig 3. In these binary choice bioassays, the volatile natural blend exhibited a marked repellent activity, mostly with a repellence index significant at the concentrations of 25 and 50%. The best results were achieved with EOs collected in the month of March at location II, coinciding with the beginning of flowering.

#### Contact toxicity by topication

When dilutions of the essential oil between 10 and 50% in acetone were applied in the ventral segments of nymphs of the 3rd and 4th instar (Fig 4), no toxicity results of interest were observed compared with Deltamethrin.

#### Chiral recognition to the enantiomers of $\alpha$ -pinene by *T. infestans*

Considering the high content of *S,S*-(-)- $\alpha$ -pinene determined in all the *E. buniifolium* EOs studied, independent of the collection time and place, it was considered important to study the behavior of both isomers in terms of repellent power. Both  $\alpha$ -pinene enantiomers with an adequate enantiomeric excess were tested to evaluate their repellent activity (for the numerical data, see [Electronic Supplementary Material](#)). As expected, the levorotatory isomer was the most active, according to its presence in the EOs under study, while its enantiomer showed a very low level as an attractor (Fig 5). In addition, all nymphs submitted to this test of choice toward *S,S*-(-)- $\alpha$ -pinene were killed at 12 h.

## Discussion

An important feature to consider in this work is the contribution made by inhabitants of different areas of the San Luis

Province who commented to the authors on the repellent properties against some insects by the plant under study. This led us to start a long-term systematic study on the chemical composition of *E. buniifolium* EOs by analyzing samples from two locations in two different harvest months over 4 years. This would give us certainty regarding the constancy in the composition of both monoterpenes and sesquiterpenes present.

We then select two places that present certain differences in terms of altitude, humidity, and solar lighting for the collection of plant material. Location I (Potrero de los Funes) is a zone of hills with a high level of solar illumination throughout the year and with a low content of ambient, and soil humidity. Location II (Suyuque Nuevo) occupies a valley near a permanent watercourse with a humid atmosphere and protected from the cold of the winter. These different environmental conditions probably contribute to the chemical composition of their oils, in addition to genetic factors that are beyond the scope of this study. However, this difference in the environment where the plant under study grows only translates into non-significant changes in its chemical composition, with the only exception mentioned in the [Results](#) section.

The increase in the relative concentration of *S,S*-(-)- $\alpha$ -pinene (March 2012, location II) accompanied by the fall in the concentration of *R*-limonene could be explained by considering the biosynthesis of this monoterpene and that of *S,S*-(-)- $\alpha$ -pinene. Both terpenes have the terpinyl cation as an intermediary and for reasons that we cannot explain, that year the synthesis derived in favor of the bicyclic system (Dewick 2002). The simultaneous decrease of the non-cyclic monoterpenes *Z*- $\beta$ -Ocimene and *E*- $\beta$ -Ocimene remains not clear for us. However, their differences were not reflected in the data obtained in the bioassays that were carried out. In general, some differences are observed in the percentage of certain minority compounds that varied with the month and year of the collection of the fresh material. This is observed, among other terpenes, for the sesquiterpenes *E*-Caryophyllene and germacrene D.

By using commercial reference drugs of both stereoisomers of  $\alpha$ -pinene, it was possible to determine that the levorotatory isomer (*S,S*-(-)- $\alpha$ -pinene) was the main component of the blend by the GC-MS technique applying chiral phase chromatography and MS techniques. This could be verified by doping the whole EOs with each of the commercial stereoisomers. This data was very useful when studying the repellent effect on the insect under study.

One of the reasons why other plant-derived EOs are not always applicable as products for insect control is their high variability that does not allow for a constant material for their use as a pesticide. This is not the situation in the present investigation, because if we take into account the values found for the 16 chemically evaluated samples, we observe

an acceptable constancy in its major components through the 4 years studied (Tables 1, 2, 3, 4). This property has been translated into bioassays where we did not find significant differences between the different localities, and months of collection. In summary, we believe with experimental bases that it is a very good material to continue the studies in search of a possible application for the control of *T. infestans* as a complementary and probably less aggressive to human health method. The choice of the insect was made searching for alternatives for the control of Chagas disease vector. This disease, as mentioned above, is still an important parasitic sickness in our region.

In this way, we began the studies by scrutinizing possible ovicidal properties. If natural substances are found with this characteristic, it is promising to look for the design of new control alternatives. According to Parra-Henao *et al* (2007), the natural hatching rate of eggs under laboratory conditions (21°C and 90% humidity) is 85% at after 15 days. In our experiments, eggs treated with solvent alone (solvent control) presented an incubation time according to the bibliographic data aforementioned. When the eggs were not treated with any chemicals, including acetone, the values were as expected according to the literature for normal hatching (data not shown); a similar behavior was observed in Deltamethrin control. Although the ovicidal activity data of the EOs show high dispersion, the essential oils from location I showed a remarkable ovicidal power when tested in the highest concentrations. Proofs of this differences were confirmed when the LD<sub>50</sub> values were determined. In the remaining cases, the ovicidal activity observed was not enough to propose the use of this natural resource as an effective control of the insect's reproductive process, which only could be suggested as a complementary of the proved and more effective methods in use nowadays.

However, these EOs seem to be more effective than extracts from other natural resources which are more abundant in nature such leaves and fruits of *Melia azedarach*. Valladares *et al* (1999) evaluated ethanolic extracts of this species on *T. infestans* and found that eggs treated with concentrations of 5.5 and 11.1% w/v had hatching rates above 80%.

Usually, the control of the vector is done by fumigation of homes and their adjacencies with pyrethroids. In this order, we try to determine the effect on nymphs of the EOs looking for a possible friendly application to the environment and less aggressive for human health. The main component  $\alpha$ -pinene (without some stereochemical reference) has shown strong fumigant toxicity against several species of insects as *Acanthoscelides obtectus* (Say) (Rice & Coats 1994, Regnault-Roger & Hamaroui 1995), which is in agreement with the results observed in our experiments.

A third aspect of the study includes the evaluation of EOs as repellent agents that have the potential to cause insects to

leave their hiding places during the day. As has been reported by other authors, the important volatility of the monoterpenes of the EOs permits that they can penetrate holes and cracks in walls, wood, chicken houses, and bricks, which are the habitat of triatomines, reaching the insect respiratory system, and causing their death (Gomes & Favero 2013). The repellence index was significant at the concentrations of 25 and 50%. Taking into account that there are no significant differences between these two concentrations, it is possible to think that at the 25% concentration, a saturation of the olfactory organs is reached. Notably, the solvent (acetone) after evaporation showed some attractor effect.

The exact interpretation of repellence that results in insects is not easy to achieve since they are the result of a large number of physiological aspects (Hansson & Stensmyr 2011). However, in order to understand in depth the mode of action of the EO under study as well as toward pure terpenes, we think that electroantennography studies are required.

The repellent activity against nymphs of the essential oils here studied is, in our judgment, the greatest potential of application for the control of the insect here evaluated.

The studies of topication or direct contact of the EOs with the nymphs did not have the success that the pyrethroids exhibit; this reduces the value of these natural products to be applied in this way. In this experiment, the pyrethroid produces 100% mortality, and this result decreasing the potential value of the EOs here assayed to be applied in this way as a control of *T. infestans*.

In our opinion, studies directed to the exploration for bioactive natural products should consider aspects of the stereochemistry of the molecules that predominate in the complex mixture that is present in an EO. The recognition of enantiomers has an important role in a large number of interactions at molecular level in drugs, taste perception, enzymatic specificity, and chemical communication between insects and plants. The phenomenon of chirality is common in nature at molecular level and has particular incidence on the detection between molecules responsible for a bioactivity and its recognition site, usually a protein or a glycoprotein complex. Numerous volatile products from plants have been identified as chiral molecules being very important floral indicators and it is clearly demonstrated that the neural complexes of insects, for example in bees, respond to the presence of a particular enantiomer (Laska & Galizia 2001). These data are fundamental when designing suitable traps to attract insect pests (Erbilgin *et al* 2001) or design suitable insect repellents. In our studies, an important fact is that we have been able to determine a non-common repellent effect that is related to aspects of the chirality of the main monoterpene present in all the EOs tested. Throughout this investigation, we have been able to determine that the levorotatory enantiomer of  $\alpha$ -pinene (*S,S*-(-)- $\alpha$ -pinene) has a marked repellent

effect. As far as our knowledge is concerned, there are no bibliographic reports on this behavior. To this repellent effect is added a very important mortality of the nymphs under study.

## Conclusions

In conclusion, the evaluated EOs showed some activity in all the bioassays performed; however, these results should be interpreted with caution. Ovicidal activity results presented high dispersion, although the oil from the higher and more illuminated hills (location I) showed good results at high doses. In consequence, it is reasonable to consider that the use of these EOs as ovicidal only could be a minor contributor in insect control.

The evaluation of the vapor phase of *E. buniifolium* EO showed high fumigant power against the nymph instar showing a high percentage of mortality which reached, in some cases, values of 92–100%. This data, added to the repellent power observed, can turn this natural product into a potential base of new preparations for the treatment outside the domestic environment in order to minimize the presence of the Chagas disease vector. The possible application of preparations based on this type of products in the future should be a complement to the traditional methods already tested for the control of this insect. Its application could contribute to decreasing the amounts of synthetic products under use.

In summary, we can say that the primary control of Chagas disease should be centered on its vector. In this sense, this research may be an adequate complementary method to achieve this objective and opens the possibility to continue studies that allow a cheap formulation to be tested in the field, although not in home spaces if there are no previous studies of toxicity in mammals.

Finally, as far as we know, the present work constitutes the first report of chiral recognition by *T. infestans* to a monoterpene. Clearly, the *S,S*-(-)- $\alpha$ -pinene was shown to be the most active as a repellent, which may lead to future studies for the proposal of a formulation of possible application in the control of this vector, looking for ways to enrich the EOs in this enantiomer.

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