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Original Article

ASSESSING *IN VITRO* ACARICIDAL EFFECT AND JOINT ACTION OF A BINARY MIXTURE BETWEEN ESSENTIAL OIL COMPOUNDS (THYMOL, PHELLANDRENE, EUCALYPTOL, CINNAMALDEHYDE, MYRCENE, CARVACROL) OVER ECTOPARASITIC MITE *VARROA DESTRUCTOR* (ACARI: VARROIDAE)

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Abstract

***Varroa destructor* (Anderson & Trueman, 2000) causes the most important parasitosis of beekeeping in the world. For this reason, prevention is needed to avoid colony death. The most typical treatments involve synthetic acaricides. However, the use of these acaricides results in the emergence of resistant populations of mites to these products and in the appearances of drug residues in products of the hives. Compounds of essential oils have emerged as an alternative to traditional acaricides; however the toxicity produced by these mixtures is currently poorly explored. The aim of this work was to assess, by means of in vitro tests with adult bees, how acaricidal action and toxic interactions in a binary mixture of essential oil compounds (Thymol, Phellandrene, Eucalyptol, Cinnamaldehyde, Myrcene, and Carvacrol) affect *V. destructor*. Calculations of LC_{50}'s of the individual compounds on *A. mellifera* and *V. destructor* made clear that the toxic effect of each compound is different for both species. Thymol and Phellandrene turned out to be lethal for mites at lower concentrations than for bees. The binary mixture of these two substances presented a different toxicity than one produced by each pure compound, as it was highly selective for mites in bioassays at 24 hours through complete exposure to both *A. mellifera* and *V. destructor*. These results make such formulations optimal substances to be considered as alternative controls for the parasitosis.**

Keywords: *Apis mellifera,* binary mixtures, essential oil compounds, toxic interactions, *Varroa destructor*

INTRODUCTION

The bees belonging to the family Apidae are key organisms for the pollination of both wild plant species and crops. However, in recent years pollinator populations have been in decline and their numbers are on global alert (Goulson et al., 2008; Maggi et al., 2013). Within the family Apidae, *Apis mellifera* (Linnaeus) is one of the most affected species (Stokstad,

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2007). Among the different causes attributed to this phenomenon, nutritional stress, negative effects of pesticides and synergistic action of different viruses and parasites are mentioned (Cox-Foster et al., 2007; Stokstad, 2007; Maggi et al., 2013).

Varroosis is a disease caused by the mite *Varroa destructor* (Anderson & Trueman, 2000), and undoubtedly is the largest problem for beekeeping. This parasite feeds on the hemolymph of the *A.mellifera* adult and brood causing major damage by its own and by introducing pathogens, primarily viruses (Rosenkranz et al., 2010; Martin et al., 2012). Because the mite is an exotic pest, the parasite/host system is not in balance and colonies of *A. mellifera* need a control treatment to survive parasitism.

At present, the control of such parasites is highly dependent on the application of such synthetic acaricides products as coumaphos (organophosphate), amitraz (formamidine), fluvalinate and flumethrin (pyrethroids), which are all molecules commonly used in beekeeping. The indiscriminate and wrong use of these acaricides has produced a strong selection pressure on different populations of mites, which has led to the emergence of pockets of resistance in different parts of the world (Elzen & Westervelt, 2002; Maggi et al., 2009, 2010, 2011 b). Additionally, synthetic acaricides can produce unwanted effects on bee colonies such as mortality and replacement of queen bees and poisoning of breeding and adult individuals (Maggi et al., 2013). The presence of resistant mite populations and the possibility of incorporating pollutants in the products of the colonies by the use of synthetic miticides (Medici et al., 2015) are the main problems to be solved in Argentinean beekeeping. Thus, *V.destructor* causes two main problems - the first being by the biological damage generated by the bees mite (parasitism itself) and the second the use and abuse of acaricides used in control (phenomena of resistance - waste - toxicity to honeybees).

Natural compounds represent a valid and useful alternative to be incorporated into a program of integrated pest management allowing rotation of existing acaricides. They have a low toxicity to mammals, little environmental effect and good public acceptance (Isman, 2000). Several of these compounds, especially organic acids and certain essential oils, demonstrate effects on parasitic mites of bees (Eguaras et al., 2001; Ruffinengo et al., 2005). Plant extracts and essential oils against *Paenibacillus larvae* (White), (Alippi et al., 1996; Fuselli et al., 2006; Gende et al., 2007, Pellegrini et al., 2014) and Nosemosis (Porrini et al., 2011) are now being evaluated to such an end. The substances contained in natural products can be potentially useful to control honeybee diseases (Gende et al., 2008), and their incorporation as a natural alternative could assist in the reduction of beehive products contamination, if properly evaluated.

Certain monoterpene constituents of essential oils are competitive inhibitors of the acetylcholinesterase nervous system. Research by Enan et al. (1998), point to the octopaminergic nervous system as the site of action in insects. As this relates specifically to *V. destructor*, numerous studies are based on the action produced by natural essences or its compounds. By 1998, more than 150 essences had already been evaluated, *in vitro* or in field (Imdorf et al., 1999), and this number has grown considerably in recent years (Ruffinengo, 2010).

Numerous major compounds of essential oils have been evaluated for their acaricidal activity. Myrcene is an acyclic monoterpene found in a variety of plants such as rosemary, mint, aniseed and lavender, and studies on the mite *Tyrophagus putrescentiae* (Schrank) showed high efficacy of this compound (Lee, 2005). Phellandrene is a cyclic monoterpene present in the essential oils of some species of the genus *Juniperus*, *Pinus* and *Schinus*. It is one of the main compounds of the essential oil of *Schinus molle* (Linnaeus), which in previous studies has shown good efficacy in laboratory assays against *V.destructor* (Ruffinengo et al., 2005). Thymol is a phenolic compound that is present in many plants as thyme, basil, rosemary, mint and sage. In the case of thyme, this compound can reach 50% of the essential oil of the plant. It presents a great capacity as an insecticide, bactericide, fungicide and nematicide. Thymol is the only compound of essential oils widely used

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in beekeeping, with between 70% and 95% efficiency against *V. destructor* (Calderone, 1999; Eguaras et al., 2004; Ruffinengo et al., 2014). Carvacrol is present in 80% of essential oils of such species as *Origanum vulgare* (Linnaeus), and in thyme. The oil of thyme has shown good acaricidal activity in previous studies of our research group (Eguaras & Ruffinengo, 2006). It also presents great bactericidal capacity, characteristic that was evidenced in studies on various strains of bacteria, such as *Escherichia coli* (von Escherich) and *Bacillus cereus* (Garcia-Garcia & Palau Garcia, 2008). Moreover, the food industry uses it as an additive for preventing bacterial contamination.

The combination of different compounds leads them to interact among one another which generates an additive effect (when the result is the sum of the results obtained by each compound or part, individually), antagonism (when the result is lesser than the sum of the results obtained by each compound or part, individually) or synergism (when the result is greater than the sum of the results obtained by each compound or part individually). From synergistic effects it is pursue the development of mixtures where the action of the combined products is greater than the isolated sum of the compounds. Besides reducing the dose of pollutants, synergistic combinations would be created to decrease the risk of resistance developing (Harris, 2002).

In the study of *V. destructor* there is not any record of trials in which toxic interactions between essential oil compounds were evaluated. In light of the current situation with control problems caused by emerging pockets of resistance to synthetic acaricides, this study would contribute to the development of tools to be incorporated into future mite control programs and pose a starting point in the study of potential synergies between essential oil compound. The aim of this work was to assess, by means of *in vitro* tests with adult bees, the acaricidal action and the toxic interactions in a binary mixture of essential oil compounds (Thymol, Phellandrene, Eucalyptol, Cinnamaldehyde, Myrcene, and Carvacrol) on *V. destructor*.

Specific objectives

- Determine individual LC_{50} values of the following essential oils compounds for bees: thymol, myrcene, eucalyptol, carvacrol, cinnamaldehyde and phellandrene,.
- Select the most promising compounds to assess their individual LC_{50} values on mites.
- Select two of the compounds tested in both species and prepare a binary mixture.
- Determine the values of mortality of mites and bees for the obtained binary mixture.
- Determine toxic interactions between the compounds of the tested binary mixture.

MATERIAL AND METHODS

Study place and biological material

The investigations were conducted from November 2013 to December 2014 at the Laboratory of Arthropods of Facultad de Ciencias Excatas y Naturales, Universidad Nacional de Mar del Plata. The biological material for laboratory tests was collected from an experimental apiary of sixteen colonies of *A. mellifera*, located on outskirts of the city of Mar del Plata, Buenos Aires, Argentina (38° 10′ 06″ S, 57° 38′ 10″ O).

In the two years before the trial, the beehives from which the biological material was obtained had been treated against *V. destructor* with a oxalic acid-based formulation (Aluen Cap). The details of this acaricides formulation can be found in a study by Maggi et al. (2015), which consisted of one application per year. The hives were treated up to ten months before the tests performed in this study. As reported by Maggi et al. (2012), the *Varroa* haplotype infesting the study area and the apiary is the Korean. Adult worker bees were collected in a jar directly from the beehive. Female mites were removed from frames of sealed brood cells in the laboratory, and the ones showing normal movement after 15 minutes of their remotion were selected for the trials.

Essential oil compounds

The compounds used in this study (Tab. 1) presented similar chemical properties such as molecular weight, density and purity.

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Table 1

Chemical properties of compounds used in this study

Compounds	Purity (%)	Molecular weight (gr/mol)	Density (gr/ml)	Brand-product ID
Thymol (T0501 – Thymol)	≥99.0	150.22	0.97	Sigma-Aldrich®
Phellandrene (W285611 – Phellandrene)	≥85	136.23	0.85	Sigma-Aldrich®
Carvacrol (W224502 – Carvacrol)	≥98	150.22	0.97	Sigma-Aldrich®
Cinnamaldehyde (W228613 – Cinnamaldehyde)	≥95	132.16	1.05	Sigma-Aldrich®
Myrcene (W276200 – Myrcene)	≥90	136.23	0.79	Sigma-Aldrich®
Eucalyptol (C80601 – Eucalyptol)	99	154.25	0.92	Sigma-Aldrich®

Determination of LC_{50} of the individual compounds on Apis mellifera and Varroa destructor

The LC_{50} (lethal concentration, 50%) is the concentration of the tested compound at which half of the sample population of a specific test-animal, after a specified period of time, dies from exposure via inhalation or respiration.

The LC_{50} of essential oil compounds were obtained individually by using the method of full exposure (Lindberg et al., 2000) modified by Ruffinengo et al. (2005). Each compound was diluted in amounts of 2.5, 5, 10, 20 and 40 µl per ml of ethanol at 96%, except for the Thymol which was the only compound that presented solid state and therefore diluted in amounts of 2, 2.5, 3, 3.5 and 4 µg per ml of ethanol at 96%. The solutions were homogeneously applied (1 ml solution/capsule) on the inner surface of petri dishes 10 cm in diameter. As a control, 1 ml of ethanol 96% solution was applied, and for each concentration, five replicates were performed. After evaporation of the alcohol, five adult worker bees were placed in each Petri dish with a feeder with water and candy (food for bees composed of powdered sugar and water). The capsules were incubated at 30°C and 70% RH. The mortality of bees at 24, 48 and 72 hours was recorded for each treatment. By probit analysis to take into account natural mortality (Abbott, 1925), the LC_{50} was estimated for each compound individually expressed in µg of compound/Petri dish.

Given the estimated LC_{50}´s obtained for bees and published reports in the reference literature, three compounds - Thymol, Carvacrol and Phellandrene were selected for the toxicity estimation of *V. destructor*. Thymol was selected because it was one of the two compounds that had a higher toxicity and today is the only one that has a wide acceptance in the beekeeping industry in relation to the fight against the mite (Calderone, 1999; Eguaras et al., 2004). Carvacrol was the other compound that showed a high toxicity, and, moreover, is in the composition of the thyme essential oil, which in previous studies, had shown good acaricidal activity (Eguaras & Ruffinengo, 2006). For its part, Phellandrene presented a low toxicity on bees and is one of the main compounds of the *S. molle* essential oil, which in previous studies (Ruffinengo et al., 2005) had shown good efficacy against *V. destructor* and a high selectivity index. To do so, the same methodology described above was repeated, adding five adult female *V. destructor* in each capsule. The corresponding selectivity rates were calculated as *A. mellifera* LC_{50} / LC_{50} *V. destructor* (Ruffinengo et al., 2005).

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Preparation of the binary mixture of compounds of essential oils and mortality records of bees and mites.

The binary mixture was made from the combination of compounds for which its toxicity were evaluated for bees and mites and that presented the most promising selectivity rates for the control of the parasitosis. To do this, the methodology described by Singh et al. (2009) was followed. The toxicity of the mixture (Compound A + Compound B) against *V. destructor* was evaluated by choosing the LC_{50} value of the most toxic compound at 24h against the mite (Compound A). The µg corresponding to the LC_{50} of compound A were placed on each Petri dish and then likewise with the same amount of µg of compound B (less toxic compound). This was done in a number of five replicates. Subsequently, five bees and five female adult mites plus a feeder with water and candy were placed in each plate. Simultaneously, we assessed the toxicity of the pure compounds again, this time to the limits specified in this new phase of experimentation (the value of the LC_{50} of the most toxic compound at 24 hours against the mite, and in a number of five replicates each). The capsules were incubated at 30°C and 70% RH for 24 hours and after this period, the mortality (as a percentage) of bees and mites obtained for each treatment were recorded.

Determination of toxic interactions between the compounds of the binary mixture

Toxic interactions between compounds of the binary mixture were analyzed through the X^2 test. The observed mortalities were compared with expected ones based on the formula described by Hummelbrunner and Isman (2001), as follows:

$$E=O_a+O_b(1-O_a)$$

where E is the expected mortality in the mixture, and O_a and O_b are the percentage values, expressed between 0 and 1, of the observed mortalities of the pure Compounds A and B respectively, at the lethal concentration of the more toxic compound involved in the sample.

The effects of the mixture were appointed antagonistic, additive or synergistic through analysis using X^2 comparisons:

$$X^2=(O_m-E)^2/E$$

where O_m is the percentage value of the observed mortality of the binary mixture, expressed in values between 0 and 1, and E is the expected mortality; X^2 with degrees of freedom = 1 and $\alpha = 0.05$ is 3.38, (value obtained from the table of values of the X^2 test). In making the statistical reading, it was compared, through this analysis, if the observed results differ from the expected results. A mixture which introduced X^2 values greater than 3.38 and if the observed mortality of the mixture was higher than the expected mortality was considered synergistic; if the observed was less than the expected, it was considered antagonistic. A mixture of X^2 values less than 3.38 was considered additive.

RESULTS

Relative toxicity of the various compounds on Apis mellifera and Varroa destructor

The results obtained showed that the compounds of essential oils differed in their bioactivity on both *A. mellifera* and *V. destructor*. Thymol (LC_{50} = 3.3 µg per capsule) and Carvacrol (LC_{50} = 2.4×10^3µg per capsule) were the most toxic compounds for *A. mellifera* at 24 hours (Tab. 2). Cinnamaldehyde showed less toxicity (LC_{50} = 7.9×10^3 µg per capsule) and Eucalyptol, Phellandrene and Myrcene were even less toxic, with LC_{50} values higher than 3×10^4 µg per capsule, at 24 hours.

Used against *V. destructor* (Tab. 3), the results showed that Thymol was the compound that presented the highest toxicity, with an LC_{50} at 24 hours of 2.60 µg per capsule. Phellandrene LC_{50} presented a lower toxicity, with a value of 1.2×10^4 µg per capsule at 24 hours. It showed a lower toxicity relative to that presented by Thymol, but greater toxicity against the mite than against the bee. As for Carvacrol (LC_{50} = 1.5×10^4 µg per capsule, at 24 hours), this was the one that presented the lowest toxicity against the mites.

From LC_{50} values obtained, the selectivity rates for Thymol, Phellandrene and Carvacrol at 24 hours were 1.27; 2.83 and 0.16 respectively.

According to the results observed in Tab. 3 and

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Table 2

Values of the LC_{50} of the individual compounds used in this study on *A. mellifera*. Thymol (LC_{50} = 3.3 μg per capsule) and Carvacrol (LC_{50} = $2.4x10^3$μg per capsule) were the most toxic compounds for *A. mellifera* at 24 hours. Cinnamaldehyde showed less toxicity (LC_{50} = $7.9x10^3$ μg per capsule) and Eucalyptol, Phellandrene and Myrcene were even less toxic, with LC_{50} values higher than $3x10^4$ μg per capsule, at 24 hours

Compounds	LC_{50} bees (μg)		
	24h	48h	72h
Thymol	3.30	3.20	3
Phellandrene	>$3.4x10^4$	>$3.4x10^4$	>$3.4x10^4$
Carvacrol	$2.4x10^3$	$1.3x10^3$	$1.1x10^3$
Cinnamaldehyde	$7.9x10^3$	$7.7x10^3$	$7.7x10^3$
Myrcene	>$3.2x10^4$	>$3.2x10^4$	>$3.2x10^4$
Eucalyptol	>$3.7x10^4$	>$3.7x10^4$	>$3.7x10^4$

to the selectivity rates estimated, the compound that demonstrated the higher toxicity against the mite was Thymol. Phellandrene showed a lower toxicity relative to Thymol, but greater toxicity against mites than against bees. Regarding Carvacrol, it showed that its toxicity was very high against bees and low relative to the other two compounds against mites. Thus, Thymol and Phellandrene were selected for the binary mixture.

Toxicity of the binary mixture on Apis mellifera and Varroa destructor

The mixture was produced using the LC_{50} value at 24 hours of Thymol as the more toxic substance (Compound A) against the mite and using the Phellandrene as the less toxic compound (Compound B). Additionally toxicities of the pure compounds at the dose of the LC_{50} of Thymol at 24 hours (2.60 μg) were evaluated again. In this part of the study (Tab. 4), there was no mortality observed of *A. mellifera* against the assessment of each pure compound, at 24 hours, at the set dose. Likewise, there was also no mortality thereof facing the mixture.

The results obtained from the evaluation of the pure compounds on *V. destructor* (Tab. 4) differed from the expected ones. Higher mortality was observed in Phellandrene than that observed in Thymol, which was contrary to that obtained before in this same study, (Section *i. Relative toxicity of the various Compounds on A. mellifera and V. destructor*, Tab. 3). Meanwhile, in the case of the mixture, the observed mortality was lower than expected. The analysis by the X^2 test for toxic interactions between the compounds of the binary mixture presented a value of X^2 = 5.51, which is greater than 3.38, being the value of mortality observed lower than the expected according to the analysis.

Table 3

Values of the LC_{50} of the compounds evaluated against *V. destructor*. Thymol was the compound that presented the highest toxicity, with an LC_{50} at 24 hours of 2.60 μg per capsule

Compounds	LC_{50} mites (μg)		
	24h	48h	72h
Thymol	2.60	2.40	2.30
Phellandrene	$1.2x10^4$	$7.4x10^3$	$6.9x10^3$
Carvacrol	$1.5x10^4$	$1.0x10^4$	$7x10^3$

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Table 4
Values of mortality observed and expected, expressed in % values between 0 and 1, for *A. mellifera* and *V. destructor*, of the pure compounds and their binary mixture, and analysis of toxic interactions between compounds

	Mortality (%)					
	Observed for pure compounds		Observed and expected for the Binary Mixture [Thym. (2.6 μg) + Phel. (2.6 μg)]			
Organism	Thymol (2.6 μg)	Phellandrene (2.6 μg)	Expected	Observed	X^2	Effect
A. mellifera	0	0	0	0	-	-
V. destructor	24	32	48.32	32	5.51	Antagonistic

DISCUSSION

Essential oils and their compounds exhibit numerous activities against arthropods including repellency (Nerio et al., 2010), deterrence of intake and oviposition (Waliwitiya et al., 2009), and toxicity when sprayed, applied topically or ingested (Regnault-Roger, 1997; Isman, 2006; Isman & Machial, 2006; Rajendran & Sriranjini, 2008). According to Neira (2003), the compounds of essential oils are a good alternative for the control of *Varroa* sp as their strong scents can alter a mite's sense of smell besides their toxicity to it. However, its main disadvantage is that there is very little difference between the lethal dose for mites and for bees (Kraus et al., 1994; Neira, 2003). The possibility of generating interactions from the combination of compounds with synergistic or additive effects is pursued to extend the modes of action of these and the existing strip between the lethal dose for bees and mites (Harris, 2002). The results presented in this study show evidence that certain compounds like Thymol and Phellandrene and their mixture cause adult mite mortality without causing bee mortality in laboratory conditions, when these are administered by the method of full exposure.

The different LC_{50} values obtained for all the substances studied with the full exposure method showed that the essential oil compounds slightly varied in their bioactivity on *A. mellifera* as on *V. destructor*. Thymol (major compound of *A. seriphioides*, among other sources) and Carvacrol (major compound of the essential oil of oregano and thyme, and other natural sources) proved to be the most toxic compounds for *A. mellifera* with LC_{50} values at 24 hours respectively of 3.30μg and $2.4x10^3$μg per capsule. Cinnamaldehyde (major compound of the essential oil of cinnamon), also showed a high degree of toxicity to the bees (LC_{50} = $7.9x10^3$μg at 24 hours), although it was minor compared to those observed for Thymol and Carvacrol. In relation to this, Kraus et al. (1994) reported the cinnamon oil to be highly toxious to bees with a 98% mortality rate. Meanwhile, Gende (2009) determined the potential of cinnamon oil to control American Foulbrood with minor toxicological risks to bees. Eucalyptol, Phellandrene and Myrcene proved to be the least toxic to them with LC_{50} values higher than $3.4x10^4$μg per capsule. Eucalyptol and Myrcene, major compounds of the essential oil of rosemary are known from previous studies in our laboratory with this essential oil to show a small difference in toxicity between bees and mites, giving values at 24 hours of LC_{50}´s greater than 20μl per capsule for both species (Maggi et al., 2011 a).

Only Thymol, Carvacrol and Phellandrene of all the compounds evaluated on bees were selected to estimate toxicity on *V. destructor*. Thymol was selected because it was one of the two compounds that had a higher toxicity and today is the only one that is widely accepted by the beekeeping industry in the fight against the mite (Calderone, 1999; Eguaras et al., 2004). Carvacrol also showed high toxicity and moreover was in the composition of thyme essential oil, which in previous studies had shown

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good acaricidal activity (Eguaras & Ruffinengo, 2006). For its part, Phellandrene presented a low toxicity on bees and is one of the main compounds of the essential oil of *S. molle*, which in previous studies (Ruffinengo et al., 2005) had shown good efficacy against *V. destructor* and a high selectivity rate.

Of these three compounds, Thymol showed greater toxicity (LC_{50} = 2.60µg at 24 hours). This is consistent with previous work in our laboratory, where the LC_{50} estimated for the essential oil of *A. seriphioides,* with Thymol as a major compound, was 1.27µl per capsule at 24 hours (Ruffinengo et al., 2005) and possible synergistic interactions between its constitutive compounds could yield a LC_{50} value lower than the one obtained for the pure compound alone. On the other hand, the Phellandrene LC_{50} values were observed to be promising (1.2×10^4µg at 24 hours), as this was one of the main compounds of the essential oil of *S. molle*, that had been reported in previous studies with successful laboratory results against *V. destructor* with a value of LC_{50} at 24 hours of 2.65µl per capsule (Ruffinengo et al., 2005). These LC_{50} values turned out to be very attractive, because they showed a lower toxicity for bees in relation to that observed with Thymol and good acaricidal activity. In turn, it was the compound that provided the greater selectivity rate indicating it could become a compound to be applied in bee colonies to minimize mortality in the controlling of *Varroa* sp.

The toxicity of Carvacrol was observed to be very high for the bees with very low values of lethal concentrations in comparison to those obtained for mites (selectivity rate less than 1). Although Lindberg et al. (2000) suggested a high efficiency of Carvacrol on *Varroa* sp, and thyme oil showed good acaricidal activity in our previous laboratory studies (Eguaras & Ruffinengo, 2006), in this study low toxicity was observed against mites in relation to the other two discussed compounds and high toxicity against bees.

As for the additional evaluation of the toxicity of Thymol and Phellandrene, the pure compounds, of the binary mixture at the Thymol LC_{50} dose at 24 hours, and the confection and evaluation of the mixture (Section *ii.* of the results: *Toxicity of the binary mixture on Apis mellifera and Varroa destructor*, Tab. 4), it was observed that:

1. Phellandrene presented higher mortality rate of mites than Thymol, which was contrary to what was previously observed in this study when bioassays were conducted against *Varroa* sp (Section *i.* of the results: *Relative toxicity of the various Compounds on A. mellifera and V. destructor*, Tab. 3). This may be due to factors such as the dynamics and mode of action of each compound (systemic, topical or evaporation), which could lead to slight variations in their bioactivity (Rosenkranz et al., 2010).
2. Significant differences were observed in the mortality rate obtained for the binary mixture (32%) in comparison to what was expected (48.32%) according to the analysis. It is concluded that no synergism occurred between this compounds and that the effect produced in this mixture was antagonistic.

The use of compound mixtures would provide a broader spectrum of activity through an increase in the expected antiparasitic activity (synergetic or additive). It is believed that the mixture of substances that act on different vital target points within the arthropod would result in a better control of the parasitosis caused by the same, when compared to the effect of each compound individually (Niestebnowanie-López, Palou & López-Malo, 2006). In this study we observed that the binary mixture evaluated on *V. destructor* presented an antagonistic effect clearly reflected in the mortality values obtained from the pure compounds and their binary mixture. This shows that not always the combination of two different substances in a binary blend powers or sums the individual effects of each one; depending on the substances involved, their bioactivity may be affected negatively by the same combination. The results obtained from the evaluation of the pure compounds and their binary mixture are still attractive because, in the given doses they differed in their toxicity on both *A. mellifera* and *V. destructor*, only mortality being observed in

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the latter. This is a very important phenomenon, as many oils with acaricide activity have adverse effects on honey bees and damage the colony (Imdorf et al., 1999; Lindberg et al., 2000). Thus, this study shows that effective control of *V. destructor* can be achieved with Thymol and Phellandrene without any harmful effects on adult bees. Likewise, other researchers have reported *Varroa* mite control with Thymol and Menthol, the main compound of peppermint oil, without any detrimental effects on bees (Steen, 1992; Schulz, 1993; Imdorf et al., 1994).

Bustos et al. (2000) stated that one of the objectives of testing different compounds is to increase the range of possibilities and prevent the emergence of drug resistance that had happened with synthetic miticides or could happen with natural products in the control of *Varroa* sp (Bakkali et al., 2008; Umpiérrez et al., 2011). In this study, it has been demonstrated that several of the major compounds of essential oils presented a good toxicity against *V. destructor* and that the formulation of a binary mixture between Thymol and Phellandrene presented promising results for further studies of this type of mixtures. New analyses and results could lead to the future production of natural blends to control the parasite.

Bioassays performed in this study provide a simple, cheap and quick method to test new acaricide treatments. Moreover, the simultaneous evaluation of the active compounds in bees and mites is useful to find better selectivity rates. Although the experience has shown remarkable *in vitro* toxicity against *Varroa* mites, further studies should be conducted in the field to assess possible effects on brood, queen mortality and possible interference in beneficial microflora within the internal environment of the colony, since monoterpenoids such as Thymol may not be safe for honey bee colonies. These compounds play a role as broad spectrum pesticides (Isman, 2006) and in previous studies, Thymol and menthol had been found to be among the most toxic of all terpenoids tested when applied to honey bees as a fumigant (Ellis & Baxendale, 1997). Despite being naturally derived, Thymol treatment can induce brood removal (Marchetti, Barbattini & D'Agaru, 1984; Floris et al., 2004) and result in increased queen mortality in field conditions (Whittington et al., 2000).

The control of the *V. destructor* population in honeybee colonies requires that treatments show an acceptable acaricidal activity without any side effects on honeybees and leave no or minimal residues in honey and wax with safety margin to the customer. Adamczyk et al. (2005) concluded that the presence of residues of essential oil compounds in honey samples did not represent a sanitary risk or a risk for human health and only changed the taste of the honey. The lack of octopamine receptors in vertebrates likely accounts for the profound mammalian selectivity of essential oils as insecticides or acaricides (Isman, 2000). In the case of Thymol, its residues evaporate upon storage of combs and wax foundations (Bogdanov, Kilchenmann & Imdorf, 1998a). However, if Thymol treatments are carried out during the whole bee season, then in some cases the residues in honey reach levels that cause a change in taste, which is not permitted according to international honey regulations (Wallner, 1997; Bogdanov et al., 1998b, 1999). This is the reason why different treatments should be coupled for a more effective result in the control of the parasite and to prevent the emergence of pockets of mite resistance to control agents and the accumulation of residues in the colonies products.

Like other alternative products for pest control it should be noted that acaricides based on compounds of essential oils are not a panacea for the protection of honey bees. However, they could contribute greatly to the regulation of parasites in a plan for integrated management, particularly when the safety of beekeepers and the environment is at stake.

Finally, in the *V. destructor*/*A. mellifera* history there is no record of trials in which toxic interactions between compounds of essential oils were evaluated. This makes the results obtained an innovative contribution to the study and allows progress in the development of tools to be incorporated in future mite control programs using natural products. In this context and regarding

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the uncontrollable *V. destructor* problems worldwide, it is of paramount importance to continue with research of this kind to work on the expansion of information on the subject and find a solution.

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