Laboratory Evaluations of *Syzygium aromaticum* (L.) Merr. et Perry Essential Oil Against *Varroa destructor*

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Abstract

The oil obtained by hydrodistillation of the floral bottom of *Syzygium aromaticum* (L.) Merr. et Perry was analyzed by GC and GC/MS. Eugenol was the main constituent in the oil (86.7%). The biological activity of the oil applied to *Varroa destructor* and *Apis mellifera* was evaluated in two laboratory tests. Mite lethality was estimated using a complete exposure method test with the oil at different concentrations, and a systemic administration method of oil at different concentrations diluted in syrup was placed in feeders for bees. The LC_{50} for complete exposure method at 24 h was 0.59 µL/dish. The inferior and superior limits obtained were 0.47 x 10^{-6} µL/dish and 1.22 µL/dish, respectively. LC_{50} estimated at 48 h showed a slight decrease as compared to that recorded at 24 h. Ratio selection (LC_{50} of *A. mellifera*/ LC_{50} of *V. destructor*) for complete exposure method was 26.46 and 13.35 for 24 h and 48 h, respectively. Regarding the systemic administration method, mites LC_{50} at 24 h was 12,300 ppm. The inferior and superior limits calculated were 9,214 ppm and 15,178 ppm, respectively. LC_{50} estimated at 48 h showed a slight decrease as compared to that recorded at 24 h. Ratio selection for systemic administration method was 3.05 and 2.22 for 24 h and 48 h, respectively. *Syzygium aromaticum* oil was found to be an attractant for *V. destructor* at 4.8% (w/w) concentration. The results showed that oil toxicity against *V. destructor* differed depending upon its administration. Nevertheless, the ratio selection calculated by this oil is expected to enable its application under field conditions with a good safety margin. This oil could also be used in combination with other oils in integrated pest management strategies in bee colonies.

Key Word Index

Syzygium aromaticum, Myrtaceae, essential oil composition, eugenol, mite lethality test, *Apis mellifera*, *Varroa destructor*.

Introduction

Natural pesticides based on essential oils may represent alternative methods for repelling flying insects in the home, protecting stored grain and controlling different types of pests, particularly those related to food production. Along these lines, the control of honeybee (*Apis mellifera* L.) parasites such as *Varroa destructor* with natural substances is vital, as honey (beehives' main product) is usually a contaminant-free natural product.

For several years, pyrethroids and organophosphates have been applied to control *V. destructor* (1). Yet, on several occasions their excessive use has led to beehive product contamination as well as to resistance by mite population (2,3). Prior studies have demonstrated that some oils may be effective in *V. destructor* control (4–7). However, in general terms, these oils have not been widely applied except to some commercial formulations. This could be attributed to the variability of results from researches and the lack of adequate substrates to incorporate in beehives.

In fact only a few investigations are available on *Syzygium aromaticum* (L.) Merr. et Perry; these showed a high mortality rates in mites when exposed to different concentrations of this oil (8). Similarly, Hope (9) showed an in vitro attractant effect of this oil on *V. destructor.* However, the aim of this work was to evaluate *S. aromaticum* bioactivity on *V. destructor* and *A. mellifera* in different laboratory tests.

Experimental

Plant material and essential oil isolation: Commercial samples of floral buds of *S. aromaticum* (Myrtaceae) were

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acquired in Mar del Plata, Argentina (July 2005). The oil was isolated from the buds using stainless steam distillation equipment for 4 h in accordance with Aldicara (10), after which it was separated from water by decantation, dried with anhydrous sodium sulphate, and kept refrigerated to avoid deterioration.

Identification of components: Oil composition was analyzed by GC and GC/MS, using a Shimadzu GC-17A chromatograph equipped with a DB-1 fused silica capillary column (60 m x 0.248 mm, film thickness 0.25 µm). Temperature was programmed from 60°C (5 min) to 220°C at 3°C/min, and the final temperature was held for 22 min; injector and FID detector temperatures were 230°C and 250°C, respectively; carrier gas was N_2 at a flow rate of 0.9 mL/min. The GC/MS analysis was performed on a Perkin-Elmer, Q-Mass 910 GC. Separation was accomplished on a 30 m x 0.25 mm fused silica capillary column (DB-5) with a film thickness of 1.0 µm. The injector and detector temperatures were 250°C, oven temperature was programmed at 60°C (5 min), 60–220°C (3°C/min) and 220°C (8 min). Carrier gas was He at a flow rate of 1 mL/min, operating at 70 eV.

The identification of components was based on comparison of their mass spectra with those reported in literature (11) and by computer search of their 70 eV mass spectra with those stored in the library of the GC/MS data system, as well as by retention indices.

Biological material: Worker bees from a regional hybrid of *A. mellifera* (*A. m. ligustica* x *A. m. mellifera*), collected from beehive combs were employed with no age differentiation. Females of *V. destructo*r were obtained from brood cells or from adult bees depending on the different evaluation tests employed.

Mite and bee lethality test: Two methods were used to analyze the effect of the oil on *V. destructor* and *A. mellifera:* the complete exposure method and the systemic administration method. In the complete exposure method, mites were able to touch, smell or intake the oil (5). The treatment was assayed in unmodified Petri dishes (140 x 20 mm). The oil (1, 2, 4, 8 and 12 µL) was diluted in 1 mL of ethanol, and the solution was applied to the bottom of the Petri dish. Five minutes later, six newly-emerged adult worker bees (between 0–3 days) and six *Varroa* mite females obtained from brood cells were used. The mites employed were adult *Varroa* females removed from brood cells, since, according to Milani (2), they vary less than those originating from adult bees. A candy was placed inside as food. Complete exposure was tested for each oil concentration, and solvent and fluvalinate were included as controls. Before and during experiments, *Varroa* females were placed in an incubator at 33–34°C and 70% RH. Five replicates were done for each experimental unit. Mite and bee mortality was controlled at 24 h and 48 h. LC_{50} values and inverse 95% fiducial confidence limits were estimated following Lindberg et al. (12). Variables were transformed with the *probit* function, and calculations were performed with PROC LOGISTIC of the SAS (13). The registered mortality values were adjusted in agreement with Abbott (14) as a function of natural mortality.

In the systemic administration method, packages (16 cm x 12 cm x 6 cm) with adult bees were employed. For ventilation, each cage had a nylon mesh on its walls. Cylindrical feeders (8 cm x 3.5 cm) placed inside each package were hung from the ceiling; holes enabled the bees to feed during the whole course of the investigation. The bottom of the package counted on a double floor and a metallic mesh that allowed removal and counting of dead mites while leaving bees undisturbed. Approximately 400 bees with no age differentiation from *V. destructor* high-prevalence colonies were introduced in each package. The bees remained with no food for 4 h. After that, the oil was administered in 10 mL of 2:1 (sugar:water) syrup diluted in 70% alcoholic solution at different concentrations: 1,250; 2,500; 5,000; 10,000; 15,000; 20,000; 25,000; 30,000; 40,000 and 50,000 ppm. Packages with no oil and treated with dimetoato were included as controls. Five replicates were done for treatment. After 24 h, the packages received only syrup, depending on demand. All packages were maintained at 22°C and 65% RH and with a synthetic pheromone queen. Bee and mite mortality was recorded at 24 h and 48 h. Once the experiment finished, the bees were killed by immersing the packages in a 70% alcoholic solution, to determine the total number of bees and mites and calculate the proportion of death attributable to treatment at 24 and 48 h. LC_{50} values and inverse 95% fiducial confidence limits were estimated in accordance with Lindberg et al. (12). Variables were transformed with the *probit* function, and calculations were done with PROC LOGISTIC of the SAS (13). Mortality values were adjusted according to Abbott (14) as a function of natural mortality.

Adult bee toxicity test and ratio selection: LC_{50} values and inverse 95% fiducial confidence limits of *A. mellifera* and *V. destructor* were estimated for both tests, according to Lindberg et al. (12). Variables were transformed with the *probit* function (15,16), and PROC LOGISTIC of the SAS was employed for calculations (13). Ratio selection was obtained as: LC_{50} of *A. mellifera*/LC_{50} of *V. destructor.* This ratio is only recognized as a selectivity indicator unless overlap occurs among the confidence intervals of the compared LC values.

Attractant and repellent effect: Different assayed counts of oil were mixed with 30 g of liquid wax to obtain the concentrations. These were: 0.6%, 1.2%, 2.4% and 4.8% (w/w). The solidified wax was placed in a 3 cm long and 1 cm wide tube that was closed at one end. One mite was placed inside the tube, which was then joined to a tube containing pure wax with no oil. Mite position could be observed through the thin wax layer and was recorded after 90 min. According to Kraus et al. (8), *Varroa* orientation behavior becomes notoriously evident in this period. Ten replicates were carried out for each treatment and for the control tubes with no oil. Categorical data analysis was conducted using PROC CATMOD procedure (13). To compare oil effects with those on control groups, specific contrasts were used.

Results and Discussion

The principal compounds of the *S. aromaticum* oil (94.9%) can be seen in Table I. Eugenol was the main constituent in the oil (86.7%). This result is in agreement with the bibliographic data reported by Alonso (17).

Mites LC_{50} obtained for complete exposure method at 24 h was of 0.59 µL/dish (Table II). The confidence interval for LC_{50} was estimated, and the inferior and superior limits

Table I. Principal compounds of *Syzygium aromaticum* oil

Compound	RI[a]	Percentage
eugenol	1349	86.7
isocaryophyllene	1418	0.5
β-caryophyllene	1433	3.2
allo-aromadendrene	1460	1.3
α-humulene	1468	0.9
eugenol acetate	1525	1.9
caryophyllene oxide	1586	0.4
Total		94.9

[a] Retention indices referred to n-alkanes, determined on DB-1 column.

Table II. Lethal concentration 50 (LC_{50}) estimated for *Varroa destructor* and *Apis mellifera*, depending on exposition time to *Syzygium aromaticum* oil for complete exposure method

Organism	24 h			48 h		
	CL_{50}[a]	Li[a]	Ls[a]	CL_{50}[a]	Li[a]	Ls[a]
V. destructor	0.59	$0.47x10^{-6}$	1.22	0.36	nc	nc
A. mellifera	15.53	nc	nc	4.87	3.19	8.46

a = μL/dish; Li = inferior limit; Ls = superior limit; nc = not considered.

Table III. Lethal concentration 50 (LC_{50}) estimated for *Varroa destructor* and *Apis mellifera*, depending on exposition time to *Syzygium aromaticum* oil for systemic administration method

Organism	24 h			48 h		
	CL_{50}[a]	Li[a]	Ls[a]	CL_{50}[a]	Li[a]	Ls[a]
V. destructor	12,300	9,214	15,178	11,698	9,582	13,342
A. mellifera	37,574	31,441	50,110	26,051	17,979	33,684

[a] ppm; Li = inferior limit; Ls = superior limit.

Table IV. Ratio selection of *Syzygium aromaticum* oil at 24 h and 48 h depending on the method employed

Method	24 h[a]	48 h[a]
Complete exposure	26.46	13.35
Systemic	3.05	2.22

[a] Ratio selection = LC_{50} of *A. mellifera* / LC_{50} of *V. destructor*.

Table V. Mite number related to position inside the test tube for each treatment. Percentage of mite values are depicted in parentheses

Treatment[a]	Pure wax	Wax + oil	Total
0 (Control)	6 (60%)	4 (40%)	10
0.6	6 (60%)	4 (40%)	10
1.2	6 (60%)	4 (40%)	10
2.4	3 (30%)	7 (70%)	10
4.8[b]	1 (10%)	9 (90%)	10

[a] Percentage of oil (w/w); [b] contrast compared oil versus control (df = 1; $p < 0.05$).

were 0.47×10^{-6} and 1.22 μL/dish, respectively. LC_{50} estimated at 48 h yielded a slight decrease with respect to that at 24 h. With respect to *A. mellifera*, LC_{50} was 15.53 μL/dish after 24 h—higher that estimated at 48 h.

From a 4 μL dose, mite mortality showed significant differences at 24 h compared with the negative control ($p < 0.05$). At 48 h, significant differences were observed in relation to the negative control for all concentrations ($p < 0.01$). Yet, no significant differences were observed in bee mortality in relation to the negative control ($p < 0.01$) at 24 h and 48 h regarding concentrations applied.

Fluvalinate: LC_{50} at 24 h was 2.82 μL/dish for mites. This value decreased to 1.97 μL/dish at 48 h. LC_{50} for bees was 1,542 and 1,389 μL/dish at 24 h and 48 h, respectively. This shows an adequate index selection (> 700) for both times. Significant differences were detected in relation to treatments ($p < 0.001$).

Mites LC_{50} obtained for the systemic administration method at 24 h was 12,300 ppm/package (Table III). LC_{50} confidence interval was estimated and the inferior and superior limits were 9,214 and 15.178 ppm/package, respectively. LC_{50} estimated at 48 h showed a slight decrease with respect to the 24 h record. The LC_{50} for *A. mellifera* after 24 h was 37,574 ppm/package, which was higher than the LC_{50} estimated at 48 h. The CL_{50} of mites and bees at 24 h and 48 h as well as fiducial limits, considering the natural mortality obtained in accordance with the systemic administration method are shown in Table III.

From the 10,000 ppm dose, mite mortality at 24 h and 48 h yielded significant differences with respect to the negative control ($p < 0.01$). At 24 h and 48 h, all doses presented significant differences in bee mortality with respect to the negative control ($p < 0.01$).

Dimetoate, systemically administered, led to high mortality at low doses. The LC_{50}/package obtained for bees was of 0.11 ppm with limits inferior and superior of 0.08 ppm and 0.13 ppm, respectively.

The ratio selection for each test at 24 h and 48 h are presented in Table IV. For complete exposure method the ratio selection was 26.46 and 13.35 for 24 h and 48 h, respectively. The ratio selection for the systemic administration method was 3.05 and 2.22 for 24 h and 48 h, respectively.

The time mites were found in the tube area with pure wax and in the tube area with both wax and oil are listed in Table V. Mite numbers in tubes containing wax and the oil at 4.8% were significantly higher than those in control tubes (χ^2obs = 5.48; df = 1; $p < 0.05$). Therefore, this oil when mixed with bee wax showed significant attractant effect. At doses of 2.4% and 4.8%, mite mortality was evinced, though not registered.

The experiments carried out with different administration approaches demonstrated that under either systemic or low complete exposition, *S. aromaticum* oil was selective regarding mites in one or more doses. Such miticide effect was greater than on other previously tested oils applying similar methodologies. In complete exposition assays, Ruffinengo et al. (5) detected a high mite mortality rate when mites came in contact with *Acantholippia seriphioides* and *Schinus molle* oils. However, although these oils yielded better results as compared to a selection of 20 oils, LC_{50} values (*A. seriphiodes* 1.27 μL/capsule and *S. molle* 2.65 μL/capsule) of *S. aromaticum* oil offer more

promising results (0.59 µL/capsule). Regarding *S. aromaticum* oil, earlier works on its toxicity by complete exposition or vapor applications have yielded no significant differences in its final efficacy (12). According to these authors, CL_{50} values were 0.958 µL/capsule quite similar to those obtained in this work. In these same experiments, fluvalinate proved to be highly selective. The order of magnitude reached by the selection rate was about 10 times higher than that of *S. aromaticum* oil. In most cases, natural substances present a low degree of selection in agreement with the variable efficacy obtained in beekeeping. Nevertheless, this does not seem to be the specific case of *S. aromaticum* oil, which yields selection rates (26.46) higher than those of some organic acids such as formic acid, currently applied to *Varroa* control (19–20). The selection ratios obtained for the complete exposition method were greater than those for the systemic administration method. This indicates that a possible administration approach to the colonies could be an application system promoting contact between mites and such oil, thereby minimizing the potential secondary miticide effects on the colony activity.

This work shows, for the first time, the systemic action of the *S. aromaticum* oil. Fifty percent of the mites introduced in bees packages and fed with solutions containing *S. aromaticum* oil (oral administration) died when the solution reached oil values of about 12,000 ppm. Even though vapors released from the oil-containing solution should not be ruled out, the packages were constantly ventilated and hence a high component of this mortality should be attributed to oral administration. Besides, syrup was consumed during the first hours and mite mortality remained constant for the following 48 h, during which no vapors were released from the essential oil.

The use of *S. aromaticum* oil in a varroosis management program seems promising. However, the carrier to be introduced to beehives needs to be determined first so that its bioactivity can be assessed under field conditions.

The toxicity tests used proved a lethal effect of *S. aromaticum* on *V. destructor*. Still this effect differs notoriously depending on the administration method applied as well as on its concentration. The complete exposure method showed a lethal effect on *V. destructor* at lower concentrations and a high ratio selection. Moreover, mite behavior was significantly affected by the oil, which in concentrations close to 5% was attractant. Selection ratio values showed that this oil is an optimum component for future formulations of this pest control.

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