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ONUR DEMİR

ALİ BİLGİLİ

MEHLİKA PULAT

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# Investigation of the acaricidal efficacy, tolerance, and residue levels of thymol-containing gelatin-based hydrogel developed for varroosis control

**Onur DEMİR**[1,2,*], **Ali BİLGİLİ**[3], **Mehlika PULAT**[4]

[1]Pharmaceutical Quality Control Laboratory, Pendik Veterinary Control Institute, Republic of Türkiye Ministry of Agriculture and Forestry, İstanbul, Turkiye
[2]Graduate School of Health Sciences, Ankara University, Ankara, Turkiye
[3]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkiye
[4]Department of Chemistry, Faculty of Science, Gazi University, Ankara, Turkiye

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**Abstract:** The aim of this study was to investigate the acaricidal efficacy, tolerability of the target species, and residue levels of a thymol-containing gelatin-based hydrogel developed as a controlled release system for varroosis control. The study was carried out on naturally infested honey bee colonies and results were compared with those of negative and positive control groups. The acaricidal efficacy of the negative control, positive control, and hydrogel treatment was 21.93 ± 2.87%, 81.09 ± 2.30%, and 70.32 ± 2.05%, respectively. For tolerability evaluations, the average number of dead bees per colony was determined to be 60.80 ± 8.01, 297.40 ± 21.4, and 86.80 ± 6.94 for the negative control, positive control, and hydrogel treatment, respectively. The average thymol residues in honey were measured as 0.030 ± 0.022 mg/kg, 5.498 ± 3.346 mg/kg, and 0.168 ± 0.110 mg/kg for the negative control, positive control, and hydrogel treatment, respectively. The average thymol concentrations in beeswax were 0.074 ± 0.047 mg/kg, 1.667 ± 0.855 mg/kg, and 0.476 ± 0.278 mg/kg for the negative control, positive control, and hydrogel treatment, respectively. The controlled release system developed in this study was found to prevent the transport of thymol out of the colony due to its structure, which is well tolerated by bees. Consequently, a single application can achieve longer infestation control. Furthermore, the lack of intervention by bees in the system ensures that bee mortality due to contact toxicity and residue levels in honey and beeswax are low. In addition to these advantages of the system, further development is required to increase the acaricide efficacy in order to make it an ideal alternative to existing varroacidal veterinary medicinal products.

**Key words:** Controlled release system, gelatin, hydrogel, thymol, varroosis

## 1. Introduction

Varroosis is caused by the *Varroa* mite (*Varroa destructor* Anderson and Trueman), which is the most common parasitic infestation of honey bee colonies and important for the agricultural sector and ecosystem [1–3]. The presence of parasitic mites in bee colonies has been associated with a reduction in colony productivity, increased susceptibility to other diseases, and even the collapse of the entire colony. It has been reported that acaricidal chemicals can be effective tools in the control of varroosis, provided that they are applied using the correct method, correct timing, and appropriate dose [4]. However, colony health may be negatively affected by improper use or failure to control the application time and dosage because of environmental influences. At the same time, other species in the ecosystem are at risk of the development of antiparasitic resistance and residues [5,6]. Thymol, which has been used for the control of varroosis for many years, inhibits the GABA-gated chloride channels of mites and bees and subsequently causes an acaricidal effect in the central nervous system through overstimulation and convulsions [7–10]. The topical 50% lethal dose ($LD_{50}$) values are 56.1, 210.3, and 150.7 µg for adult mites, adult worker bees, and bee larvae, respectively [11]. Thymol provides good acaricidal activity when inhaled at concentrations of 5–15 µg/L but has a 90% bee mortality rate at concentrations above 20 µg/L [12]. It has been reported that the application of thymol to colonies at ambient temperatures below 15 °C results in queen mortality, whereas at temperatures above 30 °C, thymol causes behavioral changes, including brood and adult bee mortality [13–15]. Bees react differently to thymol applied to colonies at different concentrations. It has been observed that thymol placed in colonies for treatment purposes is generally carried out of the hive

* Correspondence: demir.onur@tarimorman.gov.tr



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by the bees in a short time and is rarely covered with propolis. As a result, there is insufficient thymol left in the colony for control and additional thymol application is needed [9,10]. Furthermore, bees are exposed to thymol through contact and the respiratory route, which increases the total amount of exposure. The acaricidal activity of thymol is affected by temperature and humidity changes in the environment where it is applied [16]. Varroacidal efficacy values were reported to range from 66% to 98% for pure thymol in different amounts. A veterinary medicinal product reported to have rates of 74% to 99% was selected for this study as a reference product. Other veterinary medicinal products containing different amounts of thymol were reported to have rates of 66.4% to 99.5%. These values were observed in different geographical locations and seasons, in different hive types, and at different durations [17,18]. Thymol is classified as a group II nontoxic veterinary active substance and does not require a maximum residue limit [19,20]. A limit of 0.8 mg/kg is generally applied to avoid exceeding the residue threshold of 1.1 mg/kg, which can cause flavor residue in honey [7].

Controlled release systems (CRSs) aim to improve the effectiveness of active substances by enhancing their stability against various damaging factors, such as temperature, oxidation, moisture, and microorganisms. Additionally, they help minimize high volatility, allowing for the controlled application of these substances at specific intensities, speeds, and times in the treatment area [21–23]. It has been proposed that these systems may be alternatives to the currently applied mite control methods in terms of reducing the amount of active substance required for treatment, the incidence of side effects, and the frequency of application while limiting environmental damage [23–25].

Hydrogels, which play an important role in CRSs, are polymeric structures formed by chemical or physical cross-linking [26,27]. Owing to these interactions, the release of drug molecules loaded into their structures into the treatment area can be ensured through different kinetics [26]. A number of studies have been conducted on the application of hydrogels in the context of CRSs in combination with thymol [28]. Gelatin is a natural polymer used in pharmaceutical and biomedical fields for applications such as drug coating, microencapsulation, and hydrogel production, and glutaraldehyde is commonly used to provide chemical cross-linking in hydrogel production [26,27,29].

The aim of this study was to investigate the acaricidal activity, tolerability, and residue levels of a thymol-containing gelatin-based hydrogel developed as a CRS for varroosis control. Additionally, the study aimed to identify possible differences between treatment groups.

## 2. Materials and methods

### 2.1. Materials

The hydrogel production materials included powdered bovine gelatin (food grade, Alfasol, s-Hertogenbosch, the Netherlands), 25% glutaraldehyde (Merck, Darmstadt, Germany), sunflower oil (food grade, Yudum, Ayvalık, Türkiye), thymol standard (technical grade, 99% purity, Sigma-Aldrich, St. Louis, MO, USA), and distilled water. For chromatographic analyses, a thymol reference standard (99.6% purity, Dr. Ehrenstorfer, Teddington, UK), a *p*-cumenol reference standard (99.6% purity, Dr. Ehrenstorfer, Augsburg, Germany), ethyl acetate (Merck, Darmstadt, Germany), glacial acetic acid (≥99% purity, Sigma-Aldrich, St. Louis, MO, USA), acetonitrile (HPLC grade, Sigma-Aldrich, Darmstadt, Germany), ammonium formate (97% purity, Sigma-Aldrich, St. Louis, MO, USA), methanol (HPLC grade, Merck, Darmstadt, Germany), n-hexane (HPLC grade, ISOLAB, Eschau, Germany), and distilled water were used.

### 2.2. Preparation of hydrogels

A solution was prepared in a glass flask using powdered gelatin and distilled water in a volume of 2500 mL and a concentration of 10%. Another solution was then prepared in a glass flask using technical grade thymol and sunflower oil at a concentration of 50% in a volume of 250 mL. The flasks were placed in an ultrasonic bath at a temperature of 50 °C for 1 h to completely dissolve the gelatin and thymol. The solution was then removed from the ultrasonic bath. After the solution reached room temperature, 10 mL of gelatin solution (10%) was added to 15-mL centrifuge tubes, followed by 1 mL of thymol-sunflower oil solution (50%). The capped tubes were vortexed at 2000 rpm for 20 min and then 0.8 mL of 12.5% glutaraldehyde solution was added to all tubes, which were vortexed at 2000 rpm for another 2 min. The hydrogels were kept at room temperature for 24 h and then removed from the tubes and cut into 0.5-cm-thick discs with a scalpel. These discs were washed three times with distilled water and then dried to a constant weight in an oven at 30 °C. After drying, hydrogel discs loaded with thymol at an average of 25% by weight were stored in moisture- and light-proof bags under room conditions.

### 2.3. Steps taken before the field study

For experiments to be carried out in honey bee colonies, permission was obtained from the Pendik Veterinary Control Institute Animal Experiments Local Ethics Committee (Decision No. 202-17/2018). The research was conducted in September, October, and November 2021 at the apiary of the İstanbul Beekeepers Association located in the İstanbul Aydos Forest at 40°55′25.5″N, 29°15′04.9″E. Anatolian bee colonies (*Apis mellifera anatoliaca*) and Langstroth-type hives with pollen drawers were used in

this study. Before the study began, the health status of the colonies was assessed in terms of varroosis and nosemosis infestation levels, pesticide exposure was checked, and suitable colonies were selected [30].

**2.4. Determination of varroosis infestation level in colonies**

The powdered sugar shake method [3,31] and Eq. (1) were used to estimate the total number of phoretic mites in the colonies and the level of infestation based on the number of phoretic mites detected by sampling adult bees in each colony.

$$\text{Infestation (\%)} = \frac{\text{Number of detected mites}}{\text{Number of sampled bees}} \quad (1)$$

**2.5. Determination of pesticide levels in colonies**

Samples of bees, honey, and beeswax were analyzed via liquid chromatography–mass spectrometry (LC–MS/MS) to determine the presence or absence of pesticides [23]. Bee, honey, and beeswax samples were extracted using a method described in the literature [32]. The mobile phase gradient program of the LC–MS/MS system (Ultimate 3000 series, MS/MS, Thermo Finnigan Discovery Max, Thermo Fisher Scientific, Waltham, MA, USA) with 5 mM ammonium formate from line A and pure methanol from line B was used with a flow rate of 0.3 mL/min at 0–2 min, 98%:2%; 2–18 min, 60%:40%; 18–22 min, 5%:95%; 22–26 min, 98%:2%. Chromatographic separation was achieved with a 2.6-µm column (100 × 2.1 mm) (Thermo Accucore aQ, Thermo Fisher Scientific). The column temperature was set to 30 °C while the dwell time was 0.01, detector voltage was 1600 V, needle voltage was 5000 V, spray chamber temperature was 50 °C, and drying gas temperature was 300 °C. Calculations were performed with the Xcalibur workstation chromatographic program (Thermo Fisher Scientific).

**2.6. Determination of nosemosis level in colonies**

Microscopic diagnosis of nosemosis and estimation of the infestation level of colonies were carried out using the standard method [33,34]. Samples were photographed (400×) using an Axio Imager D2 microscope with a digital camera and a ZEN 3.1 (Blue Edition) imaging system (Carl Zeiss, Oberkochen, Germany), and *Nosema* spp. spore counts were determined. The nosemosis level of each colony was calculated by assuming that 1 *Nosema* spp. spore observed on the hemocytometer slide corresponded to 10,000 spores in 1 bee [33].

**2.7. Field studies**

A field study comparing acaricidal efficacy, target species tolerance levels, and thymol residue levels in honey and beeswax was planned according to the guidelines of the European Medicines Agency (EMA) [35]. Five colonies constituted the negative control group, 5 colonies constituted the positive control group (with the reference veterinary medicinal product containing 12.5 g of thymol administered 2 times at a 2-week interval), and 5 colonies constituted the experimental group (with gelatin hydrogel containing 25 g of thymol with 1 application in 4 weeks). Honey and beeswax samples were collected from the colonies before treatment for thymol residue analyses. To monitor the environmental conditions during the treatments, temperature and humidity meters (KLIMALOGG Pro, TFA Dostmann, Wertheim, Germany) were placed in the apiary area, with one in each hive selected to represent each treatment group.

This research was carried out as a comparison study between the negative control, positive control, and hydrogel-treated colonies in the first stage. In the second stage, follow-up treatment was applied for all colonies with a tau fluvalinate-containing veterinary medicinal product [36].

No thymol was applied to the hive frames in the negative control group, but 25 g of thymol equivalent reference veterinary medicinal product was used for the positive control group and 25 g of thymol-containing hydrogel was used for the test group. Sticky white papers were placed in pollen drawers and dead bee traps were placed in front of the hives.

Bee behaviors in the colonies were observed and dead bees and dead mites were counted periodically for the recommended period of 4 weeks [14]. At the end of 4 weeks, all thymol had been applied and the colonies were removed. Honey and beeswax samples were collected from the colonies for thymol residue analyses after 4 weeks. Honey and beeswax samples collected from the colonies were labeled and stored at –20 °C until the analyses were performed. Follow-up treatment with 10% tau-fluvalinate-containing licensed veterinary medicinal product (each strip with 8 g) was performed for 6 weeks according to the instructions with two strips per hive [36]. Acaricidal efficacy levels were calculated with Eq. (2), where A is the number of dead mites detected in the treatment group in the first stage treatment and B is the number of dead mites detected in the follow-up treatment.

$$\text{Acaricidal efficacy (\%)} = \frac{A}{A \times B} \times 100 \quad (2)$$

**2.8. Analysis of thymol residue levels in honey and beeswax**

For the evaluation of thymol residue in the honey, 2.5 g of honey was weighed into 50-mL tubes for blind samples, test samples, and calibration samples. Seven milliliters of distilled water was added to the tubes. The calibration samples were prepared by adding 0.25, 0.5, and 1 mL of the stock thymol solution (10 µg/mL in acetonitrile) to the tubes. To all tubes, 1 mL of *p*-cumenol solution (10 µg/mL in acetonitrile) was added as an internal standard. The tubes were vortexed at 1000 rpm for 20 min. All

samples were then passed through an SPE cartridge (SPE-R00230B-03P, SiliaPrep, SiliCycle Inc., Quebec City, Canada) activated with 6 mL of methanol (Merck) and 6 mL of water. The SPE cartridges were washed again with 6 mL of water and finally eluted with 1 mL of methanol. The samples were made up to a volume of 10 mL with 80% acetonitrile solution, passed through a 0.45-μm PTFE filter, and analyzed by HPLC-DAD [9,37].

For the evaluation of thymol residue in beeswax, 2.5 g of beeswax was weighed into 50-mL tubes for blind samples, test samples, and calibration samples. Ten milliliters of *n*-hexane was added to the tubes. To the calibration samples, 0.25, 0.5, or 1 mL of stock thymol solution (10 μg/mL in acetonitrile) was added to the tubes. To all tubes, 1 mL of *p*-cumenol solution (10 μg/mL in acetonitrile) was added as an internal standard. The tubes were vortexed at 1000 rpm for 20 min and then centrifuged at –10 °C and 4000 rpm for 20 min. The supernatants were filtered through a 0.45-μm PTFE filter and centrifuged again at –10 °C and 4000 rpm for 20 min. The supernatants were adjusted to a volume of 10 mL with 80% acetonitrile solution, filtered through a 0.45-μm PTFE filter, and analyzed by HPLC-DAD [38].

For the honey and beeswax studies, three samples were prepared and the thymol content was measured via HPLC-DAD (Dionex Ultimate 3000, Thermo Fisher Scientific). Analytical separation was achieved with a C18 (250 mm × 4.60 mm, 5 μm ACE) column. Acetonitrile and water were used as the mobile phase at a 75:25 ratio and a flow rate of 1 mL/min. The samples were injected into the system in a volume of 20 μL. Thymol peaks were detected at a retention time of 5 min and at 278 nm.

### 2.9. Statistical methods

The Kolmogorov–Smirnov test was used to assess the normality of the distribution of the data. One-way ANOVA and Tukey HSD tests were applied to the data obtained from the treatment groups, and the treatment groups that generated significant differences were determined by comparing the groups among themselves. All statistical analyses were performed using IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA) at a 95% confidence level (5% error margin, two-tailed, $\alpha = 0.05$) [39,40].

## 3. Results

### 3.1. Control findings before the field study

*Varroa* spp. mites were detected in all sampled colonies. The rate of varroosis infestation in the colonies varied between 2% and 4%. No residues were found in the honey, beeswax, or bee samples taken from the colonies at the limits of determination for the investigated pesticides [41]. *Nosema* spp. spores were not detected in any of the samples at 1 million or more.

### 3.2. Field study findings

During the study period, rainfall occurred on 12 days in total with light intensity and intervals of a few hours during the day. As shown in Figure 1, the average daily environmental and hive temperature and humidity values were not specified in the thymol veterinary medicinal product manuals [13–15].

#### 3.2.1. Acaricidal efficacy level findings

During the field study, mites on the sticky papers in the pollen drawer in the colonies were periodically counted and recorded, and the weekly average number of mites killed is presented in Figure 2.

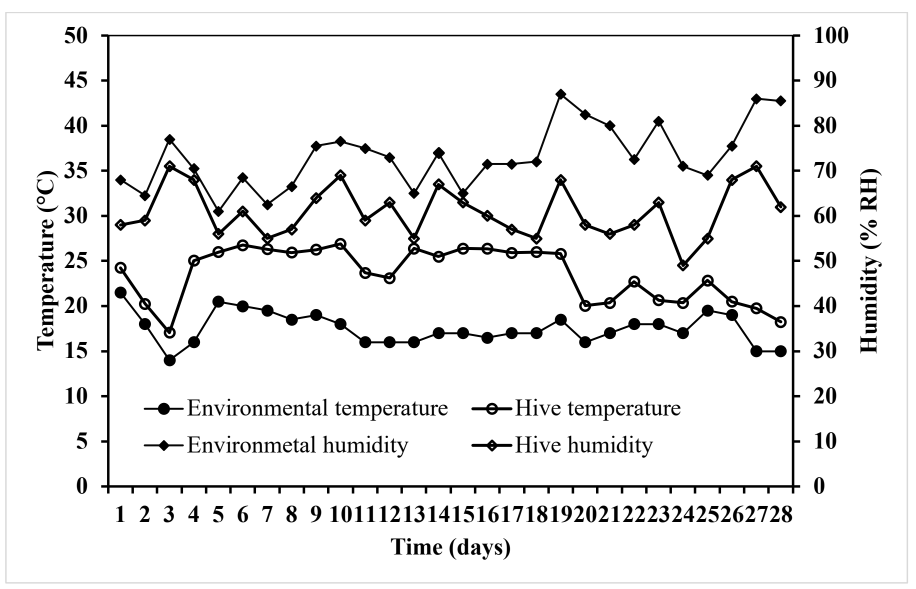


**Figure 1.** Field study temperature and humidity measurements.

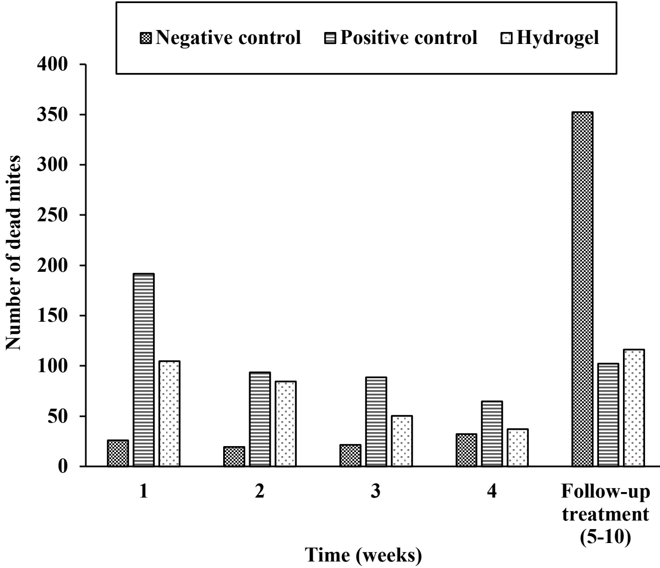


**Figure 2.** Number of mites killed in the first stage of treatment (weekly average) and in the follow-up treatment (6-week average).

According to the total number of mites killed (mean ± standard deviation) and the acaricidal efficacy levels at the end of the treatments, as presented in Table 1, there was a statistically significant difference ($p < 0.05$) between all groups in terms of acaricidal efficacy. The reference veterinary medicinal product demonstrated the highest acaricidal efficacy.

**3.2.2. Target species tolerance level findings**

During the field study, the dead bees in the traps in the colonies were periodically counted and recorded, and the weekly average number of dead bees is presented in Figure 3.

No negative effects, such as restlessness or queen loss, were observed in the colonies. Almost all of the reference veterinary medicinal product was removed from the application area within a 2-week period, and thymol crystals were observed in the pollen drawer and comb compartments. In the hydrogel colonies, no behavior of the bees to move the hydrogel from the application area or to cover it with propolis was observed. Table 2 shows the number of dead bees (mean ± standard deviation) detected during the first treatment stage.

The reference veterinary medicinal product treatment had significantly greater bee mortality than the hydrogel treatment and the negative control treatment ($p < 0.05$), while there was no significant difference ($p > 0.05$) between the negative control group and the hydrogel treatment in terms of bee mortality.

**3.2.3. Thymol residue levels in honey and beeswax**

The recovery rates of honey and beeswax were 99.93% and 100.6%, respectively. Table 3 shows the thymol residue levels (mean ± standard deviation) in honey and beeswax samples obtained from the treatment groups before and after the first stage of treatment.

There was no statistically significant difference ($p > 0.05$) between the groups in terms of the thymol residue levels in the honey and beeswax samples before treatment.

After treatment, it was determined that the honey from the positive control group's colonies contained significantly more residue than the honey from the hydrogel group and negative control group ($p < 0.05$), and the thymol taste residue limit was exceeded [7]. There was no significant difference between the colonies of the negative control group and hydrogel treatment group in terms of thymol residue in honey ($p > 0.05$).

After treatment, the amount of thymol residue in the wax samples obtained from the positive control group was significantly greater ($p < 0.05$) than that in the other treatment groups. There was no significant difference between the negative control group and hydrogel group in terms of thymol residue in beeswax ($p > 0.05$).

## 4. Discussion

Varroosis is a prevalent parasite infestation of honey bee colonies that can result in a decline in colony productivity and even the collapse of the entire colony [1,3]. Acaricidal chemicals have been demonstrated to be efficacious at maintaining varroosis infestation levels below the economic damage threshold when employed at the optimal time and at the appropriate dosage [4]. However, the improper use of these chemicals or inability to control the dosage because of environmental effects may result

**Table 1.** Mean number of mites killed by treatments and acaricidal activity levels in the treatment groups.

| Treatment groups | Mites killed in the first stage treatment (numbers) | Mites killed in the follow-up treatment (numbers) | Mean acaricidal efficacy (% ) |
|---|---|---|---|
| Negative control | 99.20 ± 18.03 | 483.80 ± 59.18 | 21.93 ± 2.87* |
| Positive control | 246.60 ± 29.34 | 349.00 ± 41.12 | 81.09 ± 2.30* |
| Hydrogel | 172.00 ± 37.67 | 288.20 ± 47.42 | 70.32 ± 2.05* |

*There was a significant difference among groups (p < 0.05. ANOVA followed by Tukey HSD test)).

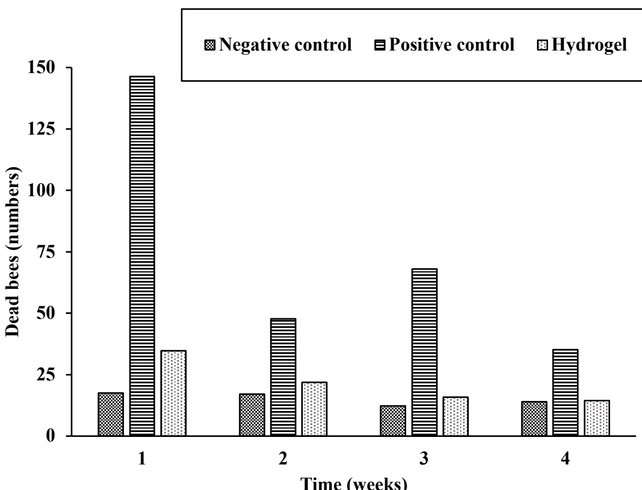


**Figure 3.** Mean number of dead bees detected in the first stage of treatment.

**Table 2.** Mean number of dead bees detected in colonies at the end of the first stage of treatment.

| Treatment groups | Colony numbers | Dead bee numbers (mean ± standard deviation) |
|---|---|---|
| Negative control | 5 | 60.80 ± 8.01 |
| Positive control | 5 | 297.40 ± 21.45* |
| Hydrogel | 5 | 86.80 ± 6.94 |

*There was a significant difference in the positive control group compared with the hydrogel and the negative control groups (p < 0.05 (ANOVA followed by Tukey HSD test)).

**Table 3.** Mean thymol residue results in honey and beeswax before and after the first stage of treatment.

| | Honey | | Beeswax | |
|---|---|---|---|---|
| Treatment groups | Before treatment (mg/kg) | After treatment (mg/kg) | Before treatment (mg/kg) | After treatment (mg/kg) |
| Negative control | 0.070 ± 0.012 | 0.030 ± 0.022 | 0.107 ± 0.067 | 0.074 ± 0.047 |
| Positive control | 0.060 ± 0.037 | 5.498 ± 3.346* | 0.077 ± 0.071 | 1.667 ± 0.855* |
| Hydrogel | 0.047 ± 0.044 | 0.168 ± 0.110 | 0.091 ± 0.069 | 0.476 ± 0.278 |

*There was a significant difference in the positive control group compared with the hydrogel and negative control groups (p < 0.05) (ANOVA followed by Tukey HSD test)).

in adverse effects such as impaired colony health, the development of antiparasitic resistance, and the presence of residues in bee products [5,18]. It has been proposed that CRSs may serve as an alternative to the currently applied mite control methods, with the potential to limit chemically induced environmental damage and reduce the amount of active substance required for treatment, the incidence of side effects, and the frequency of application [9,21,25].

This study presents the first example of applying thymol, which has been used as a varroacidal agent for many years, to honey bee colonies using a gelatin-based hydrogel developed as a CRS [28]. In addition, the advantages and disadvantages of the system were examined statistically by comparing the varroacidal activity, tolerability, and residue levels created by this system with the values of positive and negative control groups.

The acaricidal efficacy level of 81.09 ± 2.30% obtained with the reference veterinary medicinal product in this study is in agreement with the results of previous studies using this medicine (74%–99%) [18]. However, despite containing equal amounts of active ingredients, the acaricidal activity of the hydrogel showed a lower level of performance compared to the reference veterinary medicinal product. This may be attributed to the fact that, unlike the reference product, thymol is trapped in the hydrogel matrix structure in capsules surrounded by gelatin, limiting its volatility and producing lower concentrations in the air of the hive [21–23].

During field observations, no queen loss, agitation, swarming, or colony extinction was observed in the bee colonies. This is likely because of the favorable environmental and hive temperatures, as previously reported [13–15], which are conducive to thymol application. In the positive control group, the applied reference veterinary medicinal product was largely cleared from the site by the bees within 2 weeks. This reaction is consistent with observations from previous studies with reference veterinary medicinal products [10]. The higher bee mortality observed with the reference veterinary medicinal product treatment compared to the other groups was probably due to the toxicity caused by the intense thymol contact of bees during the transport of this product. The fact that higher bee mortality occurred in the first and third weeks of the reference veterinary medicinal product applications supports this assumption. The degree of bee mortality in the hydrogel group was similar to that in the negative control group. This is likely because the bees in the colonies exhibited no reaction to the hydrogel and no transport behaviors, indicating the successful production of a system compatible with honey bee colonies. Although it contains equal amounts of thymol, the fact that there was less bee death in colonies treated with hydrogel than those treated with the reference product can be attributed to the fact that the active substance distributed in the matrix structure prevents burst release and provides a more controlled dosage release [21–23]. In addition, the fact that bees did not carry the gelatin-based hydrogel outside of the hive or cover it with propolis shows that gelatin, which is reported to be compatible with various biological systems, can also be compatible with bee colonies [26,27,29].

The results for the thymol residue obtained in this study were generally similar to those of previous studies [10]. However, thymol residue, which tends to accumulate in beeswax, was detected at higher levels in honey from positive control colonies than in beeswax. This could be related to the contamination of the honey during the removal of the reference veterinary medicinal product from the colonies. In the hydrogel group, the residue levels were similar to those in the negative control group. This can be attributed to the hydrogel being well accepted by the colonies and remaining stable where it was applied.

Considering relevant factors such as the reproductive dynamics of bees and *Varroa* mites, the presence of food products in the environment, variables of beekeeping in different environmental conditions, and the development of antiparasitic resistance, it is difficult to manage this complex infestation within hives. It is clear that advanced systems are required to provide the desired benefits from acaricidal chemicals in terms of effectiveness, tolerance, and residue levels. In this study, thymol-containing gelatin-based hydrogel offered promising potential advantages over a reference product containing an equal amount of thymol. First, the hydrogel has a biocompatible structure that prevents thymol, the active ingredient, from being carried out of the colony by bees. This ensures continuity in the treatment without the need for additional applications by preventing the loss of active substance. Second, by controlling the way in which bees are exposed to chemicals, this treatment provides advantages in reducing the toxicity that may be caused by contact in bees and in limiting the formation of residues in bee products and the environment.

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**Conflict of interest**
The authors declare that there are no conflicts of interest.

**Author contributions**
The first, second, and third authors designed and planned the experiments. The first and second authors performed

the experiments and contributed to the interpretation of the results. All authors provided critical feedback and helped analyze the research and shape the manuscript.

**Availability of data**

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

**Ethical statement**

This study was carried out after the animal experiments were approved by the Local Ethics Committee of the Pendik Veterinary Control Institute (Decision Number: 202-17/2018).

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