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Determination of Efficacies of Thyme and Eucalyptus Oils and Oxalic Acid to Combat Varroosis in Honey Bees

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ABSTRACT

Objective: The subject of our study is to determine the efficacies of the natural products such as thyme oil, eucalyptus oil, and oxalic acid against *Varroa destructor* ectoparasite, which is widely observed in honey bee (*Apis mellifera* L.) colonies both in our country and worldwide **Materials and Methods:** The study was designed as five groups and these groups were separated into Group 1 (oxalic acid), Group 2 (thyme), Group 3 (eucalyptus), Group 4 (positive control), and Group 5 (negative control). Seven honey bee colonies were included in each group. The first, second, and third groups were designed as the treatment groups and the last two groups were kept as positive and negative control groups. Each group consisted of seven hives. In the treatment groups; oxalic acid was dissolved in glycerin, and thyme and eucalyptus oils were dissolved in ethyl alcohol and absorbed into special towels. Two pieces of special towels per beehive were placed on to the frames in the beehives. The treatments applied weekly for three weeks during September. The treatment efficiencies calculated with the Henderson-Tilton formula and the General Linear Model procedure determined the statistical differences between the oxalic acid, thyme oil, and eucalyptus oil treatment groups. **Results:** The treatment efficiencies determined as 91.74%, 82.25%, and 79.2% in oxalic acid, thyme oil, and eucalyptus oil treatment groups, respectively. **Conclusion:** The findings revealed that completely natural herbal extracts and oxalic acid can be used instead of synthetic chemical drugs against *Varroosis*. Toxication and unnatural deaths were not observed in honey bees throughout the trials.

Keywords: Varroosis, Oxalic acid, Thyme, Eucalyptus.

Kekik ve Ökaliptus Yağları ile Okzalik Asidin Bal Arılarında Varroosis Mücadelesindeki Etkinliklerinin Belirlenmesi

ÖZ

Amaç: Çalışmamızın amacı, bal arısı (*Apis mellifera* L.) kolonilerinde hem ülkemizde, hem de Dünyada yaygın olarak gözlemlenen *Varroa destructor* ektoparazitine karşı doğal ürünler olan timol ve ökaliptus yağları ile okzalik asidin etkinliklerini belirlemektir. **Gereç ve Yöntem:** Çalışma, beş gruptan oluşmuştur ve bu gruplar; Grup 1 (okzalik asit), Grup 2 (kekik), Grup 3 (okaliptus), Grup 4 (pozitif kontrol) ve Grup 5 (negatif kontrol) olarak isimlendirilmiştir. Her gruba, yedi adet bal arısı kolonisi dâhil edilmiştir. Birinci, ikinci ve üçüncü gruplar tedavi gruplarını ve son iki grup da pozitif ve negatif kontrol gruplarını oluşturacak şekilde tasarlanmıştır. Tedavi gruplarında; okzalik asit gliserinde, kekik ve ökaliptus yağları etil alkolde çözdürülerek özel havlulara emdirilmiştir. Havlular, kovanlardaki çerçevelerin üzerine konulmuş ve her kovana ikişer adet yerleştirilmiştir. Tedaviler, Eylül ayında haftada bir olmak üzere, toplamda üç kez uygulanmıştır. Tedavi etkinlikleri ise Henderson-Tilton formülü ile hesaplanmış olup okzalik asit, kekik ve ökaliptus yağları tedavi grupları arasındaki istatistiksel fark, Genel Lineer Model prosedürü ile tespit edilmiştir. **Bulgular:** Tedavi etkinlikleri; okzalik asit, kekik ve ökaliptus yağları tedavi gruplarında sırasıyla %91.74 %82.25 ve %79.2 olarak tespit edilmiştir. **Sonuç:** Elde edilen bulgular, Varroosis'e karşı sentetik kimyasal ilaçlar yerine, tamamen doğal bitkisel ekstraktların ve okzalik asitin kullanılabileceğini ortaya koymuştur. Saha denemeleri boyunca, bal arılarında toksikasyon belirtileri ya da doğal olmayan ölümler gözlenmemiştir.

Anahtar Kelimeler: Varroosis, Okzalik asit, Kekik, Ökaliptus.

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INTRODUCTION

Varroa destructor is one of the most frequently observed ectoparasites in honey bees worldwide (McMenamin&Genersch, 2015). It transports many viral agents like Deformed Wing Virus (DWV), Israeli Acute Paralysis Virus (IAPV), Acute Bee Paralysis Virus (ABPV) and Black Queen Cell Virus (BQCV) (Durand et al., 2023). Colonies with an untreated *Varroa* burden cannot survive over two years. *Varroosis*-infected colonies produce less honey than treated colonies (Emsen et al., 2014).

In terms of treatment, synthetic chemicals were thought to be the best in controlling *Varroa* for many years and the most important of these chemicals are pyrethroids and organophosphates (Calderone et al, 1994). The various studies have revealed that the resistance against these chemicals has developed in *V. destructor* over time. In addition, the excessive use of these chemicals has revealed the residue problem in bee products, especially honey and beeswax. Therefore, the use of organic acids, essential oils, and their derivatives,which are natural compounds are in rise (Bahreini et al., 2004).

Oxalic acid is generally used in honey bees against *Varroosis* by evaporating and trickling methods (Charriere&Imdorf, 2002). The activities vary by location, season, and mostly on the presence or absence of brood. Oxalic acid should be used in broodless conditions without side effects (Rademacher & Harz, 2006). Another way to reduce *Varroa* mites is essential oils. Among natural compounds, essential oils are the most important compounds used instead of chemical compounds against *Varroa*. The studies have shown that using essential oil against *V. destructor* causes less stress in honey bees than using organic acids (Conti et al., 2020).

Thymol (2-isopropyl-5-methylphenol) is a monoterpenoid phenol in thyme essential oil with significant acaricid activity. It causes the death of *Varroa* agent by binding to octopamine and gamma butyric acid receptors in the central nervous system (Escobar et al., 2020).

Eucalyptus essential oil finds extensive use in pharmaceuticals, perfumery, and industry (Hanoğlu et al., 2022). It has a repellent effect against insects and has also been known for its antifungal, antibacterial, and antiseptic properties for centuries (Chandel et al., 2021). Essential oils have promising efficacy in *invitro* studies but most of their usage has not been tested in field areas. Therefore, the researchers have been trying to find the most effective oil components against *Varroa* mites. This study was designed to determine the activities of organic products like thyme oil, eucalyptus oil, and oxalic acid against *Varroa* in naturally-infested honey bee colonies.

MATERIALS AND METHODS

Study area

This study was carried out in Balıkesir Province in September 2022. Balıkesir is located in the Southern part of the Marmara Region of Türkiye (39º40'N-26º28'E).

Honey bee information

The local hybrid bee breeds (*Apis mellifera* L.) were studied. The queen bees in all hives were one year old. During the period of the study, honey bee colonies averaged between 30,000 and 35,000 individuals. Before the study, the honey bee's owner confirmed that no chemical or plant extract was used to treat any pathogens.

Field experiment and detection of *Varroa* mites

There were 50 hives in the apiary where the study was carried out. First, 50 hives were examined regarding *Varroosis* positivity/negativity using the powdered sugar method. Approximately 300–350 worker honey bees from the brood combs were brushed into 900 ml *Varroa* tester jars where the circular middle part of the caps was removed and replaced with a 3.1 mm sieve. Then, about two tablespoons (15 g) of powdered sugar was added through a sieve. The jars were rolled to distribute the sugar evenly over the bees. One minute later, the jar and honey bees were shaken vigorously for about 4 minutes on a white paper plate and the displaced *Varroa* were counted (Dietemann et al., 2013). *Varroa* tester jar and *Varroa* mites are shown in Figure 1 and Figure 2, respectively. Pollen drawers were cleaned before each trial and covered with white paper to count the *Varroa* mites that fell in each application.

Figure 1. Varroa monitoring with tester jars.

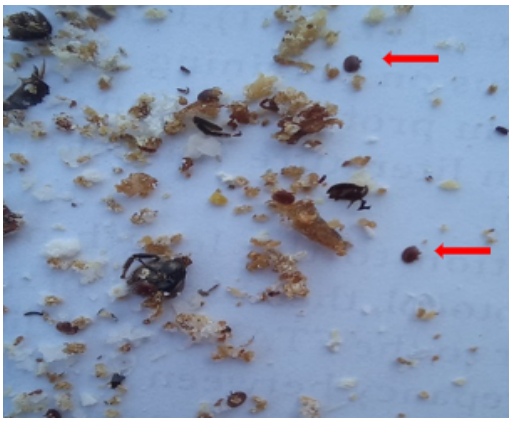

Figure 2. Varroa mitess are shown in red arrow.

Preparation of essential oils and oxalic acid

The information about the thyme oil (Yeşilvadi Botanical Products, Türkiye); 100% purity, active ingredients: p-cymene (8.25%), γ-terpinene (31.35%) and thymol (48.50%), linear formula: 2-[(CH_3)2CH]C_6 H_3-5-(CH_3)OH, molecular weight: 150.20 g/mol. Eucalyptus oil (Yeşilvadi Botanical Products, Türkiye); 100% purity, active ingredients: eucalyptol (50.22%), α-pinene (24.78%), p-cymene (9%), and β-cymene (9.24%), linear formula: $C_{10}H_{18}O$| ID 2758, molecular weight: 153.20 g/mol. Oxalic acid dihydrate; linear formula: $(COOH)_2 \cdot 2H_2O$, CAS number: 6153-56-6, molecular weight: 126.07. Seventy-two grams of oxalic acid (Sigma–Aldrich®) was dissolved in 30 ml of water at 60–70°C and then mixed with 78 ml of glycerin. Twelve pieces of paper towels were dipped in to the solution and left for 30 minutes. Each towel absorbs approximately 6 g of oxalic acid and 6.5 ml of glycerine. Two towels in each hive were placed on to the frames. Thirty-six ml of thyme oil were dissolved in 21 ml of ethanol, then 75 ml of water at 60–70°C and 78 ml of glycerin were added, respectively. Two towels in each hive are placed on to the frames. On the other hand, eucalyptus oil was prepared and applied like thyme oil (Sabahi et al., 2020).

Treatment and control groups

Thirty-five honey bee colonies in Langstroth-type hives consisting of six to seven frames were divided into five homogeneous groups. The treatments were applied weekly for three weeks during the fall season. The treatments were administered on days 0, 7, and 14. In each application, two towels were placed on to the frames in each hive. The towels used were changed every week and replaced with new ones. Powdered sugar counts were applied at day 0 (before treatment), day 7, day 14, and day 21 (post-treatment) for the determination of *Varroa* loads in honey bee colonies. Also, the dead mites that had dropped onto the pollen drawers were counted on the 1st, 3rd, 7th, 14th, and 21st days. As in the treatment groups, powdered sugar counts were performed on days 0, 7, 14, and 21 in the positive and negative control groups, and in addition, *Varroa* agents that fell into the pollen drawer were counted on days 1, 3, 7, 14, and 21 (Girisgin and Aydin, 2010).

Determination and comparison of treatment efficacy

The therapeutic efficacy of organic compounds was determined by the Henderson-Tilton formula shown below (Girisgin and Aydin, 2010).

$$\text{Corrected \%} = \left(1 - \frac{\text{n in Co before treatment x n in T after treatment}}{\text{n in Co after treatment x n in T before treatment}}\right) \text{x } 100$$

Where n= mite population, T= treated, Co= control

Statistical analysis

Statistical analyses of the study were performed in the IBM SPSS20 package program. In order to evaluate the effects of the factors used on different days together, the General Linear Model procedure was followed and interactive analysis of variance was applied by considering the 2×2 (Agent × Days) factorial experiment scheme. The statistical significance was considered when $P \leq 0.05$ (Collins et al., 2009).

Ethical considerations

Insect studies do not require ethics committee approval according to Türkiye's related law.

RESULTS

Thirty-five of 50 (70%) honey bee colonies were found to be positive and 15 of them (30%) were negative in terms of *Varroosis* by powdered sugar method. The hives with a negative test result do not mean that they are free of *Varroa*, but only that there is not enough mite load on the adult bee. Therefore, the treatment groups were selected with positive hives. Three hives were separated as treatment groups, a total of 21 hives out of 35 *Varroosis*-positive hives (60%). Seven of the remaining 14 *Varroosis*-positive hives formed the positive control group of the study. Seven out of 15 *Varroosis*-negative hives were selected as the negative group of the study considering their clinical status. Powdered sugar counts were applied at day 0 (before treatment), day 7, day 14, and day 21 (post-treatment) for the determination of *Varroa* loads in honey bee colonies and results are given in (Table 1).

Varroa count reduction was compared on days 0-7, 0-14, and 0-21. The maximum reduction was found between days 0–21 in the treatment groups. The decrease in a load of *Varroa* in oxalic acid, thyme oil and eucalyptus oil treatment groups on days 0–7 was 43.79%, 17.53% and 18.16%, respectively. The decrease in a load of *Varroa* in oxalic acid, thyme oil and eucalyptus oil treatment groups on days 0–14 was 68.21%, 45.67%, and 36.72%, respectively. The decrease in the load of *Varroa* in oxalic acid, thyme oil and eucalyptus oil treatment groups on days 0–21 was 84.88%, 67.53%, and 61.87%, respectively (Table 1).

The efficacies of natural compounds were calculated by applying the Henderson-Tilton formula to the results of the counts on days 0 and 21 and the treatment efficacies are given in Table 2. Table 2 shows the treatment efficacies were found in oxalic acid, thyme oil, and eucalyptus oil treatment groups at 91.74%, 82.25%, and 79.2%, respectively.

Table 1. Mite counts by powdered sugar method according to days in treatment and control groups.

Hive No	Oxalic Acid				Thyme Oil				Eucalyptus Oil				**Positive Control Group**			
	D.0 V.L	D.7 V.L	D.14 V.L	D.21 V.L	D.0 V.L	D.7 V.L	D.14 V.L	D.21 V.L	D.0 V.L	D.7 V.L	D.14 V.L	D.21 V.L	D.0 V.L	D.7 V.L	D.14 V.L	D.21 V.L
1	45	30	20	8	40	35	28	20	50	42	30	25	56	67	78	89
2	55	30	15	7	50	38	25	18	52	43	35	27	34	45	67	78
3	67	37	20	10	62	47	30	20	64	51	40	30	45	59	80	67
4	78	40	20	6	70	52	35	25	73	55	39	21	56	78	79	89
5	89	45	25	12	80	59	38	25	85	67	55	35	34	56	78	90
6	90	50	26	15	85	70	45	20	87	74	58	28	56	78	81	80
7	92	58	38	20	75	80	50	22	90	78	60	25	35	75	82	85
T.	**516**	**290**	**164**	**78**	**462**	**381**	**251**	**150**	**501**	**410**	**317**	**191**	**316**	**458**	**545**	**578**
Days	Treatment groups' % efficacies determined according to the days															
0-7	Oxalic Acid VCR=43.79								Thyme Oil VCR=17.53				Eucalyptus Oil VCR=18.16			
0-14	Oxalic Acid VCR=68.21								Thyme Oil VCR=45.67				Eucalyptus Oil VCR=36.72			
0-21	Oxalic Acid VCR=84.88								Thyme Oil VCR=67.53				Eucalyptus Oil VCR=61.87			
Hive No	Negative Group Count Results															
1	D.0 V.L		D.7 V.L		D.14 V.L		D.21 V.L									
2	0		2		5		12									
3	0		3		8		23									
4	0		4		12		34									
5	0		7		34		45									
6	0		12		45		56									
7	0		23		55		67									

D.0=Day 0, D.7=Day 7, D.14=Day 14, D.21=Day 21, V.L=Varroa Load, T.=Total, VCR=Varroa Count Reduction

Table 2. The Treatment efficacies according to days 0 and 21.

Days	Oxalic Acid *Varroa* Loads	VCR %	Thyme Oil *Varroa* Loads	VCR %	Eucalyptus Oil *Varroa* Loads	VCR %
Day 0	516	91.74	462	82.25	501	79.2
Day 21	78		150		191	

It was determined that the percentage change in the number of *Varroa* in the pollen drawer increased from day 1 to day 21 in all treatment groups. In the pairwise comparison of day 1 with the other days, the percentage increase in the number of *Varroa* falling into the pollen drawer among the treatment groups was found the highest in the oxalic acid treatment group (153.01%). As a comparison of 1–3 days, percentage change in the number of *Varroa* in the pollen drawer of oxalic acid, thyme oil, and eucalyptus oil groups were found as 55.42%, 15.62%, 14.63%, respectively. This rate was determined as 60.24%, 31.25%, and 32.92% in the oxalic acid, thyme oil, and eucalyptus oil treatment groups between day 1 and day 7, respectively. This ratio was found to be 125.90%, 58.33%, and 54.43% in oxalic acid, thyme oil, and eucalyptus oil treatment groups between day 1 and day 14, and 153.01%, 81.25%, and 76.82% in the oxalic acid, thyme oil, and eucalyptus oil treatment groups on day 1 and day 21, respectively.

The statistical differences were determined between the agents used to control *Varroosis* and the days ($P<0.001$).

According to the powdered sugar count results, the highest *Varroa* load was determined in the positive control group (67.38), followed by eucalyptus oil (48.53), thyme oil (42.38), oxalic acid (35.00) and negative control group (18.63), respectively. The difference between days was statistically significant (P<0.001). According to the powdered sugar count results from the beginning of the study to day 21, infestation with *Varroa* gradually decreased. The interactions between the agents used in the treatment and the days were also found to be statistically significant (P<0.001). While the average number of *Varroa* was 70.67 in the group using oxalic acid at the beginning of the study (day 0), this number decreased to 9.67 at the end of the study (day 21). The average number of *Varroa* decreased from 64.50 to 21.33 in the thyme oil group and from 68.50 to 27.67 in the eucalyptus oil group. The average number of *Varroa* gradually increased in the positive and negative control groups.

The numbers of daily average *Varroas* falling into the pollen drawer were found to be 39.80 in the oxalic acid, 17.27 in thyme oil, 14.53 in eucalyptus oil, 3.20 in positive control, and 0.97 in negative control groups, respectively. While the average number of *Varroa* falling in the pollen drawer was 22 in the oxalic acid group on day 0, this number increased to 56 at the end of the study (day 21). Similarly, from 12.17 to 23.17 in the thyme oil group; increased from 10.33 to 19.50 in the eucalyptus oil group. Both the main effects (P<0.001) and interactions between administration and days (P=0.001) were statistically significant.

DISCUSSION

The papers suggest that both essential oils and oxalic acid have potential as control agenta against *Varroosis* in honey bees. Hýbl (2021) found that several essential oils, including manuka, peppermint, oregano, litsea, and cinnamon, were effective against *Varroa* mites and safe for honey bee workers under laboratory conditions. Umpiérrez (2010) reviewed the literature on essential oils with anti-*Varroa* activity and suggested that they are good candidates for safer control. Adjlane (2020) found that two doses of oxalic acid were effective for controlling Varroa mites, and that the effectiveness of oxalic acid is directly linked to the presence of brood at the time of the intervention. Adjlane (2016) found that oxalic acid treatment was effective for controlling *Varroa* mites, but that the dose of 100 g oxalic acid caused a weakening of honey bee colonies. In the study in which oxalic acid and thymol were tested in honey bee colonies, the oxalic acid application method was the same as ours. Two towels were placed on to the frames in each hive and the treatment efficiency was determined as 78.7±3.9% (Sabahi et al., 2020), while 91.74% success was achieved in our field studies with the same method. Also, 92.4±3.2% success was achieved in the thymol absorbing method (Sabahi et al., 2020), while 82.25% success was achieved in our field studies with the same method. Overall, the papers suggest that oxalic acid and plant essential oils are effective options for controlling *Varroosis* in honey bee colonies, but that proper dosing and timing are important factors to consider.

Although the total mite reduction in the positive control seems to be higher than in the control groups, there is a significant difference compared to the first days of treatment. The increase at the end of the third week is thought to be due to the decrease in the number of brood in the hive and similar results have been obtained in another study (Girişgin and Aydın, 2010).

In our study, the highest treatment efficacy against *Varroosis* was found in the oxalic acid treatment group. Some factors such as environmental, self-cleaning properties, essential oil ingredients or population, may lead to this difference.

CONCLUSION

The results of our study suggest that thyme, eucalyptus, and oxalic acid could play an important role in the integrated management of Varroosis in honey bee colonies. Considering the increases in powder count, at least two treatments per year should be applied during the early first and late autumn periods to prevent Varroosis infestations. The possibility of using these natural agents in *Varroa* control in apiaries warrants further investigations.

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Conflict of Interest

The author declares no potential conflicts of interest concerning for to the research, authorship, and/or publication of this article.

Author Contributions

Plan, design: MO; **Material, methods, and data collection:** IK, OD; **Data analysis and comments:** YB; **Writing and corrections:** AOG.

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