

The effects of plants extracts of thyme (*Thymus vulgaris*) and lavender (*Lavandula angustifolia*) on *Varroa destructor* (Acarina: Varroidae) and *Nosema ceranae* (Dissociodihaplophasida: Nosematidae) in adult worker honey bees (*Apis mellifera*)

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Research Article

Keywords: Honey bee, Thyme, Lavender, *Varroa destructor*, *Nosema ceranae*

Posted Date: May 31st, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4407998/v1>

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Running title: Effects of plants extracts on *Varroa destructor* and *Nosema ceranae*

Authors' Contributions:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Keikhosrow Sarhangzadeh, Mohammad Yakhchali and Mostafa Moradi, Keikhosrow Sarhangzadeh and Mohammad Yakhchali, and Keikhosrow Sarhangzadeh and Mohammad Yakhchali, respectively. The first draft of the manuscript was written by Keikhosrow Sarhangzadeh and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Statements and Declarations

The authors declare that there is no conflict of interest.

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Ethics Approval

Institutional ethics committee of Urmia Faculty of Veterinary Medicine, Iran was approved the study. The study was also performed in accordance with the ethical standards.

The effects of plants extracts of thyme (*Thymus vulgaris*) and lavender (*Lavandula angustifolia*) on *Varroa destructor* (Acarina: Varroidae) and *Nosema ceranae* (Dissociodihaplophasida: Nosematidae) in adult worker honey bees (*Apis mellifera*)

Abstract

Varroa destructor is one of the important mites of the Western honey bees in Iran and throughout the world. *Nosema ceranae* is also cause of decreasing honey bee colony population. Herbal extracts are applicable replacement for chemical compounds in order to control bee mites and microsporidian agents. The aim of this study was to determine effects of hydro-alcoholic extracts of thyme and lavender in infected adult worker bees with *V. destructor* and *N. ceranae*. Hydro-alcoholic extracts and doses consisting of 2% thyme (2g/100ml) and 2% lavender (2g/100ml) were prepared and employed in treatment groups with three replicates. Dead mites were collected at 12, 24, 36, 48, and 72 hours and efficacy was measured. Twenty examined adult bees were collected at 24, 36, 48, and 72 hours and spores' reduction were determined. The highest mortality effects of plant extracts of thyme and lavender on *V. destructor* was 81% and 76% at 48h, respectively. The extracts had significant association with mortality of *V. destructor* and their efficacies were comparable to Apistan. Decreasing of number of *N. ceranae* spores was significant in comparing with each plant extracts and their efficacies were comparable to fumagilin. *Nosema* spores' reduction in bees was 4-5 fold less after extract treatment. The plant extract of thyme had higher antimicrosporidian activity against *N. ceranae* than lavender and inhibited the development of *Nosema* spores after 72h of treatment. It is concluded that the extract plants might be applicable alternative compounds in *V. destructor* and *N. ceranae* control in bees' colonies.

Keywords: Honey bee, Thyme, Lavender, *Varroa destructor*, *Nosema ceranae*

Introduction

The importance of honey bees is in pollination, increases of agricultural products and production of various products. Most of the plant and animal products needed by humans are under its influence, and with the production of honey and honeydew, royal jelly, bee venom in pharmaceuticals, wax in candle, mold, textile, and cosmetics (Sarhangzadeh and Yakhchali 2016; Ramezanipour and Yakhchali 2020). *Varroa destructor* (Acarina: Varroidae) mite is a parasitic agent with health and economic importance in honey bees (Tentcheva et al. 2004). In 1904, varroasis reported for the first from *Apis cerana* in the Java Islands of Indonesia (Ramezanipour and Yakhchali 2020). In 1964, *Varroa* infection also reported in European honey bee (*Apis mellifera* L., Apidae, Hymenoptera) (Tentcheva et al. 2004). *Varroa* entrance to Iran was first from the neighboring countries of Azerbaijan and Turkey, and spread in almost all provinces of the country (Sarhangzadeh and Yakhchali 2016). For instance, prevalence of *Varroa* infestation was 25.8% 61%, and 35.83% from East Azerbaijan province (North West of Iran), Kurdistan province (West of Iran), Urmia city (North West of Iran), respectively (Sarhangzadeh and Yakhchali 2016; Khezri and Moharami 2017; Malekifard and Yakhchali 2019).

Nosema disease is one of the most important honey bee diseases worldwide and Iran (Khuzari et al. 2018). Different species of *Nosema* (Dissociodihaplophasida: Nosematidae) is cause of nosemosis in honey bees. *Nosema apis* and *N. ceranae* are two important species when respectively reported in 1909 and 1996 (Razmaraii et al. 2013).

In recent years, transmission of *Nosema* spp. occurred from *A. cerana* to *A. mellifera* (Klee et al. 2007). Whereas honey bee mortality caused by *N. ceranae* is more compared to *N. apis* (Moeini et al. 2022). In addition, importance of *Nosema* disease in infected honey bees increases when queen bee infected with *Nosema* spp. (Paxton et al. 2007). In Iran, *N. ceranae* distribution reported molecularly for the first in 2011 (Nabian et al. 2011). *Nosema ceranae* is prevalent infection agent in Iranian honey bee farms of 17 provinces like Ilam (30%), West Azarbaijan (58.2%), and East Azarbijan (46.52%) (Mahmoudi and Nabian 2011; Mohammadian 2017; Imani Baran et al. 2021; Moeini et al. 2022).

The resistance to chemical compounds like acids and essential oils (EOS) are increasingly reported (Imdorf et al. 2003). Over one-decade consumption of acaricides like Bayvarol and Apistan, there was no acceptable reports in decreasing of *Varroa* infestation in Iranian apiaries (Pourelmi et al. 2010). For several decades, fumagillin was also an effective antibiotic to treat *Nosema* infection. Currently, its application in honey bees is prohibited in many countries because of its usage in human health and no prescription in food animals (Chaimanee et al. 2021). Thus, it was necessary to find effective herbal compounds against *Varroa* infestation and *Nosema* infection (Ramezanipour and Yakhchali 2020; Chaimanee et al. 2021). For this reason, the natural products similar to the EOS may be suitable compounds to lunch control program against *Varroa* infestation and *Nosema* infection (Damiani et al. 2009). Thyme (*Thymus vulgaris*) has anti-fungal, anti-bacterial, anthelmintic, antioxidant, and disinfectant effects and used as a syrup for digestive disorders and sore throat (Ekoh et al. 2014). Lavender (*Lavandula angustifolia*) has also analgesic, antispasmodic, relaxing, antibacterial and antiparasitic effects (Dadman et al. 2007; Denner 2009).

The beekeeping industry is currently facing with serious threats in the world (Higes et al. 2010). Treatment of honey bee parasitic diseases needs to be consider on their life cycle. Since, honey bee parasites may play important role in surviving of honey bees, colony collapse syndrome, and reproductively of queen bee (Razmaraii et al. 2013). *Varroa destructor* infestation and *Nosema* infection may be important causes of colony collapse syndrome with impact on national economy (Higes et al. 2013). Therefore, study on effects of plant extracts against *V. destructor* and *N. ceranae* are important and may be an alternative way to treat and control those (Damiani et al. 2009). Thus, the current study was undertaken to evaluate the effects of hydro-alcoholic extracts of thyme and lavender on *V. destructor* infestation and *N. ceranae* infection in adult worker honey bee colonies.

Materials and Methods

Study area and sampling condition

Of 3,200 non-migratory bee hives in different apiaries located in East Azerbaijan province, North West of Iran; 320 beehives were randomly sampled from three parts of the region, *i.e.* east (Ahar city), center (Tabriz city), and (Shabstar city) in summer 2023. A questionnaire was previously provided to record data pertaining to breeding system, number of hives in each examined apiary, nutrition, and history of previous parasitic infection, treatment, economic losses and honey production.

Parasites collection and identification

a. *Varroa*

A total number of 200 adult worker honey bees from each infected hive were randomly collected into jars containing cotton with chloroform and examined for *Varroa* infestation (Sarhangzadeh and Yakhchali 2016). The bottom of

each drawer were previously cleaned and covered with white paper. They were kept in water with a few drops of dishwashing liquid and mixed. It was left at laboratory temperature for 1 hour (h). Mites were removed and identified based on key identification described by Ilia et al. (1999).

b. *Nosema*

Nosema spores were collected according to Mussen et al. (2002). Briefly, 20 adult worker honey bees from each infected hive were removed. Thoracic and abdominal parts of each one were separated and completely crushed in sterile distilled water (SDW, 10ml) at a ratio of 1 midgut in 1 ml of water. It was filtered and centrifuged at $484 \times g$ for 5min. The clear part of the supernatant was centrifuged at $6,000 \times g$ for 30 min. The supernatant discarded and SDW (1ml) was added and mixed. A drop of the mixture was placed on a slide under a cover slip and examined microscopically at $400\times$ magnification. The intensity of *Nosema* infection in examined hives was determined using a hemocytometer cell counting slide and identified (Tunca et al. 2016; Moeini et al. 2022).

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The alcoholic extracts of Thyme and Lavender plants were analyzed to determine components of each examined extract by using Gas Chromatography-Mass Spectrometry technique (GC-MS) (Hewlett-Packard, Palo Alto, USA).

Hydro-alcoholic plant extracts preparation

Fresh thyme and lavender plants were extracted in Department of Botany, Faculty of Agriculture, Urmia University, Iran. In brief, 200gr of each plant was powdered by using electric mill and dissolved in 98% ethanol (1liter). The solution was kept at room temperature and daily shake for a week. The mixture was passed through a mesh. The extract was placed in incubator (40°C) and obtained dry plant extract. The stock concentration was provided by crushing each dried hydro-alcoholic extract and dissolved it in 20% ethanol and sugar syrup (2gr/100ml) (Moradi 2018).

Treatment and assessment procedures

a. *Varroa destructor*

Twelve naturally infested hives with *V. destructor* were selected in Gharamalek village near to Tabriz city, capital of East Azarbaijan Province. The infested hives were divided into four homogeneous groups with three replicates. Doses consisting of 2% of thyme (2g/100ml) and lavender (2g/100ml) were respectively provided to each treatment group. Two groups with spray of 2% hydro-alcoholic extracts of thyme and lavender served as treatment groups. The third group were treated with two strips of Apistan where were hanged between frames 3-4 and frames 7-8 in each hive (treated control group). The last group with sugar 1:1 (W/V) syrup spray was considered as untreated control group. Dropped dead mites were collected at 12, 24, 36, 48, and 72 hours and counted. The percentage of efficacy of each plant extract was determined based on formula described by Rasool et al. (2017) as listed below:

$$\text{Efficacy (\%)} = \frac{\text{no. of dead mites in treatment group} - \text{no. of dead mites in control group}}{\text{no. of dead mites in treatment group}} \times 100$$

b. *Nosema ceranae*

Twelve naturally infected hives with *N. ceranae* (5.361×10^6 spores/infected bee) were selected and divided into four homogeneous groups with three replicates. The extracts of 2% hydro-alcoholic thyme (2g/100ml) and lavender (2g/100ml) were respectively prepared to treatment groups. Two treated groups with spray of 2% hydro-alcoholic

extracts of thyme and lavender served as treatment groups. The third group with 1% fumagillin (2.5 gr/100 ml) was also served as treated control group. Sugar syrup was prepared as untreated control group. Twenty adult worker honey bees were collected from each examined hive at 0, 24, 36, 48, and 72 hours and reduction of number of *N. ceranae* spores counted by using cell counting slide under light microscope at magnification 400× (Khuzari et al. 2018).

Statistical evaluation

Two-way ANOVA test was used for evaluating statistical associations between obtained data using the SPSS statistical program (version 23, SPSS Inc., Chicago, IL, USA). Probability values of $p \leq 0.05$ were regarded statistically significant with confidence intervals of 95%.

Results

GC-MS analyses findings

GC-MS analyses demonstrated thyme and lavender had 36 and 23 essential components which the highest components were thymol (25.75%) and coumarin (17.04%), respectively (Table 1 and 2).

Table 1. The essential components of examined thyme (*Thymus vulgaris*).

Row	Components	RT (min)	Area (%)
1	O-cymol	6.98	1.48
2	Cineole	7.14	0.53
3	γ -terpinen	7.72	0.69
4	P-Methyl- α -methylstyrene	8.45	0.52
5	Linalool	8.67	1.87
6	Borneol	10.26	2.56
7	Thymyl methyl ether	11.75	1.13
8	Thymol	13.29	25.75
9	Isothymol	13.55	3.46
10	P-Vinylguaiaicol	13.79	0.98
11	Piperitenone	14.53	8.01
12	Syringol	14.59	0.76
13	Eugenol	14.65	0.40
14	Piperitene oxide	14.91	1.55
15	l-Caryophyllene	15.97	1.60
16	(1R)-1,6,6-Trimethyl-cis-bicyclo	16.62	14.54
17	Acetovanillone	17.44	0.52
18	Tert-butylcatechol	18.80	3.60
19	α -cadinol	20.36	0.65
20	Trans-phytol	24.24	0.60
21	Methyl butyrate	24.37	0.44
22	Cetene	25.00	0.60

23	Palmitinic acid	26.52	1.95
24	Methyl 9,12,15-octadecatrienoate	28.15	1.22
25	Denderalasin	28.58	0.52
26	Trans-10-methyl-4-ketoperhydroazul	28.65	0.88
27	α -Linolenic acid, methyl ester	28.78	1.59
28	Carvacrol	31.64	3.65
29	Cis-3a-methyl-3a,4,5,6,7,7a-hexahydro-1h-inden-1-one	32.15	0.62
30	β . monoglyceride-Palmitic acid	32.32	1.17
31	Cirsimaritin	32.59	1.01
32	Bis (2-ethylhexyl) phthalate	32.77	2.91
33	Tridecanedial acid	34.65	0.73
34	Nonanoic acid	34.79	1.11
35	Squalene	36.93	0.57
36	γ -sitosterol	54.01	2.14

Table 2. The essential components of examined lavender (*Lavandula angustifolia*).

Row	Components	RT (min)	Area (%)
1	Cineol	7.18	13.15
2	Trans-sabinene hydrate	7.97	1.71
3	Linalool	8.8	16.29
4	Camphor	9.80	4.40
5	Borneol I	10.39	11.50
6	4-Terpineol	10.59	2.91
7	Hexyl butyrate	10.77	1.85
8	α . terpineol	10.89	2.99
9	Borneol acetate	11.67	0.56
10	Linalyl acetate	12.25	2.64
11	Lavandulyl acetate	13.00	0.50
12	Thymol	13.09	1.84
13	Trans- α -bergamote	15.21	0.38
14	Caryophyllene	15.92	0.69
15	Cumarine	16.58	17.04
16	Nerol acetate	17/65	0.49
17	α -cadinol	20.33	0.77
18	Levomenol	21.11	1.94
19	4a(2H)-Naphthalenemethanol	30.76	0.79
20	Tricyclo[8.6.0.0(2,9)]	31.46	0.70
21	3-Nitrophthalic acid	32.71	0.96
22	Squalene	36.90	0.48
23	γ -sitosterol	53.66	0.43

Efficacy and assessment findings

a. *Varroa destructor*

During the course of the study, the highest mortality effects of hydro-alcoholic extracts of thyme and lavender on *V. destructor* were 81 ± 4.58 and 76 ± 6 at 48h, respectively. There was significant association between the effects of the plant extracts on mortality rate of *V. destructor* and control groups ($P < 0.001$). Mortality rate of mites had significant differences at different times ($P < 0.001$) (Table 3). The highest effects of hydro-alcoholic extracts of thyme (81%) and lavender (76%) on mortality rate of *V. destructor* at 48h was significant in comparing to treated control group of Apistan (91.33%) at 24h ($P < 0.05$) (Table 3). The plant extracts had no mortality effect on adult worker honey bees similar to Apistan. The efficacies of hydro-alcoholic extracts thyme and lavender and Apistan were 74.48%, 72.72%, and 79.30%, respectively (Table 3).

Table3. The efficacy of the hydro-alcoholic extracts of thyme and lavender on *Varroa destructor* in comparing to treated (Apistan) and untreated control groups.

Groups	Time (hours, SD* $\pm$ mean)					Efficacy (%)
	12	24	36	48	72	
Thyme	9 $\pm$ 2.65	22.67 $\pm$ 4.16	64 $\pm$ 5.29	81 $\pm$ 4.58	62.33 $\pm$ 5.13	74.48
lavender	8 $\pm$ 2	17 $\pm$ 3.61	57.33 $\pm$ 5.03	76 $\pm$ 6	65.33 $\pm$ 6.11	72.72
Apistan	52.67 $\pm$ 3.22	91.33 $\pm$ 3.51	75.33 $\pm$ 4.51	44.67 $\pm$ 6.11	30.67 $\pm$ 3.51	79.30
Control	7 $\pm$ 1	13.33 $\pm$ 3.51	12.33 $\pm$ 2.52	14.33 $\pm$ 3.06	14 $\pm$ 4	0

Notes: *SD, standard deviation.

b. *Nosema ceranae*

In examined apiaries with *N. ceranae* infection, the average spores' number per adult worker honey bee was 5.361×10^6 in treatment and control groups at 0h. The numbers of *N. ceranae* spores per each examined honey bee were decreased after treatment at different times. *Nosema* spores' reduction in infected adult worker honey bees was 4-5 fold less after extract treatment. *Nosema* spores development was inhibited after 72h of treatment. There was significant difference between *N. ceranae* spores reduction and plant extracts and their efficacies were comparable to fumagillin at 36h ($P < 0.05$). The efficacy of thyme, lavender, and fumagillin in treatment groups were 62.89%, 67.34%, and 79%, respectively (Table 4). The efficacies in plant extract-treated groups were significant and comparable to fumagillin at different times ($P < 0.001$) (Table 4).

Table 4. The effectiveness of the hydro-alcoholic extracts of thyme and lavender on *Nosema ceranae* spores in comparing to treated (fumagillin) and untreated control groups.

Groups	Time (hours, SD±mean)					Reduction of spores (%)
	0	48	36	24	72	
Thyme	5.377*±20.66	5.162±63.49	4.672±77.94	2.725±42.72	1.756±51.06	67.34
Lavender	5.345±15	5.286±93.74	4.816±89.15	2.937±71.65	1.983±14.53	62.89
Fumagillin	5.358±14.42	4.255±21.79	2.344±67.53	1.567±30.44	1.125±47.69	79
Control	5.364±19.31	5.365±26.28	5.368±37.74	5.384±21	5.412±85.44	0

Notes: *×10⁶.

Discussion

The importance of honey bees' role in pollination of agricultural crops, nutritional and medicinal products are remarkable. The most effective risk factors on productivity of honey bees' colonies and parasitic diseases are genetic and environmental factors and pests' infestation (Sarhangzadeh and Yakhchali 2016). The plant extracts have a long spectrum of acaricidal efficacy against *Varroa* infestation and other organisms, *i.e.* insects, mites, bacteria, fungi, and nematodes (Kütükoğlu et al. 2012).

Varroa is one of the important honey bee infestation throughout the world and Iran (Kar et al. 2006). A large number of colonies damages and beekeepers loses are due to *Varroa* infestation in winter and/or spring (Akyol and Özkök 2005). In Iran, acaricides like Apistan and Bayvarol are commonly applied. However, their residues in honey, royal jelly, wax and other products may be as risks for public health (Pourelmi et al. 2010). In current study, the obtained composition of the plants with GC/MS was in line with Kütükoğlu et al. (2012). In addition, applied dosages of the hydro-alcoholic extracts had significant effects on reduction of *V. destructor*. These findings were similar to Refaei (2011), Hamad et al. (2008), Pourelmi et al. (2010), and Nowar et al. (2018). According to Kütükoğlu et al. (2012) and EI-Hady et al. (2015), thyme and lavender effects on *Varroa* decreasing was 67.46% and 79.4%, respectively. However, laboratory findings were not in agreement with field trail because of differences of humidity and temperature on both laboratory and field findings (Hoppe and Schley 1984). According to Damiani et al. (2009), differences among findings obtained from researches and compositions may be also due to influences of several factors on the productions and chemical composition of EOS. Lindberg et al. (2000) reported dosages of the plant extracts may decrease ectoparasitic infestation rate and useful for pest management. With regard to this, dosages of 1% and 0.5% of lavender and thyme were respectively safe to treat *Varroa* infestation (Ariana et al. 2002). Thus, lavender and thyme may play role as safe acaricidal compounds against *V. destructor* with regard to temperature, time of the year, and colony strength effects (Kütükoğlu et al. 2012).

Chemical substances such as phenolic compounds, terpenoids and alkaloids are the main components of crude plant extracts and are complex (Boulogne et al. 2012). According to Borges et al. (2020), sulfuraphane are suitable agent to control *N. ceranae*. While it may affect growth of beneficial microbial flora in bee gut and lead to mortality

in bees (Johansson et al. 2008). In current study, *N. ceranae* spores reduction was significantly time depended in treated adult worker bees. Spores decreasing in examined adult worker honey bees was 4-5 fold less after extract treatment and their efficacies were comparable to fumagillin. This finding was in line with Chaimanee et al. (2021) who reported the higher bee mortality was in plant extract-treated groups when compared to *N. ceranae*-infected group and fumagillin-treated group. According to Roussel et al. (2015), Cecilia et al. (2010), Ptaszynska et al. (2018) and Mura et al. (2020), the mechanism of inhibition spore development, generation and adherence by some natural compounds like thymol may be due to *N. ceranae* spores' development inhibition through increasing permeability of cell walls and plasma membranes of the spore, disruption of the plasma membrane.

To date, in Iran, the effectiveness of hydro-alcoholic extract of lavender as a non-antibiotic alternative for *N. ceranae* control in worker honey bees was reported. This work also uncovered that the plant extracts may be suitable compounds for *N. ceranae* and *V. destructor* control in honey bees. Additionally, the plant extracts need to be tested at lower concentrations to evaluate their efficacies on *N. ceranae* and *V. destructor* in honey bee colonies. Assessment of the plant extracts efficacies on development and generation of *Nosema* spores may provide informative data related to the mechanisms of the plant extracts. Moreover, further study is recommended to evaluate the plant extracts residues in honey bees' products as a potential risk factors for consumer and public health.

Acknowledgements:

The authors would like to appreciate Vice chancellor at Urmia Faculty of Veterinary Medicine for assistance and support.

Statements and Declarations

The authors declare that there is no conflict of interest.

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

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