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INSECTICIDAL ACTIVITY OF SAGE (*SALVIA OFFICINALIS*) ESSENTIAL OIL TO *VARROA DESTRUCTOR* (ACARI: VARROIDAE) AND *APIS MELLIFERA* (HYMENOPTERA: APIDAE)

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Abstract

The need to find alternative systems of the fight against *Varroa* mite without application of chemicals and provide healthy bee products resulted in investigation of application of different plant essences to arthropod control. In order to perceive the sage essential oil (*Salvia officinalis*) bioactivity, contact residual toxicity of mites and bees was examined in the laboratory conditions. The chemical composition of essential oil was determined by standard GC and GC/MS methods. Different doses of the sage essential oil dissolved in acetone (0.1–10 µl/Petri dish) were applied in Petri dishes and left to dry for 20 minute at a room temperature. Following this period of time, ten honey bees and five adult female mites were added in each Petri dish and they were all maintained in controlled conditions (T = 30°C, Relative humidity = 60%). Survival of examined honey bees and *Varroa* mites was recorded two times, after 24 h and 48 h. The most prominent toxic effect on the examined *Varroa* mites was observed after 24 h and 48 h, with application of 10 µl of sage oil (the average values for dead mite individuals were 3.25 and 3.50, respectively). Recorded biological activities of the oil tested in different doses on both honey bee and *Varroa* mite revealed opportunity to proceed with further investigation by selecting the most appropriate variants and combinations of the most prominent individual components of the examined sage oil.

Key words: *Apis mellifera, essential oil, Varroa mite*

Introduction

In modern agriculture presence of harmful residues in food as a result of use of pesticides in control of harmful organisms appeared as a great problem. Beekeeping suffers damages caused mainly by ectoparasite *Varroa destructor*, but also due to application of acaricides used to protect honey bees from this mite. Following use of synthetic chemical agents against mites, harmful metabolites use to be cumulatively deposited in wax, and from there they migrate to the honey and other bee products (Kochansky et al., 2001; Anne-Claire et al., 2007; Karazafiris et al., 2008). Solution to the problem is to be found in application of so-called "green pesticides", since their basis should be of plant origin, and they have to be “environmental friendly” (Gashout HA and Guzmán-Novoa E 2009; Umpiérrez et al., 2011). Preparations composed of plant essential oils seem to be promising for replacement of the synthetic substances used in combating *Varroa* mites. This way, it can also lower the

risk of harmful residues in bee products and their appearance in the food chain. So far, over 150 different essential oils have been tested, but only few of them have showed acceptable tolerability by bees and good efficacy in controlling *Varroa* mites (Lindberg et al., 2000; Imdorf et al., 2006). The complex composition of essential oils and a very low tolerability of honey bees in laboratory studies make difficult discovering the oil that would be safe for the bees and effective against the mites (Nedić et al., 2012).

The objective of this work was to examine in laboratory conditions the miticidal effect of the commercial *Salvia officinalis* essential oil against *Varroa destructor* and tolerance of bees to contact residual toxicity of the oil.

Materials and methods

The sage oil (*Salvia officinalis*) used in experiment, was purchased as commercial samples from "Elmar d.o.o.", Trebinje, the Republic of Srpska. Analytical gas chromatography (GC-FID) was performed on the GC HP-5890 Series II apparatus, equipped with autosampler (ALS), split-splitless injector, attached to HP-5 fused silica capillary column (25 mm × 0.32 mm, 0.52 μm film thickness) and fitted to flame-ionization detector (FID). Identification of individual essential oils constituents were accomplished by comparison of their mass spectra with those from available MS libraries (NIST/Wiley) and by comparison of their experimentally determined retention indices (calibrated AMDIS) with data from the literature (Adams, 2001).

Testing of bees tolerance to contact residual toxicity of essential oils, was conducted in Petri dishes, in four replications, under controlled laboratory conditions (T = 30° C; Relative humidity = 60%). Different essential oils doses dissolved in acetone (0.1–10 μL/Petri dish) were applied in Petri dishes (11 cm in diameter) and left to dry for 20 minute at a room temperature, and then the dishes were supplemented with 10 newly emerged adult bees (0 to 3 days old) and five adult female mites. The bees were fed with 3 g of candy and watered with water from a plastic micro tube (1.5 mL). Acetone was used as control. Survival of bees and mites in each Petri dish was recorded after 24 h and 48 h. The obtained results were processed by analysis of variance (LSD test at the significance level of 5 and 1%). Means were separated by the Duncan test at $P < 0.05$. Probit analysis (E in %) was used to calculate the contact residual toxicity of the essential oil for adult bee and *Varroa* mites.

Results and discussion

Chemical composition of *Salvia officinalis* essential oil used in our experiment is presented in Table 1.

Table 1. *Chemical composition of the commercial Salvia officinalis essential oil used in the laboratory studies*

No.	Compounds	%
1.	Salvene<cis->	0.22
2.	Tricyclene	0.16
3.	Thujene<alpha->	0.18
4.	Pinene<alpha->	2.74
5.	**Camphene**	**5.46**
6.	Pinene<beta->	1.67
7.	Myrcene	0.84
8.	Terpinene<alpha->	0.15
9.	Cymene<para->	0.99
10.	Limonene	2.00
11.	**Cineole<1,8->**	**5.88**
12.	Terpinene<gamma->	0.22
13.	Mentha-2,8-dien-1-ol<trans-para->	0.30
14.	**Thujone<cis->**	**27.91**
15.	**Thujone<trans->**	**3.91**
16.	**Camphor**	**19.84**
17.	Pinocamphone<trans->	0.15
18.	**Borneol**	**3.14**
19.	Terpinen-4-ol	0.40
20.	Terpineol<alpha->	0.11
21.	Myrtenol	0.29
22.	Bornyl acetate	2.43
23.	Sabinyl acetate<trans->	0.25
24.	Myrtenyl acetate	0.21
25.	Copaene<alpha->	0.11
26.	**Caryophyllene<trans-beta->**	**4.05**
27.	Aromadendrene	0.36
28.	**Humulene<alpha->**	**7.85**
29.	Caryophyllene<9-epi-trans->	0.16
30.	Dauca-5,8-diene	0.14
31.	Viridiflorene	0.26
32.	Cadinene<delta->	0.20
33.	Caryophyllene oxide	0.63
34.	**Viridiflorol**	**3.94**
35.	Humulene epoxide II	1.46
36.	Manool<13-epi->	1.07
	Total compounds identified (%)	99.67

Total of 36 compounds was identified in the oil and the most dominant components were as follows: *cis*-thujone, camphor and 1.8-cineol (oxygenated monoterpenes), α-humulene (sesquiterpene hydrocarbon), and camphene (monoterpene hydrocarbon), all together covering 66.94% of the oil.

Results on impact of different doses of *S. officinalis* oil on honeybee (*A. mellifera*) and *Varroa* mite (*V. destructor*) are presented in tables 2 and 3.

Table 2. *Tolerability of A. mellifera to toxic effect of the commercial Salvia officinalis essential oil*

Nº of variants	Doses assayed (µL/Petri dish)	After 24h		After 48h	
		Mean *A. mellifera*	E in % (K=0)	Mean *A. mellifera*	E in % (K=0)
1.	0.1	0.00[a]	0.00	0.00[a]	0.00
2.	0.5	0.00[a]	0.00	0.00[a]	0.00
3.	1.0	0.00[a]	0.00	0.50[a]	5.00
4.	2.5	0.00[a]	0.00	0.25[a]	2.50
5.	5.0	0.50[a]	5.00	1.75[b]	17.50
6.	10.0	2.50[b]	25.00	3.25[c]	32.50
7.	0.0 (Control)	0.00[a]	0.00	0.0[a]	0.00
		$LSD_{0.05}$=0.49 $LSD_{0.01}$=0.68		$LSD_{0.05}$=0.77 $LSD_{0.01}$=1.06	

Following 24 h application, doses ranging from 0.1 to 5.0 µL *S. officinalis* oil per Petri dish had no statistically significant influence on bees mortality (Table 2). The highest tested oil dose (10 µL/Petri dish) caused bees mortality of 25%, significantly differing ($P<0.05$) from all the other doses assayed. In the same observing period (24h), between the oil doses ranging from 0.1 to 5.0 µL/Petri dish (Table 3) there were no significant differences in mortality of *Varroa* mites, and their efficacy amounted to 15.79 % of dead mites. Similarly to honeybees, the mortality of mites following application of the highest *S. officinalis* essential oil dose (10 µL/Petri dish) significantly differed from all the other oil doses tested and amounted 63.16 % of dead mites.

Table 3. *Toxic effect of the commercial Salvia officinalis essential oil on Varroa destructor*

Nº of variants	Doses assayed (µL/Petri dish)	After 24h		After 48h	
		Mean *V. destructor*	E in % (K=0)	Mean *V. destructor*	E in % (K=0)
1.	0.1	1.00[b]	15.79	1.79[b]	31.58
2.	0.5	1.00[b]	15.79	1.75[b]	31.58
3.	1.0	1.00[b]	15.79	2.75[ab]	52.63
4.	2.5	1.00[b]	15.79	2.25[ab]	42.11
5.	5.0	1.50[b]	26.39	2.25[ab]	42.11
6.	10.0	3.25[a]	63.16	3.50[a]	68.42
7.	0.0 (Control)	0.25[b]	0.00	0.25[c]	0.00
		$LSD_{0.05}$=1.19 $LSD_{0.01}$=1.64		$LSD_{0.05}$=1.21 $LSD_{0.01}$=1.66	

Following 48 h of application, there were no statistically significant differences in residual toxicity on bees in applied *S. officinalis* oil doses ranging from 0.1 to 2.5 µL per Petri dish. The applied oil doses of 5 and 10 µL/Petri dish resulted in significantly and very significantly different toxicity from all other tested doses, respectively, causing 17.50 % and 32.50% of dead bees, respectively. Toxic effect of *S. officinalis* oil used in our experiment following 48 h of application proved to be the most prominent when 10 µL/Petri dish was applied, which caused death of 68.42 % mites, significantly differing from all the other doses, while for doses ranging from 1 to 5 µL/Petri dish, mites mortality ranged from 42.11 to 52.63%.

The obtained results showed that the lower oil dose of 1 ml / Petri dish caused more dead mites in comparison to application of oil doses of 2.5 and 5 µL/Petri dish. This may be

explained by the fact that for the toxic activity of an essential oil, in addition to the age of the plant and the part of the plant used for oil extraction and method of oil extraction used, the age of the relevant harmful organism, in our case *Varroa destructor*, also plays an important role (Sampson et al., 2005). The complex composition of an essential oil, effect of its constituents, synergistic effects and variation of the content of even minor constituents can all lead to significant differences in residual toxic effect of tested essential oil.

If we stick to the criterion for selection of appropriate chemical for control of *Varroa* mite, as proposed by Lindberg et al., 2000 (i.e. it has to kill more than 70% of mites and less than 30% of bees), doses of our *S. officinalis* essential oil, ranging from 5 to 10 μL/Petri dish seem to be appropriate for further investigation on honeybees in the field conditions.

Lethal and sublethal effects of essential oils and their individual components on honeybees and *Varroa* mites have been already presented in several previous studies. Colin (1990) proved good efficacy of *Thymus vulgaris* essential oil (major oil constituents: p-cymene and thymol) and *Salvia officinalis* oil (major oil constituents: α-thujone, camphor and eucalyptol) on suppression of *Varroa* mites. Good bee tolerance to the sage oil (major oil contents: α-thujone, camphor, α-humulene, eucalyptol, caryophylene and β-thujone) in laboratory experiment of Imdorf et al. (2006) where the bees and *Varroa* mites were placed in Liebefeld cages and treated by the air enriched with sage oil (300-500 mg/L) what induced 100% mortality of the mites and 10-20% of the honey bees. In the same investigation the authors also proved that individual oil component, camphene, influenced an increase in mites mortality (from 60 to 100%) but also an increase in bees mortality (from 20 to 40%). Ruffinengo et al., (2007) presented great bees mortality due to activity of individual oil component, 1.8 cineol, while Imdorf et al. (1995) used the flow of air through the desiccator with bees and mites which contained 50-150 g / L of individual oil component, camphor, succeeded to achieve *Varroa* mites mortality without significant toxicity to the bees.

In our investigation, we have observed that following the initial toxicity of the *S. officinalis* essential oil applied in doses of 5 and 10 μL/ Petri dish, in the first 24 h the oil toxicity was pronounced and it was prolonged in the next 24 hours. Furhtermore, the oil activity was more pronounced on *Varroa* mites comparing to honeybees, which could the most probably attributed to specific composition of the *S. officinalis* essential oil used in our experiment (Table 1), particularly the monoterpene oil portion (oxigenated monoterpenes and monoterpene hydrocarbons).

Biological control is a very sensitive tool to combat harmful organisms and is considered as a safe alternative to chemical control (Onstad and McManus, 1996). However, it also carries the risk and possibility to cause adverse effects to non target organisms, thus imposing necessity to take in consideration interactions of a number of different factors and to try to quantify the final outcome. Until recently, there was little or no evidence that biological control has adverse effects on non-target species. As a result, it was considered that these measures are generally safe, and not likely to have significant effects on non-target organisms. However, as pointed out by Hovarth (1991) "the absence of proof is not a proof of the absence" of such effects. No-target effects can be direct (introduced biological agent attacks non target organisms) or indirect (through the effects on goal which is successfully controlled, the impact on the ecosystem, food changes, etc.). Fans of biological control have to accept that there are dangers and risks associated with the use of biological measures due to control of harmful agents and is therefore necessary to pay attention to the consequences of its non target impact and risk possibilities. The "impact" is what is actually happening when implementing biological measures, while the "risk" is a

priori evaluation of the possible impact of events. Therefore, the impact may be as follows: a high risk of minimal impact, or a low risk of enormous influence, or any combination of the two. In many cases, it is very difficult to evaluate a potential non-target impact or the risk of it. The combination of the overall risk and various potential impacts needs to be balanced in terms of benefits and likelihood of providing them (Cock, 2002). Therefore, our research conducted with the *S. officinalis* essential oil has been done in parallel to *A. mellifera* and *V. destructor*.

Conclusion

In order to overcome the consequences of using the synthetic preparations to protect honeybees from *Varroa* mites, such as the mite resistance and residues in honey and honey products, in the present study we have examined an alternative method with the use of essential oil of plant origin. In order to examine the oil toxic effects on the bees, we have applied the oil of *S. officinalis* at doses ranging from 0.1 to 10 μL per Petri dish.

The oil, applied at doses ranging from 1 to 5 μL, had an acceptable toxic efficacy to *A. mellifera* (from 2.50 to 17.50 %), and a somewhat satisfactory efficacy to *Varroa destructor* (from 31.58 to 52.63%). When applied at a dose of 10 μL per Petri dish, the oil caused toxicity to bees slightly above the eligibility threshold (32.50%), the same toxicity of the oil dose being more pronounced to *V. destructor* (68.42%).

The results indicate that the *Salvia officinalis* essential oil may be promising for the application against *V. destructor* which we set for our future research goals.

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