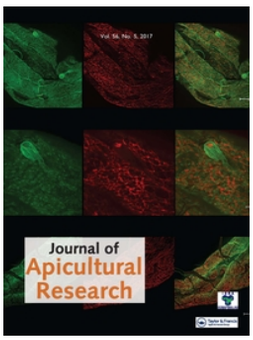

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ORIGINAL RESEARCH ARTICLE

Oral administration of essential oils and main components: Study on honey bee survival and *Nosema ceranae* development

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Diverse parasites and pathogens affect productivity and survival of honey bees. Plant secondary metabolites are potential alternative treatments, however, their effect has been little studied on microsporidian diseases. Furthermore, there is poor information about the toxicity resulting from prolonged oral administration of these substances to bees. In this research, we evaluated *in vivo* effects of different essential oils and main components (MCs) on bee survival and *Nosema ceranae* development under an *ad libitum* non-choice regimen. Substances administered on sucrose syrup to newly emerged bees at different concentrations were avidly consumed and caused different survival performances. Nevertheless, sublethal doses of substances did not control the parasite.

Administración oral de aceites esenciales y componentes principales: Estudio sobre la supervivencia de abejas melíferas y el desarrollo de *Nosema ceranae*

Diversos parásitos y patógenos afectan la productividad y la supervivencia de las abejas melíferas. Los metabolitos secundarios de las plantas son tratamientos alternativos potenciales, sin embargo, su efecto ha sido poco estudiado sobre las enfermedades microsporidianas. Además, hay poca información sobre la toxicidad resultante de la administración oral prolongada de estas sustancias a las abejas. En esta investigación, evaluamos los efectos in vivo de diferentes aceites esenciales y componentes principales (CPs) sobre la supervivencia de abejas y el desarrollo de *Nosema ceranae* bajo un régimen de *ad libitum* sin elección. Las sustancias administradas en el jarabe de sacarosa a las abejas recién emergidas a diferentes concentraciones se consumieron con avidez y causaron diferentes resultados de supervivencia. No obstante, las dosis subletales de sustancias no controlaron el parásito.

Keywords: *Apis mellifera*; *Nosema ceranae*; essential oils; main components; survival

Introduction

Controlling diseases with non-contaminant products is a challenge in veterinary research and also an urgent need at productive and environmental level. Plant extracts and derivatives such as essential oils (EOs) and its main components (MCs) are a large source of diverse compounds that shows a wide spectrum of bioactivity (Reviewed by Bakkali, Averbeck, Averbeck, & Idaomar, 2008). Furthermore, the low toxicity to humans and the capability to control stored-food pests (Shaaya, Kostjukovski, Eilberg, & Sukprakarn, 1997; Shaaya et al., 1991) and food pathogens (Smith-Palmer, Stewart, & Fyfe, 1998), make suitable its use in food obtaining processes, such as in honey production.

Beekeeping practice is seriously affected by diverse pathogens, some of them, being relatively new to honey bees (*Apis mellifera*) and therefore little co-adapted to its host, deriving in highly insidious consequences (Genersch, 2010; Higes, Martín-Hernández, & Aranzazu, 2010). For instance, *Nosema ceranae* (Microsporidia: Nosematidae) has demonstrated to be an epidemiologically relevant parasite, because a host-jump event achieved from another host (from *Apis cerana*) and the subsequent worldwide expansion that was surprisingly late discovered (Higes, Martín-Hernández, & Meana, 2006; Huang, Jiang, Chen, & Wang, 2007; Klee et al., 2007). This intracellular obligate parasite penetrates the epithelial tissue of the bee midgut throughout the extrusion of the polar tube and discharges the genetic material to develop vegetative stages, producing large quantities of resistant spores at expense of the host (Dussaubat et al., 2012; Higes, García-Palencia, Martín-Hernández, & Meana, 2007; Paldi et al., 2010).

Although the role of this parasite in honey bee colony collapse disorder and colony losses is debated (reviewed by Holt and Holt & Grozingerv, 2016), there is only one antibiotic available to temporarily treat the disease: fumagillin (Higes et al., 2011; Sarlo et al., 2011; Williams, Sampson, Shutler, & Rogers, 2008). Today, the drug is licensed in a few countries and its use restricted

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because a safe limit of residues in honey has not been yet specified (Higes et al., 2011; van den Heever, Thompson, Curtis, Ibrahim, & Pernal, 2014). Furthermore, fumagillin cannot be fed to colonies before a nectar flow, to avoid honey stores contamination. Besides, in beekeeping, traditional control using antibiotics and synthetic molecules has caused drug resistance and contamination problems (Maggi, Ruffinengo, Damiani, Sardella, & Eguaras, 2009; Maggi, Ruffinengo, Negri, & Eguaras, 2010; Mullin et al., 2010; Tian, Fadhil, Powell, Kwong, & Moran, 2012). Therefore, there is a growing research effort to develop potential alternative treatments, until now, unsuccessful or still lacking of field results (Botías, Martín-Hernández, Meana, & Higes, 2013; Chen et al., 2013; Costa, Lodesani, & Maistrello, 2010; Damiani et al., 2014; Maistrello et al., 2008; Porrini et al., 2010, 2011).

Essential oils and their MCs have been widely studied as alternative treatments for honey bee pathologies, such as varroosis (mite disease) (Damiani, Gende, Bailac, Marcangeli, & Eguaras, 2009; Damiani et al., 2010; Eguaras et al., 2005; Imdorf, Bogdanov, Kilchenmann, & Berger, 2006; Ruffinengo et al., 2005; Umpiérrez, Santos, González, & Rossini, 2011; Umpiérrez, Santos, Mendoza, Altesor, & Rossini, 2013) ascosphaerosis (fungal disease) (Craig & Wendy, 2003; Dellacasa, Bailac, Ponzi, Ruffinengo, & Eguaras, 2003) and American foulbrood (bacterial disease) (Albo et al., 2003; Alippi, Ringuelet, Cerimele, Re, & Henning, 1996; Gende, Floris, Fritz, & Eguaras, 2008). Besides this research effort, there is still scarce information about the effect of EOs and MCs on nosemosis (microsporidian disease) (Costa et al., 2010; Maistrello et al., 2008). Surprisingly, there is also a limited and methodologically heterogeneous information about the effects of prolonged systemic administration of EOs, MCs and volatile substances on honey bees (Albo et al., 2003; Ebert, Kevan, Bishop, Kevan, & Downer, 2007; Maistrello et al., 2008; Sammataro, Degrandi-Hoffman, Needham, & Wardell, 1998; Sammataro, Degrandi-Hoffman, Ostiguy, Wardell, & Finley, 2004; Sammataro et al., 2009). The wide activity range of these substances could also affect the honey bee survival hence, as a first approach, toxic effects on the host should be studied to determine safe concentrations previous to perform *in vivo* antiparasitic assays. Therefore, the aim of this study was to evaluate the effect of long term consumption of essential oils and MCs on honey bee survival and to study the antiparasitic effect of these substances on *Nosema ceranae* development.

Materials and methods

Due to the lack of bibliographic references on antiparasitic activity of essential oils on *Nosema ceranae* development, at first, the substances selection was made based on EOs concentrations reported as active against other honey bee pathogens. Values administered on previous research ranged between 4 and 1200 mg/kg (Albo et al., 2003; Ebert et al., 2007; Maistrello et al., 2008; Sammataro et al., 2009), also, dosage and administration ways applied varied strongly between authors. So, in our work, we choose concentrations overlapping these ranges.

Essential oils

The oils were obtained by hydrodistillation using a Clevenger-type European Pharmacopoeia apparatus for 2 h. On average, 100 g of vegetal material was used in each experiment, and several distillations were performed until the volume required to run all trials was reached. The oils were dried over anhydrous sodium sulphate and stored in screw-capped dark glass vials at 5.00–8.00 °C until further tests. The oils were analysed by gas chromatography–flame ionization detection (GC-FID) and gas chromatography–mass spectrometry (GC-MS).

GC analyses were done on RTX-5 ms columns (Restek, USA) (30 m × 0.25 mm i.d., 0.25 μm film thickness), operated with a constant carrier flow of 1 ml/min (He for GCMS and H_2 for GCMS). Injection volume was always 1 μl, the temperature of the GC oven was programmed from an initial temperature of 40 °C (1 min), then heated to 300 °C at 5 °C/min, and held for 1 min. The injector temperature was 250 °C and the interphase temperature was 300 °C in GCMS analyses and detector temperature was 280 °C in GC-FID analyses. GCFID analyses were run on a HP 5890 A chromatograph. GCMS analyses were done using a QP-2010 Plus Shimadzu acquiring mass spectra from m/z 28 to 350 in the scan mode (70 eV). Chemical characterization was performed by comparison of the mass spectra and arithmetic retention indexes to those reported in the Nist, 2008 and SHIM2205 databases and in the literature (Adams, 2007).

Substances and extraction details are showed in Table 1.

Main components of essential oils

The MCs administered were commercially obtained: 1,8-cineol (Fulka Analytical), β-myrcene (Sigma Aldrich), Cinnamaldehyde (Lab. SAFC), carvacrol (Sigma Aldrich) and α-phellandrene (Sigma Aldrich).

Experimental design

Three experiments (I, II and III) were performed on spring-summer, between December 2011 and February 2012.

Bees were obtained from sealed brood combs, extracted from healthy colonies placed on the Social Bees Reserch Center (CIAS) experimental apiary (38°10′06′′ S, 57°38′10′′ W). Brood combs were maintained under incubator conditions until imagoes

Table 1. Description of substances used in the assay.

EOs treatments	Extracted material	Yield (%)	Main components (Composition percentages of substances over 5%)	Concentration administered
Laurel (*Laurus nobilis* L.; Lauraceae). Origin: Henderson; Characterization: INTA, Bs As, Argentina	Dry leaves	0.20–0.25	1,8-cineole (48.1); Linalool (12.3); Terpinyl acetate (8.8); Sabinene (6,9) Main compound class: Monoterpene hydrocarbons)	333 and 6666 mg/kg
Origanum (*Origanum vulgare*; Lamiaceae). Origin: Pcia. De Mendoza; Characterization: UdelaR, Lab. Ecol. Quím. Montevideo, Uruguay	Dry leaves	0.38–1.6	Carvacrol (27.7); γ-Terpinene (10.6); Terpinen-4-ol (22.6); p-Cimene (6.3) (Main compound class: Monoterpene hydrocarbons and aromatics)	333 and 6666 mg/kg
Rosemary (*Rosmarinus officinalis*). Origin: Mar del Plata; Characterization: IZS, Roma, Italia	Dry leaves	2.31–2.38	β-myrcene (24.9); Camphor (15.2); 1,8-cineole (9.1); α-pinene (5.3) (Main compound class: Monoterpene hydrocarbons)	333 and 6666 mg/kg
Cinnamon (*Cinnamomun zeylanicum*). Origin: Italia; Characterization: IZS, Roma, Italia	Dry leaves	s/d	Cinnamaldehyde; (79.3); Eugenol (11.9) (Main compound class: aromatics	333 and 6666 mg/kg
Eucalypt (*Eucalytus aff.globulus*). Origin: Mar del Plata, Argentina. Characterization: FFyB, UBA, Bs As, Arg	Dry leaves	2.50–2.55	1,8-cineol (63.5); α-pinene (13.7); Viridiflorol (5.4) (Main compound class: Monoterpenes)	333; 3,333 and 6666 mg/kg

emergence (32 °C; 60% HR). Emerged bees were carefully manipulated, randomly confined on wooden cages with plastic mesh ($10 \times 10 \times 3$ cm^3) and fed with water and candy (powdered sugar and syrup) along two days until treatment administration.

In all 3 experiments, mortality and diet consumption were recorded and feeding treatments replaced in a daily basis. Solutions evaporation was controlled to correct the daily consumed volumes.

Experiment I – Effect? of oral administration of essential oils and MCs on Apis mellifera *survival*

This assay was performed to obtain the safest concentration to administrate volatile substances under a prolonged oral regimen.

Treatments with EOs and MCs were performed for 15 days, including the substances detailed previously (Table 1).

Volatile substances were diluted in 0.5 ml of ethanol (96% v/v) and then mixed with warm sugar syrup (14.5 ml) to obtain concentrations of 333 mg/kg or 6666 mg/kg. Also a control treatment (0 mg/kg) was included. Feeding bees with substances began two days after emergence. *Ad libitum* administration of each solution was performed on plastic Pasteur pipettes (5 ml) with truncated tip to generate a hanging drop. Three replicates of 25–35 individuals were established for each treatment.

Experiment II – Antiparasitic activity of EOs on Nosema ceranae

The highest concentrations for each EOs that did not caused lethal effects in experiment I, were selected with the aim of testing the EO antiparasitic activity during a longer assay. Therefore, five treatments with EOs were performed including Laurel (*L. nobilis*) at 6666 mg/kg, Eucalyptus (*Eucalyptus* sp.) at 3333 mg/kg, Rosemary (*R. officinalis*) at 6666 mg/kg, Origanum (*O. vulgare*) at 6666 mg/kg, Cinnamon (*C. zeylanicum*) at 333 mg/kg. Also, a control treatment (0 mg/kg) was included. In the case of the Eucalypt EO the 3333 mg/kg concentration was selected to test antiparasitic activity based on previous assays (data not shown).

Bees were obtained and confined as described previously. Three days after emergence, imagoes were individually inoculated according to Porrini, Garrido, and Eguaras (2013) with a 10 µl solution, containing 2.32×10^4 *N. ceranae* fresh spores or a solution without spores for control treatment. The *Nosema* spores used for inoculation were obtained from naturally infected colonies at the experimental apiary and molecularly characterized following Martín-Hernández et al. (2007), verifying the solely presence of *N. ceranae*.

Solutions for each treatment were prepared as described in experiment I. Feeding of confined bees with substances started one day after inoculation with fresh spores or control solution. Three replicates of 35 individuals were established for each treatment.

Fourteen and nineteen days post infection (p.i.), seven bees per replicate where sacrificed in order to individually quantify the number of spores in the midgut (Cantwell, 1970).

Experiment III – Antiparasitic activity of MCs on Nosema ceranae

Bees were obtained, confined and inoculated as described previously.

MCs concentrations that did not caused lethal effects in experiment I, were selected with the aim of testing their antiparasitic activity. Five treatments with MCs were performed including α-phellandrene at 333 mg/kg; carvacrol at 333 mg/kg; Cinnamaldehyde at 6666 mg/kg; 1–8 cineol at 6666 mg/kg; ß-myrcene at 6666 mg/kg.

Twelve days post infection (p.i.), 10 bees per replicate were sacrificed in order to quantify the number of spores. The digestive tract was removed by pinching the last abdominal segments. Midguts were sectioned, isolated and stored at −20 °C until quantification.

The number of spores in suspension (parasite intensity) was individually quantified with a haemocytometer under a light microscope in the midgut (Cantwell, 1970).

Statistical analysis

For each treatment, survival curves plotting number of live bees versus time were constructed pooling data from three cages per treatment. Gehan–Breslow nonparametric test was performed to determine whether survival curves were significantly different. Pairwise multiple comparisons were performed with Holm–Sidak method.

To analyze differences in spore loads (average of intensity values), One Way ANOVA tests were performed.

To verify the dietary preference of bees towards the experiment, average daily feed intake was compared using Kruskal–Wallis nonparametric test, because the absence of normality in data, and comparisons with control treatment were performed by means of Dunn's method.

The statistical analysis of the results were conducted applying $\alpha = 0.05$. The SYSTAT software package Sigmastat 3.5 (https://systatsoftware.com) was employed to run the statistical tests.

Results

Experiment I – Effect of oral administration of essential oils and MCs on Apis mellifera *survival*

Survival

Statistical differences were obtained between survival curves. Table 2 resumes the survival results at the end of the experiment, showing data from different treatments.

Table 2. Average survival (days) obtained at the end of the assay (experiment I).

		Average survival (days)	
	Treatment	333 mg/kg	6666 mg/kg
Essential oils	Laurel	9.667	9.610
	Origanum	9.600	9.458
	Eucalyptus	9.821	9.317*
	Cinnamon	9.886	7.243*
	Rosemary	9.473	9.593
	Control	9.560	9.585
Main components	Phellandrene	12.297	12.133
	Carvacrol	12.4	12.184*
	Cinnamaldehyde	12.97	10.868*
	1,8 cineole	13	12.718
	β-myrcene	12.571	12.059
	MC control	12.875	12.875

Notes: Statistical differences between treatments and control curves (p-values < 0.05) are indicated with asterisk (*). The same control treatment was used for both concentrations, however average survival values calculated by the statistic test are different since data analyses was made in two different groups (333 mg/kg and 6666 mg/kg).

Treatments consumption

Essential oils did not cause differences in consumption rate (Table 3, $p = 0.275$) while MCs solutions, except for carvacrol and cinnamaldehyde, were less consumed than control treatment at both concentrations ($p < 0.05$).

Experiment II – Evaluation of antiparasitic activity of EOs on Nosema ceranae

Survival

Eucalypt EO was the only treatment showing statistical differences with control. Figure 1 shows survival results.

Treatments consumption

Table 4 shows consumption rates for each treatment expressed in grams consumed per bee in 24 h. Statistical analysis showed no differences on substances consumption (ANOVA; F: 1.443; DF: 6; $p = 0.267$).

Nosema ceranae *development*

Table 5 describes spores intensity values for the EOs and Control treatments. Statistical analyses did not show differences between treatments at day 14 post infection (ANOVA on Ranks, Kruskal-Wallis; $H = 6.134$; DF: 5; $p = 0.293$) or at day 19 post infection (ANOVA, $F = 2.469$; DF: 5; $p = 0.084$). No spores were detected in "Non-infected control" treatment.

Experiment III – Evaluation of antiparasitic activity of MCs on Nosema ceranae

Survival

Carvacrol was the only treatment showing statistical differences with control. Figure 2 shows survival results.

Table 3. Average consumption rate (grams/day/bee) for experiment I.

		Average consumption (gr) ± Std. error	
	Treatment	333 mg/kg	6666 mg/kg
Essential oils	Origanum	0.019 ± 0.007	0.019 ± 0.007
	Eucalyptus	0.018 ± 0.007	0.019 ± 0.008
	Cinnamon	0.018 ± 0.007	0.014 ± 0.006
	Rosemary	0.018 ± 0.003	0.016 ± 0.006
	Laurel	0.019 ± 0.008	0.016 ± 0.005
	EO Control	**0.017** ± 0.004	**0.016** ± 0.003
Main components	Phellandrene	0.014* ± 0.004	0.020* ± 0.003
	Carvacrol	0.023 ± 0.004	0.028 ± 0.007
	Cinnamaldehyde	0.021* ± 0.005	0.022 ± 0.006
	1,8 Cineole	0.021* ± 0.005	0.020* ± 0.003
	β-Myrcene	0.021* ± 0.006	0.018* ± 0.007
	MC Control	**0.028** ± 0.003	**0.028** ± 0.003

Notes: Statistical differences between treatments and control amounts (p-values < 0.05) are indicated with asterisk (*); control values are indicated with bold.

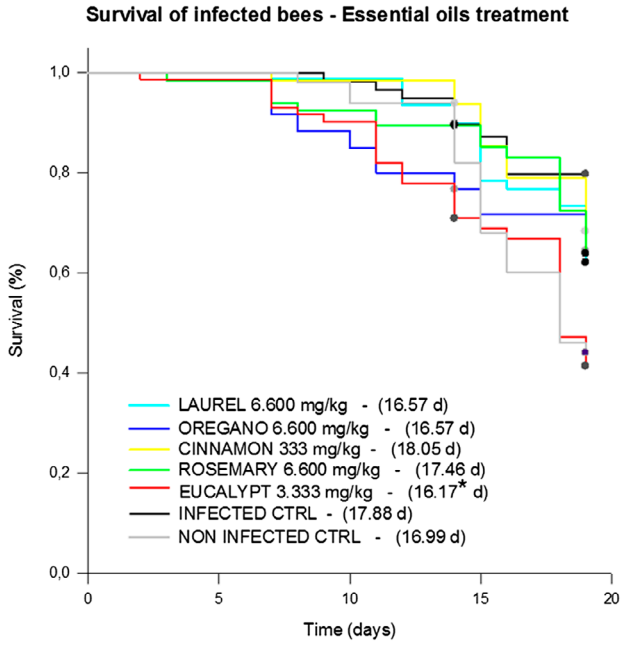

Figure 1. Survival curves for experiment II. Average of survival time (days) estimated by Gehan-Breslow test is indicated between brackets.
Note: Statistical differences between treatments and control curves (p-values < 0.05) are indicated with asterisk (*).

Table 4. Average consumption rate (grams/day/bee) for experiment II.

Treatments (EOs)	Average consumption (gr) ± Std. error
Laurel	0.021 ± 0.004
Origanum	0.017 ± 0.007
Eucalyptus	0.024 ± 0.002
Cinnamon	0.024 ± 0.004
Rosemary	0.017 ± 0.001
Infected control	0.020 ± 0.002
Non infected control	0.020 ± 0.004

Treatments consumption

Table 6 shows consumption rates for each treatment expressed in grams consumed per bee in 24 h. Statistical analysis showed no differences on substances consumption (ANOVA; F: 0.940; DF: 7; p = 0.507).

Nosema ceranae *development*

Table 7 describes spores intensity values for the MCs and Control treatments. Statistical analysis did not show differences between treatments at day 12 post infection (ANOVA on Ranks, Kruskal-Wallis; H = 5.728; DF: 5; p = 0.334). No spores were detected in "Non-infected control" treatment.

Discussion

Previous studies on the effect of essential oils and MCs on honey bees have reported several results performing different methods of administration, including in the diet a wide variety of doses and vegetal origin of the compounds. In our experiment, we performed an *ad libitum* regime, obtaining novel results about toxicity and anti-*Nosema* activity for compounds not previously tested.

Intake of substances

Although deterrent effects were previously reported for rosemary and eucalyptus oils (Detzeland & Wink, 1993), under the no-choice administration performed in our assay, the amount of treated syrup consumed together with treatments did not change significantly comparing with controls, independently of the botanical origin or the concentration of substances. However, when administering MCs, a drop in averages of consumption rates was registered for the majority of components. Nevertheless, the bees have kept on feeding until the end of the assay and therefore, we can state that survival

Table 5. Spore counts in midgut (intensity values ± Std. error) for experiment II.

EOs	Day 14 p.i. Average of spores ± Std. error	Day 19 p.i. Average of spores ± Std. error
Laurel	1,267,333 ± 575,709	4,241,667 ± 141,667
Origanum	2,682,937 ± 2,352,338	9,608,333 ± 2,345,237
Eucalyptus	512,500 ± 724,784	10,725,000 ± 2,934,493
Cinnamon	225,333 ± 315,571	12,850,000 ± 2,616,295
Rosemary	3,653,333 ± 3,755,284	10,491,667 ± 5,919,899
Infected control	850,000 ± 1,472,243	5,920,000 ± 520,000

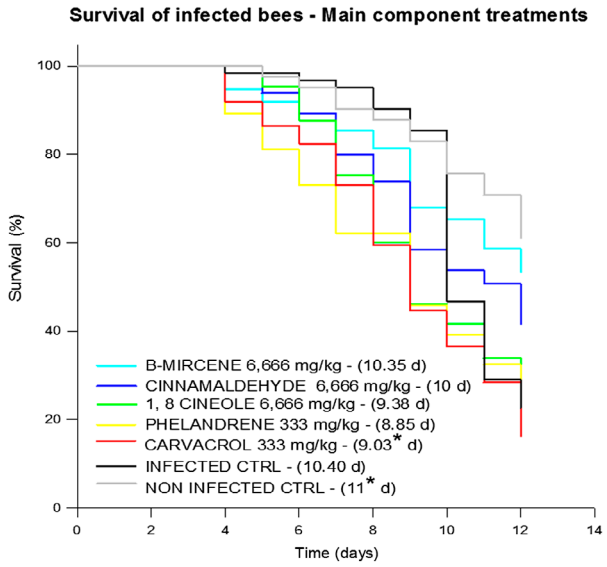

Figure 2. Survival curves for experiment III. Average of survival time (days) estimated by Gehan-Breslow test is indicated between brackets.
Note: Statistical differences between treatments and control curves (p-values < 0.05) are indicated with asterisk (*).

Table 6. Average consumption rate (grams/day/bee) for experiment III.

Treatments	Average consumption (gr) ± Std. error
Phellandrene	0.031 ± 0.011
Carvacrol	0.038 ± 0.012
Cinnamaldehyde	0.034 ± 0.006
1,8 Cineole	0.030 ± 0.002
β-Myrcene	0.025 ± 0.002
Infected control	0.035 ± 0.008
Non-infected control	0.028 ± 0.011

differences between treatments did not depend on diet consumption rates.

Administering treatments throughout a hanging drop system was useful for *ad libitum* feeding but, daily replacement of feeder content is necessary to avoid crystallization or phase formation in the pipette content which is commonly observed after 24 h of feeder recharging.

Table 7. Spore counts in midgut (intensity values ± Std. error) for experiment II.

Treatments	Day 12 p.i. Average of spores ± Std. error
Phellandrene	4,517,778 ± 1,328,130
Carvacrol	1,988,333 ± 529,187
Cinnamaldehyde	3,817,619 ± 829,455
1,8 Cineole	3,661,500 ± 1,153,953
β-Myrcene	4,874,444 ± 1,064,279
Infected Control	6,621,429 ± 1,283,672

Survival

Some EOs and many allelochemicals ubiquitous in vegetal extracts such as alkaloids, coumarins and saponins cause toxic effects on adult honey bees (Albo et al., 2003; Detzeland & Wink, 1993). In our experiment, the prolonged oral intake of some EOs (composed mostly by monoterpenes) and purified MCs, caused toxicity depending mainly on the administered concentration.

Eucalyptus oil decreased the survival of bees when administered at 6666 mg/kg (experiment I) and 3333 mg/kg (exp II). Nevertheless, a 333 mg/kg concentration of the EO and concentrations of 333 mg/kg and 6666 mg/kg of the main component of these oil (1,8-cineole, with a concentration of 63.5%) caused no detrimental effect. This last result matches with data reported by Ebert et al. (2007) for a diet including 1000 mg/kg of cineole. This suggests the presence of other main component/s of this oil that intoxicate bees at higher concentrations than the one tested. Therefore, to perform oral administration in future assays, we can suggest a secure concentration of approximately 300 mg/kg of Eucalyptus EO and at least a 6000 mg/kg for the terpenoid compound 1,8-cineole.

In the case of cinnamon oil, toxicity was registered at a 6666 mg/kg concentration. Consistently with that, the main component of this oil (cinnamaldehyde, 79.3%) caused higher mortality than control at the same concentration but no at 333 mg/kg, demonstrating a dose-dependent toxic effect of this aromatic compound. Lethal doses of this EO were studied in short time assays (Gende et al., 2009) evaluating this substance as "virtually nontoxic", but showing also a cumulative toxicity evidenced by a decrease in LD_{50} (lethal concentration 50) values along the assay. Nevertheless, in our

experiment, long time administration of a 333 mg/kg concentration was safe for both, cinnamon EO and its isolated MC (cinnamaldehyde).

The origanum EO (carvacrol, 27.6%) did not caused toxicity at any concentration in concordance to data published by Ebert et al. (2007). However, the purified carvacrol, showed toxic effects at the highest dose tested (6666 mg/kg) when administered to non-infected individuals. Furthermore, a 333 mg/kg concentration of the MC, caused higher toxicity than control in infected bees, indicating a possible combined effect of *Nosema* infection and carvacrol intake.

Rosemary oil, containing mainly β-myrcene (25%) and 1,8-cineol (9.1%), as much as those purified MCs by separately, did not caused any effect on honey bee survival at both concentrations tested. Previously reported LC_{50} of this oil (containing mainly β-myrcene 22.1% and 1,8 cineol 16.6%) tested under a complete exposure method, also evidenced a very low toxicity on worker bees (Maggi et al., 2009). These results postulate this oil as a safe substance to perform long-lasting oral administration assays at high concentrations.

Essential oils and MCs are commonly classified as harmless, toxic or benign under systemic administration. In our work, the majority of the tested substances, both EOs as MCs, did not cause lethal effects on bees under a non-choice prolonged intake, providing different safe concentrations to perform laboratory assays with honey bees. Furthermore, in presence of a chronic and debilitative infection such as nosemosis, synergic interactions affecting survival were rarely observed during the assay. This information supports the hypothesis of a safe performance of the diets to develop a field treatment for honey bee diseases.

It is noteworthy that, more than its lethal effects, the toxicity produced by a substance can be deeply studied throughout their sublethal effects. In addition, harmless or benign effects may change over time, or under many interactions with natural food components and microorganisms in the colony environment and bee luminal medium, such as those present in beebread (Anderson, Sheehan, Eckholm, Mott, & DeGrandi-Hoffman, 2011; Good, Gauthier, Vannette, & Fukami, 2014). Therefore, further experiments under semi-field or field conditions are needed if a massive administration of EOs and MCs is planned. In addition, testing the toxicity of the substances on worker bee imagoes is just a first approach, being necessary to study their effects on other castes, larval stages and worker cohorts.

Nosema *development*

The doses selected by us to test the anti-*Nosema* activity were based on the highest concentration not causing lethal effects after a prolonged ingestion. Furthermore, forcing the treatments incorporation coupled with the carbohydrate source allowed us to maintain a continuous contact of substances with the luminal epithelium in the midgut, the *Nosema* target tissue. Nevertheless, although parasitized bees were exposed to these extreme conditions along many days, no significant effects on parasite development were detected. Throughout antimicrobial assays, Gende et al. (2008, 2009) demonstrated highest effects of essential oils mainly composed by phenolic compounds over oils with terpenoid substances as major components. However, in our study, essential oils or their MCs were not associated with differential activity on microsporidia proliferation. There is only one antecedent describing EO administration under a chronic oral regime to treat *N. ceranae* (Maistrello et al., 2008): *Vetiveria zizanioides* oil, which also reported negative results when dosed at 1200 mg/kg.

Some evidence demonstrates that EOs may act as prooxidants affecting eukaryotic cell membranes and organelles, with production of reactive oxygen species (ROS) (reviewed by Bakkali et al., 2008), which has been discussed as a possible way of anti-microsporidia action by limiting parasite growth (Holt & Grozingerv, 2016). Results obtained in our work, evidence dose-dependent effects of some EOs and their MCs in the infected host, causing elevated mortality before any significant reduction in the parasite development could be obtained and consequently discouraging the use of these substances as an antiparasitic alternative.

Since laboratory trials allow the survival of workers along 25 or 30 days, prolonged assays might demonstrate deeper effects of EOs and MCs against *N. ceranae*. Nevertheless, essential oils cause alterations in honey bee behavior (Bergougnoux, Treilhou, & Armengaud, 2013; Carayon et al., 2013) which, coupled with the toxic effects caused by accumulation of substances, as demonstrated in our experiments, will possibly impose a limit in assays' length. Furthermore, if the tested substances did not affected the development of the parasite at an early stage, controlling a long term established disease seems to be unlikely.

Final comments

Despite having described unsuccessful treatments for nosemosis control, we hope our conclusions can provide technical resources to design long term pharmacological studies on *A. mellifera*. The use of plant secondary metabolites as a natural pesticide is of immense significance in view of the environmental and toxicological implications of the indiscriminate use of synthetic pesticides, mostly acaricides, and the efforts to overcoming the problem of the increasing pest resistance.

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No potential conflict of interest was reported by the authors.

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