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Essential oil from *Eupatorium buniifolium* leaves as potential varroacide

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Abstract Beekeeping has experienced a great expansion worldwide. Nowadays, several conventional pesticides, some organic acids, and essential oil components are the main means of chemical control used against *Varroa destructor*, an ectoparasite that may contribute to the colony collapse disorders. *Varroa* resistance against conventional pesticides has already been reported; therefore it is imperative to look for alternative control agents to be included in integrated pest management programs. A good alternative seems to be the use of plant essential oils (EOs) which, as natural products, are less toxic and leave fewer residues. Within this context, a bioprospecting program of the local flora searching for botanical pesticides to be used as varroacides was launched. A primary screening (driven by laboratory assays testing for anti-*Varroa* activity, and safety to bees) led us to select the EOs from *Eupatorium buniifolium* (Asteraceae) for follow up studies. We have chemical characterized EOs from twigs and leaves collected at different times. The three *E. buniifolium* EOs tested were active against *Varroa* in laboratory assays; however, there are differences that might be attributable to chemical differences also found. The foliage EO was selected for a preliminary field trial (on an experimental apiary with 40 hives) that demonstrated acaricidal activity when applied to the hives. Although activity was less than that for oxalic acid (the positive control), this EO was less toxic to bees than the control, encouraging further studies.

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Introduction

Beekeeping, now practiced for more than 4,500 years (Bradbear 2009), is an important activity not only in terms of agricultural production, but also in terms of family livelihoods (Bradbear 2004). Besides, as bees are among the main pollinators on Earth, their activity also provides a natural service (Potts et al. 2010).

The progressive death of domesticated worker bees, *Apis mellifera* L. (Hymenoptera: Apidae), has been generically named colony collapse disorder (CCD). An epizootiological study (van Engelsdorp et al. 2009) has recently concluded that no single risk factor is enough to distinguish colonies with CCD. Indeed, CCD may be correlated to many sanitary problems caused by viruses (Chen et al. 2007), mites (Sammataro et al. 2000), wax moths (Villegas and Villa 2006), beetles (Elzen et al. 1999), the American foulbrood (Hansen and Brodsgaard 1999), the European foulbrood (Roetschi et al. 2008), and other fungi (Ellis and Munn 2005). Among mites, *Varroa destructor* Anderson and Trueman (Acari: Varroidae), originally named *Varroa jacobsoni* (Anderson and Trueman 2000), is the main concern related to CCD (Dainat et al. 2012; Rosenkranz et al. 2010).

V. destructor is originally an ectoparasite of the Asian bee *Apis cerana* F., its natural host, on which it produces less damage than in *Apis mellifera* (Peng et al. 1987; Rosenkranz et al. 2010; Sammataro et al. 2000). *V. destructor* was first recorded parasitizing *Apis mellifera* in Hong Kong in 1962. From then, it took only a decade for its establishment in Europe and America (Denmark et al. 1991). The importance of *V. destructor* damage forced beekeepers to develop special management practices (Coffey 2007), as well as to use synthetic acaricides (Mehlhorn 2008). As expected, resistance to

acaricides has already been described worldwide (Maggi et al. 2010; Van Leeuwen et al. 2010). Since the discovery of the natural product thymol from essential oils (EOs) as a good control agent (Flamini and Atta-ur 2003), EOs have been the focus of several studies in regard to their potential as varroacides [reviewed by Flamini and Atta-ur (2003), Umpiérrez et al. (2011), Flamini (2006), and Imdorf et al. (1999)]. This work presents the results of one of these studies focused on local plants found in our region (Southern Cone of South-America). After a preliminary screening, we have selected the EOs from *Eupatorium buniifolium* (Asteraceae) for characterization of their chemistry and of their laboratory and field activities.

Experimental

Plant material and production of EOs

The aerial parts (fruits, leaves, twigs, and flowers when available) of the plants under investigation were collected at the times and places indicated in Table 1, where the extraction yields (EO weight/fresh plant material weight×100) are also shown. The species were identified by Prof. Eduardo Alonso-Paz (Cátedra de Botánica, Facultad de Química, Universidad de la República). All plant material was separated by their organs (fruits, leaves, flowers, and twigs) and the EOs obtained by steam distillation using a Clevenger apparatus. To perform the field bioassay, plant material was collected in Las Brujas, Canelones (34°39′51″S, 56°23′37″W) and the EO (*E. buniifolium* foliage) was obtained as previously described (Umpiérrez et al. 2012) by exogenously generated steam distillation using a 200-L alembic connected to a 50-L plant material container. After drying with anhydrous magnesium sulfate, EOs were stored under nitrogen, at −4 °C, in amber glass vials.

Experimental animals

Apis mellifera L. and *V. destructor* were collected from brood cells of organic commercial hives located in Canelones, Uruguay (34°43′30″S, 56°5′13″W) the same day that bioassays

Table 1 Toxicity of selected EOs against bees and *Varroa* at 1/10 dilution (0.26 mg/cm^3 total volume of the assay dish)

Family	Species	Common name regional/English	Collection			EO Yield (%)	Toxicity at 48 h		
			Place	Time	Organ		Bees[a]	Varroa[b]	Risk ratio[c]
Apiaceae	*Pastinaca sativa* L.	Apio Silvestre/ Parsnip	34°46′33.6″S, 56°8′16.8″W	Winter	Fruit	0.4	95±10d	50±14a	1.92
Asteraceae	*Artemisia absinthium* L.	Ajenjo/Absinthium or Wormwood	34°40′12″S, 56°3′23.76″ W	Winter	Twig	0.1	93±12d	NT	NA
	Eupatorium buniifolium Hook. et Arn.	Chirca negra/Boneset	34°40′12″S, 56°3′23.76″W	Fall	Flower	0.1	67±42c,d	87±12b	0.77
				Winter	Leaf	0.2	13±12a,b	80±20b	0.18
				Summer	Leaf	0.2	47±12b	100±0b	0.46
			34°35′45.6″S, 56°15′0″W	Summer	Twig	0.04	0±0a	100±0b	0
Verbenaceae	*Aloysia gratissima* (Gillies and Hook.) Tronc.	Cedrón de monte/ Beebrush	34°40′48″S, 56°11′20.4″W	Spring	Leaf	0.2	NT	100±0b	NA
	Lippia alba (Mill.) N. E. Br. ex Britt. and Wilson	Salvia trepadora/ Bushy Matgrass	–[d]	–[d]	Leaf	–[d]	100±0	100±0b	1
Anacardiaceae	*Schinus molle* L. (♂)	Anacahuita/ Peppertree	34°50′45.6″S, 56°7′40.8″W	Winter	Leaf		47±50b,c	NT	NA
					Twig		100±0d	100±0b	1
	Schinus molle L. (♀)		34°40′48″S, 56°11′20.4″W	Winter	Leaf		47±12b,c	87±23b	0.85
				Spring	Fruit		100±0d	100±0b	1.05
Solvent (Ethanol)							6.8±1.0a	8±4a	
Negative control							8.4±1.6a	8±2a	

Different letters within the same column indicate significant differences (ANOVA test at $P < 0.05$, pairwise comparisons with Tukey's family error)

NT not tested (*Varroa* were not included in these trials), *NA* risk ratio not available

[a] Bee toxicity is reported as dead plus knockdown animals

[b] *Varroa* toxicity is reported as dead plus fallen off from the bees

[c] Risk ratios were calculated as percentage of dead + knockdown bees/percentage of dead + dislodged *Varroa*

[d] Data are not available: the EO was provided by INIA, Las Brujas from its collection

were started. The bees in this region are predominantly hybrid bees (known as "Creole") resulting from crosses of *Apis mellifera mellifera* (European bees) with *Apis mellifera scutellata* (African bees) (Burgett et al. 1995; Carrasco-Letelier et al. 2012; Diniz et al. 2003). They were kept under controlled conditions, and fed on a sugar/honey preparation (Ruffinengo et al. 2005) throughout the experiments. Nurse bees (4 to 11 days old) were used for the laboratory assays.

Laboratory bioassays

The initial screening of the EOs for selective activity was performed with both arthropods simultaneously in each experimental unit. The bioassays were performed following the design of the "vapor only" dish bioassay previously described (Lindberg et al. 2000). Briefly, the activity of EO vapors against both arthropods was evaluated using a two-chamber system [made with two bases of plastic Petri dishes 9×1 cm, separated by a perforated lid (ca. four holes/cm^2)]. Five nurse bees and five adhering *Varroa* (one per bee) were placed into the upper chamber. In the lower chamber, a filter paper (36 cm^2) treated with 0.5-mL of either ethanol (solvent control) or the EO ethanolic solutions (10 % weight/volume; treatment) was placed. In this manner, a final concentration of 0.26 mg/cm^3 (dish volume) was achieved. An additional negative control without solvent was run to assess natural death (N=5 in all cases). Assays were run for 48 h, incubating the plates at 20–22 °C and 60–70 % RH. Toxicity to bees and mites was recorded as dead and knocked-down (non-responsive) bees and dead and fallen off mites at 24 and 48 h. A risk ratio was determined for the results of the EO screening as percentage of dead + knocked-down bees/percentage of dead + dislodged *Varroa* (i.e., percentage of individuals intoxicated). From these ratios, the EOs for further studies were chosen, and their lethal doses (LD_{99}), their knockdown doses for bees (KD_{99}) and their doses needed for 99 % mites dislodgement from the bees (FD_{99}) were determined following the same experimental protocol. For the selected EOs, selectivity indices were calculated as *Apis mellifera* LD_{99}/*V. destructor* LD_{99} and *Apis mellifera* KD_{99}/*V. destructor* FD_{99} following the procedure previously reported (Ruffinengo et al. 2005). For comparative purposes, the LD_{99}, KD_{99}, and FD_{99} were also obtained for formic acid and thymol (both from Sigma), two control agents commonly used in organic practices by beekeepers.

Field bioassay

An experimental apiary kept by the Beekeeping Unit of Experimental Station Alberto Boerger, INIA-La Estanzuela (34°20′22.20″S, 57°41′14.93″W, Colonia, Uruguay) was used to perform a 21-day field assay. Forty Langstroth hives (with only brood chambers) were used following a complete randomized design (regarding previous infection rate and natural bee death) to apply four treatments: (1) oxalic acid (OA), (2) amitraz, (3) *E. buniifolium* leaf EO, and (4) negative control (no product applied). OA is usually used in organic practices of beekeeping and amitraz is a conventional pesticide. Treatments were as follows: (1) OA was applied in 50 mL of sucrose syrup (6.2 %) by dripping between frames. After the initial application, two re-applications were made at 7-day intervals (as it is usually done by local beekeepers). (2) Amitraz was applied as recommended by the manufacturer (two Amivar® strips per hive). (3) *E. buniifolium* leaf EO was applied as an aqueous emulsion (TWEEN® 20, 2 %) applied on Floral foam bricks (4.5×4.5×0.95 cm, Oasis®) that were placed on the top of the frames. Two applications were done: first application, 4.3 g per hive and second application, 8.6 g at day 12 of the assay. The mortality of bees and mites was monitored daily. Bee mortality was measured with traps at the entrance of the hive. Fallen *Varroa* were collected with technical floors (drilling 3 mm^2) bottom-lined with a paper treated with Vaseline®. At the end of the 21-day period, to calculate the treatment efficacy, a last treatment with coumaphos and flumetrin was carried out in each hive to kill and count surviving mites as recommended (European Working Group CA3686 2001).

Chemical characterization

For the identification of EOs constituents, a Shimadzu 2010 gas chromatograph coupled to a Shimadzu QP2010 plus mass spectrometer was used. In all cases, injections were 1 μL of EO diluted in dichloromethane (10 mg/mL). The analyses were performed with an OPTIMA-5-MS column (30 m×0.25 mm id×0.25-μm film thickness; Macherey-Nagel). The analytical conditions were as follows. Gas carrier: helium (1 mL/min); oven temperature: from 40 °C (isothermally held for 2 min) to 240 °C (5 °C/min, and held for 1 min), and then increased to 320 °C (10 °C/min, held for 5 min); injector and detector temperatures were 250 °C; injector mode was split (30:1); ionization potential 70 eV; scan range 40–350 m/z. The identification of constituents of EOs was done by comparison of the calculated Retention Indices (RI) with those reported by Adams (2007) and Pherobase (El-Sayed 2012) and by comparison of fragmentation patterns with those contained in NIST 05 and SHIM 2205 mass spectrometer libraries. The relative amount (uncorrected) of each constituent was estimated from the corresponding peak area expressed as the percentage of the total peak area in the chromatogram.

Statistical analyses

The results of the screening assays (Table 1) were analyzed by an ANOVA test follow by pairwise comparisons using a

Table 2 *E. buniifolium* EOs toxicity on bees and *Varroa* as mean ± std error (in percent) as a function of the time of collection, organ extracted, and time of the experiment (N=5 each)

EO kind	Bees				*Varroa*			
	Knockdown + dead		Dead		Fallen + dead		Dead	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Fall flower	67±24	67±24	60±20	67±24	47±18	87±7	7±7	80±10
Summer leaf	47±7	47±7	40±7	47±7	53±13	100±0	40±12	80±12
Winter leaf	13±7	13±7	0±0	13±7	73±7	80±12	53±18	80±12
Summer twig	0±0	0±0	0±0	0±0	87±13	100±0	33±7	100±0

EOs were applied at 0.26 mg/cm^3 (dish volume)

Tukey's test at P <0.05 using the MINITAB 12.2 software package. In these assays, mortality caused by the control solvent was compared with the natural death rate applying t tests for non-paired data (Zar 1999). The mortality of either arthropod was not different for the assays where the solvent was used (6.8±0.9 and 8.7±0.2 % of dead bees and *Varroa*, respectively) compared to natural death (8±2 and 8.4±0.3 % of dead bees and *Varroa* respectively) during the treatment (P >0.6, for both arthropods and t tests on transformed data). Since this mortality was <10 %, no correction on data was performed in further analyses (Abbott 1925).

When comparing discrete data on toxicity from the *E. buniifolium* foliar EO (Table 2), results were analyzed with the Fisher Exact text (2-tailed; with a level of significance of P <0.05). The procedure was done following Zar (1999). The LD_{99}, KD_{99}, and FD_{99} were calculated by regression analyses using the Statgraphics Plus package (Table 3).

Unless otherwise indicated, all data are presented as means ± standard error. Chemical data were subject to Principal Component Analysis (PCA) performed on compound class using the statistical software PAST. To improve comparison among data (Zar 1999), the relative areas (in percent) were transformed as arcsin $\sqrt{p}$, where p is the proportion of each compound class.

Results and discussion

Screening of EOs

The activity of the EOs against bees and mites and their risk ratios are shown in Table 1. Since toxicity for bees (as knockdown plus dead) did not change from 24 to 48 h for all EO except that from *Schinus molle* fruits, Table 1 only shows the results at 48 h. Likewise, the number of dislodged plus dead *Varroa* did not change from 24 to 48 h (although the number of dead *Varroa* did increase as fallen *Varroa* were dying—data not shown). All EOs showed some degree of toxicity to mites, with that from *Pastinaca sativa* being the least active. At the same time, this EO exhibited high toxicity against bees (96 %, Table 1), with a concomitant high-risk ratio (1.92). This EO was therefore eliminated from follow up studies. In general, since risk ratios were calculated as percentage of dead bees/percentage of dead *Varroa*, EOs

Table 3 Lethal doses 99 (LD_{99}), knockdown doses 99 (KD_{99}), and falling off doses 99 (FD_{50}) at 48 h of EOs from *E. buniifolium* leaves and two positive controls used often in organic practices of beekeeping

	LD_{99} (mg/cm^3)[a]		KD_{99} (mg/cm^3)[a]	FD_{99} (mg/cm^3)[a]	Selectivity index	
	Bees	*Varroa*	Bees	*Varroa*	LD_{99b}/LD_{99v} [b]	KD_{99b}/FD_{99v} [c]
Summer leaves EO	0.7 (0.4–1.1)	0.3 (0.2–0.5)	0.7 (0.3–1.2)	0.2 (0.1–0.3)	2	3.5
Winter leaves EO	–[d]	0.07 (0.05–0.08)	–[d]	0.05 (0.04–0.07)	–[e]	–[e]
Formic acid	0.02 (0.01–0.04)	0.07 (−0.03–0.18)	0.02 (0.01–0.04)	0.04 (0.01–0.08)	0.3	0.5
Thymol	0.08 (0.04–0.11)	0.07 (0.02–0.13)	0.08 (0.04–0.11)	0.07 (0.02–0.13)	1.1	1.1

Parentheses show the 95 % confidence interval calculated by linear regression analyses (P <0.05).

[a] Mass/application volume

[b] LD_{99b}/LD_{99v}: *A. mellifera* LD_{99}/*V. destructor* LD_{99}

[c] KD_{99b}/FD_{99v}: *A. mellifera* KD_{99}/*V. destructor* FD_{99}

[d] Bee mortality was 0 at the highest tested doses

[e] A security index was not calculated for this EO as there was no bee mortality at the highest tested dose

with high-risk ratios were eliminated. For instance, the *E. buniifolium* floral EO exhibited good acaricidal activity but also produced high-bee mortality (risk ratio=0.77).

On the other hand, *E. buniifolium* twig EO was the most active against *Varroa* (100 % mortality) and innocuous to bees (0 % mortality, risk ratio=0), followed by the Table 6 EO from leaves which exhibited a good acaricidal activity with apparently some degree of toxicity towards bees (risk ratios equal to 0.18 and 0.46 for winter and summer foliar EO respectively). In the case of EO from winter leaves, bee mortality was not significantly different from the mortality found in the controls (Table 1).

Toxicity of *Eupatorium buniifolium* EOs in laboratory tests

Table 2 shows the detailed results for all *E. buniifolium* EOs in terms of percentages of dead and knockdown effects. Clearly, the effect of EOs differed between the bees and the mites. Whereas bee toxicity did not change from 24 to 48 h, EOs produced more fallen off and dead mites with time indicating either a cumulative or a delayed effect.

The EO from *E. buniifolium* twigs exhibited excellent attributes (Table 2) as it was not toxic to bees, and the effect on *Varroa* was high even at 24 h (87±13 and 100±0 % fallen off plus dead *Varroa* at 24 and 48 h, respectively). However, this EO had low distillation yield (0.04 %) precluding subsequent studies as obtaining large amounts for field assays would have been too costly and time-consuming. Furthermore, although the twig EO seems to kill more *Varroa* (100±0 %) than EOs from both summer and winter leaves (80±12 %, Table 2), these differences were not significant (Fisher's Exact Tests, P=0.11); indicating that the foliar EO (disregarding their time of collection) was as good as the EO from twigs as a varroacide. On the other hand, toxicity against bees did differ among these products, and even though bee mortality was lower for the twig EO (0±0 %) than the mortality caused by the EO from summer leaves (47±7 %, Table 2), it was not significantly different from the bee mortality caused by winter foliar EO (13±7 %, Fisher's Exact Test P=0.11). Finally, the foliar EOs did not show differential activity against *Varroa* related to the time of collection of the plant material (Table 2. Fisher's Exact Tests, P=0.11).

Therefore, since the EOs obtained from *E. buniifolium* leaves collected at different times showed low-risk ratios during the observation time as well as better yields, they were chosen to evaluate the dose-dependent effect (as well as their field activity). The LD_{99}, KD_{99}, and FD_{99} for the EOs from leaves are shown in Table 3. Both *E. buniifolium* leaf EOs exhibited better selectivity indices than formic acid and thymol, two varroacides commonly used in organic beekeeping. Although both, formic acid and thymol were *c.a.* four times more toxic than the summer-leaf EO towards *Varroa*, they also were more toxic against bees (35 and 8 times more toxic respectively). At the same time, formic acid and thymol had the same toxicity against *Varroa* than the winter-leaf EO, but this EO produced no bee mortality at the highest doses tested.

Activity of *Eupatorium buniifolium* EO in field tests

The activity of the *E. buniifolium* EO applied to hives compared to the negative and positive controls (amitraz and OA) at the end of the 21-day experiment is shown in Table 4. This table also shows mortality at day 7 before OA re-application. Even though acaricide activity was better for both positive controls compared to the *E. buniifolium* EO, the latter showed no toxicity against bees (not significantly different compared to the negative control). This was not the case for OA, which caused bee mortality significantly higher than all other treatments at day 7 and at the end of the bioassay. Therefore, when applied in the field, this EO would have a lower-risk ratio than OA.

Chemical characterization of EOs from *E. buniifolium*

The chemical compositions of the different EOs produced from *E. buniifolium* are shown in Table 5 with the exception of the flower EO that was not analyzed due to its bee toxicity (Table 1) [a figure showing the three gas chromatography/mass spectrometry (GC/MS) traces are included as supplementary material]. The compounds

Table 4 Cumulative mortality on hives (mean ± standard error) of *Varroa* and bees as a consequence of treatment with the four products tested (N=10 per product)

	7-day period		21-day period		
Product	Bees	*Varroa*	Bees	*Varroa*	Effectiveness (%)
E. buniifolium	3±1a	259±88a	9±1a	630±194a	32±4a
Amitraz	18±4a	1,532±411b	28±6b	2,243±556b	96±1b
Oxalic acid	102±20b	485±110a	182±39c	1,451±343a,b	74±4c
Negative control	6±2a	67±21a	18±4a,b	644±228a	21±4d

Different letters within the same column indicate significant differences (ANOVA test at P <0.05, pairwise comparisons with Tukey's family error)

Table 5 Chemical composition of the different tested EOs from *E. buniifolium*. Data obtained by GC/MS

Compound class	Compound	RI bibliog.	Summer leaves			Winter Leaves			Twigs		
			Simil. (%)	Area (%)	RI calc.	Simil. (%)	Area (%)	RI calc.	Simil. (%)	Area (%)	RI calc.
Heterocycle	N-methyl pirrol	X	–	–	–	–	–	–	97	0.12	<800
Alcohols	3Z-hexenol	850	93	0.03	849	–	–	–	–	–	–
	2Z-Hexenol	859	86	0.01	860	–	–	–	–	–	–
	1-Hexanol	863	93	0.06	863	–	–	–	93	0.11	863
Aldehydes	Hexanal	801	–	–	–	–	–	–	96	0.20	<800
	2E-hexenal	846	92	0.03	844	93	0.09	843			
	Nonanal	1,100	91	0.03	1,101				89	0.02	1,102
Ketones	2-Methyl-4-heptanone	918	87	0.01	914	92	0.02	914	–	–	–
	3-methyl-4-heptanone	918	–	–	–	91	0.01	919	–	–	–
Non-oxygenated	Tricyclene	921	–	–	–	91	0.07	917	–	–	–
Monoterpenes	NI	X	X	0.01	918	–	–	–	X	0.01	919
	α-Thujene	924	95	0.14	924	96	0.53	922	94	0.11	925
	α-Pinene	932	97	8.20	931	96	14.75	930	97	5.53	932
	Camphene	946	97	0.56	945	96	1.60	944	97	0.46	947
	2,4(10)-Thujadiene	953	–	–	–	–	–	–	86	0.03	952
	Sabinene	969	97	1.52	971	95	3.71	970	95	0.81	973
	β-Pinene	974	97	2.65	974	96	5.53	973	96	2.12	976
	β-Myrcene	988	96	0.92	989	96	2.28	988	95	0.55	991
	NI	X	–	–	–	X	0.02	997	–	–	–
	4-Carene or 2-carene	1,001	86	0.02	1,000	–	–	–	95	0.11	1,002
	α-Phelandrene	1,002	–	–	–	85	0.02	1,001	81	0.01	1,005
	α-Terpinene	1,014	93	0.07	1,016	95	0.33	1,014	92	0.10	1,017
	Ocymene	1,022	86	0.02	1,022	86	0.04	1,021	93	0.07	1,024
	Limonene	1,024	94	2.19	1,028	93	3.88	1,027	94	1.61	1,030
	(Z)-β-ocymene	1,032	89	0.06	1,037	89	0.08	1,036	–	–	–
	(E)-β-ocymene	1,044	96	2.40	1,048	95	2.16	1,046	–	–	–
	γ-Terpinene	1,054	96	0.15	1,059	97	0.54	1,058	96	0.17	1,060
	Terpinolene	1,086	96	0.36	1,089	95	0.69	1,088	94	0.12	1,090
	NI	X	X	0.02	1,360	X	0.02	1,359	–	–	–
Oxygenated	Linalool	1,095	87	0.04	1,099	88	0.05	1,098	–	–	–
Monoterpenes	Z-p-menth-2-en-1-ol	1,118	–	–	–	83	0.03	1,120	–	–	–
	α-Campholenal	1,122	–	–	–	–	–	-	90	0.03	1124
	NI	X	–	–	–	X	0.03	1,138	–	–	–
	E-pinocarveol	1,135	–	–	–	–	–	–	90	0.06	1,139
	E-verbenol	1,140	–	–	–	–	–	–	92	0.06	1,144
	Pinocarvone	1,160	–	–	–	–	–	–	87	0.03	1,160
	Borneol	1,165	89	0.08	1,166	94	0.35	1,164	91	0.21	1,165
	Z-pinocamphone	1,172	–	–	–	–	–	–	82	0.02	1,171
	Terpinen-4-ol	1,174	97	0.31	1,177	96	0.80	1,175	95	0.35	1,176
	P-cymen-8-ol	1,179	80	0.01	1,181				84	0.02	1,181
	α-Terpineol	1,186	96	0.17	1,189	96	0.44	1,188	96	0.19	1,188
	Myrtenal	1,195	–	–	–	–	–	–	91	0.04	1,191
	Myrtenol	1,194	–	–	–	–	–	–	87	0.03	1,194
	NI	X	X	0.02	1,231	X	0.03	1,230	–	–	–
	NI	X	–	–	–	–	–	–	X	0.05	1,293
	NI	X	–	–	–	–	–	–	X	0.01	1,333

Table 5 (continued)

Compound class	Compound	RI bibliog.	Summer leaves			Winter Leaves			Twigs		
			Simil. (%)	Area (%)	RI calc.	Simil. (%)	Area (%)	RI calc.	Simil. (%)	Area (%)	RI calc.
	Eugenol	1,356	81	0.09	1,355	81	0.16	1,354	–	–	–
Non-oxygenated	δ-Elemene	1,335	93	1.69	1,341	95	0.53	1,342	93	0.20	1,342
Sesquiterpenes	NI	X	–	–	–	–	–	–	X	0.06	1,354
	α-Terpinyl acetate	1,346	–	–	–	–	–	–	82	0.03	1,359
	α-Copaene	1,374	84	0.06	1,382	89	0.08	1,381	94	0.14	1,381
	β-Elemene	1,389	95	0.45	1,389	94	0.25	1,388	91	0.14	1,388
	β-Bourbonene	1,387	–	–	–	–	–	–	89	0.04	1,390
	NI	X	–	–	–	96	5.86	1,395	–	–	–
	β-Isocomene	1,407	81	0.05	1,400	–	–	–	85	0.13	1,398
	NI	X	–	–	–	–	–	–	X	0.02	1,416
	β-Caryophyllene	1,417	96	1.75	1,428	97	2.57	1,426	97	2.81	1,426
	β-Copaene	1,432	–	–	–	82	0.12	1,435	90	0.25	1,435
	γ-Elemene	1,434	97	0.63	1,439	82	0.10	1,437	88	0.14	1,437
	α-Guaiene*	1,437	96	0.40	1,445	94	0.40	1,443	–	–	–
	1(5),11 Guaiadiene	1,453	–	–	–	–	–	–	96	1.17	1,443
	6,9-Guaiadiene	1,442	92	0.50	1,450	90	0.46	1,448	91	1.28	1,448
	NI	X	X	0.30	1,457	X	0.10	1,451	–	–	–
	E-muurola 3,5-diene	1,451	–	–	–	89	0.36	1,455	86	0.76	1,455
	α-Caryophyllene	1,452	95	0.62	1,463	95	0.91	1,461	95	1.42	1,461
	E-9-Epi-caryophyillene	1,464	–	–	–	–	–	–	94	1.49	1,468
	NI	X	–	–	–	X	0.42	1,469	–	–	–
	4,5-Di-epi-aristolochene	1,471	–	–	–	83	0.13	1,476	–	–	–
	γ-Muurolene	1,478	90	0.20	1,471	90	1.23	1,482	91	1.58	1,483
	α-Amorphene	1,483	90	0.70	1,484	–	–	–	–	–	–
	Germacrene D	1,484	95	11.14	1,491	92	8.53	1,489	91	12.32	1,489
	β-Selinene	1,489	–	–	–	–	–	–	94	1.55	1,494
	α-Selinene	1,498	91	0.70	1,496	94	1.46	1,494	90	0.45	1,497
	δ-Selinene	1,492	88	0.23	1,499	–	–	–	–	–	–
	E-muurola-4(14),5-diene	1,493	81	0.43	1,502	–	–	–	–	–	–
	Bicyclogermacrene	1,500	96	3.24	1,506	94	3.49	1,503	94	4.62	1,504
	α-Muurolene	1,500	–	–	–	–	–	–	94	1.57	1,506
	E-β-Guaiene*	1,502	92	7.39	1,516	89	5.14	1,513	–	–	–
	NI	X	–	–	–	–	–	–	X	9.09	1,514
	γ-Cadinene	1,513	89	0.57	1,522	91	0.54	1,520	92	0.82	1,520
	δ-Cadinene	1,522	91	2.24	1,530	91	2.07	1,528	92	3.74	1,528
	E-iso-γ-Bisabolene	1,528	–	–	–	–	–	–	82	0.07	1,533
	3,7(11)Eudesmadiene	1,545	–	–	–	–	–	–	86	0.36	1,537
	NI	X	X	0.62	1,538	–	–	–	–	–	–
	α-Cadinene*	1,537	80	0.72	1,545	90	0.17	1,542	92	0.60	1,543
	α-Calacorene	1,544	–	–	–	–	–	–	91	0.33	1,546
	Germacrene B	1,559	95	2.72	1,567	95	1.51	1,564	93	2.28	1,564
	Guaiazulene	1,779	–	–	–	–	–	–	80	0.08	1,778
	NI	X	X	0.26	1,571	–	–	–	–	–	–
Oxygenated	Elemol	1,548	95	1.81	1,554	93	0.75	1,551	93	0.63	1,552
Sesquiterpenes	NI	X	–	–	–	–	–	–	X	0.40	1,557
	NI	X	X	0.22	1,579	–	–	–	–	–	–

Table 5 (continued)

Compound class	Compound	RI bibliog.	Summer leaves			Winter Leaves			Twigs		
			Simil. (%)	Area (%)	RI calc.	Simil. (%)	Area (%)	RI calc.	Simil. (%)	Area (%)	RI calc.
	Spathulenol	1,577	–	–	–	86	0.87	1,581	91	2.90	1,582
	Germacrene D-4-ol	1,574	87	0.83	1,582	–	–	–	–	–	–
	Caryophyllene oxide	1,582	–	–	–	88	0.63	1,587	90	2.91	1588
	Viridiflorol	1,592	88	1.01	1,593	83	0.36	1,591	–	–	–
	NI	X	–	–	–	X	0.44	1,596	X	0.66	1,597
	Globulol	1,590	87	0.79	1,598	–	–	–	–	–	–
	NI	X	–	–	–	X	0.54	1,608	X	0.69	1,608
	NI	X	X	0.64	1,611	X	2.24	1,616	X	0.57	1,614
	NI	X	X	2.31	1,619	–	–	–	–	–	–
	Junenol	1,618	–	–	–	–	–	–	83	0.24	1,624
	1-Epi-cubenol	1,627	84	0.97	1,627	82	0.34	1,624	–	–	–
	NI	X	X	0.88	1,634	X	0.75	1,631	X	1.95	1,631
	γ-Eudesmol	1,630	90	0.37	1,639	89	0.35	1,636	89	0.44	1,636
	α-Epi-muurolol	1,640	–	–	–	–	–	–	92	2.21	1,645
	10-Epi-muurolol	1,640	–	–	–	92	1.52	1,645	–	–	–
	α-Cadinol	1,652	93	1.54	1,648	89	0.71	1,656	92	3.36	1,659
	α-Muurolol	1,644	84	0.46	1,652	85	0.48	1,649	83	0.86	1,649
	β-Eudesmol	1,649	–	–	–	84	0.71	1,656	86	1.12	1,656
	Himachalol	1,652	89	4.42	1,662	–	–	–	–	–	–
	NI	X	–	–	–	–	–	–	X	0.37	1,665
	NI	X	X	0.31	1,669	–	–	–	–	–	–
	NI	X	–	–	–	–	–	–	X	0.64	1,675
	NI	X	–	–	–	–	–	–	X	0.14	1,682
	NI	X	X	0.20	1,685	–	–	–	–	–	–
	NI	X	X	0.25	1,688	–	–	–	–	–	–
	4(15),5,10(14) Germacratrien-1-α-ol	1,685	–	–	–	–	–	–	81	1.68	1,689
	8-Cedren-13-ol	1,688	–	–	–	82	2.00	1,689	–	–	–
	(Z)α-bergamotol	1,693	81	4.66	1,692	–	–	–	–	–	–
	NI	X	X	1.69	1,701	93	1.11	1,698	–	–	–
	Shyobunol	1,688	–	–	–	–	–	–	94	1.48	1,698
	NI	X	X	1.47	1,705	X	0.54	1,702	–	–	–
	NI	X	–	–	–	X	0.28	1,709	X	0.31	1,710
	NI	X	X	0.74	1,712	X	0.11	1,712	X	0.49	1,712
	NI	X	–	–	–	–	–	–	X	0.18	1,720
	NI	X	–	–	–	–	–	–	X	0.37	1,723
	NI	X	X	0.10	1,726	X	0.16	1,727	X	0.64	1,727
	NI		X	0.15	1,730	–	–	–	–	–	–
	NI		–	–	–	–	–	–	X	0.08	1,750
	NI		–	–	–	–	–	–	X	0.08	1,758
	2α-Hidroxi-amorfa-4, 7(11)-diene	1,779	80	0.12	1,776	–	–	–	82	0.18	1,769
	14-Hydroxy-δ-cadinene	1,803	80	0.03	1,805	–	–	–	84	0.03	2,026
Sulfurated sesquiterpene	Mintsulfide	1,740	85	0.08	1,749	–	–	–	–	–	–
Diterpene	NI	X	X	0.09	2,015	X	0.09	2,011	–	–	–
	NI	X	X	0.03	2,038	X	0.02	2,035	–	–	–
	NI	X	X	0.02	2,051	X	0.02	2,058	–	–	–

Table 5 (continued)

Compound class	Compound	RI bibliog.	Summer leaves			Winter Leaves			Twigs		
			Simil. (%)	Area (%)	RI calc.	Simil. (%)	Area (%)	RI calc.	Simil. (%)	Area (%)	RI calc.
	NI	X	X	0.02	2,061	–	–	–	–	–	–
	NI		X	0.22	2,213	–	–	–	–	–	–
Non identified structures	Total			6.04			4.01		–	7.22	–

NI non identified compound, *% Simil*. refers to the percentage of similarity when comparing the mass spectrum obtained with the spectra from the Adams (2007) database, *% Area* compound concentration from the TIC chromatogram (not normalized). *RI* retention index (calc.: calculated; bibliog.: data from the Adams (2007) and Pherobase (El-Sayed 2012) databases), *X* absence.

* Tentative identification

identified from the three EOs account for ca. 80 % of the total chromatogram area. Regardless of their oxidation state, sesquiterpenes accounted for more than 50 % of the composition (65, 75, and 75 % for the EOs from summer leaves, winter leaves, and twigs, respectively, Table 6). The main compounds in these EOs (α- and β-pinene, sabinene, limonene, β-ocymene, germacrene D and B, E-β-guaiene, δ-cadinene) were similar to the ones previously reported by Lorenzo et al. (2005) and Umpiérrez et al. (2012) which were obtained in both studies from plant material collected in Canelones, Uruguay. However, these essential oils do differ in their composition compared to the ones reported by Lancelle et al. (2009) and Ruffinengo et al. (2005) which were collected in the Andino Cuyana and Andino Patagónica areas. In turn, these locations belong to different global ecological zones (FAO 2001). As it has been suggested, these differences may be due to the existence of different chemotypes in *E. buniifolium*, similar to other Asteraceae (Umpiérrez et al. 2012). In the work from Ruffinengo et al. (2005), the EO obtained was monoterpene-rich and when tested as a varroacide, was not found to be very active.

Table 6 Main compound class in the different tested EOs from *E. buniifolium* (as percentage of total uncorrected area). These data were subjected to principal component analyses

	Summer leaves	Winter leaves	Twigs
Total	90.26	93.69	95.73
Identified compounds	79.71	80.94	78.92
Non identified compounds[a]	10.55	12.75	16.81
Heterocycles	–	–	0.12
Short-chain alcohols	0.10	0.00	0.11
Short-chain aldehydes	0.06	0.09	0.22
Short-chain ketones	0.01	0.03	0.00
Non-oxygenated monterpenes	19.29	36.25	11.81
Oxygenated monterpenes	0.72	1.86	1.10
Non-oxygenated sesquiterpenes	37.61	36.43	49.54
Oxygenated sesquiterpenes	25.97	14.89	25.61
Sulfur compounds	0.08	–	–
Diterpenes	0.38	0.13	0.00
Non identified structures	6.04	4.01	7.22

[a] Non identified: compounds either belonging to a particular compound class or non-identified structures

The EO from winter leaves contained higher amounts of monoterpenes (regardless of their oxidation state) than the other two EOs (Tables 5 and 6). The presence of a sulfur-containing monoterpene in the EO from summer leaves is noteworthy. Sulfur-containing compounds are common in the Asteraceae, especially as acetylenic thiophenes (Bicchi et al. 1992; Szarka et al. 2006). The sesquiterpene mintsulfide is ubiquitous across plant families: it has been described from members of the Anacardiaceae (Kossouoh et al. 2008), Apiaceae (Baser et al. 2006; Baser et al. 2000), Asteraceae (Baser et al. 2001; El-Shamy et al. 2000; Kalemba 1998; Kalemba et al. 2001; Miyazawa et al. 2008; Yanming et al. 2005), Lamiaceae (Javidnia et al. 2006a; Javidnia et al. 2006b; Kukic et al. 2006; Tirillini et al. 2004), Lauraceae (Ciccio and Chaverri 2008), and Meliaceae (Asekun and Ekundayo 1999) among others. In particular for Asteraceae, the EOs from *Tanacetum* spp. (Baser et al. 2001), *Grindelia* spp. (El-Shamy et al. 2000), *Solidago* spp. (Kalemba 1998; Kalemba et al. 2001), *Seriphidium transiliense* (Yanming et al. 2005), *Aster ageratoides* (Miyazawa et al. 2008) and *Eupatorium cannabinum* subsp. *corsicum* (Paolini et al. 2005) possess this compound.

Table 7 Eigenvalues and percentage of variability for the components found by PCA using the correlation matrix with the compound class

CP 1	Eigenvalues	Percentage of variability
1	7.11	64.62
2	3.89	35.38
3	1 E-30	1 E-29
4	8 E-65	7 E-64
5	0	0

The PCA performed on compound class (Table 6) from these three EOs showed two components that explain almost 100 % of the data variation (64 and 35 % for component 1 and 2, respectively, Table 7). Furthermore, since components 3 and 4 had eigenvalues less than the Jolliffe cut-off (0.7) (Hammer Ø et al. 2009), only components 1 and 2 were considered to explain the data. According to these results (Fig. 1), the EOs from leaves and twigs are well characterized with the variables studied. Winter-leaf EO exhibited more monoterpenes (either as hydrocarbons and oxygenated) and ketones. Summer-leaf EO is characterized by the presence of oxygenated sesquiterpenes, sulfur-containing compounds, and diterpenes. Finally, the twig EO exhibited greater amounts of non-oxygenated sesquiterpenes, aldehydes, and heterocycles. From our results, one could speculate that the differences in activity may be explained by the presence of sulfur-containing sesquiterpenes and more diterpenes in the summer-leaf EO (Tables 2 and 3).

EOs from other Asteraceae has also been previously studied as varroacides (Umpiérrez et al. 2011). These include *Tagetes minuta* (Eguaras et al. 2005; Ruffinengo et al. 2005, 2007), *Wedelia glauca* (Ruffinengo et al. 2005), *E. buniifolium* (Ruffinengo et al. 2005), *Heterotheca latifolia* (Ruffinengo et al. 2007, 2002), *Heterothalamus alienus* (Ruffinengo et al. 2006), and *Artemisia dracunculus* (Ariana et al. 2002). An ethanolic extract from *Baccharis flabellata* (Damiani et al. 2009) was also tested as a varroacide. In the cases of previously tested EOs where the chemical composition is reported, a rough generalization might be that these EOs were monoterpene-rich [e.g., in *Tagetes minuta* main constituents were ocimenes and tagetenes (Eguaras et al. 2005); in *W. glauca* (Ruffinengo et al. 2005), limonene and pinenes in *Heterothalamus alienus* (Ruffinengo et al. 2006), β-pinene] with the exception of the EO from *Heterotheca latifolia* (Ruffinengo et al. 2007) which mainly contained bicyclic oxygenated monoterpenes (camphor and borneol). Even though these chemical differences may account for the differences in activity among the EOs previously reported, as well as the one studied here, more studies are clearly needed to confirm if that is the case.

Considering that our laboratory and field results represent our first attempt to use *E. buniifolium* EO as a control agent in hives, these appear promising. Future studies will focus on dosages, application mode, and controlling the geometry of the hives. Regarding the dosage, it is worth noting that calculations on how much to apply were based on LD_{99} in laboratory tests and on the hive volume. In the future, doses will have to take into account air circulation inside the hives, temperature, and bee behavior in relation to thermoregulation triggered by stress factors (Stabentheiner et al. 2010) as temperature variations may change the effectiveness of the applied chemicals.

A more detail study must also be carried out on the chemical variation of the EO from *E. buniifolium*. On one hand, previous results for *E. buniifolium* EO from other

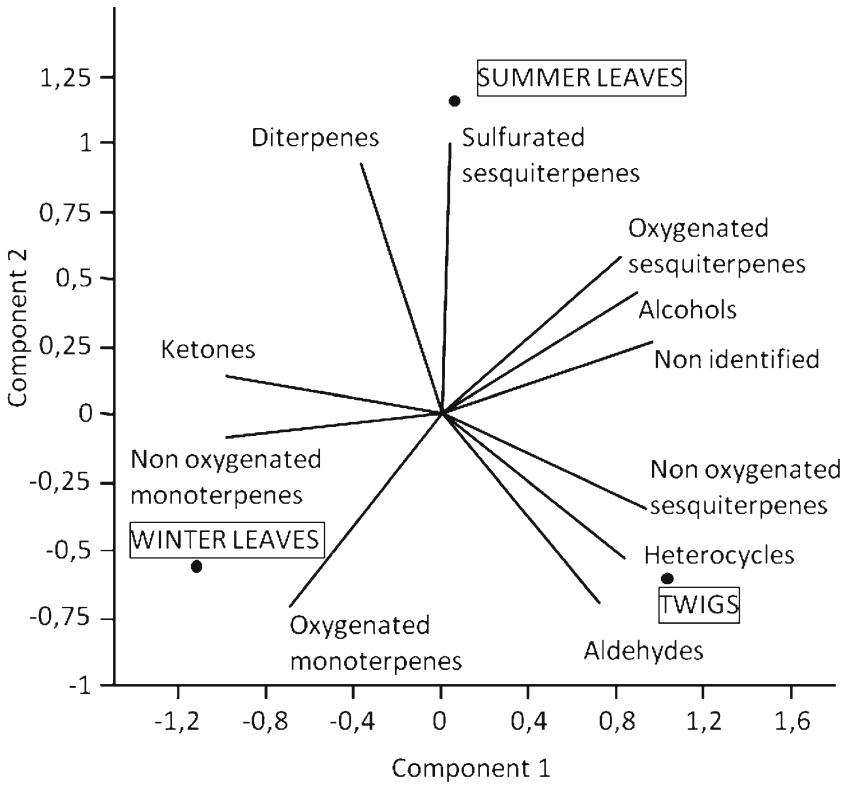

Fig. 1 PCA on compound class for the *E. buniifolium* EOs. Only components 1 and 2 were considered as they accounted for more than 80 % of the variation[57]. Coordinates (component 1; component 2) are as follows: summer leaves (0.05; 1.15), winter leaves (−1.02; −0.53) and summer twigs (0.97; −0.62)

investigators (Lancelle et al. 2009; Lorenzo et al. 2005; Ruffinengo et al. 2005) compared to our present and previous results (Umpiérrez et al. 2012) may be pointing, as stated, to the existence of chemotypes with variable varroacide activity. On the other hand, our results showed differences related to collection time and among the products extracted from different tissues (Table 4). It would be interesting to produce an EO from leaves and twigs together to try to improve activity.

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