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The Effects of Using Different Organic Compounds against Honey Bee Mite (*Varroa destructor* Anderson and Trueman) on Colony Developments of Honey Bee (*Apis mellifera* L.) and Residue Levels in Honey

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Abstract: Two organic compounds (thymol and oxalic acid) with three delivery methods (dust, trickled and vermiculite) were applied to 30 infested honey bee (*Apis mellifera* L.) colonies to investigate the effects of treatments on colony development and to determine residues in honey. Bee population, number of mites in brood cells and brood area of groups were determined in autumn, before and after the research. It was observed that treatments did not cause damage to amount of brood, bee population and adult bee mortality. However, there was no significant difference in the level of parasitization inside cells among treatments. A significant positive correlation was found between number of mites in cells and amount of brood reduction ($r = 0.38$, $p = 0.03$). There was also, a positive correlation between number of mites inside cells and bee mortality. Conversely, a negative correlation was found between number of mites in cells and bee population ($r = -0.41$, $p = 0.001$). Residues of thymol found in honey collected from the beehives ranged from 0.021-0.288 for thymol in dust, from 0.119-0.311 for thymol solution in sugar syrup and from 0.041-0.277 mg kg^{-1} for thymol solution in alcohol. The range of oxalic acid content in honey was 6.63-34.99 in oxalic acid treated groups. These results stayed under the acceptable limit of the World Health Organization.

Key words: *Varroa destructor*, honey bee, thymol, oxalic acid, colony performance, residue levels

INTRODUCTION

The varroa mite (*Varroa destructor* Anderson and Trueman, 2000), an ectoparasite of honey bees, can cause substantial damage to a honey bee colony if not treated. In general, varroatosis reduces the life span of bees, including the queen and reduces the bee population. *V. destructor* feeds on the hemolymph of adults and immature honey bees causing a reduction of up to 60% in the protein content of the hemolymph, a 30% reduction of hemocytes, 25% of body weight loss, a reduction on colony development and production activity, wing and limb deformity in adults (Shimanuki *et al*., 1992). A range of organic compounds that occur naturally in the honey bee colony environment and that are present in honey can be used to control *V. destructor*. Up to the present, a few organic products have shown potential effectiveness against *Varroa*, oxalic acid and thymol essential oil are among them, which have no negative effect on the development of colonies (Melathopoulos and Gates, 2003; Floris *et al*., 2004; Espinosa-Montano and Guzman-Novoa, 2007). However, these organic substances occur also in honey naturally. The replacement of synthetic acaricide treatments by oxalic acid and thymol minimizes the risk of residues in bee products such as honey, wax and propolis (Bogdanov *et al*., 1999; Moosbeckhofer *et al*., 2003).

The concentration of organic acids such as oxalic acid, formic acid and lactic acid vary with in a wide range, according to honey origin. The content of oxalic acid varies from 1-225.

The maximum residue level for thymol in the honey is 0.8 and this doesn't represent a risk for human health. Bogdanov *et al*. (1998) reported that the test threshold for thymol was between 1.1 and 1.3.

The present study aimed, at investigating the effects of different organic compounds used for the fall control of the honey bee mite *V. destructor* on colony performance and assessing their residue levels in honey.

MATERIALS AND METHODS

The experimental apiaries were located at the Ataturk University, Erzurum, Turkey. Thirty honey bee colonies were divided into 10 groups of 3 colonies and kept in standard Langstroth hives. Before the experiment, colonies were initially equalized to contain a similar amount of brood, bee population strength (ca. 8 frames

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covered with bees) and food stores (2 combs containing honey and pollen). All colonies were artificially infested with varroa mites in accordance with the method by Emsen *et al.* (2007).

Organic compounds used in the present research, consisted of thymol, oxalic acid and the mixture of those 2 products and applied with three delivery methods. The release devices per colony were as follows: Treatment 1 consisted of Thymol applied in Dust (TD). Six gram of powdered thymol was mixed with 24 g of confectionary sugar. Thirty gram of the layer of this mix was stratified on paper and placed over the frames. Treatment 2 consisted of Thymol applied with the Trickled method (TT). A thymol/sugar syrup solution was prepared from 6 g thymol and 100 mL sugar syrup. This solution was trickled onto the bees trying to cover as much area and bees as possible. Treatment 3 consisted of Thymol in Vermiculite method (TV). In this method 6 g of thymol was diluted in 10 mL alcohol and infused 2 vermiculite blocks. Pieces of blocks were cut 5 by 7.5 by 0.7 cm. Treatment 4, 5 and 6 consisted of oxalic acid applications with the same delivery methods described above. For treatment 4, 2 g of oxalic acid was mixed with 100 mL sugar syrup and trickled Onto the bees (OT). Treatment 5 consisted of 2 g of Oxalic acid and 28 g of powdered sugar (OD). This mix was applied over the surface of a half newspaper sheet and placed over the frames of the brood chamber. Treatment 6 consisted of Oxalic acid in Vermiculite method (OV). In this method, 2 g of oxalic acid was dissolved in 10 mL alcohol and embedded into one vermiculite block. Treatments 7-9 were applied as above, but using a mixture of thymol and oxalic acid at the doses (6 g of thymol and 2 g of oxalic acid) previously described (TOT, TOD and TOV). Treatment 10 was composed of control hives that did not receive any chemical treatment. Control Colonies (CC) received only 100 mL sugar syrup trickled on the bees, 30 g of powdered sugar and 2 dry vermiculite blocks. All the treatments were applied weekly during 4 occasions.

Bee traps were established to determine the number of bees dying inside the hive. For this purpose, at the start of the treatments bee traps were placed at the hive entrance to collect dead worker bees. The traps were designed to restrain worker bees from removing dead bees from a hive. They flew through a wire mesh with 1cm openings attached to the lid and in the process dead bees dropped in a tray at the bottom of the trap (Illies *et al.*, 2002). The number of dead worker bees was counted twice a week during the treatments.

Measurements of worker brood area were determined by measuring capped brood to the nearest cm^2 using Adobe Photoshop® CS2 9.0 (Emsen, 2006). For this, a digital camera was set up onto the stand so that it was oriented directly towards the hive frame holder. The frame to assure the comb was supposed to be fully in the picture. The picture was taken by focusing the camera. The frame holder was rotated so that the other side of the frame can be photographed. When the first frame was done, that frame was removed, replaced it in the hive and continued to the next frame. Measurements were taken before and after the treatments.

Before and after the treatments, bee population was estimated as the number of combs covered with bees and multiplied by 2972 in accordance with the method by Valle *et al.* (2004).

To estimate the mite infestation levels of sealed worker brood, 4×4 cm pieces of comb containing brood were cut with a knife and put in plastic bags. Before, during and after the treatment, the number of mite from treated and control hives was counted in 50 cells/brood sample.

Before and end of the treatments, honey samples were collected from each treated and control hive, to estimate the presence of thymol and oxalic acid residues. 4×4 cm pieces of honey samples were collected supers and brood chambers by using a sharp knife. The samples of comb were kept in identified plastic bags and then stored at -20°C until analysis.

Statistical analysis: The data were analyzed by Analysis of Variance (ANOVA). Data on bee population, amount of brood area, the number of mite inside cells, dead bees and honey samples were log (10) transformed to reduce the heterogeneity of variance. Paired and Wilcoxon tests were used to compare the differences between group means in honey residues, bee population and brood area, before and after the treatments. Since, these data were not distributed normally, nonparametric methods were used. Duncan (1995) test was used to determine the significant differences between group means in an analysis of variance setting. The numbers of mite in cell were log transformed and correlated with brood area, bee population and adult dead bees using Pearson correlation analyses (Sokal and Rohlf, 1981).

RESULTS

Table 1 shows that before the treatment no statistically significant differences existed between the experimental groups and control groups regarding the size of sealed brood area ($F_{9,20}$ = 0.311, p = 0.962), bee population ($F_{9,20}$ = 0.746, p = 0.615) and the infestation level inside cells ($F_{9,20}$ = 0.489, p = 0.738). However, at the end of treatments, differences were not significant between treated groups and control. Conversely, the

Table 1: Sealed brood area, number of bee population, mite infestation level and dead bees (mean±SD) in the experimental hives groups before and after treatment

	Sealed brood area (cm^2)		Number of bee population			Worker brood infestation (%)				
Treatment	Before treatment	After treatment	Before treatment	After treatment	t	Before treatment	During treatment	After treatment	t	Number of dead bees
TD	3067.787±66.6^{ns}	1042.326±41.4^{ns}	41191.92±2423.4^{ns}	29720.00±0.0^{ns}	ns	11.33±2.9^{ns}	14.7±11.7^{ns}	10.7±4.3^{ns}	ns	125.042±16.5^{ns}
TT	2886.167±278.5	1027.906±38.0	39824.61±1166.8	27738.67±1981.3	ns	14.67±5.2	13.33±9.4	8.67±3.7	ns	167.083±25.5
TV	2947.867±366.2	1036.127±39.4	40498.27±1782.3	28729.33±990.6	ns	17.33±3.5	18.00±4.1	25.3±17.3	ns	158.250±25.7
OD	2861.963±275.3	969.300±31.3	39151.15±1347.3	29720.00±0.0	ns	13.33±7.3	17.33±2.4	16.00±4.1	ns	159.125±11.1
OT	2914.037±264.6	999.377±54.3	37130.19±2936.3	27738.66±1981.3	ns	18.0±14.0	8.00±3.0	6.67±4.6	ns	148.417±20.5
OV	3149.910±99.4	993.010±63.2	39824.61±1166.8	28729.33±990.6	ns	11.33±7.1	12.00±10.0	28.0±14.1	ns	222.417±78.6
TOD	2881.140±168.7	1002.753±21.1	38477.49±2694.6	27738.66±1981.3	ns	7.33±3.3	10.67±4.0	5.33±2.4	ns	166.583±22.5
TOT	3129.387±103.6	1030.523±80.4	41845.76±2333.6	28729.33±990.6	ns	12.67±3.5	17.3±10.4	23.3±14.8	ns	146.292±10.8
TOV	3116.967±68.5	1034.720±49.5	43193.07±1347.3	26748.00±1715.8	ns	7.33±5.3	9.33±4.7	14.0±6.9	ns	206.000±23.8
Average treated c.	2992.343±57.67	1011.752±13.66	40094.19±610.78	28432.13±394.98	**	12.4±1.8	12.8±2.0	15.9±2.9	ns	164.983±9.97
CC	2968.193±97.3	981.483±71.1	39824.80±2020.9	28729.33±990.6	ns	11.33±6.5	7.33±4.3	21.3±11.6	ns	150.625±33.9

t: Comparisons between before and after treatments, **p<0.01, ns: not significant

Table 2: Levels of thymol residues in honey before and after treatments

		Thymol before	Thymol after		After treatment	
Treatments	n	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	r	Maximum residues	Minimum residues
TD	3	0.077±0.037^{ns}	0.181±0.081^{ns}	ns	0.288	0.021
TT	3	0.098±0.038	0.222±0.053	ns	0.301	0.119
TV	3	0.067±0.020	0.190±0.074	ns	0.277	0.041
TOD	3	0.082±0.014	0.201±0.084	ns	0.319	0.037
TOT	3	0.071±0.018	0.210±0.046	ns	0.285	0.126
TOV	3	0.061±0.013	0.172±0.043	ns	0.258	0.116
Average treated c.	18	0.039±0.001	0.160±0.02	**	0.299	0.033
CC	3	0.047±0.006	0.049±0.005	ns	0.058	0.040

r: Comparisons of thymol residues between before and after treatments, **p<0.01, ns: not significant

Table 3: Levels of oxalic acid residues in honey before and after treatments

		Oxalic acid before	Oxalic acid after		After treatment	
Treatments	n	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	r	Maximum residues	Minimum residues
OD	3	17.28±5.01^{ns}	23.15±6.63^{ns}	ns	32.99	10.52
OT	3	16.69±4.60	24.09±6.52	ns	33.09	11.42
OV	3	19.83±3.82	27.06±5.05	ns	34.99	17.68
TOD	3	15.69±5.01	20.29±5.35	ns	27.84	26.04
TOT	3	18.06±5.02	22.25±6.02	ns	30.30	10.46
TOV	3	13.99±3.97	25.23±6.50	ns	34.28	12.60
Average treated c.	18	16.92±1.64	23.68±2.13	**	34.99	9.60
CC	3	10.39±1.95	11.37±2.88	ns	34.28	5.64

r: Comparisons of oxalic acid residues between before and after treatments, **p<0.01, ns: not significant

average of brood area and bee population had a significant difference between before and after the treatments.

During the experiment, dead bees were collected from bee trap and counted twice a week in 4 occasions. There was no significant difference between treated groups and control. The effect of thymol, oxalic acid and the mixture of two products applied with three delivery methods didn't seem to be toxic to bees.

Significant positive correlations were found for the number of mite inside cell and the brood area reduction ($r = 0.38$; $p = 0.03$; $n = 30$) and the number of mite inside cell and adult bee mortality ($r = 0.39$, $p = 0.03$, $n = 30$). There was a significant negative correlation ($r = -0.41$; $p = 0.001$, $n = 30$) between the number of mite in cells and bee population. These results indicate that varroa mite infestations definitely cause a reduction in the amount of brood and adult bees in colonies as well as an increase in adult bee mortality.

Thymol and oxalic acid residues collected before and after the treatments, from the control and treated groups. The thymol and oxalic acid residue levels in honey were used as controls (average 0.047±0.006 and 10.39±1.95, respectively). Concerning the colonies treated with thymol, the average residue level of thymol in honey was 0.039±0.001 and 0.160±0.02 before and after the treatments, respectively (Table 2). There was no significant difference between treated and control groups.

After thymol treatments, the residue levels of thymol found in honey had increased but was below the taste threshold (1.1 and 1.3).

The average level of oxalic acid residues in all groups was 16.92±1.64 and 23.68±2.13 before and after the experiment, respectively (Table 3). There was a significant difference between treated colonies before and after treatments. The paired-samples t-test ANOVA considering as variables the residues and the time of sample collection (before and after the treatments) showed statistically significant differences between acaricides ($p<0.01$). Although, there was an increase in the level of oxalic acid in the honey after the treatment no treatment was significantly different than the others.

DISCUSSION

In the present study, thymol, oxalic acid and a mixture of thymol and oxalic acid had no significant negative effect on colony development between the treated colonies. A reduction in brood area recorded after the treatment for thymol treatment groups could be explained by a partial brood removal by bees during the treatment and a natural decrease in colony size usually depends on season, as observed previously by Imdorf *et al.* (1995) and Rice *et al.* (2002).

Nanetti *et al.* (2003), oxalic acid is considered good tools for fighting the mite but may cause a reduction on brood. Conversely, Imdorf *et al.* (1997) reported that trials with the trickling method of oxalic acid didn't show a significant decrease on brood area.

There was no difference in bee population. The bee population decreased in all treatments at the end of the experiment but no treatment was significantly different than the others (Imdorf *et al.*, 1999; Skinner *et al.*, 2001; Mutinelli and Baggio, 2004; Charriere *et al.*, 2004). On the other hand, Nanetti *et al.* (2003) reported that a 4.6% oxalic acid dehydrate solution had negative effects on bee population.

A research showed that Thymovar didn't cause any worker bee mortality but oxalic acid had a significant different ($p<0.001$) compared with the control group (Cornelissen and Gerritsen, 2006).

It has been observed that the organic compounds applied with three delivery methods didn't reduce the mite infestation level inside cells. In previous studies, researchers reported that using organic products such as thymol and oxalic acid had no significant effect to the number of mite inside cells (May-Itza *et al.*, 2007; Charriere and Imdorf, 2002; Gregorc and Planinc, 2002).

It was obvious that mite infestation level had significant impacts on the brood area, adult bee and bee population size of bees. This observation was supported with the similar results obtained by Fries *et al.* (2006).

Concerning residues, using organic compounds with three delivery methods didn't represent a sanitary risk for human health and honey quality. According to European Food legislation, thymol is in group of the non-toxic veterinary drugs, which do not require a MRL (Maximum Residue Level).

Therefore, the taste threshold detected at the concentration level of 1.1 (Bogdanov *et al.*, 1998) was used as a maximum. In our study, the residue level of thymol found in honey stayed under the taste threshold (1.1-1.3).

In previous studies, researchers used homemade thymol preparations such as thymol in olive oil or in ethanol. In one study, 15 g of thymol dissolved in 20 mL of olive oil and ethanol, applied in a vermiculite pad of spongy material of 8×5×2 cm. The residues of thymol solutions found in honey ranged between 0.03, 6.30, 0.05 and 6.20 mg kg^{-1}, respectively (Adamczyk *et al.*, 2005).

These high residue levels obtained by the researchers could be explained by the amount of thymol and climates. Treatments with other thymol-based products such as Apilife VAR and Apiguard (Schulz, 1993; Trouiller, 2004) were similar to our findings ranged between 0.19 and 0.17.

Nanetti *et al.* (2003) reported that the residue level of oxalic acid in honey was 76.3 were found in honey after autumn treatment, but were still within the natural content levels of honey from various botanical origins. Likewise, using high concentrations of oxalic acid (7% oxalic acid dehydrate, 25-30 mL/hive) did not even raise the oxalic acid content of the honey after the treatment.

CONCLUSION

Our results showed that using thymol, oxalic acid and a mixture of 2 products with three different delivery methods did not cause any damage to colony strength. The average thymol and oxalic acid residues were below the taste threshold.

Therefore, residues of thymol and oxalic acid posed no risk for health. Finally, organic compounds used in the present study, can be considered to be good alternatives for synthetic acaricides, especially because they do not represent a sanitary risk.

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