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# Laurel leaf extracts for honeybee pest and disease management: antimicrobial, microsporidicidal, and acaricidal activity

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**Abstract** A diverse set of parasites and pathogens affects productivity and survival of *Apis mellifera* honeybees. In beekeeping, traditional control by antibiotics and molecules of synthesis has caused problems with contamination and resistant pathogens. In this research, different *Laurus nobilis* extracts are tested against the main honeybee pests through an integrated point of view. In vivo effects on bee survival are also evaluated. The ethanol extract showed minimal inhibitory concentration (MIC) values of 208 to 416 µg/mL, having the best antimicrobial effect on *Paenibacillus larvae* among all substances tested. Similarly, this leaf extract showed a significant antiparasitic activity on *Varroa destructor*, killing 50 % of mites 24 h after a 30-s exposure, and on *Nosema ceranae*, inhibiting the spore development in the midgut of adult bees ingesting  $1 \times 10^4$  µg/mL of extract solution. Both ethanol extract and volatile extracts (essential oil, hydrolate, and its main component) did not cause lethal effects on adult honeybees. Thus, the absence of topical and oral toxicity of the ethanol extract on bees and the strong antimicrobial,

microsporidicidal, and miticidal effects registered in this study place this laurel extract as a promising integrated treatment of bee diseases and stimulates the search for other bioactive phytochemicals from plants.

## Introduction

*Apis mellifera* is the most economically valuable honeybee, crucial in maintaining biodiversity by pollinating numerous plant species (Klein et al. 2007). However, many pests are threatening the health and optimal maintenance of honeybee populations. An exceptionally diverse set of parasites and pathogens including mites (*Varroa destructor*, *Acarapis woodi*), microsporidia (*Nosema* spp.), fungi (*Ascosphaera apis*), bacteria (*Paenibacillus larvae*, *Melissococcus plutonius*), viruses, and scavengers can affect them at any life stage (Genersch 2010). They adversely impact bee productivity and survival (Morse and Flottum 1997). Sustainable development of disease prevention and control strategies could be made that consider cures for more than one pest affecting beehives simultaneously. In many countries, traditional control and prevention of bee diseases are made using antibiotics and synthetic molecules such as oxytetracycline, fumagillin, and organophosphate, formamidine, and pyrethroid acaricides for American and European foulbrood, *Nosema*, and *Varroa* control, respectively. However, the prolonged chemotherapy in bee colonies has caused contamination of honey and wax (Bogdanov 2006; Martel et al. 2007) and, more disturbingly, resistant pathogens (Maggi et al. 2009, 2010a; Evans 2003). These facts have driven the study on control alternatives with natural substances such as plant extracts and their components (Damiani et al. 2009, 2011; Gende et al. 2008a, 2010; Porrini et al. 2011).

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Most plants synthesize secondary metabolites with important biological properties. These are not essential to basic metabolic processes of the plant but have a key role in its defense mechanism (Jacobson 1989). The current trend in phytotherapy research is to select plants with phytochemicals able to be extracted and to use them as natural antimicrobials or antiparasitics that are safer for the environment and human health (Bidlack 2000; Jones and Kingkorn 2006; Kayser et al. 2003; Schmahl et al. 2010).

*Laurus nobilis* (Lauraceae) dried leaves and their essential oil are used as a valuable spice and flavoring agent in the culinary and food industries. Traditionally, it is used in herbal medicine for treating and preventing many human and veterinary illnesses (Patrakar et al. 2012; Semmler et al. 2009). Active fractions from plant material can be obtained by several procedures (Zeković et al. 2009) such as distillation, maceration, and decoctions, among others (Sarker et al. 2006). From laurel leaves, different extracts according to their chemical and biological properties can be obtained. The potential of laurel leaf extract as an antimicrobial and antifungal agent (Ertürk 2006; Santoyo et al. 2006; Simić et al. 2004), and their antioxidant properties (Simić et al. 2003) have been evaluated. In honeybee pathology research, laurel essential oil was reported to inhibit *P. larvae* growth (Damiani et al. 2008). Promising results have been obtained from other laurel extracts and their components against *N. ceranae* and *V. destructor* (Damiani et al. 2009; Lindberg et al. 2000; Porrini et al. 2011). Thus, the aim of this study was to evaluate the biological activity of *L. nobilis* leaf extracts obtained by different procedures—and a main component—against *P. larvae*, *Nosema ceranae*, and *V. destructor* through an integrated point of view. Also, the biosecurity level for bees in contact and ingesting each substance is estimated.

## Materials and methods

### Plant material and extracts of *L. nobilis*

*L. nobilis* leaves (Fig. 1) were collected in Henderson (36°18' S; 61°42' W), Buenos Aires Province, Argentina. The essential oil (EO) and hydrolate of laurel were provided by E. Tkacik Company. Both were obtained by steam distillation of dried leaves and were sent to be characterized chemically by the supplier. The 1,8-cineol (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane), the main compound of both substances, was purchased by Sigma-Aldrich (Fluka Analytical). The laurel leaf ethanol extract (EE) was obtained from the same plant material that was crushed and macerated with 80 % (v/v) ethanol according to Porrini et al. (2011). All substances were stored in screw-capped dark glass vials at 4 °C until further testing.



**Fig. 1** *L. nobilis* leaves

### Honeybees and pathogens for experimental procedures

*A. mellifera* colonies emplaced in an experimental apiary of the National University of Mar del Plata, Argentina (38°10' 06" S, 57°38'10" W) were used. The apiary had a main group of healthy colonies, and some colonies parasitized with *V. destructor*. *N. ceranae* spores were obtained from foraging bees.

### Effects on *P. larvae*

#### *Microorganisms and culture media*

A collection of *P. larvae* strains from the Arthropods Laboratory, National University of Mar del Plata, was used. The strains had been isolated from the larvae with American foulbrood corresponding to apiaries from five different locations in Argentina. These were identified by biochemical and molecular analysis and were maintained on Mueller–Hinton broth, yeast extract, glucose, and sodium pyruvate (MYPGP) agar with 15 % (v/v) glycerol until use (Gende et al. 2010).

#### *Determination of minimal inhibitory concentrations by microdilution method*

Vegetative cells of *P. larvae* were suspended in distilled sterile water (0.5 McFarland scale). Bacterial isolations were exposed to serial dilutions of each individual antimicrobial agent according to Gende et al. (2008a). The hydrolate, EO, EE, and 1,8-cineol were tested. The



concentrations range was set between 26 and 3,333 ppm. Blends were incubated at  $32\text{--}35\text{ }^{\circ}\text{C}\pm 0.5$  for 48 h. After that, 10  $\mu\text{L}$  of a solution 0.01 % (w/v) resazurin sodium salt (Sigma-Aldrich) was added per well, and the plate was returned to incubation for 90 min. The inhibition of bacterial growth was confirmed due to a blue hue in the well. The lowest concentration of the antimicrobial substance that showed inhibition was considered as the minimal inhibitory concentration (MIC) (Lennette et al. 1987). Each treatment was made in triplicate.

#### Effects on *V. destructor*

##### Mite collection

Female adult mites of *V. destructor* were collected from parasitized capped honeybee brood by opening and inspecting brood cells.

##### Topical activity

The topical toxicity on mites of hydrolate, EO, and 1,8-cineol was evaluated by the complete exposure method (Ruffinengo et al. 2005). Dosages were calculated in microliters diluted in 96 % (v/v) ethanol applied to the bottom of Petri dishes (from 1.25 to 25  $\mu\text{L}$  for 1,8-cineol; from 5 to 100  $\mu\text{L}$  for EO; and 25, 50, 100, 1,000, and 2,000  $\mu\text{L}$  for hydrolate). Once the solvent evaporates, five mites were placed in every device. An hour later, some bee pupae were included for feeding mites. Mites in cages with no substance were included as controls.

Due to its natural viscous condition, the topical application of the EE on mites was made according to Damiani et al. (2010). The soft extract was dissolved in dimethyl sulfoxide (DMSO)–ethanol–water solution (1:5 DMSO/55 %v/v ethanol). Six mites remained in contact with each extract solution (1, 2, 4, and 5  $\mu\text{g}$  of EE in 200  $\mu\text{L}$  of solution) for 30 s. Then, parasites were transferred to a clean Petri dish containing bee pupae as food. Controls treating mites with DMSO/55 % (v/v) ethanol were used.

For both treatments, five replicates were made, and the incubation was set at  $28\pm 0.5\text{ }^{\circ}\text{C}$  and 60 % RH. The activity of mites was observed under dissecting microscope at 1 and 24 h (for EE) and, at 24, 48, and 72 h (for the other ones) after the beginning of treatments. Statistical analyses were performed with specific software for the calculation of  $\text{LC}_{50}$  values (lethal concentration that kills 50 % of the exposure animals), and 95 % inverse confidence limits (for details, see Damiani et al. 2009).

#### Effects on *N. ceranae* development

##### *Nosema* strains

*Nosema* spores used for inoculation were purified from a strain developed from confined workers and molecularly characterized according to Medici et al. (2012).

##### Oral administration and microsporidial activity

Capped brood combs were obtained from healthy honeybee colonies and maintained at  $32\text{ }^{\circ}\text{C}\pm 1$  and 60 % RH until hatching. The newly emerged bees were confined and maintained in experimental wooden cages according to Porrini et al. (2011). Three replicates of 25–30 bees were used. Three days after emergence, they were supplied different diets: 1,8-cineol (333, 3,333, and 6,666 ppm), hydrolate ( $1\times 10^5$ ,  $3\times 10^5$ , and  $6\times 10^5$  ppm), EO (333 and 6,666 ppm), and EE ( $1\times 10^4$  and  $1\times 10^5$  ppm). Control treatments supplying only sugar syrup 60 % (w/v) were settled. Treatments were replaced daily. To correct the eaten solution volumes, consumption and evaporation were controlled. Individual infection was also achieved 3 days after emergence with nonanesthetized bees (Porrini et al. 2013) by using 10  $\mu\text{L}$  of sugar solution containing  $2.03\times 10^4$  spores and then supplied ad libitum to one of the diets detailed previously. At 7 and 19 days postinfection (p.i.), five bees per replicate were sacrificed to quantify the individual number of spores in the midgut (Cantwell 1970).

A Duncan multiple comparison test ( $\alpha=0.05$ ) was performed to test differences in spore loads. For each treatment, survival curves plotting the number of live bees versus time were made. The Gehan–Breslow nonparametric test was performed to determine significant differences in survival curves. Pairwise multiple comparisons were made with the Holm–Sidak method. The dietary preference of bees was evaluated by comparing the average daily feed intake using a Kruskal–Wallis nonparametric test and contrasting with control treatment by means of Dunn's method. All statistical analyses were conducted applying  $\alpha=0.05$ .

#### Effects on adult honeybees

Toxicity effects on adult bees of the different substances were evaluated by means of oral and topical administration.

##### Contact toxicity test

The toxic effects caused by the contact with 1,8-cineol, hydrolate, and EO were evaluated by the method of complete exposure (detailed in **Topical activity** for mites). Dosages were calculated in microliters per cage; thus, 1.25, 2.5, 5, 10, 25, 50, 100, and 200  $\mu\text{L}$  were diluted in 2 mL of 96 % (v/v) ethanol.

The hydrolate was also proved at 1,000  $\mu\text{L}$  in ethanol and pure (2,000  $\mu\text{L}$ ). Once the solvent evaporated, five nurse bees were placed in every device. Bees in cages with no substance were included as controls.

Due to the viscosity of the EE, their toxic effect evoked by contact was evaluated by the method of topical application, where a solution is applied to the dorsum of the thorax of each individual bee (EPPO 2010). Different concentrations were tested: 2.5, 5, 7.5, 10, and 20 % w/v, using as diluent DMSO/55 % (v/v) ethanol (1:5). Two microliters of the reconstituted extract were placed on the thorax of immobilized nurse bees. Then, five treated bees were included per cage. Negative and positive controls were maintained applying 2  $\mu\text{L}$  of a solution of DMSO/55 % (v/v) ethanol (1:5) and increasing concentrations of dimethoate (from 0.038 to 0.6  $\mu\text{g}/\text{bee}$ ), respectively.

In both bioassays, a device with sugar and water was placed as food for bees. Five replicates were made. The incubation was at  $28 \pm 1$  °C and 60 % RH. Mortality of bees was assessed at 24, 48, and 72 h. The  $\text{LC}_{50}$  values were determined as in “Topical activity”.

#### Oral toxicity test

Newly emerged honeybees were maintained under long-term consumption of the different substances with the same conditions set in “Oral administration and microsporidial activity”. Bees fed with increasing concentrations of dimethoate in sugar syrup 60 % (p/v) were included as positive control. Mortality rates and the survival curves were statistically estimated and analyzed as in “Oral administration and microsporidial activity”.

## Results

#### Antimicrobial activity

Results for antimicrobial activity of the tested substances against *P. larvae* strains isolated from different Argentinean geographic areas are shown in Table 1. The most active one was the EE showing the lowest MIC values, from 208 to

416  $\mu\text{g}/\text{mL}$ . The MIC values for the EO ranged between 600 and 1,200  $\mu\text{g}/\text{mL}$ . The hydrolate and 1,8-cineol showed very low antimicrobial activity against all tested strains.

#### Acaricidal activity

The  $\text{LC}_{50}$  values for mites obtained at each observation time for different treatments are shown in Table 2. The  $\text{LC}_{50}$  values for mites exposed to the EE were determined after topical contact during 30 s. This laurel extract showed the highest acaricidal activity at 24 h after treatment. Higher amounts of the EO were needed to cause mortality of completely exposed mites. When the hydrolate and 1,8-cineol effects were tested, no significant differences were registered in mite mortality with respect to control treatment. Neither  $\text{LC}_{50}$  values could be determined.

#### Microsporidial activity

The EE was the unique derivative from *L. nobilis* that caused significant anti-*Nosema* activity. As was indicated by Porrini et al. (2011), differences in the number of *N. ceranae* spores developed in the midgut of bees that ingested this extract occurred at 7 and 19 days after artificial inoculation. At day 7 p.i., the higher EE concentration caused significantly lower spore counts than the control treatment ( $6.60 \times 10^4$  spores vs.  $2.20 \times 10^6$  spores on average, respectively;  $P=0.037$ ) but high bee mortality (Table 3). Scavenging of individual midgut homogenates at day 19 indicated that  $1 \times 10^4$   $\mu\text{g}/\text{mL}$  of EE solution significantly inhibited *N. ceranae* development ( $3.02 \times 10^6$  vs.  $7.16 \times 10^6$  spores on average in control treatment;  $P=0.017$ ) causing no significant mortality of the host. Long-term consumption of 1,8-cineol, hydrolate, and EE showed no significant effect against the development in vivo of *N. ceranae* in the midgut of bees (all  $P<0.05$ ).

Effects on healthy and *Nosema*-infected bees after chronic oral exposure

Table 3 resumes the results obtained after prolonged oral administration of all substances. Oral ingestion of EE caused

**Table 1** MIC values of 1,8-cineol and different extracts from *Laurus nobilis* for five *Paenibacillus larvae* strains

MIC values are expressed in micrograms per milliliter. Analyses were made in triplicate

	<i>Paenibacillus larvae</i> strains				
	Mechongue	La Plata	Sierra	Típica	Vidal
Ethanol extract	208	208	208	208	416
Essential oil	1,200	800–1,000	600–700	700–1,000	600
Hydrolate	3,333	>3,333	3,333	>3,333	>3,333
1,8-Cineol	3,333	>3,333	3,333	>3,333	3,333

**Table 2** LC<sub>50</sub> values + 95 % confidence limits estimated for 1,8-cineol and laurel extracts against *Varroa destructor* at 1, 24, 48, and 72 h after treatment

	LC <sub>50</sub> for mites + (95 % confidence limits)			
	1 h	24 h	48 h	72 h
Ethanol extract <sup>a</sup>	3.2 (2.82–3.66)	2.68 (1.5 × 10 <sup>-4</sup> –6.02)	N.E.	N.E.
Essential oil <sup>b</sup>	N.E.	64.68 (39.52–580.27)	32.66 (23.03–52.63)	11.69 (2.33–58.31)
Hydrolate <sup>b</sup>	N.E.	>1,962	>1,962	>1,962
1,8-Cineol <sup>b</sup>	N.E.	>23.03	>23.03	>23.03

N.E. not estimated

<sup>a</sup> LC<sub>50</sub> values (lethal concentration that kills 50 % of the exposure animals) are expressed in micrograms in 200 µL of ethanol extract solution

<sup>b</sup> LC<sub>50</sub> values (lethal concentration that kills 50 % of the exposure animals) are expressed in micrograms per Petri dish

significant mortality of infected bees at the lowest concentration. Also, some mortality was registered when hydrolate was administered at 3 × 10<sup>5</sup> µg/mL solution. EE and 1,8-cineol did not cause mortality at any concentration.

#### Topical toxicity on honeybees

Topical effects on bees exposed to contact with different substances are shown in Table 4. Only contact with laurel EO was lethal for worker bees; its toxic effect increased throughout the observation times. The hydrolate, EE, and 1, 8-cineol showed no mortality effects on bees exposed even when they were tested at very high concentrations.

#### Discussion

According to Eguaras and Ruffinengo (2006), the growth control of pathogens and parasites in honeybee colonies should be done in the context of an integrated pest management (IPM) including four pillars: (1) measures to reduce the growth and development of pathogens and parasites populations; (2) monitoring and control when required; (3) tolerant host search; and (4) treatments with environmental and toxicologically friendly substances. In this work, we investigated different extracts obtained from leaves of *L. nobilis* with potential bioactivity against *P. larvae*, *N. ceranae*, and *V. destructor*. Also, we tested the biosecurity level for bees subjected to the contact and ingestion of each substance.

**Table 3** Survival and consumption (means ± se) results obtained from *Nosema*-infected and noninfected bees fed with laurel derivative substances

	Concentration (µg/mL)	Mean survival (days) ± se		Mean consumption (µL/bee/day) ± se	
		Uninfected bees	Infected bees	Uninfected bees	Infected bees
Ethanol extract <sup>a</sup>	0	n/t	17.30±0.36	n/t	21.60±1.20
	1 × 10 <sup>4</sup>	n/t	16.10±0.71	n/t	27.61 <sup>b</sup> ±3.08
	1 × 10 <sup>5</sup>	n/t	7.80 <sup>b</sup> ±0.18	n/t	22.95±9.15
Essential oil	0	9.58±0.50	17.98±0.32	12.38±2.93	12.28±3.52
	333	9.66±0.20	n/t	14.33±5.53	n/t
	6,666	9.61±0.14	17.7±0.28	11.95±3.41	15.24±3.52
Hydrolate	0	9.58±0.32	13.47±0.25	36.58±9.08	n/t
	1 × 10 <sup>5</sup>	9.45±0.28	n/t	33.40±1.58	n/t
	3 × 10 <sup>5</sup>	8.09 <sup>b</sup> ±0.40	12.95±0.34	37.60±20.14	n/t
	6 × 10 <sup>5</sup>	9.05±0.354	n/t	32.95±2.95	n/t
1,8-Cineol	0	12.87±0.14	10.43±0.19	14.92±2.25	28.53±6.61
	333	13.00±0.01	n/t	15.53±3.14	n/t
	3,333	12.50±0.3	n/t	15.21±3.05	n/t
	6,666	12.71±0.22	9.38±0.29	14.85±2.95	24.79±1.58

n/t not tested

<sup>a</sup> Extended data available in Porrini et al. (2011)

<sup>b</sup> Express differences with control group

**Table 4** LC<sub>50</sub> values + 95 % confidence limits estimated for 1,8-cineol and laurel extracts against *A. mellifera* at 24, 48, and 72 h after topical application treatment

	LC <sub>50</sub> for bees + (95 % confidence limits)		
	24 h	48 h	72 h
Essential oil <sup>a</sup>	108.12 (93.13–125.76)	89.94 (71.92–112.46)	78.06 (67.53–89.09)
1,8-Cineol <sup>a</sup>	>184.2	>184.2	>184.2
Hydrolate <sup>a</sup>	>1,962	>1,962	>1,962
Ethanol extract <sup>b</sup>	>400	>400	>400

<sup>a</sup> LC<sub>50</sub> values (lethal concentration that kills 50 % of the exposure animals) are expressed in micrograms per Petri dish

<sup>b</sup> LD<sub>50</sub> values expressed in micrograms per bee

Previous investigations about the biological activity of numerous derivatives of plants against these bee pathogen agents have been evaluated on each pest individually (Damiani et al. 2009, 2011; Gende et al. 2008a; Porrini et al. 2011; Umpiérrez et al. 2013). Only some studies have evaluated the overall effect on more than one disease. Combined therapies on *V. destructor* and *P. larvae* with essential oils of *Eucalyptus globulus* and *Rosmarinus officinalis* were studied by Gende et al. (2010) and Maggi et al. (2011), respectively. Thus, we put emphasis on the necessity of beekeepers to control the three major bee diseases, adding to the increasing demand for bioactive botanical agents and a never-ending search for natural, biologically active products.

Different kinds of extracts can be obtained from leaves and other parts of a plant. According to the extraction technique, each individual extract comprises a complex and unique mixture of different phytochemicals (plant secondary metabolites) (Ong 2004). Essential oils (EO) are volatile fractions obtained by steam or water distillation of medicinal and aromatic plants. The major constituents of EOs are monoterpenes and sesquiterpenes (Mohamed et al. 2010). The aqueous solutions obtained as by-products of distillation are known as hydrolates, which also contain many bioactive hydrophilic compounds (Lima et al. 2006). Alcohol terpenes and phenols are reported to be present in these condensed waters (Di Leo et al. 2009). In the present research, we tested the EO and the hydrolate of *L. nobilis* obtained by steam distillation of laurel leaves that showed 48.1 and 39.5 % of 1,8-cineol as main compound, respectively. Similar oil composition was reported by Hokwerda et al. (1982) and Ozek et al. (1998). However, nothing was found in the literature data about other laurel hydrolate compounds. To extract as many compounds as possible from plants, an aqueous alcoholic mixture is employed. The result is referred to as a “total” extract that contains polar compounds such as flavonoid glycosides, tannins, and some alkaloids, among others (Sarker et al. 2006). Most researchers conclude that due to more than 150 chemical

components constituting an individual botanical extract (Cutler and Cutler 1999), the full extract has to be considered an active “compound.” Furthermore, in spite of the modern chemical analytical procedures available, only rarely do phytochemical investigations succeed in isolating and characterizing all secondary metabolites present in a plant ethanol extract (He 2000; Ong 2004). From the four tested substances, the *L. nobilis* EE was the only one that showed significant inhibitory effect on *P. larvae* growth. This value was slightly higher than the best MIC values (among 25 and 150 µg/mL) from EOs of other plants, such as *Cymbopogon citratus*, *Thymus vulgaris*, and *Cinnamomum zeylanicum* (Alippi et al. 1996; Floris et al. 1996; Gende et al. 2008a). But the EE presented better antimicrobial activity compared with other natural substances such as propolis (Antúnez et al. 2008), *Melia azederach* extract, and other EOs (Gende et al. 2008b). The differences in the antimicrobial activity could be ascribed to the chemical composition of the EE-rich flavonoids, terpenoids, saponins, and other compounds, which are absent in EOs. Flavonoid-rich phytochemical preparations of plant extracts from diverse species have also been reported to inhibit microbial growth (Cushnie and Lamb 2005).

There are few records on the administration of botanical origin substances for *Nosema* control (Maistrello et al. 2008; Pohorecka 2004). Both EO and hydrolate of laurel as well as 1,8-cineol—their main component—showed no significant effects against in vivo development of *N. ceranae*. This lack of antiparasitic effect was also recorded for EOs and their main components from other plant species (Porrini 2013). However, the significant antimicrosporidial activity of laurel EE, coupled with its palatability and low toxicity noticed during the assay, makes its inclusion feasible as an agent for nosemosis control. It has been reported that EEs often have low antimicrobial action in relation with EOs (Gende et al. 2008b; Nanasombat and Lohasuphawee 2005). However, in our case, the volatiles isolated from *L. nobilis* did not show significant anti-*Nosema* activity, and the total extract did. By means of forced incorporation of treatments with carbohydrate sources, the intestinal environment parasitized by *N. ceranae* is saturated; but, in some cases, it could cause accumulation of substances with toxic and lethal effects in the long term. This is the case of the registered data for the highest concentration of EE and for the 3,333-ppm concentration of hydrolate whose explanation will require further investigation. Thus, it is possible to classify substances as lethal or nonlethal in long-term administration.

The tendency registered for antimicrobial and antimicrosporidial activity was also reported in *Varroa* toxicity assays. During tests of the bioactivity of laurel hydrolate, mite mortality effects were not registered, even applying the substance in its pure state. The same result was reported for 1,8-cineol. Higher concentrations were not tested because it would exceed the limit of the desired security level. For laurel EO, increased mite



mortality was recorded over increased observation times. However, the  $LC_{50}$  was 7-fold higher than other laurel EO reported (Damiani et al. 2009) and 113-fold higher than the EO with the better acaricidal activity recorded in laboratory conditions by the complete exposure method after 24 h of treatment (Maggi et al. 2010b). The laurel EE showed a strong acaricidal activity even just an hour after treatment. This bioactivity was greater than that recorded for mites exposed to propolis extracts (Damiani et al. 2010) and similar to EEs of *Minthostachys verticillata* and *Baccharis flabellata* (Damiani et al. 2011).

Toxic effects due to contact of bees with 1,8-cineol, hydrolate, and EE were not recorded. However, to kill bees, it needs less than double the amount of EO to kill 50 % of mites after 24 h of contact. Thus, the absence of topical and oral toxicity on bees of the laurel EE and its strong antimicrobial, microsporidicidal, and miticidal effects suggest that this botanical extract is promising in the integrated treatment of bee diseases and inspire the search for other bioactive phytochemicals of plants.

In recent decades in Argentina, formal institutions that promote bee health have tried to implement different strategies to control bee parasites and pathogens in the context of an IPM. Brenner et al. (1998) explain the IPM by using a variety of management tools including traditional toxic substances. Unfortunately, the most widely conventional pesticides have unacceptable levels of risk to human health and the environment (Dhaliwal and Arora 2001). In addition, susceptibility to drugs is a resource that should be preserved. The use of botanical pesticides in IPM offers several advantages over synthetic ones. The natural phytochemicals exert a wide range of effects on metabolism, behavior, and physiology of pests, and, therefore, it is difficult for them to develop resistance to these pesticides. Research indicates that botanical pesticides are biodegradable, in contrast to the long persistence of many synthetic insecticides (Guleria and Tiku 2009). A large number of different plant species representing different geographical areas around the world have secondary metabolites that can cause a range of acute and chronic toxic effects. In addition, the high degree of biodegradation exhibited in most phytochemicals makes them eco-friendly and attractive as replacements for synthetic chemicals (Cutler and Cutler 1999; Isman and Machial 2006). However, the evaluation of phytochemicals is still in its early stage, and much more research is needed to characterize promising agents and discover new ones. Only a few plants have been evaluated in relation to the natural source available worldwide (Bidlack 2000), so there is important incentive for future research. In honeybee health, there is an unexplored area for future research involving alternative natural substances to control pests. Reduction of parasite infestation and bacterial loads in honeybee colonies involves treatments with products showing acceptable bioactivity in laboratory conditions with no side effects on

honeybees that minimize residues in honey and wax and that constitute a viable alternative to reduce resistance phenomena in bee colonies (vanEngelsdorp et al. 2013).

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