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Research Article

In vivo evaluation of the efficacy of *Cymbopogon citratus* (lemongrass), *Allium sativum* (garlic), *Leptospermum scoparium* (manuka), and *Litsea cubeba* essential oils on Varroosis and Nosemosis co-infection in honey bees under field conditions

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Abstract

In this study, the efficacy of four different essential oils; *Cymbopogon citratus* (CC, Lemongrass), *Allium sativum* (AS, Garlic), *Leptospermum scoparium* (LS, Manuka), and *Litsea cubeba* (LC) against mite and microsporidian infections was evaluated in hives simultaneously infested with *Varroa destructor* and *Nosema* spp. Changes in mite and microsporidian loads were analyzed to determine the biological control potential of these plant-based treatments. Forty-two hives were divided into six groups (four treatment, two control), each consisting of seven hives. Essential oils were applied to the frames four times at weekly intervals using a spray method in the treatment groups. The *Nosema* load was determined using the digestion method, and the *Varroa* load was determined using the powdered sugar method. The number of falling mites was calculated using sticky paper placed on the bottom frame of the hive. Nosemosis treatment efficacy was 57.64% (CC), 58.97% (AS), 62.54% (LS), and 66.55% (LC). For *Varroa*, efficacy was 77.92%, 77.77%, 75.91%, and 79.42%, respectively. All treatment groups showed significant reductions in mite numbers from day 0 to 28 ($p<0.001$). Sticky board counts revealed a progressive decline between days 7 and 28 in AS, LS, and LC groups ($p<0.001$). *Nosema* spore counts also significantly decreased in all treatment groups ($p<0.001$). In this study, essential oils were found to be effective against *Varroa* infestation. Chemical residues in bee products, especially honey, can be prevented by using plant extracts instead of chemical agents in *Varroa* infestations.

Keywords: Efficacy, essential oil, nosemosis, treatment, varroosis

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Introduction

Honeybees are essential pollinators for both natural ecosystems and agricultural activities (Rajagopalan et al 2024). Their ecological significance related to the resources they provide for human consumption, primarily honey, along with pollen, propolis, bee bread, royal jelly, and beeswax.

In the last two decades, the population of honeybee colonies has markedly declined due to various factors. The most important of these factors are climatic change, nutritional deficiencies, pesticide exposure, habitat loss and infectious diseases (Brunet and Fragoso 2024). The health of honeybee colonies is primarily threatened by two parasites: the ectoparasite *V. destructor* and the intracellular microsporidia *Nosema apis* and *Nosema ceranae* (also known as *Vairimorpha apis* and *Vairimorpha ceranae*). If untreated, these parasites can lead to substantial colony losses (Ostap-Chec et al 2024, Warner et al 2024). *V. destructor* feeds on the fat tissue of adult and larval honey bees, but may also prefer small amounts of hemolymph, and acts as a vector for many viral pathogens. The most important of these pathogens is Deformed Wing Virus (DWV). DWV negatively affects the average lifespan, immunity and flight capacity of honey bees (Oz et al 2023; Tlak-Gajger et al 2025). Nosemosis affects the digestive system of adult honey bees, causing nutritional stress, immunosuppression, reduced foraging efficiency, and impaired digestion. In addition, it also diminishes the host's energy reserves, including ATP and carbohydrates, and interferes with

amino acid and protein metabolism (Kunat-Budzyńska et al 2025). *Varroa* and *Nosema* co-infections create a synergistic effect and increase morbidity rates in the colony (Bahreini and Currie 2015).

Synthetic acaricides against *Varroa* and antimicrobials against *Nosema* are widely used. In controlling *Varroa* infestations, hives are treated with pharmaceuticals such as coumaphos, amitraz, tau-fluvalinate, and flumethrin through various methods (Almecija et al 2024). Fumagillin, an antibiotic derivative used for treating Nosemosis, has been prohibited or significantly restricted in numerous countries due to its genotoxic effects (Manea-Karga et al 2025). It has been determined that long-term use of these acaricides and antibiotics poses a serious threat to public health, particularly due to the risk of residues in honey and beeswax (Gruznova et al 2025).

In addition, continuous use of chemical compounds at sublethal doses against Nosema and *Varroa* infections in honey bees has adverse effects on neurophysiology, reproductive systems, and detoxification mechanisms (Frost et al 2013). The emergence of chemical-resistant mite populations diminishes treatment efficacy, prompting the development of alternative therapeutic methods in response to this issue (Lester 2023). Researchers are including natural extracts, particularly essential oils, into their studies on *Varroa* and *Nosema* treatments due to their natural and eco-friendly characteristics. Essential oils contain compounds such as terpenoids, aldehydes and phenolics, which have proven acaricidal, antimicrobial, antioxidant, and antifungal properties (Nwanade et al 2021). The volatility, biodegradability, and minimal mammalian toxicity of essential oils make them attractive for application in apiculture. Promising results have been obtained in laboratory and field studies on the efficacy of thymol, eucalyptol, menthol and carvacrol against *Varroa* and *Nosema* (Bava et al 2023a).

This study was designed to determine the effectiveness of four different essential oils *Cymbopogon citratus* (CC), *Allium sativum* (AS), *Leptospermum scoparium* (LS), and *Litsea cubeba* (LC) in field conditions on *Varroa* and *Nosema* co-infections. The research has established a basis for creating ecological and sustainable alternative treatment methods for honey bee pathogens by assessing pathogen loads pre- and post-treatment.

Material and Methods

This study was approved by the Balıkesir University Animal Experiments Local Ethics Committee (Approval no: 2025/6-6).

Study area

The study was conducted in Balıkesir province of Türkiye. The study took place in an apiary with 100 hives. Balıkesir is located in the Marmara Region of Türkiye.

Honey bee information

The local hybrid bee species (*Apis mellifera* L.) were examined. All queen bees in the hives were one year old. Throughout the study period, honey bee colonies averaged between 60,000 and 70,000 individuals. Prior to the study, the owner of the honey bee verified that no chemicals or plant extracts were used to combat any pathogens.

Varroa field experiment

The powdered sugar technique was employed to ascertain the Varroosis status of 100 hives, categorizing them as either positive or negative (Bava et al 2023b). Approximately 350 worker bees were collected from the combs using a brush and placed in a 900 ml *Varroa* Test Apparatus (VTA). Fifteen grams of powdered sugar were added to the VTA. The VTA was shaken vigorously by hand for 4 min to allow sugar to penetrate the bees' bodies. After waiting for one min, the VTA was uncovered, the powdered sugar was sieved and the *Varroa* agents falling on the white paper were counted with the naked eye and the phoretic *Varroa* load in the hives was calculated (Dietemann et al 2013). The VTA and the macroscopic appearance of *Varroa* are shown in Figures 1 and 2, respectively. Prior to each trial, the white paper in the pollen traps was placed to facilitate the counting of *Varroa* mites.

Nosema field experiment

Thirty honey bees were collected from the outer frame of the hives to detect Nosemosis. The honey bee samples were stored in a laboratory deep freezer for one day to guarantee their immobilization. The digestion method assessed 10 stationary honey bees from each hive for the presence of *Nosema* spores (positive or negative). In this procedure, the abdomens of 10 honey bees were excised from their bodies using a scalpel. The abdomens were placed in a mortar and subsequently mashed. One milliliter of distilled water was added to each abdomen in the mortar. The honey bees' abdomens were compressed using a baguette for approximately five min. The solution was homogenized using a Pasteur pipette, and one drop was analyzed at 40x10 magnification under a light microscope (Nikon Eclipse E100®, Japan) to identify *Nosema* spores (Özüiçli et al 2024a). The microscopic appearance of the *Nosema* spore form is shown in Figure 3.

General information about essential oils

The essential oils used in the study were purchased from Adenaş A.Ş. (Balıkesir). The essential oils were obtained through steam distillation. The information about CC; 100% purity, active ingredients: α-citral (42%), β-citral

Figure 1. *Varroa* tester apparatus

(41%), β-myrcene (11%), geraniol (1.8%), geranyl acetate (1.2%), citronellal (1%), citronellol (0.8%), linalool (0.6%), and elemol (0.6%), linear formula: $(CH_3)_2C=CHCH_2CH_2C(CH_3)=CHCHO$, molecular weight: 152.23 g/mol. AS; 100% purity, active ingredients: diallyl disulfide (24%), diallyl trisulfide (20%), allyl methyl trisulfide (20%), dimethyl trisulfide (12%), diallyl sulfide (9%), 3 vinyl 1,2 dithiin (8%), diallyl tetrasulfide (7%), and linear formula: $CH_2=CHCH_2S(O)CH_2CH=CH_2$, molecular weight: 162.27 g/mol. LS; 100% purity, active ingredients: α-selinene + cadinene + alamenene (62%), calamenene (16%), leptospermone (12%), and flavesone + iso leptospermone (10%), linear formula: $CH_3CH(CH_3)C_6H(OCH_3)_2(C=O)_2$, molecular weight: 184.23 g/mol. LC; 100% purity, active ingredients: e-citral (48%), z-citral (43%), and d limonene (9%), linear formula: $CH_3C(CH_3)=CHCH_2CH_2CH=C(CH_3)CHO$, molecular weight: 152.23 g/mol. The chemical composition and active ingredient ratios of the essential oils were provided by the manufacturer (Certificate of Analysis, COA), which was based on Gas Chromatography–Mass Spectrometry (GC–MS) analysis performed according to ISO 3515:2002 standards.

Figure 2. *Varroa* agents macroscopic appearance are shown in rectangle

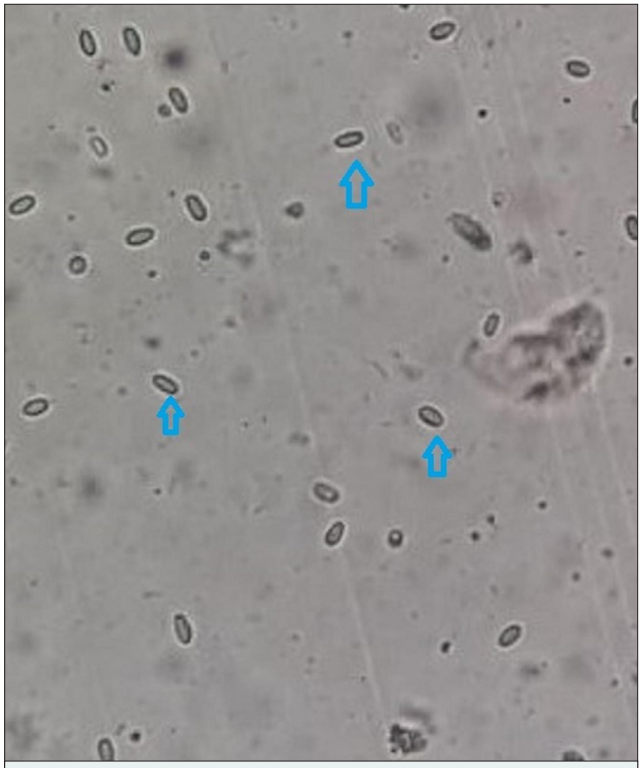

Figure 3. Microscopic appearance of *Nosema* spore forms (indicated by blue arrow)

Preparation of essential oils

The essential oils of CC, AS, LS, and LC were dissolved by combining them with polyethylene glycol 400 (PEG-400) in a 1:1 ratio. The target concentrations were achieved by mixing sugar syrup into the essential oils dissolved in PEG-400. In the treatment, 3% concentrations of each essential oil were used. Fifteen milliliters of each essential oil was taken and 15 ml of PEG 400 was added to it. Four hundred seventy milliliters of sugar syrup was added to the 30 ml mixture to obtain a 500 ml mixture. A 500 ml mixture was applied in spray form to hives, each containing 8 frames. Each frame received 62.5 ml of solution per application. Considering that the treatment groups consisted of 7 hives, a cumulative volume of 3,500 ml of stock solution was formulated for each application (7x500). A total of 14,000 ml of solution was used, which was resulting from four applications to the treatment groups, calculated as 3,500 ml multiplied by four.

Essential oils treatment and control groups

Forty-two honey bee colonies in Langstroth-type hives, each containing eight frames, were categorized into six homogeneous groups: four essential oil treatment groups, one phoretic *Varroa* and *Nosema* negative control group,

and one phoretic *Varroa* and Nosema positive control group. Each group contained seven hives. Essential oil treatments were applied as a spray once a week for four weeks. Essential oil application was performed on days 0, 7, 14, and 21. Five hundred milliliters of essential oil solution were administered to each hive weekly. Mite counts in the powdered sugar were assessed on days 0 (pre-treatment) and 28 (post-treatment) to evaluate phoretic Varroa infestations in honey bee colonies. Counts of mites in the powdered sugar were conducted on days 0 and 28 for both the negative and positive control groups, parallel to the treatment groups, while *Varroa* agents collected in the pollen trap were recorded on days 7, 14, 21, and 28 (Girişgin and Aydın 2010). The digestion method was applied to determine Nosema spore forms on days 0 (pre-treatment) and 28 (post-treatment) (Özüiçli et al 2024a). During the formation of the groups, numerical homogeneity of phoretic *Varroa* and *Nosema* loads was achieved in all seven hives on day zero. Essential oils were administered to treatment groups in the evening to prevent robbing in honey bee colonies.

Determination of treatment efficacy for Varroa in treatment groups

The Henderson-Tilton formula was applied to evaluate the therapeutic efficacy of the essential oils (Girişgin and Aydın 2010).

$$\text{Corrected \%} = \left(1-\frac{\text{n in Co before treatment x n in T after treatment}}{\text{n in Co after treatment x n in T before treatment}}\right) \text{X100}$$

(where n = mite population, T = treated, Co = control).

Counting of *Nosema* spp. spores

A Neubauer thoma slide was used for counting Nosema spp. spore loads. The results of counting days were assessed with the formula (N=Sx4x106/80) (Shimanuki and Knox 2000).

Determination of treatment efficacy for Nosema in treatment groups

The efficiency of treatment groups was determined with the following formula (Özüiçli et al 2024a):

$$\text{Percent reduction test= 100- (}\frac{\text{Final Number of Nosema spores}}{\text{Initial number of Nosema spores}}\text{ x 100)}$$

Day 28 was designated as the final count of *Nosema* spores, whereas day 0 was established as the initial count (baseline) of *Nosema* spores.

Statistical analysis

The data were analyzed using repeated measures analysis of variance (ANOVA) in SPSS 20. To determine between-subjects differences, an Epsilon value was first calculated using Mauchly's test of sphericity. The correct p-values were determined by applying the necessary corrections based on the Epsilon value found. To determine between-subjects differences, the LSD post-hoc test was applied. Statistical significance was accepted at $p<0.05$. The data are presented as mean and standard error (Blanca et al 2023).

Results

IThe study included 100 hives. Thirty-five hives were positive for both *Nosema* and phoretic *Varroa*, 20 hives were negative for phoretic Varroa and Nosema, 27 hives were positive for phoretic *Varroa* only, and 18 hives were positive for *Nosema* only. Total Nosema spores decreased as ($1,570x10^4$-$665x10^4$=9,050,000), ($1,560x10^4$-$640x10^4$=9,200,000), ($1,495x10^4$-$560x10^4$=9,350,000), and ($1,435x10^4$-$480x10^4$=9,550,000) in CC, AS, LS, and LC groups, respectively. In the positive control group, the *Nosema* spore count increased from 13,050,000 to 16,700,000. In the negative control group, the *Nosema* spore count, which was 0 on day 0, increased to 28,050,000 on day 28. In the treatment groups (CC, AS, LS, and LC), the percentage decreases in the *Nosema* spore count on day 28 compared to the *Nosema* spore count on day 0 were determined as 57.64%, 58.97%, 62.54% and 66.55%, respectively (Table 1). According to the powdered sugar counting method results, phoretic *Varroa* load in live bees decreased from 259 to 90 in the CC treatment group, from 283 to 99 in the AS group, from 277 to 105 in the LS group, and from 318 to 103 in the LC group. In the positive control group, the phoretic *Varroa* load, which was 270 on day 0, increased to 425 on day 28, and in the negative control group, the phoretic *Varroa* load, which was 0 on day 0, increased to 213. Treatment efficiencies in the CC, AS, LS, and LC treatment groups were determined as 77.92%, 77.77%, 75.91%, and 79.42% (Table 2). According to the pollen trap count results, the number of phoretic *Varroa* counted in the pollen trap in all treatment groups decreased gradually starting from day 7, and on days 14, 21, and 28. The number of phoretic *Varroa*, which was determined as 303 on day 7 in the CC treatment group, was determined as 214 in the last count. On days 7 and 28, the numbers of phoretic *Varroa* in the AS, LS, and LC treatment groups were determined as (175;116, 166;97, and 199;115) for the day and treatment group, respectively (Table 3). In the negative control group, the number of phoretic *Varroa* in the pollen trap, which was 0 on day 7, gradually increased on days 14, 21 and 28 and was determined to be 51 on day 28. In the positive control group, the number of phoretic *Varroa* in the pollen trap, which was 55 on day 7, was determined to be 66 on day 28. According to the counts performed on days 0 and 28, *Varroa* loads decreased significantly in the CC, AS, LS, and LC groups ($p<0.001$). In particular, the average mite load in the CC group, which was 37.00±5.72 at the beginning, decreased to 12.85±1.27 at the end of day 28. In contrast,

Table 1. Day 0 (before treatment) and Day 28 (after treatment) *Nosema* count results and treatment efficacies

Hive No	CC		AS		LS		LC		PC		NC	
	D.0	D.28	D.0	D.28	D.0	D.28	D.0	D.28	D.0	D.28	D.0	D.28
1	3.50	1.55	4.50	2.25	4.00	2.10	3.75	1.75	2.30	2.75	0	3.00
2	1.25	4.50	1.20	0.35	1.00	0.20	1.15	0.40	1.35	1.90	0	2.55
3	1.55	3.50	1.40	0.50	1.35	0.30	1.20	0.25	1.45	2.15	0	4.00
4	1.85	5.50	1.70	0.65	1.65	0.75	1.60	0.50	1.60	2.00	0	3.50
5	2.25	9.50	2.10	0.80	2.00	0.65	1.80	0.70	1.75	2.50	0	5.50
6	2.55	1.55	2.30	1.10	2.45	1.00	2.25	0.65	2.10	2.60	0	2.00
7	2.75	1.25	2.40	0.75	2.50	0.60	2.60	0.55	2.50	2.80	0	7.50
Total	15.70	6.65	15.60	6.40	14.95	5.60	14.35	4.80	13.05	16.70	0	28.05
Days	*Nosema* percentage reductions in treatment groups compared to days 0 and 28											
0-28	CC NCR: 57.64				AS NCR: 58.97		LS NCR: 62.54		LC NCR: 66.55			

C: *Cymbopogon citratus*; AS: *Allium sativum*; LS: *Leptospermum scoparium*; LC: *Litsea cubeba*; PC: Positive Control; NC: Negative Control; D.: Day; NCR: *Nosema* Count Reduction. All values are expressed as $\times 10^6$ spores/mL.

an increase was observed in the PC group, with the load rising to 60.71±8.09. In the NC group, where Varroa mites were not present at the beginning, the number reached 30.42±2.68 by the end of day 28. No correction was necessary, as the sphericity assumption was met (ε=1.000) (Table 4). Weekly observations based on *Varroa* count results falling into the pollen trap showed that essential oil applications reduced mite numbers over time. In the AS, LS, and LC groups, a gradual and significant decrease in mite numbers was observed between days 7 and 28 ($p<0.001$). In the LS group, the numbers decreased from 23.71±3.77 to 13.85±2.27. Although a decrease was observed in the CC group, it was slower compared to the other groups. Since the epsilon value was 0.528, the Greenhouse-Geisser correction was applied (Table 5). Applications of essential oils have produced favorable outcomes regarding *Nosema* spore load. On day 0, spore counts were 2.24×10^6 (CC), 2.21×10^6 (AS), 2.13×10^6 (LS), and 2.05×10^6 (LC); by day 28, these values diminished to 0.95×10^6, 0.91×10^6, 0.80×10^6, and 0.68×10^6, respectively. The positive control group exhibited an increase, with the spore count rising to 2.38×10^6. The maximum *Nosema* load (4.00×10^6) was recorded in the negative control group. No correction was required as sphericity was obtained (ε=1.000) (Table 6).

Discussion

Essential oil activity studies are generally conducted on single infections (either *Nosema* or *Varroa*). At the same time, the studies are usually conducted in a laboratory environment, and field studies are limited. This study aims

Table 2. Day 0 (before treatment) and Day 28 (after treatment) powder sugar count results and treatment efficacies

Hive No	CC		AS		LS		LC		PC		NC	
	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.	D.0 V.L.	D.28 V.L.
1	35	10	40	10	45	10	40	18	40	65	0	26
2	30	18	38	20	38	19	38	16	30	44	0	25
3	42	14	58	24	54	26	56	24	48	58	0	34
4	18	10	26	8	26	8	33	7	23	40	0	45
5	26	13	25	9	19	7	26	6	18	40	0	26
6	43	16	55	16	55	20	67	20	67	89	0	28
7	65	9	41	12	40	15	58	12	44	89	0	29
Total	259	90	283	99	277	105	318	103	270	425	0	213
Days	*Varroa* percentage reductions in treatment groups compared to days 0 and 28											
0-28	CC VCR: 77.92				AS VCR: 77.77		LS VCR: 75.91		LC VCR: 79.42			

D.: Day; V.L:. *Varroa Load,* VCR: *Varroa* Count Reduction; CC: *Cymbopogon citratus*; AS: *Allium sativum*; LS: *Leptospermum scoparium*; LC: *Litsea cubeba*; PC: Positive Control; NC: Negative Control

Table 3. Treatment groups pollen trap count results

Hive No	CC				AS				LS				LC			
	D.7	D.12	D.21	D.28	D.7	D.12	D.21	D.28	D.7	D.12	D.21	D.28	D.7	D.12	D.21	D.28
1	35	33	30	28	20	18	15	14	20	15	13	12	25	20	18	15
2	50	40	38	35	28	20	18	16	25	20	15	14	30	28	20	18
3	60	45	41	36	35	30	25	23	30	28	20	17	40	35	30	25
4	28	25	20	18	10	9	7	6	10	7	6	5	11	9	10	8
5	25	20	18	16	15	14	12	10	13	10	9	8	16	11	10	6
6	60	56	50	45	37	38	35	30	38	32	22	22	45	40	32	25
7	45	41	40	36	30	30	20	17	30	26	21	19	32	30	23	18
Total	303	260	237	214	175	159	132	116	166	138	106	97	199	173	143	115

D.: Day; CC: *Cymbopogon citratus*; AS: *Allium sativum*; LS: *Leptospermum scoparium*; LC: *Litsea cubeba*; PC: Positive Control; NC: Negative Control

to fill these gaps in phoretic Varroosis and Nosemosis co-infections. Based on this point, in this study, the efficacies of four different essential oils (CC, AS, LS and LC) were evaluated against phoretic *Varroa* and *Nosema* co-infections under field conditions.

The simultaneous presence of these two pathogens increases stress levels in honey bees. The increase in stress levels is caused by *Varroa* mites feeding on the fat tissue and hemolymph of honey bees, and in *Nosema*, damage to the digestive system. As a result, honey bee colonies experience adverse conditions such as physiological, biological, and behavioral changes (decrease in vital functions such as feeding and self-cleaning), and a shortened lifespan. Moreover, the inability of worker bees to fulfill their responsibilities within the colony results in diminished colony strength, disruption of larval care, adverse impacts on royal jelly synthesis, and potential colony collapse. Additionally, honey and other apicultural products are decreased in colonies afflicted by *Nosema* and *Varroa* in comparison to healthy hives. This results in significant ecological and economic losses (Kurze et al 2018, Panek et al 2018, Abban et al 2024).

From the past to the present, mainly chemical compounds have been preferred in the treatment of *Varroa* and *Nosema*. In *Varroa* management, organic acids (formic acid and oxalic acid) and commercial products derived from chemical active ingredients (amitraz, flumethrin, coumaphos) are employed. Despite the short-term efficacy of these chemicals, issues such as improper dosage and non-adherence to usage frequency can lead to risks including residue in bee products, particularly honey, adverse effects on bee health, and harm to non-target organisms over time (Bogdanov et al 2002, Yu et al 2015, Pohorecka et al 2018). Non-compliance with the prescribed chemical dosages results in the development of pesticide resistance in *Varroa* populations, leading to economic losses and adversely impacting the sustainability of control activities (Lester 2023). Fumagillin, an antibiotic in the treatment of Nosemosis, has been used worldwide for a long time, but its use has been banned or restricted in most countries

Table 4. Descriptive statistics and group comparisons of *Varroa* mite loads determined via powdered sugar method

	Day 0	Day 28
	Varroa count	
CC[a]	37.00±5.72	12.85±1.27
AS[a]	40.42±4.80	14.14±2.28
LS[a]	39.57±5.07	15.00±2.66
LC[a]	45.42±5.66	14.71±2.52
PC[b]	38.57±6.29	60.71±8.09
NC	0.00	30.42±2.68
Epsilon value	1.000	
p values (between-subjects)*	0.002	
p values (within-subjects)**	<0.001	

a,b: Shows the differences between treatments. *: LSD test was applied; **: *Sphericity assumed; CC: Cymbopogon citratus; AS: Allium sativum; LS: Leptospermum scoparium; LC: Litsea cubeba;* PC: Positive Control; NC: Negative Control

Table 5. SPSS results of *Varroa* counts falling into the pollen trap				
Hive No	Day 7	Day 14	Day 21	Day 28
	Varroa count			
CC[a]	43.28±5.43	37.14±4.61	33.85±4.42	30.57±3.96
AS[b]	25.00±3.86	22.71±3.87	18.85±3.45	16.57±3.01
LS[b]	23.71±3.77	19.71±3.56	15.14±2.33	13.85±2.27
LC[ab]	28.42±4.60	24.71±4.44	20.42±3.28	16.42±2.81
Epsilon value	0.528			
p values (between-subjects)*	0.011			
p values (within-subjects)**	<0.001			

a,b: Shows the differences between treatments. *: LSD test was applied. **: Greenhouse-Geisser correction was applied. CC: *Cymbopogon citratus*; AS: *Allium sativum*; LS: *Leptospermum scoparium*; LC: *Litsea cubeba*

due to the developing resistance and residues in honey and beeswax (Higes et al 2010, Manea-Karga et al 2025). On the other hand, Peirson and Pernal (2024) reported that Fumagillin inhibited both *Nosema* species and no resistance development was observed based on the results of field trials. Fumagillin-treated colonies had fewer infected bees and lower *Nosema* spore counts than untreated colonies. Furthermore, no reports of fumagillin causing significant adverse effects on honey bee colonies were identified among the field trials. There have been many reports of improvements in colony survival, size and productivity with treatment.

In studies, it has been reported that genotoxic effects occur in honey bees due to chemical use and the residue in honey is at a level that may adversely affect human health (Mitkovska et al 2025). Essential oils, on the other hand, have been frequently tested against bee pests in both laboratory and field studies by researchers in recent years due to their easy degradability, minimal residue problems, low risk of resistance development and usefulness to bee biology. Essential oils have strengthening effects in honey bee colonies with their low toxicity, stress-reducing effects and immune system supportive properties (Pătruică et al 2023). With these positive effects, essential oils are considered as ecological pest control agents of the future in addition to honey bee pests. Essential oils contain phytochemical components such as aldehyde groups, phenolic compounds, and monoterpenoids, which have a wide range of properties, such as disrupting cell integrity in pathogenic microorganisms, inhibiting neural transmission, and creating negative effects on metabolic processes, especially energy metabolism (Jabin et al 2020).

Previous studies have been conducted by researchers to test the effects of CC, AS, LC, and LS on *Varroa* and *Nosema* infections. The main compounds of CC include citral and geraniol. In one study, citral and geraniol were tested on the fungus *Saccharomyces cerevisiae* and the use of these compounds resulted in both the monoterpenoids induced the osmotic stress in *S. cerevisiae* measured as a change in reduction in pH, $[H]^+$ concentration, with elevation in $[Na]^+$ and $[K]^+$ leakage into the media.

Table 6. SPSS *Nosema* digestion counts results		
	Day 0	Day 28
	Nosema count	
CC	$2.24x10^6±0.29x10^6$	$0.95x10^6±0.19x10^6$
AS	$2.21x10^6±0.41x10^6$	$0.91x10^6±0.23x10^6$
LS	$2.13x10^6±0.37x10^6$	$0.80x10^6±0.24x10^6$
LC	$2.05x10^6±0.34x10^6$	$0.68x10^6±0.18x10^6$
PC	$1.86x10^6±0.16x10^6$	$2.38x10^6±0.13x10^6$
NC	0.00	$4.00x10^6±0.27x10^6$
Epsilon value	1.000	
p values (between-subjects)*	0.311	
p values (within-subjects)**	<0.001	

*: LSD test was applied; **: *Sphericity assumed; CC: Cymbopogon citratus; AS: Allium sativum; LS: Leptospermum scoparium; LC: Litsea cubeba;* PC: Positive Control; NC: Negative Control

Geraniol treatment reduced the levels of metabolites, dehydroergosterol (DHE) and H_2O_2, in a time-dependent manner whereas citral only affected their levels at 120 h. Energy dispersive X-ray spectroscopy (EDAX) studies suggest that both the monoterpenoids treatment differentially modulated the cellular elemental contents results (Gaonkar et al 2018). In another study, CC essential oil was dissolved in different solvents and tested on *Ascosphaera apis*. According to the study results, although CC dissolved in 70% alcohol had a toxic effect on *Ascosphaera apis*, no negative effect was observed on honey bees (Albo et al 2016). In a study testing the effectiveness of CC on *Varroa* infection, it was reported that CC was effective against *V. destructor* and had low toxicity against larvae and adults. In addition, it has been reported that citral, a compound found in CC essential oil, may act as an acetylcholinesterase (AChE) inhibitor (Sabahi et al 2018). There are only a limited number of studies investigating the effectiveness of CC on Nosemosis. In one study, hives infected with Nosemosis were fed different pollen substitute diets containing leaves of medicinal plants such as lemon grass (*C. citratus*), coriander (*Coriandrum sativum*), moringa (*Moringa oleifera*), and tulsi (*Ocimum sanctum*). Forty worker bee samples were collected from each colony, and Nosema spore counts were performed on days 8, 16, 24, and 32. According to the study results, the *Nosema* spore count in the treatment group (50,000±28867.5 spores/bee) was significantly lower than that in the control group (550,000±28,867.5 spores/bee) (Gawali and Waykar 2025). In a study conducted on AS (garlic oil) was tested together with onion and fresh garlic, and the highest *Varroa* drop in the pollen trap was observed in the fresh garlic treatment group (94.29%) (Mazeed and El-Solimany 2020). In another study, AS, cinnamon oil, amitraz, peppermint oil, and lavender oil were tested against *Varroa* infestation. The study results indicate that the most effective treatments for *Varroa* mite reduction per colony, following garlic oil, cinnamon oil, and amitraz, were (4.262±1.572), (4.128±1.840), and (2.728±0.723), respectively, with minimal variation among them. Peppermint oil and lavender oil exhibited the lowest mean number of fallen *Varroa* mites compared to control colonies, with values of 0.728±0.163 and 0.066±0.066 fallen mites per colony, respectively, in the control group (0.066±0.066) (Aljedani 2021). In a study on Nosemosis, AS, thymol, eucalyptus, nettle, and laurel essential oils were tested. According to the study results, the most effective essential oil against Nosemosis was thyme (Average Spore Count=11,990,381.8±5,578,263.5), and the second most effective essential oil was garlic (Average Spore Count=12,009,222.2±8,420,472.1) (Yılmaz et al 2020). A study evaluating the impact of essential oils, specifically Manuka (*L. scoparium*), on *Varroa* in a laboratory setting identified peppermint and L. scoparium as the most effective oils (selectivity ratio (SR>9), followed by thyme and litsea (SR>5), and carrot and cinnamon (SR>4). Additionally, these oils showed a trend toward increasing selective ratio values over time. All of these oils yielded better results than thymol (SR<3.2), which is widely used in beekeeping for Varroosis infestations (Hýbl et al 2021). In another study on Varroosis, *Origanum vulgare subsp. viridulum*, *Thymus capitatus* and *Thymus longicaulis* neutralized 94%, 92%, and 94% of parasites, respectively, at 2 mg/mL, demonstrating the highest level of efficacy. Also, *Salvia rosmarinus* showed lower efficacy against Varroosis, achieving a rate of 38%. Interestingly, no side effects were observed in toxicity tests conducted on honey bees (Bava et al 2023c). No studies have been found on the effectiveness of LS essential oil against Nosemosis. In a study, thyme, peppermint, and eucalyptus were tested in spray form under field conditions against *Nosema* infection. Efficacy rates of 84% were observed in the thyme group, 77.45% in the peppermint group, and 76.10% in the eucalyptus group (Özüiçli et al 2023). Thymol, *Artemisia absinthium* essential oils, and nanoparticle ozone were applied to honey bee colonies in spray and oral forms to combat *Nosema* infection. In the study, 89.47% efficacy was observed in the spray form (200 ml-2,000 ppm nanoparticle ozone+100 ml 3% thymol+700 ml sugar syrup mixture), and 85.95% efficacy in the oral form treatment group (250 ml of 2% thymol+200 ml of 2% *A. absinthium*+500 ml of sugar syrup) was determined (Özüiçli et al 2024a). In a study on *Varroa*, essential oils of Thyme, *Cinnamomum verum*, *Melaleuca viridiflora*, and *Syzygium aromaticum* were dissolved in glycerine, impregnated into strips, and placed between frames using toothpicks. The acaricidal efficacy was found to be 73.5% in *C. verum*, 71.9% in the thyme, 71.3% in *M. viridiflora*, and 67.4% in the *S. aromaticum* treatment group (Özüiçli and Baykalır 2024). In another study on *Varroa* infestation, thyme, eucalyptus, and oxalic acid were impregnated into special towels and placed on the frames in honey bee colonies. According to the study results, the efficacy was 91.74% in the oxalic acid group, 82.25% in the thyme group, and 79.2% in the eucalyptus treatment group (Özüiçli et al 2024b). Similarly, no studies have been found on the effectiveness of LC essential oil against Nosemosis. According to this study results, the treatment efficacy in Nosemosis infection was found to be 57.64% in the CC treatment group, 58.97% in the AS treatment group, 62.54% in the LS treatment group, and 66.55% in the LC treatment group. On the day 28 of the study, *Nosema* spore counts in the positive control colonies increased compared to day 0. In the negative control group, *Nosema* loads, which were 0 on day 0, increased on day 28 (Table 1). The treatment efficacy in *Varroa* infestations was determined to be 77.92% in the

CC treatment group, 77.77% in the AS treatment group, 75.91% in the LS treatment group, and 79.42% in the LC treatment group (Table 2). The differences in the results of this study compared to others may originate from various factors. This study specifically examined co-infections of *Nosema* and *Varroa*, rather than single infections. *Nosema* infection affects the digestive system, while *Varroa* mites target fat tissue and hemolymph, thereby increasing stress levels in colony members. As a result, detoxification and suppression of the immune system may have reduced the effectiveness of essential oils. Researchers typically conduct *Nosema* and *Varroa* studies in laboratory environments with controlled temperature and humidity conditions. This study was conducted under field conditions with variable parameters (temperature fluctuations, rainfall, wind, UV exposure). These circumstances may have altered the dispersion and retention duration of the volatile compounds present in the essential oils within the hive. Essential oils are usually applied in the form of vapour, strips or sugar syrup. This study utilized a spray application, and the frequency and volume of the essential oils administered may have influenced the variations in results. The genetic structure, physiology, and past exposure to chemicals of the honey bees used in the studies may also have contributed to these differences. Similarly, the methods of obtaining essential oils, storage conditions, and shelf life may also have contributed to these differences. At the beginning of the study, the group that was negative for *Varroa* and *Nosema* was found to be positive for both agents at the end of the study. This indicates the transfer of *Varroa* and *Nosema* agents between hives. Robbing behavior between hives may have played an important role in this transfer. Additionally, beekeepers should always adhere to hygiene and disinfection rules, taking into account the risk of contamination. The dosage of essential oils is also significant. Exceeding the dosage may disturb the queen bee and other colony members due to the potent odor, potentially leading to hive abandonment. At the end of the study, no toxic effects or deaths were observed in honey bees.

Conclusion

This study is a field study evaluating the effectiveness of four different essential oils in a stressful environment caused by simultaneous *Varroa* and *Nosema* infections, as opposed to single infections. This study provides a broad perspective on both colony health and pest control. Based on the findings obtained at the end of the study, it has been revealed that completely organic treatment strategies that enhance honey bee welfare can be developed against Nosemosis and especially Varroosis, and the use of chemicals could be limited.

Declarations

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Competing Interests

Authors declare that there are no conflicts of interest related to the publication of this article.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author.

Ethical Statement

Balıkesir University Local Ethics Committee for Animal Experiments, approval no: 2025/6-6. Number Ethics Committee Decision

Author Contributions

Motivation/Concept: MO, ET, YB; Design: MO; Control/Supervision: MO; Data Collection and Processing: MO; Analysis and Interpretation: MO, YB; Literature Review: MÖ, ET, YB; Writing the Article: MO, ET; Critical Review: MO, YB

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