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ORIGINAL RESEARCH ARTICLE

Essential oils against *Varroa destructor*: a soft way to fight the parasitic mite of *Apis mellifera*

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Essential oils (EOs) extracted from the aromatic plants *Artemisia annua*, *Artemisia verlotiorum*, *Cinnamomum verum*, and *Citrus reticulata* were investigated as repellents against the honey bees parasitic mite *Varroa destructor*. In laboratory tests, all EOs except *C. reticulata* exerted significant repellent activity against the mite after 24 h exposure. *C. verum*was the most effective EO against *V. destructor* (median effective concentration EC_{50} =1.30 μL L^{-1}), and the least toxic against honey bees (EC_{50} = 13.29 μL L^{-1}). Because of its high selectivity ratio (*A. mellifera* LD_{50}/*V. destructor* EC_{50} = 10.22), *C. verum* EO was then tested to control varroosis in colonies in field trials. The results of open field tests confirmed the efficacy observed in the laboratory. After one week of treatment, colonies treated with the EO showed a significant reduction of *V. destructor* infestation (about 65% at the dose of 25.0 μL L^{-1}) and no negative effects on *A. mellifera*. Overall, **o**ur experiment indicated that *C. verum* EO could be used to effectively control varroosis in the hive with no side effects on the bee colonies.

Keywords: acaricide; beekeeping; essential oils; honey bee; repellent effect; Varroa mite

Introduction

The honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), is the most economically relevant pollinator of crop monocultures worldwide (McGregor, 1976; Watanabe, 1994), with an estimated economic value of billions of dollars in the USA (Allen-Wardell et al., 1998; Gallai et al., 2009). The adoption of honey bee colonies is often the only solution for farmers to ensure adequate pollination to their crops (Klein et al., 2007). However, their management is becoming increasingly problematic (Delaplane et al., 2000; Matheson et al., 1996; Williams et al., 1991) due to the spread of honey bee pests (Chen et al., 2004; Downey & Winston, 2001; Evans et al., 2003; Higes et al., 2006) and to the improper and extensive use of pesticides and herbicides (Ingram et al., 1996; Maini et al., 2010). Among pests, the major threat for apiculture is varroosis, caused by the mite *Varroa destructor* Anderson & Trueman (Acari: Varroidae) an obligatory ectoparasite, that feeds on the hemolymph of immature and adult bees (Rosenkranz et al., 2010; Sammataro et al., 2000). *V. destructor* parasitization reduces the weight of emerging adults, affects cuticle chemical composition (Bowen-Walker & Gunn, 2001), and suppresses the immune response system (Yang & Cox-Foster, 2005). *V. destructor* is also a vector of several debilitating viruses, including the Deformed Wing Virus, the Kashmir bee virus and the Israeli acute paralysis virus (Bowen-Walker et al., 1999; Chen et al., 2004; Di Prisco et al., 2011; Martin et al., 2012; Shen et al., 2005). As a consequence, *V. destructor* shortens the life span of the bees, causing the decline and, eventually, the loss of colonies (Rosenkranz et al., 2010).

In commercial beekeeping, the control of *V. destructor* is based on frequent treatments by synthetic acaricides, such as organophosphates, pyrethroids, and formamidines. Those chemicals, however, although easy to apply and economically convenient, induce the development of mite resistant strains (Lodesani et al., 1995; Lodesani & Costa, 2005; Milani, 1999). Furthermore, these synthetic agents are persistent in the environment (Rosenkranz et al., 2010) and, because of their lipophilic nature they often accumulate in the hive products (Wallner, 1999). Eco-compatible natural substances such as organic acids (formic acid, oxalic acid, and the monoterpene phenol), are also available. However, they require high doses to be effective and may cause the swarming of colonies and the contamination of hive products (Bogdanov et al., 1999; Carayon et al., 2014).

Among natural substances, essential oils (EOs) of aromatic plants represent one of the most promising alternatives to synthetic chemicals to be used as pest control agents (Athanassiou et al., 2014; Bedini et al., 2015; Bedini, Bougherra et al., 2016a; Bedini et al., 2017; Benelli et al., 2014; Bougherra et al., 2015; Campolo et al., 2014; Conti et al., 2012), with minimal or absent side effects (Benelli et al., 2015; Lima et al., 2014; Rajendran & Sriranjini, 2008; Regnault-Roger et al., 2012). For these reasons, a large number of EOs

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have been tested against *V. destructor* showing different efficacy (Gashout & Guzmán-Novoa, 2009; Imdorf et al., 1999; Lindberg et al., 2000), but very few of them have been tested in the hive under field conditions.

The aim of this work was, therefore, to evaluate the efficacy of *C. verum* EO for the control of varroosis in the hive. For this purpose, the *C. verum* EO was firstly evaluated in the laboratory for its ability to induce the detachment of the *V. destructor* mites from the host and for its toxicity against *A. mellifera* in comparison with the EOs of *Artemisia annua* L., *A. verlotiorum* Lamotte (Asteraceae), and *Citrus reticulata* Blanco (Rutaceae) and then it was tested in the hive, under field conditions.

Materials and methods

Essential oils chemical analyses

A. annua and *A. verlotiorum* EOs were extracted from the flowering aerial parts of plants collected along the river Arno near Pisa (Italy), during summer 2015. *C. reticulata* EO was extracted from the pericarp of fruits purchased from a local market in Pisa (Italy). *C. verum* EO, extracted from the bark, was purchased from Sigma Aldrich (Milan, Italy). The EOs were chemically analyzed by gas chromatography-electron impact mass spectroscopy (GC-EIMS) as described by Bedini, Flamini et al. (2016b). Analyses were performed with a Varian CP-3800 gas chromatograph, equipped with an HP-5 capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were injector and transfer line temperatures at 220 and 240 °C, respectively, oven temperature programed from 60 to 240 °C, at 3 °C/min, carrier gas helium at 1 mL/min, injection of 0.2 μL (10% HPLC-grade *n*-hexane solution), and split ratio 1:30. All the injections were performed in triplicate. For all the EOs, three independent samples were prepared and analyzed. EO constituents identification was obtained comparing the retention times with those of the series of *n*-hydrocarbons and by computer matching against commercial (NIST, 2014 and ADAMS) and home-made library mass spectra (built up from pure substances and components of known oils and mass spectra literature data) (Adams, 2007).

Insects and mites

V. destructor mites and *A. mellifera* drone pupae and adult workers were collected in the late spring 2016 in hives of the beekeeping farm La Mieleria Del Castello Snc (Nozzano, Lucca, Italy). Drone pupae were obtained from the capped brood. Adult worker bees were collected from the hive frames into 750 mL glass jars by a brush. Adult bees and frames with capped drone brood were immediately transferred in the laboratory.

In-vitro bioassays

The repellent effect of EOs against *V. destructor* mites was assessed, along with their toxicity against adult bees. The repellent effect of EOs was evaluated on the basis of the relative number of mites detached from parasitized drones. The toxicity against adult bees was evaluated on the basis of worker bees mortality. The parasitized drone pupae were obtained as described by Dietemann et al. (2013). Upon arrival of the frames in the laboratory, the drone cells were uncapped, the brood was removed with soft forceps and drone pupae were carefully checked for the presence of attached mites. The pupae with attached were put in a container with a source of humidity and utilized in the bioassay. Three drone pupae, with *V. destructor* mites attached, were put on a water-wetted filter paper disk and placed on the bottom of a jar (750 mL) in the lid of a 4 cm Petri dish. Ten not parasitized worker bees were put in the same jar and provided a solution of water and honey (20%) in an Eppendorf® tube and a twig to ensure support to the bees during the test.

A filter paper square (2 × 2 cm) was treated with 50 μL of *A. annua, A. verlotiorum, C. reticulata,* or *C. verum* EOs ethanol solutions at concentrations ranging from 0 to 10%. After ethanol evaporation, the treated filter paper was attached on the inner side of the jar lid to obtain a final EO concentration in the jar atmosphere of 0.00 (control), 0.33, 0.66, 1.33, 3.33, 4.66, and 6.66 μL of essential oil L^{-1} of air. A gauze was fixed with a rubber band on the opening of the jar underneath the lid. The gauze was intended to prevent the direct contact of the bees with the EOs. After 24 hours, the drone pupae were examined and gently prodded with a probe. Reacting pupae were scored as alive. Mite detachment from the host was considered valid only if the drone pupae were alive at the end of the experiment. The number of dead adult bees was recorded as well. Since no mortality was registered in the control treatment, the adult bee mortality percentage rates were not corrected. The possibility of transmission of the parasite between pupae and workers was evaluated by checking the number of mites at the beginning and the end of the trial. No Varroa mites were detected on the body of workers both at the beginning and at the end of the experiment. Each experiment was repeated three times.

In-field bioassays

C. verum, the most effective EO against *V. destructor* mites in laboratory assays, was selected for subsequent in-field bioassays. Bioassays were conducted at the beekeeping farm La Mieleria Del Castello Snc (Nozzano, Lucca, Italy) in July 2016. The daily average temperature ranged from 17 to 29 °C with relative humidity (RH) of about 70%. Eighteen hives were randomly chosen among the ones showing active colonies. All selected

Table 1. Chemical composition (%) of *Artemisia annua*, *Artemisia verlotiorum*, *Citrus reticulata*, and *Cinnamomum verum* essential oils used in the bioassays.

Constituents[a]	*LRI*[b]	*A. annua*	*A. verlotiorum*	*C. reticulata*	*C. verum*
α-Pinene	941	5.7	1.2		0.6
Camphene	955	2.4			0.3
Sabinene	976	1.8	0.3	3.0	
β-Pinene	982	1.1			0.3
Myrcene	993	2.8	1.9		
Yomogi alcohol	999	1.4			
α-Phellandrene	1006				1.4
p-Cymene	1028	0.2	0.4		2.0
Limonene	1032			83.6	3.9
1,8-Cineole	1034	18.8	7.1		
Artemisia ketone	1063	22.1			
cis-Sabinene hydrate	1070	0.3	1.0		
Artemisia alcohol	1085	5.9			
Linalool	1101			6.0	4.6
2,6-Dimethyl phenol	1108		5.2		
Chrysanthenone	1127		34.3		
trans-Pinocarveol	1141	2.2			
Camphor	1145	16.9			
β-Pinene oxide	1158	1.5	2.2		
Pinocarvone	1164	3.0			
4-Terpineol	1179	1.2	0.7	0.6	0.4
(E)-Cinnamaldehyde	1268				58.7
Perilla aldehyde	1273		1.0	0.3	
Eugenol	1358		0.5		5.5
α-Copaene	1377	0.2			2.2
β-Caryophyllene	1419	1.8	12.6		9.5
(E)-Cinnamyl acetate	1444				5.8
α-Humulene	1455		1.3		
γ-Muurolene	1477		9.9		
Germacrene D	1482	2.2			
Bicyclogermacrene	1495	0.5	1.2		
Caryophyllene oxide	1582	0.3	4.0		1.0
Selin-11-en-4-α-ol	1653		3.0		

[a]Chemical constituents $\geq$ 0.2% only are reported.
[b]LRI: linear retention index on DB-5 column.

colonies had a similar amount of sealed brood, estimated as no more than one frame (about 9000 cells). EOs were introduced in the hives by plywood sticks (4 × 20 cm) previously prepared in the laboratory. The sticks were treated with 2 mL of ethanol solutions containing 0 (control), 37.0, 150.0, 250.0, 500.0, and 1000.0 μL of *C. verum* EO. The ethanol was then completely evaporated in a fume hood and the sticks were packed in aluminum foil and stored at −20 °C until use. Two treated sticks were inserted in each hive ($40 \times 40 \times 50 = 80.000\ cm^3$). The sticks were suspended by a toothpick between the frames to obtain a final EO concentration in the hives of 0 (control), 3.1, 6.3, 12.5, and 25 $\mu L\ L^{-1}$ air. Fallen *V. destructor* mites were collected with trays bottom-lined with baking paper treated with mineral oil inserted at the bottom of hives. The toxicity of the *C. verum* EO against workers was assessed by under basket traps applied to each hive (Accorti et al. 1991; Porrini et al. 2002). Because of the EOs volatility, the treatment was stopped after seven days. After seven days, the trays and the under baskets were replaced with new ones and the fallen mites and dead bees counted. Each treatment was replicated three times. At the end of the trial, a last treatment (follow-up treatment) with a double dose of oxalic acid (Apibioxal®, Chemicals Laif, SpA, Vigonza, PD, Italy) was applied to each hive to kill and count surviving mites according to the guidelines of the European Working Group for the coordination of research on integrated Varroa control (Commission of the European Communities, 2002). The double dosage of oxalic acid was preferred considering the possibility of mite resistance mechanisms to synthetic acaricides (Maddaloni & Pascual, 2015; Milani, 1999; Pietropaoli & Formato, 2018) and to reduce the risk of an overestimation of the efficacy of EO treatments. Untreated colonies of the control group used to verify the natural mite mortality and fallen off due to natural defence mechanisms of the host (e.g., grooming behavior, Varroa sensitive hygiene activity, etc.) received the same follow-up treatment. The efficacy of EO treatments was calculated, according to the Commission of the European Communities (2002), with the following formula:

$$D\% = 100 * (A - (A * B/100))/(A + C)$$

where:
D% = relative percentage of *V. destructor* mites fallen off;

Table 2. Detachment of *Varroa destructor* mites in *in-vitro* bioassays after 24 h of exposition to *Artemisia annua*, *A. verlotiorum*, *Citrus reticulata*, and *Cinnamomum verum* essential oils (EOs).

Dose[a]	*A. annua*[b]	*A. verlotiorum*	*C. reticulata*	*C. verum*
0.00	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
0.33	10.00 ± 10.00ab	4.00 ± 4.00ab	5.00 ± 5.00a	8.00 ± 4.90a
0.66	21.33 ± 6.11b	0.00 ± 0.00a	4.00 ± 4.00a	39.33 ± 3.06b
1.33	26.67 ± 11.74bc	21.33 ± 6.11bc	0.00 ± 0.00a	57.33 ± 8.84bc
3.33	35.00 ± 4.08c	29.33 ± 7.48c	0.00 ± 0.00a	63.33 ± 4.94bc
4.66	55.33 ± 4.67c	48.33 ± 8.50c	4.00 ± 4.00a	69.33 ± 10.87bc
6.66	100.00 ± 0.00d	92.00 ± 4.90d	6.67 ± 6.67a	85.33 ± 6.46c

[a] $\mu L\ L^{-1}$.
[b] Values represent the mean percentage of detached mites from *A. mellifera* drones ± standard error. Values within each plant species followed by different letters are significantly different by Tukey B test ($p < 0.05$).

A = number of *V. destructor* mites found on the tray after treatment with *C. verum* EO;
B = percentage of *V. destructor* mites fallen off in the control treatments;
C = number of *V. destructor* mites fallen off after the follow-up treatment.

Statistical analyses

Data about the activity of EOs against *V. destructor* mites and adult bees were processed by one-way ANOVA with the EO dose as factor. To fulfill ANOVA assumption, data were normalized by arcsine transformation. Averages were separated by Tukey's post-hoc test. $P < 0.05$ was used as the significance level of differences between means. Median effective concentrations (EC_{50}) for mites detachment and bees mortality were estimated by Log-probit regression. EC_{50} values were pairwise compared by relative median potency (RMP) analysis. A selectivity ratio was calculated according to Ruffinengo et al. (2005) as *A. mellifera* LD_{50}/*V. destructor* EC_{50}. Values were considered significant indicators of selectivity only if the 95% confidence intervals of the EC_{50}values did not overlap. Differences among EO doses in bees mortality in field tests were tested by Kruskal-Wallis procedure. Analyses were performed using the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA).

Results

EOs chemical analyses

The chemical analyses of the essential oil extracted from the bark of *C. verum* permitted to identify 46 constituents, corresponding to 99.4% of the entire oil composition. The main constituent was *(E)*-cinnamaldehyde (Table 1). The main chemical class was represented by phenylpropanoids (71%). Analysis of the *A. verlotiorum* led to the identification of 39 components, which constituted 93.4% of the total compounds of the EO. The dominant chemical class was the oxygenated monoterpenes, that represented 48.6% of the whole oil. The main compound of *A. verlotiorum* EO was the oxygenated monoterpene chrysanthenone, followed by the bicyclic sesquiterpene hydrocarbon β-caryophyllene (Table 1). Oxygenated monoterpenes (48.6%) were the most represented chemical class in this oil. In the case of *C. reticulata* EO, 18 volatiles, accounting for 99.9% of the whole composition, were identified. The EO was dominated by monoterpene hydrocarbons, mostly represented by limonene (Table 1). The essential oil of *Artemisia annua* was characterized by high levels of artemisia ketone, 1,8-cineole, and camphor, followed by artemisia alcohol and α-pinene (Table 1). Oxygenated monoterpenes prevailed in its composition (75.4%).

In-vitro bioactivity against V. destructor

The results of the *in-vitro* bioassays are reported in Table 2. No detachment of *V. destructor* mites was recorded in the control treatment and excluding *C. reticulata*, all tested EOs showed a clear repellent activity against *V. destructor*. The ANOVA test indicated a significant effect of the EO ($F_{(3,\ 115)} = 37.000$; $p < 0.001$) and the dose ($F_{(1,\ 115)} = 123.826$; $p < 0.001$). According to the probit model, EOs EC_{50} values ranged from 1.30 to 24.43 $\mu L\ L^{-1}$ for *C. verum* and *C. reticulata*, respectively (Table 3). RMP analysis showed that the *C. verum* EO was the most effective, although a statistically significant difference was obtained only between *C. verum* and *C. reticulata* EOs (Log RMP= −1.265; CI = −0.473, −3.211).

In-vitro toxicity against A. mellifera *adults*

The toxicity of the EOs evaluated *in-vitro*, against adult bees was dose-dependent. After 24 h, mortality was significantly affected by the EO ($F_{(3,\ 105)} = 13.130$; $p < 0.001$) and by its concentration ($F_{(1,\ 105)} = 113.692$; $p < 0.001$). EOs EC_{50}, calculated by probit model, ranged from 2.34 to 13.29 $\mu L\ L^{-1}$ for *A. verlotiorum* and *C. verum,* respectively (Table 3). Relative toxicity, calculated by RMP analysis, showed that *C. verum* EO was the least toxic against *A. mellifera* adults, with statistically significant differences between *C. verum* and *A. verlotiorum* EOs (Log RMP= 0.659; CI = 0.176, 2.15), and between *C. verum* and *A. annua* EOs (Log RMP= 0.539; CI = 0.114, 1.794). Selectivity ratios for each treatment are shown in Table 3. *A. annua* and *C. verum* EOs resulted more effective against *V. destructor* than *A.*

Table 3. Median effective concentration (EC_{50}) of *Artemisia annua*, *Artemisia verlotiorum*, *Citrus reticulata*, and *Cinnamomum verum* essential oils (EOs) against *Apis mellifera* workers and *Varroa destructor* mites in laboratory (Lab) and field (Field) tests.

	V. destructor		*A. mellifera*		
EO	EC_{50} (95% CI)	χ^2 (df)	EC_{50} (95% CI)	χ^2 (df)	SR
A. annua	2.32 (1.72–3.32)	**6.65** (4)	3.01 (2.60–3.37)	**1.48** (2)	1.30
A. verlotiorum	3.12 (2.32–4.65)	**6.56** (4)	2.34 (2.03–2.64)	**2.17** (2)	0.75
C. reticulata	24.43 (65.64–580.93)	2.64 (4)	5.52 (4.189–8.637)	**6.47** (3)	0.23
C. verum (Lab.)	1.30 (0.79–1.93)	**0.85** (4)	13.29 (9.67–29.76)	**0.19** (2)	10.22*
C. verum (Field)	11.77 (9.31–16.00)	**5.61** (3)	–	–	–

[a]Concentration of the extract that causes the detachment of 50 % of the *V. destructor* mites.
[b]Concentration of the extract that kills 50 % of the exposed bees.
Data are expressed as μL L^{-1}; CI, Confidence Interval; df, degrees of freedom; SR, selectivity ratio; *indicate that 95% confidence intervals of the EC_{50} values did not overlap. χ2 values in bold indicate $p < 0.05$.

mellifera (SR = 1.30 and 10.22, respectively), while, *A. verlotiorum* and *C. reticulata* resulted more effective against *A. mellifera* than *V. destructor* (SR = 0.75 and 0.23, respectively). However, because of the overlapping of the 95% confidence intervals, only *C. verum* SR resulted significantly selective. No mortality of bees was recorded in the control treatment.

In field bioassays

Under open field conditions, *C. verum* EO induced a detachment of *V. destructor* mites ranging from 16.8% to 65.4% at the concentration of 0.075 and 2 mL/hive (corresponding to 0.94 and 25 μL L^{-1} air), respectively (Table 4). The ANOVA indicated that the effect was dose-dependent ($F_{(5,\ 12)} = 71.665$; $p < 0.001$). The estimated mean effective dose (EC_{50}), calculated by the probit model, was 11.77 μL L^{-1} (Table 3) (corresponding to 0.94 mL EO/hive). The number of fallen off *V. destructor* mites after the EO treatment and after the follow-up treatment is given in Table 4. No swarming and no significant differences in bees mortality among *C. verum* EO doses were observed in honey bee colonies (Kruskal-Wallis, $\chi^2_{(5)} = 2.268$, $p = 0.811$). The number of dead workers recorded in the under basket traps during treatments with *C. verum* EO ranged from 37.3 ± 6.2 and 52.7 ± 21.3 for 3.75 and 0.94 μL L^{-1}, respectively.

Discussion

The composition of the EO of *C. verum* is in good agreement with previous chemical analyses reported in the literature, with (*E*)-cinnamaldehyde, β-caryophyllene, (*E*)-cinnamyl acetate, and eugenol as the main constituents. The high content of phenols may explain the effectiveness of this EO, as previously observed for other mite species (Perrucci et al., 1995).

The *in-vitro* toxicity tests showed that all EOs, except for *C. reticulata,* exerted a strong repellent effect on *V. destructor*, causing the detachment of mites from *A. mellifera* drone pupae. A dose-dependent toxic effect of EOs against honey bees was also observed. According to our data, however, the repellent effect of EOs against *V. destructor* mites was not correlated with their toxicity against *A. mellifera*. In particular, *C. verum*, whose repellence against *V. destructor* mites was the strongest among the EOs tested, was also the EO with the lowest toxicity against adult honey bees with a high selectivity ratio (SR = 10.22). In line with our findings, a highly selective toxic activity of EOs was also found by Ruffinengo et al. (2005) for the EO extracted from *Shinus molle* L. (Anacardiaceae) that showed a significant selectivity ratio (SR > 16). Similarly, Kraus and Berg (1994) observed a significant increase of the mortality in *V. destructor* mites and no significant toxic effect against the bees by 0.1% cinnamon EO mixed to the wax of artificial cells. No mortality of adult bees was also observed by Imdorf et al. (1999) after 24 h exposure to cinnamon EO at 2.9 μL L^{-1} air. Higher toxicity against *V. destructor* than *A. mellifera* was also observed for EOs of *Thymus kotschyanus* Boiss. & Hohen (Lamiaceae), and *Eucalyptus camaldulensis* Dehn. (Myrtaceae) by Ghasemi et al. (2011). Actually, in this work, we found significantly different toxicity against *A. mellifera* among EOs. Such a difference is probably due to the specific susceptibility of the insect to different chemical components of EOs. In fact, Brasesco et al. (2017), investigating the toxicity of pure compounds of EOs found that cinnamaldehyde, the main component of cinnamon EO, was much less toxic than thymol against *A. mellifera* adults. The toxic effects of EOs on *V. destructor* mites are confirmed by recent findings showing that the exposition to vapors of *Syzygium aromaticum* (L.) Merr. et Perry (Myrtaceae) EO affects water-soluble proteins content and the activity of protective and detoxifying enzymes (SOD, GST) (Li et al., 2017). On the contrary, EOs and their components appear to be well tolerated by honey bees. Recently, Gunes et al. (2017) examined the impact of natural compound treatments on *A. mellifera* brain heat shock proteins (HPS 70) and observed that the EOs components thymol and menthol induced an HPS 70 concentration that was not different from the control bees.

Although numerous EOs and their chemical components have been proven to be toxic to the *V. destructor* mite in the lab, only a few of them have been tested in the hive under field conditions. In our work, the *C.*

Table 4. Falling-off of *Varroa destructor* mites in field tests after 7 d of exposition to *Cinnamomum verum* essential oil (EO) and after the follow-up treatment (oxalic acid).

	EO		Oxalic acid		
Dose[a]	Mites[b]	Mean ± SE	Mites[b]	Mean ± SE	D% ± SE
0.00	96	110.33 ± 53.88	1060	1004.33 ± 368.54	–
	25		340		
	210		1613		
0.94	251	142.67 ± 55.00	796	578.67 ± 109.01	16.80 ± 2.73
	105		485		
	72		455		
3.75	80	218.67 ± 87.69	113	340.33 ± 149.8	36.40 ± 0.96
	195		285		
	381		623		
6.25	50	122.00 ± 49.96	70	125.33 ± 27.67	42.40 ± 5.56
	218		154		
	98		152		
12.50	63	139.33 ± 57.50	40	72 ± 25.32	59.00 ± 1.68
	252		122		
	103		54		
25.00	552	487.33 ± 73.33	222	190.67 ± 25.57	65.40 ± 0.60
	569		210		
	341		140		

[a]$\mu L\ L^{-1}$ air.
[b]Number of mites fallen from the hives; D%, relative percentage of *V. destructor* mites fallen off; SE, standard error.

verum EO showed to be effective also in the field, while no toxic effect against the honey bees (swarming or colony depopulation) was observed. The effective concentration of cinnamon EO observed in field tests was about nine-times greater than those obtained in the laboratory (EC_{50} = 11.8 and 1.30 $\mu L\ L^{-1}$ air, respectively). This difference, however, was not unexpected, considering the very different conditions under which the tests were carried out (closed system *vs.* open system, ventilation by worker honey bees in the hive, etc.). The observed lower efficacy may be also due to the sealed brood that does not allow the penetration of the EOs.

Overall, our findings agree with previous experiments in the field. Recently, Romo-Chacón et al. (2016) applied 1.16 and 1.50 mL of oregano EO, by impregnated absorbent cotton placed on top of the combs, with efficacies of 57 to 74% of Varroa mite's mortality. Moreover, Islam et al. (2016), after 24 h treatment with 2.5 mL of mint, thyme, lemon grass, and rosemary EOs, obtained from 57 to 87% mortality of *V. destructor* mites compared to 75% of mortality obtained by formic acid treatment. Ramzi et al. (2017), testing in the hive EOs extracted from *Thymus satureioides* C. and B. and *Origanum elongatum* E. and M. (Lamiaceae) of different origin and at different vegetative stages of growth, found a variable efficacy (from 50 to 94% at the dose of 3 mL of EO per hive) that was dependent on the composition and, in particular, on the relative content in borneol and carvacrol.

Conclusions

Varroosis is one of the most problematic disease of honey bees, causing great economic losses to beekeepers and affecting important ecosystem services provided by bees as pollinators. The efficacy of *C. verum* EO against the *V. destructor* mite, coupled with its very low toxicity against the honey bees, could represent an alternative "soft" way to fight the parasite. However, further studies are needed to establish the optimal EO application methods and formulations to increase and extend its efficacy. Controlled-release formulations, i.e. by microencapsulation or sol-gel encapsulation, may be able to ensure a more constant release of volatile EO compounds, to reduce the initial concentration of the bioactive substance, and to maximize the duration and effectiveness of the treatment.

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Disclosure statement

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