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EVALUATION OF ACTIVITY OF SOME THYMOL-BASED FORMULATIONS FOR CONTROLLING HONEY BEE VARROOSIS.

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ABSTRACT

Two under-experimentation thymol-based formulations (tablets, Var-stop and strips, Var-away) were tested on twelve beehives in a commercial private apiary located in El-Minia governorate. The apiary trial was carried out during September – October 2005. The main goals were to determine their effectiveness against *Varroa distructor*, taking into account natural mite mortality in control hives and to determine the residues of thymol in honey and wax. Both formulations significantly reduced the levels of mite infestation of adult bees, their varrocidal effectiveness according to the recommendations of the *European Group for Integrated Varroa control* were 97%and 93% for Var-stop and Var-away, respectively. Residues found in honey collected from the nest were 2.1 mg/kg for Var-stop and 1.7 mg/kg for Var-away. The residues were higher in wax (371 mg/kg for Var-stop and 475 mg/kg for Var-away).

INTRODUCTION

Varroosis of the honey bee (*Apis mellifera* L.) is a disease caused by the most destructive ectoparasitic mite *Varroa distructor* (Anderson and Trueman, 2000), which was first described on the Asian honey bee, *Apis cerana*. The new host infestation (*Apis mellifera*) occurred in the 1950,s when European productive bees were introduced to Asia . Since this time, varroosis has been spreading in all continents, thus requiring effective disease control methods and chemical treatments because of the great danger of the disease for beekeeping (Colin, 1990). The first *V. distructor* mite detection in Egypt was in September 1987 at El-Arish region. By September 1989, this mite had become widespread, and by the autumn 1990 a heavy infestation was found in many parts of Egypt, and many apiaries were nearly destroyed (Abou-Zeid and Ghoniemy, 1993). Different products; chemical and natural; are currently used for controlling this mite infestation. However, this mite still cause severe damage in apiaries because of its higher reproduction rate than its host (DE Ruijter, 1987), and the adaptation between the mite and the host's life cycle (Haenel and Koeniger 1986). Additionally, *V. destructor* is developing resistance to the most common chemical acaricides fluvalinate and coumaphose (Elzen *et al.*,1998. Milani 1999, Elzen *et al.*, 2000). Other synthetic Varroa treatment such as flumethrine and bromopropylate leave toxic residues (Lodesani *et al.*,1992). Thus, there is an urgent need for an alternative treatments such as organic acids and essential oil constituents that are effective, and do not leave toxic residues in honey and wax. Essential oil constituents such as thymol (5-methyl-2-isopropyl phenol) has demonstrated a varroacidal activity not only in laboratory assays, but also in

field in Europe (Imdorf *et al.*,1999) and in north America (Calderone,1999).Different thymol-based formulations have been studied to improve acaricide effectiveness, attempting to control the phenol release by different carriers. Thymol formulations significantly reduced the levels of the mite infestation of adult bees and sealed brood (Floris *et al.*, 2004).The environmental conditions, colony condition, and the carrier of the active ingredient play an important role in the Thymol effectiveness against *Varroa* (Baggio *et al.*, 2004). Some of these formulations such as Apilife VAR, Thymovar, and Apiguard are registered as a control of *Varroa* in different countries.

The present work aimed to determine the efficacy of the two under-experimentation thymol-based formulations Var-stop and Var-away, and the thymol residues in honey and wax under Egyptian conditions.

MATERIALS AND METHODS

Apiary The present investigation was conducted in a commercial private apiary located in El-Minya governorate. Before the trial, natural mite mortality was monitored for three days to obtain three homogeneous experimental groups. The trial was carried out during September – October 2005 on twelve colonies, divided to three groups of four hives (two treated and one control).The colonies were nearly the same strength, and were headed by first hybrid Carniolen queens.

Hives The colonies were housed in Langstroth hives, with a removable sticky bottom insert, protected with a net of 8 mesh.

Administration Mode.

The colonies were treated as follows:

Var-stop 90% thymol tablets, each contains 10g of thymol .Three tablets (once a week for three weeks) were divided into two parts and placed on the top of the hive frames.

Var-away 42.5% thymol strips, each contains 5g of thymol .Six strips (twice a week for three weeks) were placed on the top of the hive frames. If present, the residues of previous doses of the two formulations were removed when further administrations were applied. After treatment, further oxalic acid (3%) treatment (control treatment) was carried out to kill surviving mites and residual number of mites was collected for 14 days after oxalic acid treatment.

The dead mites were counted twice a week. Total acaricidal effectiveness calculated according to the formula published by *European Group for Integrated Varroa control.* (Anonymous, 1999).

$$Efficacy(\%) = \frac{No.Tr. \times 100}{No.Tr. + No.ControlTr.}$$

Where *(No. Tr.)* is the number of mites killed by the treatment and *(No. Control Tr.)* is the number of mites killed by the control treatment.

The efficacy of *n*th application is given by the following formula,

$$A_n(\%) = \frac{N_n \times 100}{T_N - T_{n-1}}$$

Where N_n is the number of mites collected during the days following the *n*th application (A_n), *TN* the total number of collected mites (3 application +oxalic acid control) and T_n-1 the number of collected mites before the *n*th application (Colin,1990).

To determine thymol residues, honey and wax samples were collected before and after treatment. Honey samples were collected from unsealed cells of two comps in the brood nest. Wax samples were collected by cutting 3cm^2 from two wax frames.

Chemicals: The two formulations; Var-stop and Var-away; were obtained from the Central Agricultural Pesticides Laboratory (Dokki- Giza-Egypt).Thymol 99 % was purchased from Loba Chemie (India).The HPLC grade solvents; acetone, methanol, and diethyl ether; were purchased from Merck (UK).

Thymol Extraction :-

Thymol Extraction Procedure from honey and wax according to Floris *et al.* (2004).

Extraction Procedure from honey:

One gram of honey was weighed in a 15 ml screw- capped tube and dissolved in 2 ml of water. Two milliliters of diethyl ether were added, and the tube was agitated (10 min) in a rotary shaker. The phases were allowed to separate, and an aliquot of the extract was injected into a GC.

Extraction Procedure from wax.

0.5 g of wax was weighed in a 25 ml screw- capped tube, 5 ml of methanol/ water (1:1) were added, and the tube was plunged in hot water at 70 °C until the wax dissolved, and then agitated for 1 min in vortex. After cooling at room temperature, 5 ml of diethyl ether were added, and the tube was agitated for 15 min in a rotary shaker. The phases were allowed to separate, the ether extract was centrifuged and an aliquot was injected into a GC.

Recovery Assay. Untreated samples of honey and wax were fortified with 0.1 mg/kg thymol and processed according to the procedure described above.

Apparatus and Chromatography.

Thymol residues in honey and wax samples were analyzed using a HP-GLC 5890 gas chromatograph equipped with a flame ionization detector (FID) and split- split less injector. Separation was carried out on a HP-1, a methyl silicon gum column 30 m × 0.53 mm, 0.88 µm film thickness. The air and hydrogen flows for the FID flame were at 100 and 75 ml/ min, respectively. The carrier gas (nitrogen) flow rate was 4 ml/ min. The oven

temperature was programmed as follows: 60°C hold 5 min, raised to 130 °C (2°C/min) hold 4 min, raised to 180 °C (10°C/min).

Statistical analysis.

The data were analyzed by analysis of variance (ANOVA). When the F-tests were significant, means were separated applying least significant difference (LSD) and Duncan's test (SPSS 7.5.1 1996).

RESULTS AND DISCUSSION

Efficacy

Results in Table (1) show a significant difference in the treatment efficacy against Varroa mite between the three experimental groups, Var-stop , Var-away ,and untreated group ($P < 0.001$, $\alpha = 0.05$, One way ANOVA). However, there is no significant difference between the two formulations, Var-stop and Var-away ($P = 0.401$, $\alpha = 0.05$). The efficacy was 97.8% ± 0.8 for Var-stop and 95.75% ± 1.5 for Var-away. The mean numbers of collected mites/colony during treatment were 433.5 ± 267.9, 322.2 ± 75.2, and 96.5 ± 43.6, for Var-stop , Var-away, and untreated group, respectively.

There is no significant difference in efficacy between the two formulations in the first application ($P = 0.479$, SE = 5.410, $\alpha = 0.05$), and the third application ($P = 0.242$, SE = 6.969, $\alpha = 0.05$). However, there is a significant difference between them in the second application ($P = 0.014$, SE = 4.009, $\alpha = 0.05$).

The obtained results are in agreement with those of Baggio *et al.* (2004) for the thymol-based acaricide Apilife VAR. The treatment was more effective than that described by Mattila and Otis (1999), and Gregorc (2005) for the thymol-based acaricide, Apiguard. It is also higher than the efficacy obtained by Lindberge *et al.* (2000) for the essential oil constituent, thymol, in a laboratorial study. However, it was lower than the efficacy of Apilife VAR in the study carried out by Floris et al. (2004).

Side effects

Marked population decrease of the treated colonies and a severe disturbance to the bees were recorded in the colonies treated with Var- away. It could be explained by an intense release of thymol resulted from using two strips of Var-away and the repeat of application three times.

Table (1): Total acaricidal effect of the two under- experimentation formulations,(Var-stop and Var-away).

	No. during Tr.	No. after Tr.	TN	Efficiency of applications in (%)						TA (%)	M ± S.E. (%)	TA. Ab. (%)	M ± S.E. (%)
				1st.	M±S.E.	2 nd.	M±S.E.	3 rd.	M±S.E.				
Var-stop 90%	219	0	219	73.1	57.6 ± 6.2 a	66.1	60.7 ± 3.0 a	100.0	90.9 ± 5.4 a	100.0	98.4 ± 0.8 a	99.3	97.8 ± 0.8 a
	314	8	322	58.4		52.2		87.5		97.5		96.9	
	821	1	822	42.3		60.8		99.5		99.9		99.3	
	540	20	560	57.0		63.9		77.0		96.4		95.8	
Var-away 42.5%	272	5	277	57.0	61.6 ± 1.5 a	58.8	48.4 ± 3.7 b	89.8	82.2 ± 6.5 a	98.2	96.4 ± 1.3 a	97.6	95.7 ± 1.5 a
	321	11	332	62.7		49.2		82.5		96.7		96.1	
	267	4	271	63.1		44.0		92.9		98.5		97.9	
	429	35	464	64.0		41.9		63.9		92.5		91.4	
Control	104	494	598	6.5	5.1 ± 1.3 b	6.8	4.8 ± 0.7 c	5.2	4.6 ± 0.6 b	17.4	13.9 ± 2.2 b	17.4	13.9 ± 2.2 b
	135	618	753	8.1		4.8		6.2		17.9		17.9	
	34	375	409	2.4		3.0		3.1		8.3		8.3	
	113	832	945	3.4		4.9		4.1		12.0		12.0	

Means followed by different letters are significantly different at $\alpha = 0.05$ (ANOVA followed by LSD test).
M ± S.E. = Mean ± Standard Error.
No. during Tr.= Number of collected Mites during treatment.
No. after Tr.= Number of collected mites after treatment.
TN = Total Number of collected mites (experimental treatment and verification).
TA (%) = Efficiency of the treatment (3 applications) in (%).
TA. Ab.(%) = Efficiency of the treatment (3 applications) according to the Abbott's

Thymol residues in honey.

Results in Table (2) show a significant difference in the thymol residues levels among experimental groups (P = 0.013, α = 0.05, one way ANOVA). However, there is no significant difference in the the thymol residues levels between Var-stop group and Var-away group (P = 0.744, α = 0.05).

The mean thymol residues in honey ± standard error were 2.10 ± 0.22 mg/kg for Var-stop, 2.02 ± 0.12 mg/kg for Var-away, and 1.32 ± 0.08 mg/kg for control group.

Table (2) : Honey thymol residues in treated and control colonies.

	Thymol residues in honey (mg/kg)				
	1	2	3	4	Mean ± S.E.
Var-stop	1.6	2.7	2.0	2.1	2,10 ± 0,22 a
Var-away	2.3	2.1	1.7	2.0	2,02 ± 0,12 a
Control	1.3	1.4	1.5	1.1	1,32 ± 8, 53 b

Means followed by different letters are significantly different at α = 0.05 (ANOVA followed by LSD test).

Mattila and Otis (1999) found that the mean thymol residue in Apiguard treated colonies was 3.3mg/kg in honey from the honey super and 0.90 mg/kg in honey from the brood chamber.

Floris *et al.* (2004) found that the residues varied from 0.12 to 4.03 mg/kg for ApilifeVar and from 0.40 to 8.8 mg/kg for Apiguard.

Residues of thymol found in honey collected from treated beehives ranged from 0.75 to 8.20 mg/kg for Apilife VAR (Adamczyk *et al.*, 2005).

Piasenzotto *et al.* (2002) found that thymol content, evaluated by the SPE-GC method, ranged between 0.02 mg/kg and 0.91 mg/kg in honey samples collected from hives that had been treated with a thymol-based acaricides.

The thymol residue in the honey from the colonies treated with Thymovar was 0.384 mg/kg, (Donders and Cornelissen, 2005).

The obtained results were lower than those of Mattila and Otis (1999), Floris *et al.* (2004), and Adamczyk *et al.*(2005). However, they were higher than those obtained by Piasenzotto *et al.* (2002), Donders and Cornelissen, (2005).

Phenol and thymol are naturally occurred in botanical honey (Eucalyptus, Rosemary, Citrus, Heather and Biercol). Phenol exists in the five honey samples at levels between 15 and 318 ng/g, while thymol was only found in Heather and Biercol honey at levels between 142 and 346 ng/g (Vinas *et al.*, 2006).The WHO views thymol as generally safe up to concentration of 50 mg/kg (Imdorf *et al.*, 1995). It has been demonstrated that, unlike other chemical acaricides, thymol does not accumulate in the honey or the wax, even after long-term use, if the instructions are followed. (Bogdanov *et al.*, 1998).

In many countries, according to the National regulations, no official limits of thymol residue(max. min. levels) have been established for honey and wax (Wallner, 1999). However, foreign odors or tastes are not allowed in honey according to the European food legislation. Taste threshold was detected in honey at the concentration level of 1.1mg/ Kg.

Thymol residues in wax.

Results in Table (3) show no significant difference in the thymol residues levels among experimental groups (P = 0.132, α = 0.05, one way ANOVA).The mean thymol residues in wax ± standard error were 371.5 ± 165.4 mg/kg for Var-stop, 475.0 ± 140.9 mg/kg for Var-away, and 87.5 ± 3.2 mg/kg for control group.

Table (3) : Wax thymol residues in treated and control colonies.

	Thymol residues in wax (mg/kg)				
	1	2	3	4	Mean ± S.E.
Var-stop	212	867	181	226	371,50 ± 165,43 a
Var-away	582	806	152	360	475,00 ± 140,99 a
Control	96	84	89	81	87,50 ± 3,27 a

Means followed by different letters are significantly different at α = 0.05 (ANOVA followed by LSD test).

Our thymol residues results were higher than those obtained by Floris *et al.* (2004), who reported average wax residues of 21.6 mg/kg in the colonies treated with Apilife VAR and 147 mg/kg for Apiguard.

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تقييم فعالية بعض مستحضرات الثيمول فى مكافحة الفاروا فى نحل العسل

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أجريت هذه الدراسة على أثنى عشرة طائفة من طوائف نحل العسل فى منحل تجاري بمحافظة المنيا فى شهري سبتمبر وأكتوبر عام ٢٠٠٥ وذلك لاختبار اثنين من مستحضرات الثيمول (فار- ستوب فى صورة أقراص فار – أواى فى صورة شرائط) .

تهدف هذه الدراسة إلى تقييم فاعلية المستحضرين فى مكافحه أكاروس الفاروا فى طوائف نحل العسل كما تهدف إلى تقدير متبقيات الثيمول فى العسل والشمع ، كلا المستحضرين خفض بشكل معنوى مستويات الإصابة بالفاروا على النحل البالغ وكانت فعالية المستحضرين طبقا للبروتوكول المنشور بواسطة المجموعة الأوربية لمكافحة الفاروا هى ٩٧ % لمبيد الفار – ستوب و ٩٣ % لمبيد الفار – أواى .

وجد أن متبقيات الثيمول فى العسل هي ٢,١ ملجم / كجم بالنسبة للطوائف المعاملة بمبيد الفار – ستوب و ١,٧ ملجم / كجم بالنسبة للطوائف المعاملة بمبيد الفار – أواى .

المتبقيات كانت أعلى فى الشمع حيث بلغت ٣٧١ ملجم / كجم فى شمع الطوائف المعاملة بالفار – ستوب و ٤٧٥ ملجم / كجم فى شمع الطوائف المعاملة بمبيد الفار- أواي.