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Molecular Responses of *Varroa destructor* to Thyme Essential Oil Exposure

Tuğçe Olgun*[1], Metin Erdoğan[2], Miray Dayıoğlu[1], Ünal Karik[3]

[1]Apicultural Research Center, Aegean Agricultural Research Institute, Izmir, Türkiye, [2]The Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar, Türkiye, [3]Department of Medicinal and Aromatic Plants, Aegean Agricultural Research Institute, Izmir, Türkiye

***Correspondence**:
tugce.olgun@tarimorman.gov.tr
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ABSTRACT

Varroa mites (*Varroa destructor*) continue to pose a serious threat to honey bee (*Apis mellifera* spp.) health, exhibiting significant resistance development to synthetic acaricides. This study investigated the efficacy of thyme (*Origanum onites* L.) essential oil (TEOV) as botanical alternative, focusing on mite survival across different feeding contexts and the underlying molecular responses. Adult mites were exposed to three TEOV doses (0.1, 1, and 10 ppm), across different feeding contexts (GA;larvae, GB;fat body, and GC;live honey bee). Mortality exhibited dose and time dependency, particularly at 24 h post-exposure to TEOV. The most responsive host tissue was found to be the mites feeding on larvae and fat body tissue. Interestingly, the susceptibility of the mites was found to be influenced by the context of feeding, which suggests that nutrition affects the susceptibility of TEOV. At the molecular level, TEOV elicited delayed but dose-dependent transcriptional responses: pheromone receptor transcription factor-like (PRTF-like) was strongly upregulated at higher doses after 24h, consistent with activation of stress- or detoxification pathways, additionally Ionotropic glutamate receptors (IGRs)- Ionotropic Receptor 25a-like (IR25a-like) exhibited a general, significant elevation in response to TEOV exposure, suggesting an effort to activate an adaptive chemosensory response. In contrast, the expression of GABA-activated RDL (Dieldrine Resistance) receptor (GABA-RDL) was not generally affected at any dose or time of observation. The result reveals that the neurotoxic action of TEOV is not through transcription-regulated pathway via this receptor. No activity of the mite genes was detected in the control group indicating that any gene activity changes are attributable to treatment with thyme. The selectivity ratio (SR) was calculated as 17.0. The materials have shown their mortality rates, gene expression and physiology by two mechanisms: a fast action as neurotoxicants and long-term stress for TEOV. All results demonstrate its potential as a plant-derived acaricide.

Keywords: Varroa PRTF-like gene, Ionotropic glutamate receptors – IR25a-like gene, Neurotransmitter gene, *Origanum onites* L., *Varroa destructor*

INTRODUCTION

With respect to the problem of Varroa mites in global apiculture, the ectoparasitic mite *Varroa destructor* is still very much a major threat. The Varroa mite's feeding on the fat bodies of adult bees and their larvae severely compromises the colony's immune system and temperature control, and also serves as a means for the transmission of numerous viruses to the host, all of which are contributing to the disintegration of honey bee colonies around the world (**1**). Traditionally, beekeepers have turned to synthetic chemicals, which include pyrethroids,

organophosphates and formamidines, to try and control the Varroa mite, but this has in turn intensified the mites' nasty effects on the colonies. Furthermore, the rapid development of resistance in *V. destructor* populations means that these treatments aren't working very well anymore, and creates issues with toxic residues in honey products and pollution of the environment (**2,4**).

In response to these challenges, finding alternative, sustainable, and eco-friendly methods for controlling Varroa mites has become increasingly important. Well-known as an effective and environmentally friendly alternative, essential oils (EOs) derived from medicinal and aromatic plants have taken center stage in the fight against the Varroa mite (**5**). Among these, the carvacrol-rich thyme (*Origanum onites* L.) essential oil (TEOV) is a strong fumigant that can almost completely kill off the Varroa mite, even in low doses. Previous studies show that TEOV and its active constituents may interfere with the neural signals of the mite, much like they do in arthropods, and we're aware that the monoterpenoid compounds in TEOV have neuroactive properties (**6–8**).

Despite lots of studies on the lethal effects of TEOV, we don't yet fully understand the molecular basis of its effectiveness in the case of V. destructor. This understanding will help identify the molecular targets and pathways connected to toxicity and tolerance. Recent studies show that several gene families involved in neurotransmission, chemosensory sensing, and detoxification play crucial roles in how mites respond to chemical stressors (**7–10, 11–13**).

Key targets include genes related to GABA neurotransmission, especially the GABA-activated RDL (Resistance to Dieldrin) receptor. This receptor belongs to the cys-loop ligand-gated ion channel family and mediates inhibitory synaptic signaling in arthropods. Thymol, a monoterpenoid phenol structurally related to carvacrol, is known to allosterically modulate RDL receptors, increasing chloride influx and causing neuronal hyperpolarization and paralysis. Although carvacrol is dominant in TEOV, electrophysiological studies in V. destructor have shown that thymol can disrupt GABAergic signaling via the RDL receptor pathway in EO-induced toxicity (**8–10**).

Another gene associated with the chemosensory receptor is IR25a-like gene (chemosensory receptor-associated gene), a member of the ionotropic glutamate receptor (IGR) superfamily, which functions as a co-receptor involved in olfaction, thermosensation, and hygrosensation. These receptors are typically expressed in peripheral sensory neurons and facilitate host detection and environmental cue perception. In Drosophila melanogaster, IR25a has been reported to form complexes with other IR genes, thus facilitating the detection of volatile acids and amines. In the Varroa mite, it is thought that IR25a-like expression mediates host-seeking behavior, and that its disruption under EO exposure may reflect a disruption of the sensory processing mechanism (**14,15**).

The pheromone receptor transcription factor-like (PRTF-like) gene is less understood but is another promising candidate related to chemosensing or detoxification. In other arthropods, this gene is linked to protein transport, detoxification, and the formation of sensory structures. In Varroa mite, PRTF-like expression likely contributes to detoxification or stress response pathways and may increase when exposed to compounds like thymol or carvacrol. The level of this gene's expression after essential oil treatment could indicate a stress response or its involvement in signaling pathways related to chemical exposure (**11–13**).

Together, these genes, GABA-RDL (linked to neurotransmitter), IR25a-like (chemosensory receptor-related), and PRTF-like (related to detoxification or developmental signaling) serve as important indicators for understanding the physiology-altering activities of essential oils. Monitoring the expression levels of these genes can help us understand how Varroa mites detect and respond to essential oil exposure, as well as how they might develop tolerance. Studying these gene responses will strengthen our understanding of how thyme works and support the creation of integrated pest management strategies that use gene-targeted diagnostics and biologically sound control agents.

Many studies have shown that thyme essential oil and its active ingredient, thymol, kill V. destructor effectively (**16,17**). However, there is limited research on how thymol affects the molecular or transcriptomic aspects in Varroa mites. No studies have reported changes in gene expression in Varroa mites after exposure to essential oils (**5,7,8,13**). In contrast, researchers have better described how thymol affects honey bee physiology. For instance, thymol exposure influences the expression of stress-related genes like Heat Shock Protein 70 (HSP70) in honey bee brains and activates stress pathways (18). Additionally, thymol has been shown to affect immunity and detoxification genes, including vitellogenin and cytochrome P450 (cyp450), especially during prolonged exposure (**19,20**). These findings highlight a significant lack of understanding about how thyme essential oil affects V. destructor on a molecular level.

This study aimed to evaluate the gene responses of V. destructor to thyme (*Origanum onites* L.) essential oil in controlled laboratory conditions. It has investigated three candidate gene profiles: GABA-RDL, PRTF-like, and IR25a-like, regarding different feeding scenarios-including honey bee larvae, fat body, and live honey bee-exposed to varying doses of EO, such as 0.1, 1, and 10 ppm, and exposure time, including 10 min, 30 min, 1 h, and 24 h. By correlating gene expression profiles with Varroa mite mortality, this study aimed to elucidate how TEOV toxicity works and to support the development of EO-based, selective acaricides for sustainable Varroa management.

Materials And Methods

Ethical Approval

At the time the study was designed (2022-2023) and conducted, experimental work involving honey bees and invertebrates was not explicitly subject to mandatory ethical approval under national regulations. However, all

procedures were carried out in accordance with internationally accepted principles of animal welfare, minimizing harm and distress.

Mite Collection and Husbandry

Varroa (*Varroa destructor*) mites were collected from capped brood cells of *Apis mellifera* from hives located at the Apiculture Research Center within the İzmir Aegean Agricultural Research Institute (İzmir, Türkiye). The hives were intentionally left untreated for Varroa mite control and drone cell frames were used to stimulate Varroa mite reproduction. Prior to mite collection, the bee frames were kept in İzmir at a temperature range of 27– 32°C and 50% ± 2% relative humidity under a 14:10 h light-dark cycle. Adult female Varroa mites were collected from newly emerged adult bees or pupae after uncapping sealed brood cells. They were then transferred onto feeding substrates consisting of larvae, fat bodies, and live honey bees in petri dishes.

Thyme Essential Oil Extraction

Essential oil of thyme (*Origanum onites* L.) was extracted from air-dried plant material using a Clevenger-type apparatus (Borosil, India) via hydrodistillation for three hours. The resulting oil was then dried over anhydrous sodium sulfate and stored in the dark at +4°C until analysis (**21**).

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

Thyme essential oil at the Western Mediterranean Agricultural Research Institute (Antalya, Türkiye) was analyzed as using a capillary column (HP Innowax Capiller; 60.0 m × 0.25 mm × 0.25 μm) by gas chromatography (Agilent 5975C) equipped with both a flame ionization detector and mass spectrometry (Agilent 5975C). Essential oil was diluted 1:50 (v/v) in hexane prior to injection. The GC-MS/FID analysis was conducted using a split mode of 50:1. Essential oil was diluted 1:50 with hexane. GC-MS/FID analyses were performed in split mode (50:1). The injection volume was 1 μL with an injector temperature of 250°C. Helium (99.9%) was used as the carrier gas at a constant flow rate of 1 mL/min. The oven temperature was as follows: 60°C for 10 min, increased to 250°C at 20°C/min, and held at 250°C for 8 min. Mass spectra were recorded from 35 to 450 amu using electron ionization at 70 eV. The relative abundance of each component was calculated from GC–FID peak areas, and compound identification was carried out using the Wiley 7n, NIST 05, and Flavor and Fragrance Natural and Synthetic Compounds libraries (version 1.3).

Treatment management of In-vitro groups

All in vitro experiments were conducted under controlled laboratory conditions at 27 ± 1°C and 75 ± 2% relative humidity. Thyme essential oil (Origanum onites L.), obtained by hydrodistillation as described above, was used directly in all bioassays. Based on GC–MS analysis, carvacrol constituted 74.67% (0.7464 μg/μL) of the total oil composition. Therefore, treatment concentrations were standardized and expressed as carvacrol-equivalent concentrations within the whole essential oil preparation.

The essential oil was diluted in ≥99.8% ethanol (Sigma Aldrich, product code: 51976) to obtain working solutions corresponding to final carvacrol-equivalent concentrations of 0.01 μg/μL (10 ppm), 0.001 μg/μL (1 ppm), and 0.0001 μg/μL (0.1 ppm) (**22**). For bioassays, 100 μL of the 10 ppm working solution (containing 1 μg carvacrol equivalent, corresponding to 0.25 μg/cm^2 based on filter paper surface area) was applied to 2 × 2 cm Whatman No. 3 filter papers placed in 10-cm Petri dishes. Lower doses were prepared via serial dilution: a 1:10 dilution of the 10 ppm stock for the 1 ppm solution, and a 1:10 dilution of the 1 ppm solution for the 0.1 ppm dose.

Design of Bee-Varroa Test Groups

Experimental groups were designed as follows (In each experimental group, 15 Varroa mites were tested for each dose):

Group A (G.A): Larvae: One honey bee larva and one adult Varroa mite were placed in each petri dish. The Varroa mite was expected to climb onto the larva and initiate feeding. Subsequently, a filter paper impregnated homogeneously with essential oil was then placed in petri dish. 0.1, 1, and 10 ppm doses of thyme essential oil were tested in that group where Varroa mites feed on larvae.

Group B (G.B): Fat body: An incision was made along the laterodorsal abdomen of the honey bee, and the internal organs were removed. Fat body tissue located along the inner dorsal abdominal wall was transferred into a petri dish along with an adult mite. The mite was expected to enter the fat tissue and begin feeding. The filter paper impregnated homogeneously with essential oil was placed in petri dish. 0.1, 1, and 10 ppm doses of thyme essential oil were tested in that group where Varroa mites feed on fat body of honey bees.

Group C (G.C): Live honey bees: One live honey bee and one adult Varroa were placed in each petri dish. After the mite initiated feeding on the host, the filter paper impregnated homogeneously with essential oil was placed in petri dish. 0.1, 1, and 10 ppm doses of thyme essential oil were tested in that group where Varroa mites feed on live honey bees.

Group D: Solvent Control (ETOH) (n=15): The Filter paper impregnated homogeneously with ≥99.8% ethanol was placed in the petri dish. Feeding substrates identical to Groups A, B, and C were provided to the Varroa mites.

Group E: Negative control (NG) (n=15): No essential oil and ethanol was applied. Feeding substrates identical to Groups A, B, and C were provided to the Varroa mites.

After treatment initiation, a camera system recorded mite behavior for 10 minutes, and the time of mite death was noted. The time of the death of Varroa mite was recorded. Mortality in each petri dish was subsequently monitored at 30 min, 1 h, and 24 h under incubator conditions of 27 ± 1 °C and 75 ± 2% relative humidity (**23**).

RNA Isolation and cDNA Synthesis

Varroa samples were collected, transferred to cryotubes, rapidly frozen in liquid nitrogen, and stored at −80°C until analysis. Total RNA was extracted using the GeneJet RNA Purification Kit (Thermo, K0732, USA) according to the manufacturer's protocol. RNA concentration was measured with the Qubit RNA HS Assay Kit (Thermo Fisher, Q32855, USA) using a Qubit 2.0 Fluorometer (Invitrogen, USA), and RNA integrity was assessed with the Multiskan Go spectrophotometer (Thermo Scientific, USA). To eliminate potential genomic DNA contamination, the isolated RNA samples were treated with RNase-free DNase I (Thermo Fisher Scientific, EN0525).

To assess the presence of chemoreceptor-, chemosensor-, and neurotransmission-related genes in Varroa RNA, primers were designed using reference mRNA sequences obtained from NCBI and the FastPCR Professional v6.1.2 software (**24; Table 1**). Primer sets were evaluated for potential hairpins or dimers using the same program, and only those free of secondary structures were selected for real-time PCR amplification.

Table 1. Primer sequences, product size, annealing temparure (Ta), and NCBI numbers

Transcript (Function)	Primer Sequence (5′-3′)	Product Size (bp)	Ta (°C)	NCBI No.
Varroa PRTF-like gene (Chemosensing gene)	F: CCTGCTCTTACTGATGCGTTCC R: AATCGTCCACTGTGGCGTTAGC	135	62	LC060528.1
Ionotropic glutamate receptors (IGRs)- IR25a-like (Chemoreceptor gene)	F: CGCATTCATTGCTGATGCGAC R: AGCTTCAGAATGGCGCTTGAA	156	54	MF069507.1
GABA activated RDL receptors (Neurotransmitter gene)	F: AGTATCAGCGCCGTCTCCGAA R: TTCTGCGCCTACGGTCATCTGAC	129	62	KY748053.1
40S ribosomal protein S18 (Housekeeping gene)	F: ACGAAGGTCATGTATGCCCTCAC R: CTTCCTGTTCCGACAGCTCAC	121	62	XM_022831401.1
Heat shock protein 83-like (Housekeeping gene)	F: TCTGTTGAAGTGGCATACGCA R: CGGATCTTATCGAGAGCATCG	182	62	XM_022830434.1
NADH dehydrogenase [ubiquinone] flavoprotein 1 (Housekeeping gene)	F: CAATCTGTCGCCGTGGTGGAGC R: CACCCGGAATCACGCACAGCA	204	62	XM_022837623.1

The RNA samples were immediately used to cDNA synthesis using the VitaScript™ FirstStrand cDNA Synthesis Kit (Procomcure Biotech GmbH, PCCSKU1301). A total of 2 µg of RNA was brought to 15 µL with DEPC-treated, RNase-free water. The reaction mixture consisted of 4 µL 5× VS reaction buffer (containing $MgCl_2$, dNTP mix, random primers and oligo-dTs), 1 µL of VitaScript Enzyme Mix (contains VitaScript Reverse Transcriptase and RNase inhibitor blend), and 1 µg of total RNA. The final reaction volume was adjusted to 20 µL with RNase-free, DEPC-treated water cDNA synthesis was performed by incubating the reaction at 42°C for 60 min, followed by enzyme inactivation at 80°C for 10 min. Synthesized cDNA samples were stored at −20°C until use in real-time PCR.

For gene expression analyses, each Varroa mite was processed individually. For each treatment and control groups, 15 adult female mites were used as independent biological replicates (n = 15). RNA extraction and cDNA synthesis were performed separately for each individual sample. During real-time PCR, each biological replicate was analyzed in triplicate as technical replicates.

Real-time PCR for Gene Expression Analysis

Optimal annealing temperatures (Ta, °C) for each primer pair were determined using gradient PCR. A binding temperature of 54°C was used for the IR25a-like gene, while 62°C was used for *NADH*, *PRTF-like*, *40S*, *GABA*, and *HSP83* genes (**Table 1**).

Diluted cDNA was used for real-time PCR analyses to allow equal comparison of expression levels. First, to establish the optimal cDNA dilution and PCR efficiency cDNA was diluted from 1:2 then finally 1:8 was chosen for analysis.

The PCR efficiency of each primer set was validated before the experiments. HSP83-like, 40S ribosomal protein, and NADH dehydrogenase genes were used as the initial set of reference genes based on their stability in *Apis mellifera* in response to varying experimental conditions as reported in a previous study (**25**). NADH dehydrogenase was used as the most appropriate reference gene in the RT-qPCR reaction based on the efficiency of the primer pair in amplification as well as the stability of the gene in the experimental treatments. Amplicon sizes for IR25a-like, PRTF-like, GABA, and NADH primer sets were validated by agarose gel electrophoresis.

Real-time PCR reactions were performed using 2X Magic SYBR Mix (Procomcure Biotech GmbH, PCCSKU1107). For each 10 µL PCR, 2 µL cDNA diluted 1:8, 5 µL of SYBR Green Master Mix, 0.125 µL of forward primer (5 µM), and 0.125 µL of reverse primer (5 µM) were added. The final reaction volume was adjusted to 10 µL using RNase-free water. The reactions were programmed as follows: 1 cycle of an initial denaturation at 95°C for 5 min,

40 cycles of PCR at 95°C for 10 s, 62°C (except IR25a-like gene) for 30 s, and 72°C for 30 s, 1 cycle of melting curve analysis at 95°C for 15 s, 60°C for 1 min, and 95°C for 2 s, and 1 cycle of cooling at 40°C for 10 min. Reactions were performed using a LightCycler 480 II real-time PCR system (Roche, Switzerland). Melting curve analysis was performed for each sample and gene region to verify specificity and confirm the absence of primer dimers or genomic DNA contamination. Real-time PCR reactions were performed in triplicate for each biological sample.

Statistical Analysis

Mortality rates of the Varroa mites (*V. destructor*) were tested at varying concentrations (0.1, 1, 10 ppm) of thyme (Origanum onites L.) essential oil, a negative control, and a solvent control. Three different feeding contexts were tested: G.A: Larvae, G.B: Fat body, and G.C: Live honey bees. The survival rates of the mites were recorded at 10 min, 30 min, 1 h, and 24 h after treatment. The experiment was conducted in triplicate with 15 mites per replicate.

Mean survival proportions along with the standard error (±SE) were calculated. Since the survival data are not normal due to the binary nature of the data (0 = dead, 1 = alive), a Generalized Linear Model (GLM) was used to analyze the data. A logistic regression model was fitted to the data. In this model, the independent variables are the dose of the essential oil, the feeding context, time, and the interactions between them. The dependent variable is the survival proportion. Since the survival data are in a binary form, a binomial distribution along with a logistic link function was used.

For gene expression analysis, two-way ANOVA was conducted to evaluate the main effect and interaction between factors in every target gene (GABA, PRTF, and IR25a-like) based on treatment (TEOV vs. NG) and time (10 min, 30 min, 1 h, 24 h). Tukey's HSD post hoc tests were used to assess mortality times and compare all possible pairs of treatments. Varroa mite survival rates were analyzed by the Student's t-test to calculate significant differences among groups. Python (v3.8) was used to perform all statistical analyses.

Fold increase or decrease in mRNA expression was calculated using the 2^−ΔΔCt method in the REST2000 software package (**26**). The thyme essential oil was dissolved in ethanol; thus, an ethanol treatment group served as a control in these experiments. Means and standard errors (SEM) were calculated in each experiment, where gene expression levels in each group of Bee Varroas in the test groups over time points are calculated. Heatmaps are used to represent gene expression using Seaborn in Python (v3.8).

An analysis of probit was conducted to determine the median lethal time (LT_{50}) for Varroa mites exposed to thyme essential oil, as well as honey bees (*Apis mellifera*). Mortality rates were observed for Varroa mites and honey bees at 4-time intervals:10 min, 30 min, 1 h, and 24 h (n = 15 for both species).

A generalized linear model with a probit linkage function was constructed using maximum likelihood estimation to obtain LT_{50} values. The selectivity ratio (SR) was calculated as the ratio of honey bee LT_{50} to Varroa LT_{50}, and represented the treatment's relative safety for bees (**27**). Analyses were performed in Python using the stats model's library (v0.14.0). The convergence and goodness-of-fit of the model were examined to obtain reliable estimates.

RESULTS

Chemical Composition of Thyme Essential Oil

The chemical composition of the thyme (*Origanum onites* L.) essential oil used in the bioassays is presented in **Table 2**. Carvacrol was identified as the predominant component.

Table 2. Main components of thyme (*Origanum onites* L.), used as acaraside against *Varroa destructor*

Main Components for Thyme	%
Linalool	0.58
terpinen-4-ol	0.72
beta-caryophyllene	0.54
Borneol	1.71
alpha-thujene	1.27
alpha-pinene	0.54
camphene	0.31
beta-myrcene	1.54
cis-sabinene hydrate	0.90
thymol	6.06
carvacrol	74.80
beta-bisabolene	1.20
alpha-terpinene	1.02
p-cymene	3.74
limonene	0.37
gamma-terpinene	4.28
Total	99.45

Survival Rate of Varroa mites Treated with Thyme Essential Oil Over Time Across Feeding Types

Changes in the survival rates of Varroa (*V. destructor*) mites over time showed significant differences between the treatment, dose, and feeding context (**Figure 1**). A Generalized Linear Model was used to confirm that time was an important predictor of mortality: compared to the initial baseline of 10 min, survival declined significantly over time, reaching an all-time low at 24 h ($z = 9.25$, $p < 0.001$). During the initial exposure periods (10 min to 1 hour), survival was found to be excessively high in almost all groups, indicating that in these periods, exposure does not cause mortality. A noticeable divergence in survival was first apparent after the 24 h time point.

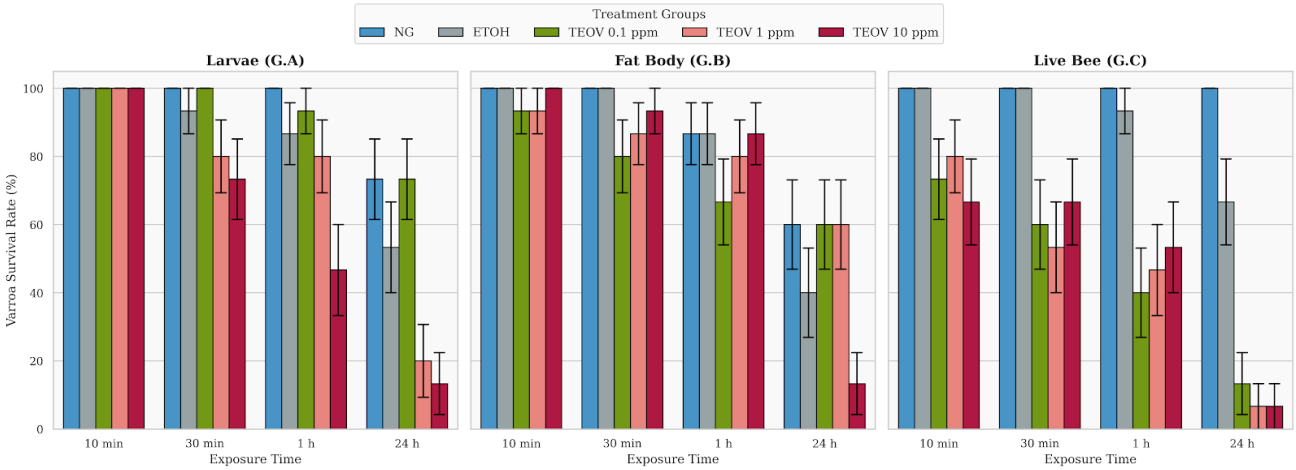

Figure 1. Time and dose dependent survival rates of *Varroa destructor* mites after being exposed to thyme (*Origanum onites* L.) essential oil at varying levels of feeding. Bar graphs are provided to demonstrate the average survival rates of mites after being exposed to thyme essential oil at varying levels of feeding. In the three bar graphs represents the type of food provided to the mites: honey bee larvae (G.A), fat body tissue (G.B), and live honey bee adults (G.C). The x-axis represents the time interval at which the mites are exposed to thyme essential oil: 10 min, 30 min, 1 h, and 24 h. The y-axis respresents the Varroa survival rate (%). The type of treatment is represented differently in terms of color: NG (Negative Control) in blue, ETOH (Solvent/Ethanol) in gray, and 0.1 ppm, 1.0 ppm, and 10.0 ppm thyme essential oil in green, light red, and dark red, respectively. The error bar represents the standard error of the mean (SEM). The statistical significance was determined through a Generalized Linear Model (GLM) binomial error distribution with a logit link function. Sample size was n = 15 mites per dose–time–feeding group combination

In the negative control group (NG), the survival of the mites remained strong even after 24 h, averaging 77.8% in total (100% survival in the live honey bee feeding group, G.C.). The effect of the ~22.2% background mortality in the NG group is consistent with the widely accepted in vitro bioassay for Varroa mites and can be accounted for by the natural environmental and nutritional stress of isolating obligate parasites from their colony micro-environment (**25**). In the solvent control group (ETOH), the survival rate was moderately reduced to an average of 53.3% after 24 h. The 24.5% absolute difference in survival between the NG and ETOH groups represents direct experimental evidence that the solvent component of ethanol inherently contributes to background toxicity, independent of essential oil exposure. Although the background mortality rate is low, the long-term exposure to the ethanol solvent component results in a base-level of handling stress.

In sharp contrast, the survival of Varroa was significantly and dose- and time-dependently reduced by the thyme essential oil (TEOV). The highest dose, 10 ppm TEOV, significantly increased total mortality compared to the 0.1 ppm baseline ($z = 2.64$, $P = 0.008$). At 24 h, this dose aggressively reduced the mean Varroa survival rate to 11.1%. At this high dose, Varroa survival was reduced to 13.3% in both G.A, where larvae were present, and G.B, where fat body was present, and to a critical minimum of 6.7% where mites fed on live honey bees (G.C). Pairwise post-hoc tests at 24 hours confirmed that this 10 ppm dose was significantly more deadly than the 0.1 ppm dose ($P = 0.0003$).

On the other hand, the lower concentrations of TEOV produced moderate results after 24 hours. The 1 ppm and 0.1 ppm doses produced overall average survival rates of 28.9% and 48.9%, respectively. It is noteworthy that the biological interface played a part in this phenomenon; the GLM showed a significantly higher baseline susceptibility to treatments in mites feeding on live honey bees (G.C) compared to mites feeding on larvae (G.A) ($z = 5.66$, $P < 0.001$). Further post-hoc tests at 24 h confirmed this phenomenon; survival in mites feeding on live honey bees was significantly lower than in both larvae (Bonferroni-adjusted p = 0.007) and fat body (Bonferroni-adjusted p = 0.0004) contexts. This phenomenon strongly supports the conclusion that the 10 ppm dose of TEOV has a highly potent acaricidal effect by 24 h, far surpassing natural levels of mortality and solvent-induced stress in the control groups.

Varroa Mite Mortality Treated with Thyme Over Time Across Feeding Types

Generalized Linear Model (GLM) with binomial error structure revealed that time of exposure, dose of essential oil, and feeding conditions were significant predictors of Varroa mortality (**Table 3**). Compared to the baseline of 10 minutes, mite mortality significantly increased with time, reaching a peak at 24 h ($z = 9.25$, $P < 0.001$). In addition, the 10 ppm dose of thyme essential oil was a significant predictor of increased mite mortality ($z = 2.64$, $P = 0.008$) compared to the baseline of 0.1 ppm, whereas the intermediate 1.0 ppm dose had no significant effect ($P = 0.176$). Notably, mites feeding on live honey bees (G.C) had a significantly higher susceptibility ($z = 5.65$, $P < 0.001$) than those feeding on larvae (G.A). The fat body (G.B) context showed no difference from G.A ($P = 0.482$).

In order to determine the actual strength of these effects and to establish which groups differ significantly at the critical 24-h exposure interval, Estimated Marginal Means were calculated and then subjected to subsequent post-hoc analyses for pairwise group comparisons with Bonferroni correction (**Tables 4 and 5**). In terms of the essential oil

doses used in the experiment, it was found that Estimated Marginal Means demonstrated a dose-response effect in survival rates. The survival rate of mites decreased from an estimated 48.89% (±7.45 SE) for the lowest dose used in the experiment (0.1 ppm) to 11.11% (±4.68 SE) for the highest dose used (10.0 ppm). The dramatic reduction in survival rates was found to be highly statistically significant through subsequent post-hoc testing, where it was established that 10.0 ppm was an extremely effective acaricide. The survival rate for mites exposed to an intermediate dose of 1.0 ppm was found not to differ statistically from either extreme of 0.1 ppm or 10.0 ppm, with adjusted p-values of 0.1550 and 0.1050 respectively.

Table 3. Summary of the Generalized Linear Model (GLM) results evaluating the main effects of exposure time, dose, and feeding context on *Varroa destructor* mortality

Factor / Variant	Coefficient	Standard Error	z-value	*P*-value	Direction of Effect
Time: 30 min	+1.0502	0.367	2.862	0.004 **	Increases mortality
Time: 1 h	+1.6884	0.357	4.728	< 0.001 ***	Increases mortality
Time: 24 h	+3.4486	0.373	9.254	< 0.001 ***	Strongly increases mortality
Dose: 1.0 ppm	+0.3693	0.273	1.354	0.176 (n.s.)	Marginal effect
Dose: 10.0 ppm	+0.7149	0.271	2.637	0.008 **	Increases mortality
Feeding: G.B (Fat body)	-0.1983	0.282	-0.703	0.482 (n.s.)	No difference with G.A
Feeding: G.C (Live Honey Bee)	+1.5413	0.273	5.655	< 0.001 ***	Strongly increases mortality

The model applies a binomial error distribution with a logit link function. The baseline reference levels for the model are: Time: 10 min, Dose: 0.1 ppm, and Feeding Context: G.A (Larvae). A positive coefficient estimate (β) indicates a higher probability of mite mortality compared to the baseline. Statistical significance: ns = not significant ($p > 0.05$); ** $p < 0.01$; *** $p < 0.001$

Table 4. Estimated Marginal Means (EMMs) of *Varroa destructor* survival rates at the 24-h exposure interval across TEOV doses and feeding contexts

Factor	Group / Level	Estimated Mean Survival (%)	95% Confidence Interval (%)
TEOV Dose	0.1 ppm	48.89±7.45	34.28 – 63.50
	1.0 ppm	28.89±6.76	15.64 – 42.14
	10.0 ppm	11.11±4.68	1.93 – 20.29
Feeding Context	G.A (Larvae)	35.56±7.14	21.56 – 49.55
	G.B (Fat Body)	44.44±7.14	29.92 – 58.97
	G.C (Live Bee)	8.890±4.24	0.57 – 17.21

The estimated marginal means (EMMs) and standard errors (SE) are based on the proportions observed at the 24-hour mark from the thyme essential oil (TEOV) treatment groups

Table 5. Post-hoc pairwise comparisons of *Varroa destructor* mortality at the 24-h exposure interval using Estimated Marginal Means (EMMs)

TEOV Dose Comparisons (at 24 hours)	Adjusted p-value (Bonferroni)	Significance	Conclusion
0.1 ppm vs. 1.0 ppm	0.1550	ns	No significant difference
0.1 ppm vs. 10.0 ppm	0.0003	***	10.0 ppm induces significantly higher mortality
1.0 ppm vs. 10.0 ppm	0.1050	ns	No significant difference
G.A (Larvae) vs. G.B (Fat Body)	1.0000	ns	No significant difference
G.A (Larvae) vs. G.C (Live Bee)	0.0070	**	Mites on G.C are significantly more susceptible
G.B (Fat Body) vs. G.C (Live Bee)	0.0004	***	Mites on G.C are significantly more susceptible

For the pairwise comparison, it was specifically done at the 24-h time point, where the difference in mortality rates was most apparent. P-values were adjusted for multiple testing using the Bonferroni correction to ensure strict control over Type 1 error rates. Statistical significance: ns = not significant ($P > 0.05$); ** $P < 0.01$; *** $P < 0.001$

The biological interface also revealed a significant impact on the susceptibility of the mites. With regard to the assessment of the feeding context, the group of mites exposed to TEOV while feeding on live honey bees (G.C) recorded a particularly low estimated survival rate of merely 8.89% (±4.24 SE). In contrast, the group of mites exposed to TEOV while feeding on larval (G.A) and fat body (G.B) substrates recorded relatively higher estimated survival rates of 35.56% (±7.14 SE) and 44.44% (±7.41 SE), respectively (**Table 4**). A pairwise comparison confirmed the group of mites exposed to TEOV while feeding on live honey bees (G.C) to be particularly more susceptible to the treatment compared to the group of mites exposed to TEOV while feeding on larval (adjusted $P = 0.0070$) and fat body substrates (adjusted $P = 0.0004$). Moreover, the low difference in the survival rates between the two groups of mites exposed to TEOV while feeding on larval and fat body substrates did not reach statistical significance (adjusted $P = 1.0000$) (**Table 5**).

Time-Dependent Effects of Thyme on *GABA*, *PRTF*, and *IR25a-like* Gene Expression in Varroa Mites

Two-way ANOVA did not reveal significant main effects of EO treatment or exposure time on *GABA* expression ($P > 0.3$). However, a significant effect of feeding contexts was detected ($F = 7.68$, $df = 2$, $P = 0.03$), indicating that variation

in *GABA* transcript levels was more strongly influenced by the feeding interface than by the treatment itself or the duration of exposure. Tukey's HSD post hoc tests did not identify significant pairwise differences among feeding groups (**Table 6**).

For *IR25a-like*, EO treatment produced a modest but statistically significant main effect (F = 5.31, *P* = 0.044), whereas neither time nor the interaction term was significant (*P* > 0.2). This suggests a general downregulatory effect of thyme essential oil on *IR25a-like* expression, although no strong temporal trends were observed. Tukey's post hoc test results confirmed that no significant result was obtained from the analysis (**Table 6**).

Tablo 6. Analysis of variance (ANOVA) for the effects of Thyme essential oil treatment (EO), exposure time, and their interactions with feeding group on the *GABA*, *PRTF* and *IR25a-like* genes in *Varroa destructor*

Gene	Factor	Effect	Sum of Squares	df	F-value	*P*-value
GABA	EO × Time	EO	148,312	1	0,9841	0,344591
GABA	EO × Time	Time	18,8263	3	0,0416	0,987996
GABA	EO × Time	EO:Time	453,6936	3	1,0034	0,430947
GABA	Feeding group × EO	Feeding group	702,5334	2	3,9067	0,05225
GABA	Feeding group × EO	EO	0,102	1	0,0011	0,973735
GABA	Feeding group × EO	Feeding group:EO	281,6504	2	1,5662	0,252036
GABA	Feeding group × Time	Feeding group	738,5257	2	7,6809	0,02988
GABA	Feeding group × Time	Time	260,8634	3	1,8087	0,262312
GABA	Feeding group × Time	Feeding group:Time	769,5626	6	2,6679	0,15049
IR25a-like	EO × Time	EO	713,8426	1	5,3147	0,043852
IR25a-like	EO × Time	Time	721,1388	3	1,7897	0,212609
IR25a-like	EO × Time	EO:Time	681,4888	3	1,6913	0,231433
IR25a-like	Feeding group × EO	Feeding group	184,0158	2	0,4283	0,662049
IR25a-like	Feeding group × EO	EO	459,7364	1	2,14	0,171485
IR25a-like	Feeding group × EO	Feeding group:EO	51,4845	2	0,1198	0,888219
IR25a-like	Feeding group × Time	Feeding group	201,7998	2	0,2613	0,779982
IR25a-like	Feeding group × Time	Time	352,3922	3	0,3041	0,821946
IR25a-like	Feeding_group × Time	Feeding group:Time	590,942	6	0,255	0,936751
PRTF	EO × Time	EO	4231,847	1	4,0491	0,071905
PRTF	EO × Time	Time	1324,26	3	0,4224	0,74111
PRTF	EO × Time	EO:Time	1732,968	3	0,5527	0,657827
PRTF	Feeding group × EO	Feeding group	4114,914	2	3,2541	0,077595
PRTF	Feeding group × EO	EO	5757,556	1	9,1062	0,011705
PRTF	Feeding group × EO	Feeding group:EO	1125,607	2	0,8901	0,438217
PRTF	Feeding group × Time	Feeding group	2969,692	2	0,9107	0,459992
PRTF	Feeding group × Time	Time	457,9222	3	0,0936	0,960332
PRTF	Feeding group × Time	Feeding group:Time	5227,698	6	0,5344	0,766356

For *PRTF*, no significant EO and time effect was found (p = 0.072), and time alone has no significant effect (*P* = 0.74). In contrast, EO treatment showed a significant main effect across feeding groups (F = 9.11, *P* = 0.012), indicating that *PRTF* expression was influenced by essential oil exposure in a manner dependent on the feeding contexts. However, pairwise post hoc comparisons did not reveal significant differences among specific treatment–time combinations (**Table 6**).

When the results are seen holistically, a conclusion could be drawn that the thyme essential oil was selecting certain genes in the *Varroa destructor* genes. *GABA* expression was dependent on feeding groups rather than treatment or time, *PRTF* responded to EO exposure in a feeding group, and *IR25a-like* displayed a limited, steady effect after TEOV treatment. Overall, the transcriptomic effects of thyme essential oil are seen as subtle based on the statistical findings, where biological variation in the groups of mites that fed is thought to be responsible.

A heatmap analysis was performed to compare expression levels of *GABA, PRTF*, and *IR25a-like* in *Varroa destructor* mites exposed to thyme essential oil (TEOV) versus the no-treatment control (NG). Across all feeding groups (G.A, G.B, G.C) and time points, gene expression levels were consistently higher in the TEOV-treated mites compared to the NG group (**Figure 2**).

The heatmap (Figure 2) illustrates clear gene- and feeding group–specific transcriptional responses following TEOV exposure, although the strength of statistical support varied across genes.

For *GABA*, although numerical values varied, ANOVA results indicated that there were no significant main effects of TEOV treatment (F = 0.98, *P* = 0.344) or exposure time (*P* = 0.988). In the G.A group, the relative expression value decreased sharply from 34.06 at 10 min to 2.05 at 30 min (a 94% reduction; fold-change 0.06), partially recovered at 1 h (10.82), and declined again by 24 h (4.90). In G.B, the levels remained low throughout the time course, from 3.06 at 10 min to 0.20 at 24 hours. G.C had delayed induction, with levels peaking at 24.04 at 1 hour, then dropping to 0.46 at 24 hours. In the NG groups, the levels of *GABA* remained low, except for G.A, which had increased levels at 24 hours, peaking at 26.72. Most importantly, ANOVA test revealed that only the feeding group itself was significant in the expression variation (F = 7.68, *P* = 0.030), which suggests that the *GABA* transcription in this timeframe is primarily controlled by the biological interface and not the essential oil treatment.

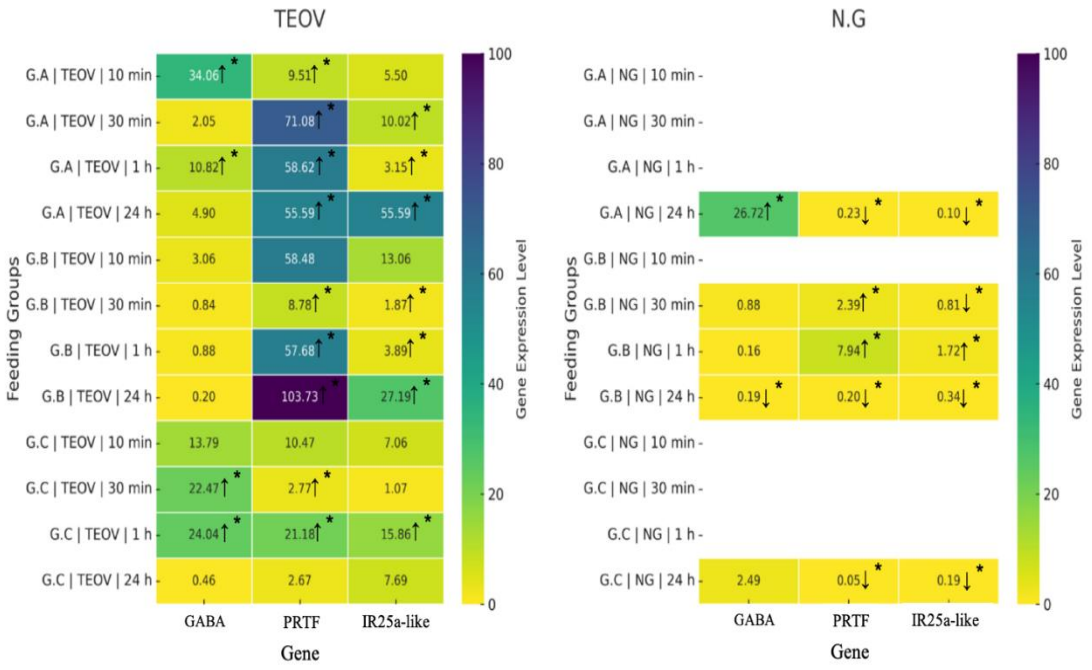

Figure 2. Gene expression levels of *GABA, PRTF,* and *IR25a-like* in *Varroa destructor* mites treated with Thymol (*Origanum onites* L.) essential oil (TEOV) after different feeding groups (G.A, G.B, G.C) and time points (10 min, 30 min, 1 h, and 24 h). *GABA*; *GABA-RDL* (neurotransmitter-related), *PRTF*; *PRTF-like* (detoxification or developmental signaling), and *IR; IR25a-like* (chemosensory receptor-related). The results are expressed as log2 fold change values compared to the control group. Gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method and was normalized to the internal reference gene [*NADH*]. The color gradient represents the degree of change, where positive values (warm colors) represent an upregulation and negative values (cool colors) represent a downregulation of gene expression. Asterisks (*) indicate statistically significant differences ($p < 0.05$) using Two-Way ANOVA. For these significant alterations, upward (↑) and downward (↓) arrows explicitly indicate the direction of regulation (upregulation and downregulation, respectively

In contrast, *PRTF* had the strongest and most significant response to the treatment. TEOV treatment had the highest significant main effect on *PRTF* expression, which was highly significant (F = 9.11, *P* = 0.012). Expression of G.A increased from 9.51 at 10 min to 71.08 at 30 min (7.5-fold increase), remained high at 1 h (58.62), and 24 h (55.59). For G.B, expression was reduced at 30 min (8.78) but rapidly recovered and peaked to 57.68 at 1 h and reached its peak at 24 h (103.73). By contrast, this response was much weaker in the G.C group; it showed only a slight increase at 1 h (21.18) before falling by 24 h (2.67). In turn, the NG groups remained at low values throughout (<8). In addition to this, ANOVA was used to confirm the significance of the interaction between the feeding group and the EO treatment (*P* = 0.021), which validated the fact that *PRTF* is indeed upregulated by TEOV; however, the degree to which it is upregulated is largely dependent on the context of the feeding.

For the *IR25a-like* gene, ANOVA results did confirm a small but significant main effect of the TEOV treatment (F = 5.31, *P* = 0.044) compared to the low NG groups (<2 Although these numeric values tended to increase over time for each of the feeding groups by the 24th hour, with values of 55.59 for G.A, 27.19 for G.B, and 7.69 for G.C, no statistically significant main effect was found for time (*P* = 0.212) or for the feeding group (*P* = 0.662), according to the ANOVA test. Thus, while TEOV causes a statistically significant elevation of *IR25a-like* expression levels when compared to untreated mites, no statistically significant variation was found for individual time points or for the individual feeding.

Taken together, these results show that TEOV triggers clear, yet distinct, genetic responses in *Varroa destructor*. *PRTF* expression is significantly and reliably upregulated in a context-dependent manner by the treatment. *IR25a-like* expression is also observed to have a significant overall elevation in response to TEOV. On the other hand, the expression of *GABA* is observed to be statistically unaffected by the essential oil. Therefore, it can be concluded that any interruption in the *GABA* system is likely to be post-transcriptional in nature.

Toxicity of Thyme on Varroa mites and Honey Bees

Probit analysis on time and mortality rates revealed a significant difference in LT_{50} values between *Varroa destructor* and *Apis mellifera* exposed to the highest dose of

thyme essential oil (10 ppm). The estimated LT_{50} was 79.1 minutes for Varroa mites and 1346.9 minutes for honey bees. The selectivity ratio (SR = LT_{50}_bee / LT_{50}_Varroa) was 17.0 (**Table 7**). These results indicate that under the tested conditions, honey bees would need almost 17 times longer exposure time compared to Varroa mites to reach a 50% mortality rate.

Table 7. Estimated LT_{50} values for *Apis mellifera* and *Varroa destructor* exposed to thyme (*Origanum onites* L.) essential oil

Insect	LT_{50} (min)	95% CI Lower (min)	95% CI Upper (min)
Honey bee (*A. mellifera*)	1346.87	–6,465,704.64	6,468,398.37
Varroa mite (*V. destructor*)	79.10	–59.39	217.58

DISCUSSION

This study, supported by both survival experiments and gene expression analyses, demonstrates that carvacrol-rich thyme (*Origanum onites* L.) essential oil (EO) can be an effective acaricide in the control of *Varroa destructor*. The survival rate of the Varroa mites was heavily dependent on the dose, time, and the feeding group of the mites (larvae, fat body, or live bees). The most profound acaricidal effect was observed at the 24th hour mark. In the case of the larvae feeding group, the survival rate was significantly reduced at the highest dose of 10.0 ppm. This observation agrees with the literature that the larvae have a higher lipid content than the adults, which can affect the absorption and distribution of the acaricide (**1**). It was observed that while the mites feeding on the fat body tissue group had a similar baseline tolerance compared to the larvae group, the mites feeding on the live honey bees had the highest susceptibility to the essential oil. In the live bee group, the overall survival rate was observed to plummet to as low as 9% at the 24th hour mark.

Additionally, our control group results could clearly identify and separate the variables affecting in vitro baseline mortality. The 77.8% survival rate of our negative control group (NG) at 24h clearly illustrates the background mortality rate due to handling and ex vivo environmental stress (**27**). It is noteworthy that the pronounced decline in survival rate of 53.3% in our ethanol control group (ETOH) clearly illustrates the mild toxicological effects of ethanol itself in a 24h continuous test. The acaricidal efficacy of 10 ppm TEOV, which strongly reduced survival rates to ~11%, far exceeded background stress effects caused by handling and solvent stress.

Gene expression profiling is an essential component in understanding the dynamics of the toxicity of carvacrol-rich essential oil. Heat map clustering, facilitated through proper ANOVA modeling, is important in understanding the intricacy of the feeding group differences. It is pertinent to note that, despite the fact that essential oils such as TEOV are well-documented for their intrinsic neurotoxicity, our statistical analysis revealed the complete absence of a treatment main effect on the transcription of the *GABA* receptor gene. The absence of a transcriptional response, combined with the high mortality rates at 10.0 ppm, suggests that the acaricidal efficacy of TEOV on the GABAergic system is likely to be independent of transcriptional mechanisms within the first 24-hour window. Rather, it is highly likely that the rapid neurotoxicity of carvacrol occurs through post-translational mechanisms, such as direct allosteric modulation of *GABA* receptor proteins, leading to immediate overstimulation and paralysis.

In contrast to *GABA*, a strong and persistent upregulation of the *PRTF* gene was found, especially in mites that fed on larvae and fat bodies. Although the precise function of *PRTF* in arthropod detoxification has not been completely elucidated, it is probably a regulatory sequence that orchestrates the defense strategy, which includes the management of oxidative stress and the activation of the cytochrome P450 pathway. The statistical analysis confirmed the significant main effect of the essential oil treatment on *PRTF* expression (P = 0.012), indicating that the strong upregulation of this gene is a consequence of the activation of a general cellular stress and detoxification response to carvacrol toxicity. The fact that there is a strong expression of *PRTF* despite the high mortality rate clearly indicates that the adaptive defense strategy of Varroa is ultimately inefficient against the toxic potential of TEOV. In addition, the *IR25a-like* gene showed a slower and more gradual response. The mild but significant overall effect of the EO treatment (P = 0.044) indicates that the *IR25a-like* gene is not a direct detoxification gene but rather a chemosensory or adaptation gene that is activated during TEOV exposure.

The low expression levels of these genes within the unexposed negative control (NG) groups confirm that these gene expression changes are specifically induced by exposure to TEOV. Furthermore, whereas the universal effect of the treatment was evident by the survival experiments, the gene expression changes were significantly influenced by the feeding group. This underlines an important methodological truth: the biological interface of the mite during feeding determines not just its inherent sensitivity to acaricide but also which specific defense pathways are activated.

The synthesis of the mortality and gene expression data provides insight into the complex mode of action of carvacrol within thyme oil on *Varroa destructor*. The rapid mortality rates induced by the high doses of thyme oil, combined with the absence of *GABA* gene expression changes, strongly suggest acute post-transcriptional neurotoxicity. At the same time, the differential upregulation of *PRTF* and *IR25a-like* gene expression points to the activation of secondary cellular stress and adaptation responses. This provides new insights into the determinants of successful treatment outcomes on the molecular level, including the influence of nutrition while confirming the established acaricidal efficacy of monoterpenoid phenolics like thymol and carvacrol (**6, 8, 27–31**).

Finally, the calculated selectivity ratio (SR = 17.0) would indicate a quite broad safety margin of honey bees in this

application. However, as bees and mites coexisted in the experimental environment, it can be assumed that behavioral interactions have likely biased the results. Future studies should include separate exposure systems and colony-level validation studies under field conditions. Potential for improving the utility of mite feeding behavior in optimizing the delivery of this mode of action and further enhancing the sustainability and utility within Integrated Pest Management (IPM) programs for carvacrol-containing plant acaricides.

These findings confirm that thyme essential oil rich in carvacrol exhibits potent acaricidal activity against *Varroa destructor* and are supported at both the survival and gene expression levels. The peak mortality rates in either larvae or fat body feeding groups emphasize how the feeding interface and duration of exposure are critical in defining treatment efficacy.

At the molecular level, carvacrol targets cellular stress response and chemosensory pathways. The lack of a prominent transcriptional response in the *GABA-RDL* gene, despite its acute acaricidal efficacy, suggests that the interaction with the inhibitory nervous system is a post-translational rapid event at the protein level rather than at the transcriptional level. On the other hand, the significant upregulation of the PRTF in both larval and fat body feeding groups indicates the strong activation of detoxification systems, and the overall upregulation of the IR25a-like gene indicates the mite's effort to activate an adaptive response based on sensory inputs.

These transcriptional changes have revealed that carvacrol does not act only through an acute toxic mode of action but is multifunctionally disrupting the mite's physiology. These results indicate that treatments based on carvacrol can reduce the infestation of Varroa mite in honey bees within a safe margin. Future work should focus on optimizing delivery systems, testing exposure routes under realistic colony conditions, and validating gene-level responses in field applications to strengthen the role of plant-derived essential oils in integrated pest management programs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, Data Curation, Investigation, Resources: T.O., M.E.; Formal Analysis: T.O., M.E., M.D.; Funding Acquisition: T.O.; Methodology: T.O., M.E., M.D., U.K.; Supervision: M.E.; Writing – Original Draft: T.O.; Writing – Review & Editing: T.O., M.E., M.D., U.K. All authors have read and approved the final version of the manuscript.

ARTIFICIAL INTELLIGENT DECLARATION

The authors declare that they are responsible for the accuracy and integrity of all content of the manuscript, including part generated by AI, and it is not used as a co-author.

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