Evaluation of the preparation, characterisation, and release properties of Thymol-Containing Gelatin-Based Hydrogels for Varroosis control

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ABSTRACT

The aim of this study was to investigate the swelling and degradation behavior of a controlled-release system using gelatin, thymol loading capacity, characterization, morphology, and thymol release level in relation to the recommended therapeutic dose for varroosis control. In this context, a series of hydrogels were first produced using a glutaraldehyde (GA) crosslinker and swelling tests were performed. Thymol loading was performed on the appropriate hydrogels, with swelling values between 269% and 431%. Thymol loading efficiency was determined to be between 20.07% and 29.80%. The chemical structures of the hydrogels with and without thymol loading were compared by Fourier transform infrared spectrometry (FT-IR) and it was determined that thymol was loaded into the structure. The morphological structures of the thymol-loaded and unloaded hydrogels were examined by scanning electron microscopy (SEM). It was observed that the non-thymol-loaded hydrogel had larger pores than the thymol-loaded hydrogel. A model release environment and measurement system were developed to predict the release type, level, and duration of the controlled release system in the hive air environment. In this model release environment, release tests were carried out for four weeks using different thymol application systems and the measurements were compared. As a result, it was found that the controlled thymol release system developed for the control of varroosis showed a more stable release compared to existing application systems.

Introduction

Varroosis is an external parasitic disease of bees characterized by the invasion of honey bee colonies by the *Varroa destructor* mite, which feeds on the haemolymph of adult and young bees and causes various damages to the colony (4). When measures are not taken against mites, colony productivity decreases, susceptibility to other diseases increases and colonies may die in more advanced stages. Many control methods have been developed against the mite, and chemical control methods are the most important of these. Thymol is a chemical used for varroosis control since the 1980s (1, 13, 35).

When *Varroa* mites inhale thymol, which sublimates and passes into the hive air, GABA-gated chloride channels are blocked. Thus, the acaricidal effect occurs as a result of excessive stimulation and convulsions in the central nervous system (2, 9, 10, 15). When thymol is applied to colonies, acaricidal activity varies in relation to environmental temperature and humidity (14). When thymol-containing drugs are applied to colonies at ambient temperatures lower than 15 °C, queen death may occur, and when applied at temperatures higher than 30 °C, agitation, flight, brood, and adult bee mortality may increase (3, 5, 7). Thymol is a chemical with a narrow therapeutic window that acts on *Varroa* mites and honey bees by the same mechanism. The topical LD_{50} of thymol is 56.1 µg for adult mites, 210.3 µg for adult worker bees and 150.7 µg for bee larvae (17). Thymol is effective

against the mite by inhalation at a concentration of 5–15 µg/L, but at an air concentration of 20 µg/L, it causes 90% mortality in bees (20).

Controlled release systems (CRS) are drug delivery systems that allow the active substance to be administered at the desired location, concentration, rate and duration. In CRS, the active substance is located in a drug reservoir coated with a film or in a matrix in which the drug is dispersed. From here, slower and more stable release processes are realized with different kinetics (11). CRS can provide benefits such as administering the dose within the therapeutic range, minimizing the amount of drug required, reducing side effects, and decreasing the frequency of administration (25, 27). In addition, CRS can increase the stability of the active substance against harmful effects such as temperature, oxidation, moisture, and microorganisms and can limit its high volatility (31, 8). On the other hand, CRS may have various disadvantages, such as toxicity or non-biocompatibility of the polymer used, unpredictable and poor correlation between in vitro release and in vivo release, unwanted degradation products, difficulty in dosage adjustment and cost (18).

Hydrogels are indispensable materials for CRS. Hydrogels are cross-linked, three-dimensional, hydrophilic polymeric structures that are insoluble in water and can absorb at least 20% or more of their dry mass. Cross-links between polymers can be shaped chemically (covalent or ionic) or physically (crystallinity) and they are insoluble in water thanks to these bonds (28). Hydrogels are widely used in biomedical fields such as contact lenses, artificial heart, muscle, and skin materials due to their biocompatibility (28), as well as in the pharmaceutical industry as support materials for controlled drug release systems and enzyme immobilizations (29).

Gelatine is a natural polymer used in pharmaceutical and biomedical fields such as drug coating, microencapsulation, and hydrogel production. In order for the materials to be produced from its aqueous solutions to be used for a long period of time, gelatine must be subjected to cross-linking, which increases both its thermal and mechanical stability. The most widely used chemical crosslinker for this purpose is glutaraldehyde (GA). The swelling and failure behavior of hydrogels can be controlled by changing the crosslinker and polymer ratios (30).

The aim of this study was to develop and characterize controlled thymol-releasing hydrogel systems for use in varroosis control. To this end, hydrogel systems were developed using gelatin polymers, GA crosslinkers, and thymol as the active substances. Swelling and thymol loading tests were carried out on the produced hydrogels. In addition, their chemical structures were examined by Fourier transform infrared spectrometry (FT-IR) and their morphological structures were examined by scanning electron microscopy (SEM). Finally, release tests of a thymol-loaded hydrogel were carried out in a model release environment for four weeks. It was planned to select the appropriate hydrogel for varroosis control studies in honey bee colonies.

Materials and Methods

Materials: Bovine gelatin (Food grade, Alfasol), GA (Technical grade, 25%, Merck), sunflower oil (Food grade, Yudum), thymol (Technical grade, 99% purity, Sigma-Aldrich), and distilled water were used for hydrogel production. For chromatographic analyses, thymol reference standard (99.6% purity, Dr. Ehrenstorfer), acetonitrile (HPLC grade, Sigma-Aldrich), n-methyl-2-pyrrolidone (NMP) (Technical grade, BASF), and distilled water were used.

Preparation of Hydrogels: 8% and 10% gelatin solutions were prepared using distilled water. The solutions were kept in an ultrasonic bath at 50 °C for one hour to completely dissolve the gelatin. Gelatin solutions and GA solutions were added into Falcon tubes at different ratios, as shown in Table 1. The tubes were mixed at 1000 rpm for 2 minutes and kept at room temperature for 24 hours. The hydrogels were removed from the tubes and cut into 0.5-cm-thick discs with a scalpel. The discs were washed three times successively with distilled water to remove gelatin and GA that did not enter the crosslinking reaction. The hydrogel discs were placed in an oven (IN 260, Memmert) set at 30 °C. The discs were weighed daily with a precision balance (AK160, Mettler Toledo, Ohio, USA), and the drying process was terminated when a constant weight was reached. The discs were stored in moisture- and light-proof bags until they were used in further studies.

Table 1. Gelatin Hydrogel Preparation Conditions.

Hydrogel Code	Gelatin (%)	GA (%12.5 mL)	GA/Gelatin Ratio
G-1	8	0.2	6.25×10^{-3}
G-2	8	0.4	12.50×10^{-2}
G-3	8	0.8	25.00×10^{-2}
G-4	8	1.6	50.00×10^{-2}
G-5	8	2.4	75.00×10^{-2}
G-6	8	3.2	1.00
G-7	8	4.0	1.25
G-8	10	0.2	5.00×10^{-2}
G-9	10	0.4	10.00×10^{-2}
G-10	10	0.8	20.00×10^{-2}
G-11	10	1.6	40.00×10^{-2}
G-12	10	2.4	60.00×10^{-2}
G-13	10	3.2	80.00×10^{-2}
G-14	10	4.0	1.00

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Swelling Test: The dried hydrogel discs were placed in sample containers containing 100 mL distilled water and placed in an oven (IN 260, Memmert) set at 30 °C. The discs were removed from the water at regular intervals and dried superficially with filter paper. The weighed discs were returned to the water and placed in the oven. Weighing was continued until a constant weight was reached for each disc. The test was carried out on three samples. The mean percentage swelling values (S%) were calculated by Equation 1 (29) (md: dry weight, mw: wet weight).

$$S\ (\%)= \frac{mw-md}{md} \times 100 \quad (1)$$

Thymol Loading and Loading Efficiency Determination: Thymol-loaded samples were prepared by using G-3 and G-10 hydrogels, which were prepared with gelatin solutions at different concentrations and exhibited the most ideal properties in terms of swelling and structural stability. Although thymol's solubility in water is very low, its solubility in vegetable oils is high (6). Accordingly, thymol was added to the gelatine solutions in two different ways before the cross-linking step, following the procedure, described in the section on the preparation of hydrogels. In the first group (T), pure thymol in solid form was added at weights of 0.5 g, 1 g, and 1.5 g. In the second group (OT), 1 mL, 2 mL, and 3 mL of 50% thymol-sunflower oil solutions were added to the tubes, respectively. Then, thymol-loaded hydrogels were produced by adding GA crosslinker. Table 2 presents the prepared samples and their codes.

Table 2. Preparation Conditions of Thymol Loaded Gelatin Hydrogel.

Hydrogel Code	Gelatin (%)	GA (%12,5, mL)	Thymol (g)	Thymol/Sunflower Oil Solution (mL)
(G-3)-T1	8	0.8	0.5	-
(G-3)-T2	8	0.8	1.0	-
(G-3)-T3	8	0.8	1.5	-
(G-3)-OT1	8	0.8	-	1.0
(G-3)-OT2	8	0.8	-	2.0
(G-3)-OT3	8	0.8	-	3.0
(G-10)-T1	10	0.8	0.5	-
(G-10)-T2	10	0.8	1.0	-
(G-10)-T3	10	0.8	1.5	-
(G-10)-OT1	10	0.8	-	1.0
(G-10)-OT2	10	0.8	-	2.0
(G-10)-OT3	10	0.8	-	3.0

The hydrogel discs were extracted with acetonitrile to determine thymol loading efficiencies. Three samples were prepared for each hydrogel series and the thymol content was measured using HPLC-DAD (Thermo Scientific, Dionex Ultimate 3000). Analytical separation was achieved with a C18 (250 mm x 4.60 mm, 5 µ ACE) column. Acetonitrile and water were used as the mobile phase in a 75/25 ratio at a flow rate of 1 mL/min. Samples were injected into the system in a volume of 20 µL. The detection of thymol peaks was performed in 5 minutes and at 278 nm. The drug loading efficiency of the hydrogel series was calculated by Equation 2 (38).

$$\text{Loading Efficiency}\ (\%)= \frac{\text{Total Thymol Mass in Hydrogel}}{\text{Total Hydrogel Mass}} \times 100 \quad (2)$$

FT-IR Analysis: Fourier Transform Infrared Spectroscopy (FT-IR) analyses (Spectrum Two™, Perkin Elmer) were performed to determine whether cross-linking between gelatin and GA was present and to prove the presence of thymol loaded in the hydrogel structure. For this purpose, direct spectra of dry samples of pure gelatin, G-10 hydrogel, and thymol-loaded (G-10)-OT1 hydrogel were taken. The scans were carried out in the wavelength range 4000-450 cm1 with a MIR TGS detector at 4 cm^{-1} resolution and 0.2 cm^{-1} scan rate conditions and the spectra were compared.

Scanning Electron Microscopy (SEM) Analysis: For the determination of hydrogel morphology, surface topography, and microstructure, 1 cm-thick discs were formed from thymol-loaded hydrogel ((G-10)-OT1) and non-thymol-loaded hydrogel ((G-10)-O1) with the help of a scalpel. The hydrogel discs were allowed to swell in distilled water at room temperature for 48 hours. The discs removed from the water were kept in the refrigerator at -20 °C overnight and frozen. The frozen discs were placed in a lyophilization device (FreeZone1, Labconco, Canada) and dried at -40 °C for 10 days (22). From the dried discs, smaller sized samples were prepared with a razor blade. The samples were coated with a 3.0 nm (±0.5 nm) thick layer of gold (Au) in a coating device (Cressington Sputter Coater 108 Auto, Watford, UK). The coated samples were imaged by SEM (VEGA3 SBU-EasyProbe, TESCAN, Czech Republic) under 15.0 kV (19).

Release Study: Release studies of thymol-loaded (G-10)-OT1 hydrogel were performed in order to examine the kinetics of thymol release under laboratory conditions and to evaluate the suitability in terms of the therapeutic window. A model release environment shown in Figure 1 was created with variables such as temperature, humidity, and hive ventilation that are effective on the pharmacological and toxicological processes of thymol in

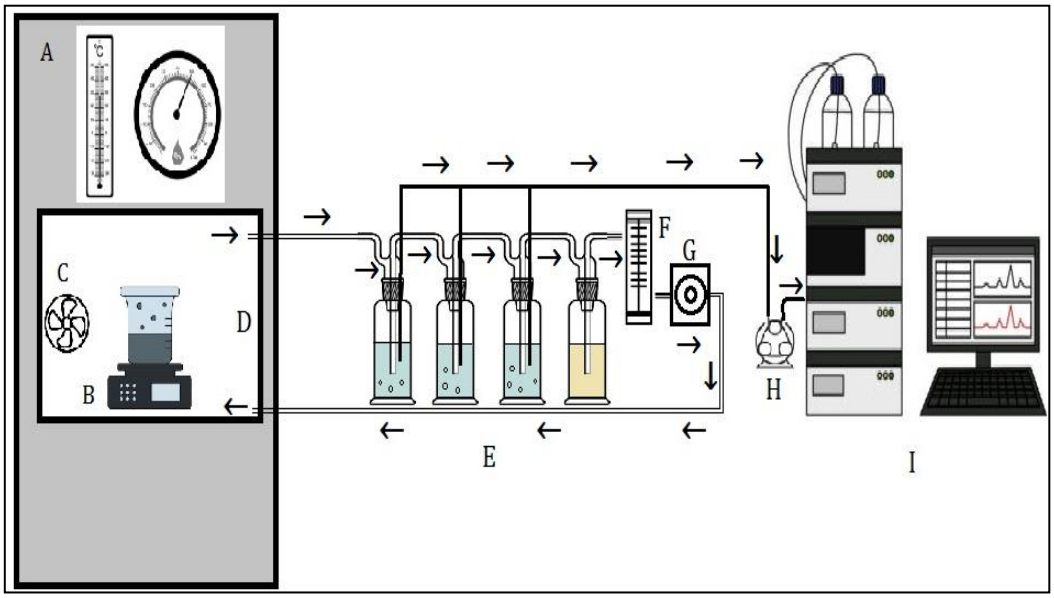

Figure 1. Release System: A-acclimatisation cabinet, B- balance, C-fan, D-hive, E-gas washing bottles, F-flow meter, G-air vacuum pump, H-peristaltic pump, I-HPLC DAD system.

colonies in nature (12, 14, 16, 26, 33, 34, 36). In this setup, bee, honey, and beeswax compartments and variables that may affect the release level, such as fluid dynamics, were ignored. For the release test, three experimental groups containing equal weights of thymol were formed. A reference drug containing thymol approved for use in the control of varroosis, pure thymol, and hydrogel ((G-10)-OT1) samples containing 12.5 g of thymol were used for four weeks.

A plastic hive with Langstroth hive dimensions was placed in an air-conditioned cabinet (Weiss WK 111–180). A fan, data logger, and balance equipment were installed in the hive. Samples were placed on the balance sheet. The hive was isolated in such a way that air inlets and outlets were provided only by pipes. The fan was operated at 400 rpm (37). The temperature of the chamber was periodically increased by 5 °C from 10 °C to 35 °C with software (Simpati 4.50) and then decreased again until the temperature reached 10 °C, creating a 24-hour temperature cycle. Hive air passed through pipes (A-60-G, Tygon) was vacuumed by an air pump (Aco 9601, Hailea) at an average speed of 0.42 L/min, adjusted by a flowmeter (LZT 4 T). The vacuumed air was sequentially passed through three gas washing bottles containing 500 mL volumes of a 10% NMP solution. The air was then returned to the hive through a gas scrubber bottle containing silica gel to trap excess moisture. The humidity level inside the hive was monitored in terms of 40%–60% relative humidity limits. The total amount of thymol retained in the solutions sent from the gas washing bottles to the HPLC loop system by capillary tubes and peristaltic pumps was measured daily by the HPLC-DAD device. At the end of four weeks, thymol adsorbed on the hive wall was collected according to a method given in the literature (32). In addition, the weight changes of the samples were measured daily with a balance.

Results

Swelling Test Results: Among the hydrogels synthesized with different ratios of gelatin and GA composition, it was aimed to determine at least two hydrogels that provided the highest level of swelling while maintaining their robustness as a result of swelling tests. As shown in Figure 2 and Figure 3, it was determined that the %S initially increased with time and then remained constant at the end of 48 h. The average %S values were determined as 431% (± 42.90) for the most swelling hydrogel G-1 and 269% (± 17.61) for the least swelling hydrogel G-14. It was determined that all hydrogels completed the 48-hour swelling test without disintegration and with an average swelling of 349% (± 53.94). Two hydrogels (G-3 and G-10) with the highest level of swelling, robust structure, and homogenous appearance were selected to be tested in thymol loading studies.

Thymol Loading Results: The thymol loading efficiency results for the synthesized hydrogels were compared as shown in Figure 4. The average thymol loading efficiency was calculated to be 25.26% (± 3.05). In general, it was observed that in hydrogels loaded with pure solid thymol, the thymol was oriented towards the outer surface of the hydrogel. Thymol loaded as dissolved in sunflower oil was found to be homogeneously distributed in the hydrogel matrix structure. The hydrogel coded (G-10)OT1, which exhibited a robust structure and homogeneous appearance as well as above-average loading efficiency, was selected for use in release trials.

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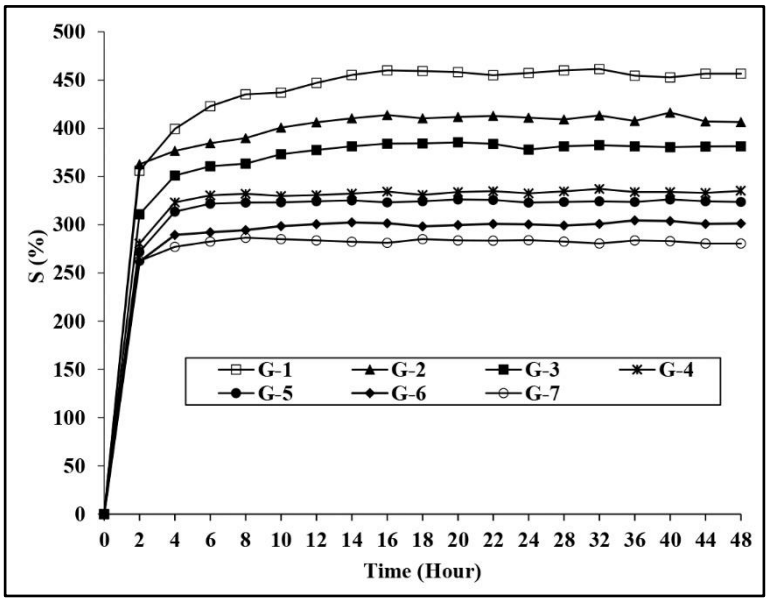

Figure 2. Swelling-Time Graph of Hydrogels (8%).

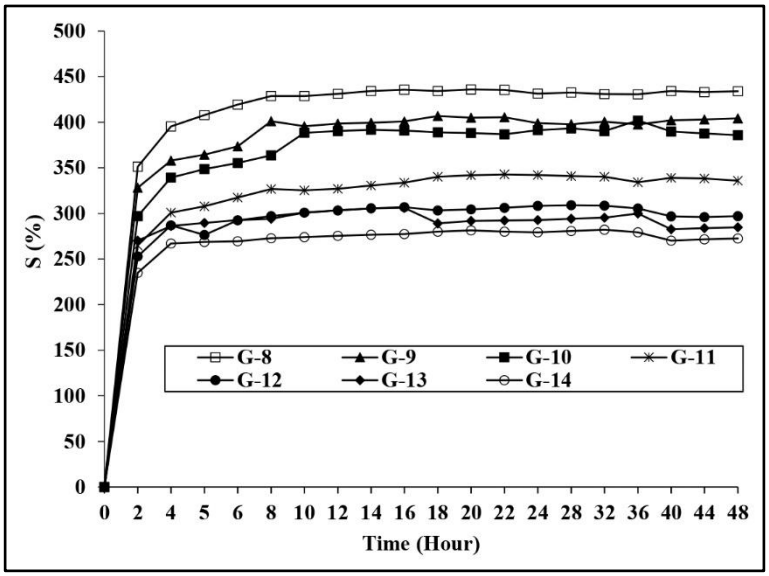

Figure 3. Swelling-Time Graph of Hydrogels (10%).

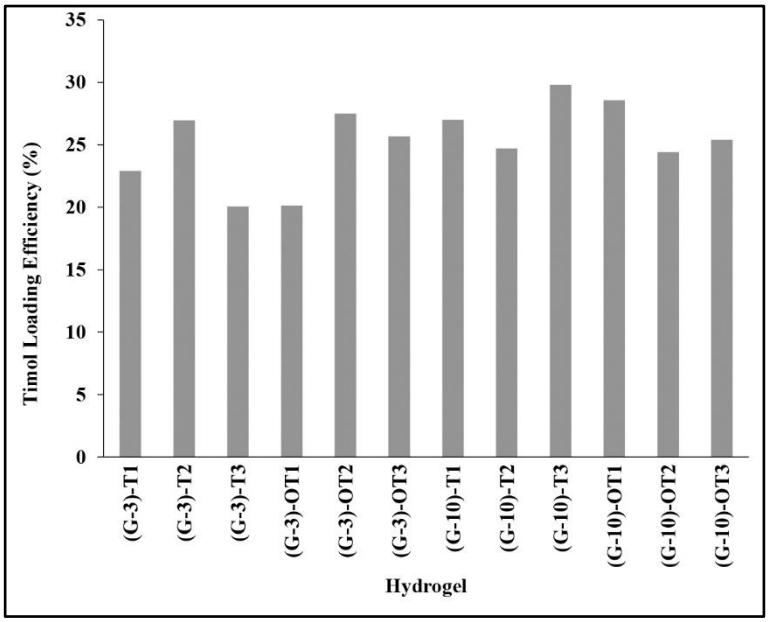

Figure 4. Timol Loading Efficiency Graph of Hydrogels.

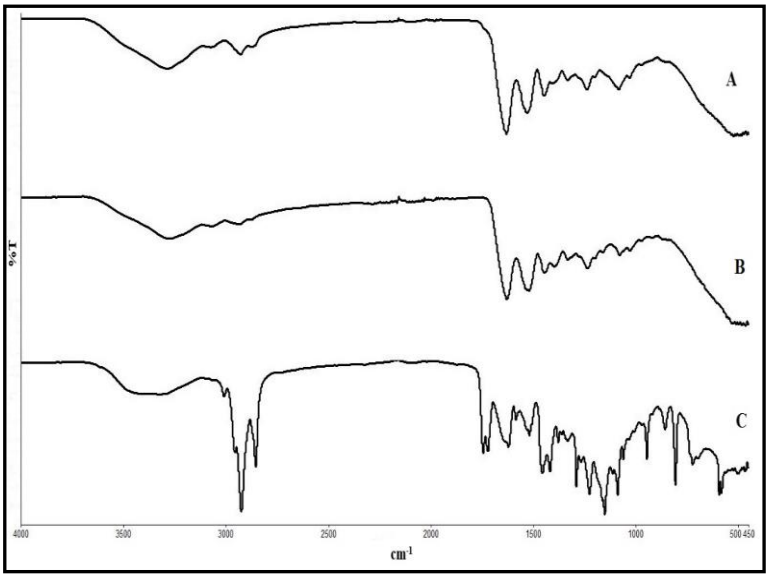

Figure 5. FT-IR Spectra of gelatin (A), Hydrogel (G-10) (B), Thymol Loaded Hydrogel (G-10)OT1 (C).

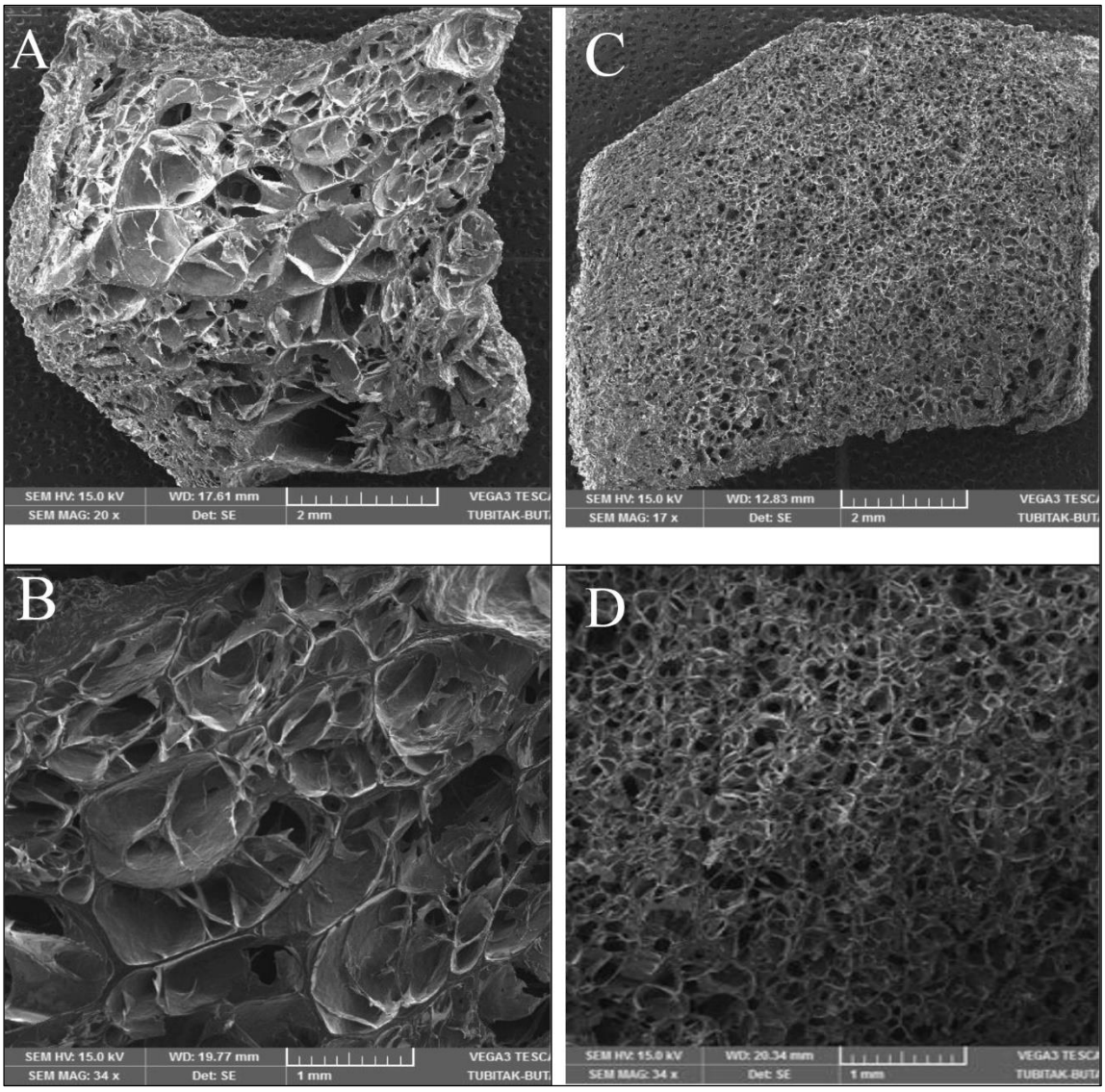

Figure 6. SEM Images of Non-Thymol Loaded Hydrogel (G-10)O1 (A- 2 mm, B-1 mm) and Thymol Loaded Hydrogel (G-10)OT1 (C- 2 mm, D-1 mm).

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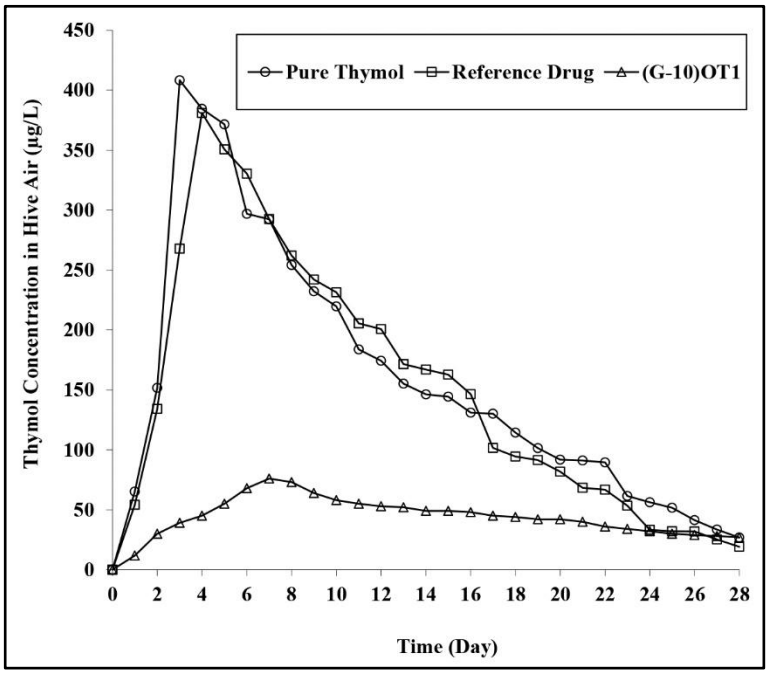

Figure 7. Time Graph of Thymol Concentration in Hive Air.

FT-IR Results: The spectra of pure gelatin, G-10 hydrogel, and (G-10)-OT1 hydrogel were compared as shown in Figure 5. The spectra of gelatin and G-10 hydrogel are generally similar to each other. After the crosslinking reaction, the narrowing of the amine (N-H) peak at 3283 cm^{-1} of gelatin and the decrease in the primary amine content of gelatin in the hydrogel (G-10) structure were observed. In the spectrum of (G-10)-OT1 hydrogel, characteristic C-H bond peaks of thymol were observed at 2924 cm^{-1} and 2854 cm^{-1}.

Scanning Electron Microscopy Results: When evaluated in terms of macroscopic findings, it was observed that the hydrogels obtained after freezing at -20 °C and the subsequent lyophilization process were suitable for disintegration. As shown in Figure 6, when compared to microscopic findings, there are significant differences between the hydrogels in terms of pore size and internal structure appearance. It was determined that there were pores in the matrix structure with an average diameter of 280 µm (±17.74) for the non-thymol-loaded (G-10)O1 hydrogel and 78.39 µm (±11.63) for the thymol-loaded (G-10)OT1 hydrogel.

Results of Release Study: At the end of the release studies, it was determined by gravimetric measurements that 5.28 g of pure thymol, 5.38 g of reference drug and 1.87 g of hydrogel ((G-10)OT1) occurred due to the sublimation of thymol. The amounts of thymol released from pure thymol, the reference drug, and thymol-loaded gelatin hydrogel (G-10)OT1 for four weeks and measured daily were converted to determine the amount of thymol in 1 L of in-hive air, and the in-hive air thymol concentration time plot is shown in Figure 7. The in-hive air thymol concentrations were calculated as the lowest 26 µg/L, 19 µg/L, 27 µg/L, the highest 408 µg/L, 381 µg/L, 76 µg/L, and the mean 160.72 µg/L (± 109.30), 153.53 µg/L (± 107.88), and 44.81 µg/L (± 14.95) for pure thymol, reference drug, and hydrogel, respectively. The release continued continuously for four weeks in all three groups.

Discussion and Conclusion

According to the results of the swelling tests, it was determined that increasing the crosslinker ratio in the hydrogel led to lower swelling values. In this respect, results consistent with published studies were obtained (29, 30).

As a result of the cross-linking reaction between gelatin and GA, the aldimine bond (CH=N) is formed between the amino groups of gelatin and the aldehyde groups of GA, and the primary amine content of gelatin is reduced. In addition, in the comparison of the samples, only in the spectrum of (G-10)OT1 hydrogel, characteristic C-H bond peaks of thymol were observed at 2924 cm^{-1} and 2854 cm^{-1}. As a result, the FT-IR findings in terms of cross-linking with GA in the hydrogel structure and the presence of thymol in the matrix structure are consistent with the literature (24, 39).

Using the hydrogel technique with gelatin, a natural polymer, a thymol-loadable CRS was formed with an average yield of 25.26% (± 3.05). The pores in the hydrogel matrix structure were measured at 280 µm

(±17.74) for (G-10)O1 hydrogel and 78.39 µm (±11.63) for thymol (G-10)OT1 hydrogel. In terms of these properties, it is evaluated that the results are compatible with the previous studies on controlled release of thymol (8, 23, 25). When evaluated in terms of macroscopic findings, it is in accordance with the literature that the hydrogels obtained after freezing at -20 °C and subsequent lyophilization exhibit a structure suitable for disintegration (22). This may be thought to be due to thymol, which is the only known difference between the two hydrogel compositions. Probably, thymol in the structure creates a difference in the heat transfer rate in the freezing process and therefore larger ice crystals are formed in the non-thymol structure during freezing. Larger ice crystals also push the gelatin chains more and cause larger pore sizes (19, 22).

As a result of gravimetric measurements, pure thymol and the reference drug, which initially contained equal amounts of thymol, released thymol at similar weights. Similarly, it is noteworthy that the lowest and highest concentrations reached in the in-hive air thymol concentration-time graphs were very similar for pure thymol and the reference drug. This indicates that the reference drug formulation was designed for rapid release of thymol. The observation of thymol concentrations above the therapeutic window in all three release trial groups is probably due to the unpredictable and poor correlation between in vitro and in vivo test results (21). However, disadvantages such as these may be experienced in the development stages of CRS (18). This situation may be caused by factors that cannot be established in vitro but may be important in the pharmacokinetics of thymol. Hydrogel release was lower than that of pure thymol and the reference drug in both gravimetric measurements and in vitro air thymol concentration-time graph evaluation. In addition, the closeness of the average, highest, and lowest thymol concentration values obtained with hydrogel release indicates that a more stable release is exhibited compared to the other groups. It is evaluated that the controlled thymol release system developed in terms of a slow, continuous, and constant rate of thymol release, more efficient use of the active substance, and minimizing environmental damage is compatible with the literature (25, 27).

In conclusion, the controlled thymol release system developed and tested in this study for the control of varroosis infestation was found to exhibit more stable release compared to existing application systems. The controlled release system has promising potential advantages in terms of the effective use of highly volatile active substances with a narrow therapeutic window, such as thymol, in the control of varroosis and the protection of colony health.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

First, second and third author conceived and planned the experiments. First and second author carried out the experiments and contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

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

Ethical Statement

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

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Akyol E, Özkök D** (2005): *Varroa (Varroa destructor) Mücadelesinde Organik Asitlerin Kullanımı*. Uludag Aricilik Dergisi, **5**, 167-174.
2. **Anonymous** (2001): A Review of Treatment Options For Control of Varroa Mite in New Zealand, Report to the Ministry of Agriculture and Forestry New Zealand. Available at: (https://www.bobsbeekeeping.com.au/image/bee-resources/varroa-treatment-options.pdf) (Accessed Sep,15, 2021).
3. **Anonymous** (2014): Summary of products characteristics-ApiLife Var bee-hive strips for honey bees. The Veterinary Medicines Directorate U.K.
4. **Anonymous** (2015): Varroa mites (Varroatosis or Varroosis). Apimondia, IZSLT - Istituto Zooprofilattico Sperimentale del Lazio e della Toscana, 8416, Available at: (http://www.fao.org/teca/en/technologies/8416) (Accessed, Sep, 22, 2021).

5. **Anonymous** (2018): Summary of products characteristics-Apiguard gel (%25 Thymol) for beehive use. The Veterinary Medicines Directorate U.K.
6. **Anonymous** (2018): Thymol. Compound Summary for CID 6989, Pub Chem-Open Chemistry Database.
7. **Anonymous** (2020): Mutual Recognition Procedure Publicly Available Assessment Report For A Veterinary Medicinal Product Thymovar. DEFRA.
8. **Bhalerao YP, Wagh SJ** (2018): *A Review on Thymol Encapsulation and Its Controlled Release Through Biodegradable Polymer Shells.* IJPSR, **9**, 4522-4532.
9. **Bisrat D, Jung C** (2020): *Insecticidal Toxicities of Three Main Constituents Derived from Trachyspermum ammi (L.) Sprague ex Turrill Fruits Against the Small Hive Beetles, Aethina tumida Murray*. Molecules, **25**, 1100.
10. **Bogdanov S, Imdorf A, Kilchenmann V** (1998): *Residues in wax and honey after Apilife VAR® treatment*. Apidologie, **29**, 513-524.
11. **Chen EY, Liu WF, Megido L, et al** (2018): Chapter 3-Understanding and utilizing the biomolecule/nanosystems interface. Editor(s): Vuk Uskoković, Dragan P. Uskoković, In Micro and Nano Technologies, Nanotechnologies in Preventive and Regenerative Medicine, Elsevier, 207-297.
12. **Çetin U** (2004): *Isı Değişimlerinin Arı Kayıplarına Etkileri*. Uludag Aricilik Dergisi, **4**, 171-174.
13. **Daş YK, Aksoy A** (2015): Arıcılıkta Hatalı İlaç Kullanımının Sağlık ve Ekonomi Üzerine Etkileri, Marka Bal Olma Yolunda Samsun Sempozyumu. Available at: (https://www.researchgate.net/publication/322682624), (Accessed, Nov,26, 2018).
14. **Emsen B, Dodologlu A** (2011): *Efficacy of Different Organic Compounds Against Bee Mite (Varroa destructor Anderson and Trueman) in Honey Bee (Apis mellifera L.) Colonies*. AJAVA, **10**, 802-805.
15. **Floris I, Satta A, Cabras P, et al** (2004): *Comparison Between Two Thymol Formulations in the Control of Varroa destructor: Effectiveness, Persistence, and Residue*s. J Econ Entomol, **97**, 187-191.
16. **Fragkou D, Stevenson V** (2012): Study of Beehive and its potential "biomimicry" application on capsule hotels. Conference: People and Buildings in Tokyo, Japan.
17. **Gashoutl HA, Guzmán-novoa E** (2009): *Acute toxicity of essential oils and other natural compounds to the parasitic mite, Varroa destructor, and to larval and adult worker honey bees (Apis mellifera L.)*. J Apic Res, **48**, 263-269.
18. **Huynh CT, Lee DS** (2014): Controlled Release, Encyclopedia of Polymeric Nanomaterials. Editors; Kobayashi S, Müllen K, Springer, Berlin, Heidelberg. 1-12.
19. **Imani R, Rafienia M, Emami SH** (2013): *Synthesis and characterization of glutaraldehyde-based crosslinked gelatin as a local hemostat sponge in surgery: An in vitro study*. Biomed Mater Eng, **23**, 211–224.
20. **Imdorf A, Kilchenmann V, Bogdanov S, et al** (1995): *Toxizität von Thymol, Campher, Menthol und Eucalyptol auf Varroa jacobsoni Oud und Apis mellifera L im Labortest*. Apidologie, **26**, 27-31.
21. **Imdorf A, Bogdanov S, Kılchenmann V, et al** (2006): *Toxic Effects of Essential Oils and Some of Their Components On Varroa Destructor Oud And Apis Mellifera L Under Laboratory Conditions*. Animal Production and Dairy Products (ALP) Swiss Bee Research Centre, **495**, 1-18.
22. **Kang HW, Tabata Y, Ikada Y** (1999): *Fabrication of porous gelatin scaffolds for tissue engineering*. Biomaterials, **20**, 1339–1344.
23. **Kaur R, Kukkarb D, Bhardwajc SK, et al** (2018): *Potential use of polymers and their complexes as media for storage and delivery of fragrances*. JCR, **285**, 81–95.
24. **Korkmaz N, Demirbağ S, Tözüm MS, et al** (2014): *Fabrication of Cross-Linked Gelatin Electrospun Nanofibers Containing Rosemary Oil For Antibacterial Application*. ACC Journal, **20**, 39-48.
25. **Maes C, Bouquillon S, Fauconnier ML** (2019): *Encapsulation of Essential Oils for the Development of Biosourced Pesticides with Controlled Release: A Review*. Molecules, **24**, 25-39.
26. **Meikle GW, Weiss M, Maes PW, et al** (2017): *Internal Hive Temperature as a Means of Monitoring Honey Bee Colony Health in a Migratory Beekeeping Operation Before and During Winter*. Apidologie, **48**, 666–680.
27. **Moretti MDL, Passino GS, Demontis S, et al** (2002): *Essential Oil Formulations Useful as a New Tool for Insect Pest Control*. AAPS PharmSciTech, **3**, 1-11.
28. **Özgündüz Hİ** (2002): Akrilik Asit-Akrilamid-Poli (Vinil Alkol) İçeren Yarı-Ipn Tipi Hidrojellerin Şişme Özellikleri ve Lipaz Salım Davranışları, Yüksek Lisans Tezi, Gazi Üniversitesi, Ankara.
29. **Pulat M, Akalın GO** (2013): *Preparation and Characterization Of Gelatin Hydrogel Support for Immobilization of Candida rugosa Lipase*. Artif Cells Nanomed Biotechnol, **41**, 145–151.
30. **Pulat M, Yoltay N** (2016): *Smart Fertilizers: Preparation and Characterization of Gelatin-Based Hydrogels for Controlled Release of Map and An Fertilizers*. Agrochimica Pisa, **60**, 249-261.
31. **Ruffinengo SR, Maggi MD, Fuselli S, et al** (2014): *Bioactivity of microencapsulated essentials oils and perspectives of their use in the control of Varroa destructor*. Bulletin of Insectology, **67**, 81-86.
32. **Rushworth ID, Higgitt C, Margaret SM, et al** (2014): *Non-invasive multiresidue screening methods for the determination of pesticides in heritage collections*. Herit Sci, **2**, 1-8.
33. **Southwick EE, Moritz RFA** (1987): *Social Control of Air Ventilation in Colonies of Honey Bees, Apis Mellifera*. J Insect Physiol, **33**, 623-626.
34. **Sudarsan R, Thompson C, Kevan PG, et al** (2016): "Beehive Ventilation: We Need to Know More and Do Better". Available at: (https://tr.scribd.com/document/388551388/Hive-Ventilation-Linton-pdf). (Accessed Jan, 05, 2019).
35. **Tihelka E** (2018): *Effects of synthetic and organic acaricides on honey bee health*. Slov Vet Res, **55**, 119-140.
36. **Toledo VAA, Nogiueira-Couto RH** (1999): *Thermoregulation in colonies of africanized and hybrids with Caucasian, Italian and Carniolan Apis mellifera honey bees.* Braz Arch Biol Technol, **42**, 425-431.
37. **Xie K, Tashkin DP, Luo MZ, et al** (2019): *Chronic toxicity of inhaled thymol in lungs and respiratory tracts in mouse model.* Pharmacol Res Perspect, **7**, 1-10.

38. Xu H, Hou Z, Zhang H, et al (2014): *An Efficient Trojan Delivery of Tetrandrine by poly(N-vinylpyrrolidone)-block-poly(ε-caprolactone) (PVP-b-PCL) Nanoparticles Shows Enhanced Apoptotic İnduction of Lung Cancer Cells And İnhibition of its Migration and Invasion*. Int J Nanomedicine, **9**, 231-242.

39. You Y, Sun X, Cui Q, et al (2016): *The Retention and Drainage Behavior of Cross-linked Gelatin with Glutaraldehyde in a Papermaking System*. Bioresources, **11**, 6162-6173.