Evaluation of The Efficiency of Some Plant Extracts (nettle ،thyme) and Vitalactic B Supplement in Controlling the Pathogen *Nosema ceranae*.

Hayder Abdelabbas Hadi[1] and Radhi Fadhil Al-Jassany[2]

[1]Department of plant protection, College of Agricultural Engineering Sciences, University of Baghdad Iraq, e-mail: hydrhady123@gmail.com

[2]Department of plant protection, College of Agricultural Engineering Sciences, University of Baghdad Iraq, e-mail : radhi.f@coagri.uobaghdad.edu.iq

Abstract

The pathogen *Nosema ceranae* is one of the most important pathogens that led to significant losses in honeybee colonies in Iraq in the last five years Due to the inability of beekeepers to identify it due to the lack of clear and distinctive signs for it, where the effect of plant extracts and *Lactobacillus* microbial preparation was studied and showed that nettle extract 1% had a good effect in reducing the number of spores of the pathogen for infected workers of honeybees during the Autumn 2021 , but the greatest effect is for the microbial preparation of *Lactobacillus* bacteria while in the spring 2022 a treatment of nettle 1% was added with the bacterial preparation of *Lactobacillus* bacteria, and it was the most effective treatment, as the percentage of inhibition was 97% and reducing the number of spores from 1.8×10^6 spores to 50×10^3spores.

Keywords: honeybee ; *Nosema ceranae* ; netlle extract ; thyme extract

Introduction

Pathogens, pests, Pesticides, climate change and biodiversity loss are just a few of the stresses that the European honeybee, *Apis mellifera*, is currently facing. (18) .One of these pathogensis *Nosema* spp. And the type C *Nosema ceranae*، a parasite of the Asian *Apis ceranae* ،has gradually in filtrated the hives of the European honeybee ،*Apis mellifera*، on a global scale (5) .*Nosema ceranae* is an internal parasite of honeybees 'midgut that is extremely common in *Apis mellifera* colonies but for which little epidemiological data is available .(12) .The use of fumagillin, the only known reliable treatment for the naturally occurring *Nosema apis* ،increased as a result ،but field results for *N.ceranae* were mixed .Although fumagillin inhabits microsporidia reproduction, disease prevalence and hive performance in treated apiaries were comparable to untreated apiaries six months after treatment (10). Fumagillin residue scan be found in hives ،and its break down products may constitute a health concern to humans (17). The demonstrated in laboratory conditions that at their commended fumagillin concentration (250g/L) , mature *N.ceranae* spore proliferation was similar in treated and untreated bees ،and that a10 fold lower fumagillin concentration induced *N.ceranae* hyper proliferation (13) .The properties of medicinal herbs have been known since ancient times, and these herbs have been utilized to treat diseases in traditional healing from the past to the present .The chemicals and active molecules found in these plants have been identified thanks to the advancement of science .As a result, methods of action against microbes that cause disease in humans, animals, and plants have been identified .There as on for this is that scientists are looking for natural or environmentally friendly molecules that can be utilized as effective and safe pesticide alternatives (1 , 2). As a result, the necessity for innovative techniques that integrate honeybee health conservation with government food safety criteria reenergized the hunt for

alternative solutions. Recently ,studies on the effect of veterinary medications or commercial nutritional supplements on the composition of the honeybee gut microbiota were published (15).

Several studies have tested plant extracts and organic substances in recent years ,reporting their efficacy in the biocontrol of nosemosis (4,11,16). During the winter ,food consumption per honeybee frame was 22% lower in these colonies .During the spring, honeybee colonies that received probiotic feed supplement were slightly weaker than the control colony, but they out number edit by 23.5 percent in terms of sealed brood (14) .In Turkey, a study confirmed the ability of nettle and thyme extracts to reduce spores of pathogen *N.ceranae* (20).This aimed to test different plant extraction from nettle ,thyme and microbial preparation.

Material and methods

Preparation of extract for (Nettle *Urtica dioica* and Thyme *Thymus vulgaris* (8). It was extracted in the Plant Extracts Laboratory of the Plant Protection Department / Ministry of Agriculture in Abu Ghraib/ Baghdad , Where the weight of 50g of the powder plant (leaves) was placed in a beaker ,then 350ml of ethyl alcohol 80% was added to it . The beaker and its contents were placed in the moving incubator for 24 hours at a temperature of 35°C, and then placed in a centrifuge at a power of 2500 cycle /minute for 10 minutes ,then the liquid was taken and filtered with Whatman No.1 filter paper, then put in a Rotary Vacuum Evaporator to evaporate the filtrate and get a thick liquid, then put it at room temperature 37°C for 3-4 days to get the powder and then keep In a clean, dark bottle

Preparation of the concentrations used in the study

The stock solution was prepared from plant extracts (thyme,nettle) by dissolving 1 gm of dried extract in 10 ml of ethyl alcohol .The required concentrations were prepared according to the following equation:(9)

C1V1=C2V2

C1:the first concentration(concentrated),
C2:the second concentration(diluted),
V1:the first volume before dilution,
V2:the second volume after dilution.

Laboratory studies

To determine the most effective concentrations, several concentrations (1%, 2%, 5%) of alcoholic extracts and plant powders (nettle and thyme) were evaluated ،Where laboratory cages with measurements (15,15,15) cm were used to put honeybees in them at a temperature of 34°C and humidity (60%±10%). 100 bees infected with the pathogen *N.ceranae* were placed from the infected colony prepared for this purpose. The number of spores was calculated before placing the bees in each cage,and the number of spores was calculated after (1,3,7,14) days (4)(19).

Field studies

Pathogenicity caused by the pathogen *N.ceranae* of healthy bees colonies.

1-Twenty five healthy colonies were prepared for infection with the pathogen *N.ceranae* in the fall of 2021 ,the colonies contain fertilized queens in the spring of 2020, and contain six combs and were placed in the bee department in the Rashidiya are until the field study is conducted.

2-The colonies were examined to ensure that they were not infected by conducting laboratory tests by optical microscope

3-infected samples with *N.ceranae* were selected and counting the number of spores by means of a hemocytometer, taking 25ml of solutions containing anumber of spores up to 10,000,000 or more (6).

4-Dilute the standard total solution by placing it on a sugar solution (1sugar :1water) by 25 liters to obtain the infection solution for the purpose of pathogenicity to the colonies.

5-Laboratory samples of bees from the study hives were examined to confirm the occurrence of infection after three

days and the tests showed the presence of the pathogen *N.ceranae.*

Performing various treatments

The treatment process was carried out with plant extracts and other treatments prescribed in the study (three colonies for each treatment) by adding nettle powder 1%with powder sugar, thyme powder 5% with powder sugar. Nettle extract 1% with sugar solution, thyme plant extract 5% with sugar solution, microbial preparation(using commercial product Vitalactic™ B) made from *Lactobacillus* bacteria 5% with sugar solution, and Arwen treatment10% (Arwen is commercial manufactured from Eucalyptus oil ,Laurel oil ,Thyme extract, Mint extract, Lavender extract) with sugar solution at a rate of 10% (sugar solution was used in a ratio of (1sugar:1water).

The samples taken from the treated colonies were examined after (1,3, 7, 14, 30) days of treatment, and the laboratory examination was conducted using an optical microscope and a hemocytometer slide and using the aforementioned equation.

Statistical analysis

Statistical analysis were performed using randomized complete block design with LSD test to determine the differences between treatments (thyme extract, thyme powder, nettle extract, nettle powder, microbial preparation) in autumn and in spring there were significant variation between the treatments.

Results

The laboratory study showed the superiority of the nettle extract 1% treatment over the rest of the treatments with inhibition rate 97%, and the treatment thyme extract 5% over the rest of the treatments in cages for raising honeybees in the laboratory with inhibition rate 91% .Where these concentrations outperformed the rest of the concentrations used in the experiment as for the rest of the treatments, nettle extract were 5%, the workers honeybees died after 3 days, and nettle extract were 2%, the workers died after 7 days , While the two treatments of thyme extract 1% and 2% recorded a decrease in the number of spores, but the treatment of thyme 5% had the most decrease in the number of spores of the pathogen Compared to the rest of the concentrations of thyme extract(see table 1).

Table 1. Number of pathogen spores 1000x *Nosema ceranae* when treated with different concentrations of nettle and thyme extracts after different time periods of treatment of worker honeybees in the laboratory

	Time (day)				Inhibition rate%
Treatment	1 day	3 days	days 7	days 14	
Nettle extract 1%	1570	1170	120	50	97
Nettle extract 2%	1020	120	died	--------	
Nettle extract 5%	783	died	---------	--------	
Thyme extract 1%	1780	1770	1700	1580	13
Thyme extract 2%	1720	1580	1320	880	51
Thyme extract 5%	1680	1580	1380	150	91
Control	2030	2730	6170	1330	
LSD 5%	68.9**	66.2**	60.4**	43.2**	

The laboratory study also showed the superiority of the treatment of nettle powder 1% and thyme powder 5% over the rest of the concentrations with inhibition rate 48% and 43% respectively , while the nettle powder 2%, and nettle powder 5% all workers treated with these concentrations died after 7 and 3 days, respectively, while the two treatments of thyme powder for the two concentrations 1% 2%, they reduced the number of spores by less than the other treatment, which is thyme powder 5%, so it was chosen for field tests with the treatment of nettle powder 1% (table 2)

Table 2. Number of spores (1000 x) of the pathogen *Nosema ceranae* when treated with different concentrations of nettle and thyme powders after different time periods of treatment of worker honeybees in the laboratory

	Time (day)				Inhibition rate%
Treatment	1 day	3 days	days 7	days 14	
Nettle powder1%	1680	1520	1370	900	48
Nettle powder2%	1350	1020	died	--------	
Nettle powder 5%	1130	died	---------	--------	
Thyme powder1%	1650	1650	1580	1480	13
Thyme powder2%	1620	1530	1320	1080	36
Thyme powder5%	1680	1580	1380	970	43
Control	1930	2420	5970	11330	
LSD 5%	63.4**	50.5**	71.5**	114.6**	

The field study in autumn (2021) using different treatments on honeybee colonies infected with the pathogen *N.ceranae* showed a decrease in the number of spores in varying degrees. The results of the field studying in two months in Autumn showed after first month that the treatments of nettle extract 1% and the microbial preparation of *Lactobacillus* bacteria 5% were superior, as the number of spores of the pathogen decreased with an inhibition rate of 71% , 74% respectively, as for the rest of the treatments there was a decrease in the number of spores of the pathogen with varying inhibition rates , while after the second month the inhibition rate are 94% ,96% (see table 3,4)

Table 3. Number of spores (1000x) of the pathogen *Nosema ceranae* with different concentrations of nettle,thyme(extracts and powders), microbial preparation and Arwen treatment after different time periods from the treatment of honeybee workers (autumn treatments) The first spray in the field / 2021 (number of spores in all workers before treatment (1820 ± 50 spore)

Treatment	Time (day)					Inhibition rate%
	1day	3 Days	7 Days	14 Days	30 Days	
Thyme powder 5%	1780	1720	1670	1600	1520	16
Nettle powder 1%	1760	1700	1640	1520	1430	23
Nettle extract 1%	1670	1470	1220	970	570	71
Thyme extract 5%	1720	1570	1400	1070	830	55
Microbial preparation 5%	1650	1450	1300	850	470	74
Arwen 10%	1610	1470	1270	970	570	70
Control	1870	1920	2100	2540	3000	
LSD 5%	62.4**	79.2**	83.5**	66.3**	86.4**	61.4**

Table 4. Number of spores (1000x) of the pathogen *Nosema ceranae* with different concentrations of nettle,thyme(extracts and powders), microbial preparation and arwen treatment after different time periods from the treatment of honeybee workers (autumn treatments) The second spray in the field / 2021

Treatment	Time (day)					Inhibition rate%
	1day	3 Days	7 Days	14 Days	30 Days	
Thyme powder 5%	1470	1450	1350	1270	1150	36
Nettle powder 1%	1340	1300	1210	1150	650	48
Nettle extract 1%	470	350	250	180	100	94
Thyme extract 5%	860	700	580	470	350	81
Microbial preparation 5%	450	320	250	150	100	96
Arwen 10%	460	380	370	250	80	96
Control	3160	3500	3900	4500	5600	
LSD 5%	61.4**	108.7**	108.7**	170.9**	342.3**	

While the field study in spring (2022) using different treatments on honeybee colonies infected with the pathogen *N.ceranae* showed a decrease in the number of spores in varying degrees. The results of the field studying in two months in spring showed after first month that the treatments of nettle extract 1% with the microbial preparation of *Lactobacillus* bacteria 5% were superior, as the number of spores of the pathogen decreased with an inhibition rate of 71% ,as for the rest of the treatments there was a decrease in the number of spores of the pathogen with varying inhibition rates , and the nettle extract 1% treatment recorded an inhibition rate 67% , as for the microbial preparation of *Lactobacillus* bacteria 5% recorded 68%.In the end of second month of spring

the inhibition rate the treatments of nettle extract 1% with the microbial preparation of *Lactobacillus* bacteria 5% were superior, as the number of spores of the pathogen decreased with an inhibition rate of 97% ,as for the rest of the treatments there was a decrease in the number of spores of the pathogen with varying inhibition rates ,and the nettle extract 1% treatment recorded an inhibition rate 92% , as for the microbial preparation of *Lactobacillus* bacteria 5% recorded 94%.The results showed that there were significant differences between the treatments at the 5%.A percentage of inhibition was calculated as follows:

Inhibition rate (%) = number of spores before treatment - number of spores after treatment ÷ number of spores before treatment(see table 5,6).

Table 5. Number of spores (1000 x) of the pathogen *Nosema ceranae* with different concentrations of extracts and powders of nettle, thyme, microbial preparation and Arwen treatment after different time periods from the treatment of honeybee workers (spring treatments first spraying in the field 2022) the number of spores in all workers before Treatment (1600 + 100 spores)

Treatment	Time (day)					Inhibition rate%
	1day	3 Days	7 Days	14 Days	30 Days	
Thyme powder 5%	1600	1520	1430	1200	1150	29
Nettle powder 1%	1600	1500	1370	1200	1030	38
Nettle extract 1%	1520	1330	1190	870	530	67
Thyme extract 5%	1600	1430	1300	1070	830	50
Microbial preparation 5%	1550	1250	1030	830	510	68
Microbial preparation 5% and Nettle extract 1%	1550	1250	1000	820	470	71
Arwen 10%	1530	1300	1150	880	500	70
Control	1700	1820	2050	2430	2870	
LSD 5%	58.1**	64.1**	59.3**	60.7**	61.2**	

Table 6. Number of spores (1000 x) of the pathogen *Nosema ceranae* with different concentrations of extracts and powders of nettle, thyme, microbial preparation and Arwen treatment after different time periods from the treatment of honeybee workers (spring treatments second spraying in the field 2022)

Treatment	Time (day)					Inhibition rate%
	1day	3 Days	7 Days	14 Days	30 Days	
Thyme powder 5%	1120	1090	1080	1050	1020	37
Nettle powder 1%	970	950	940	910	860	42
Nettle extract 1%	520	510	470	280	120	92
Thyme extract 5%	870	810	770	600	450	74
Microbial preparation 5%	510	490	360	210	100	94
Microbial preparation 5% and Nettle extract 1%	500	450	400	210	50	97
Arwen 10%	490	470	400	350	250	85
Control	3000	3000	3200	4100	4550	
LSD 5%	84.6**	83.2**	94.7**	81.8**	197.1**	

Discussions

The plant extracts proved their effectiveness in treating the pathogen *Nosema ceranae*, as the plant extract of Nettles reduced the number of spores from 1520,000 spores to 530,000 spores after the first month and to 120,000 spores after second month, While the plant extract of thyme reduced it from 1,600,000 spores to 830000 spores after the first month and to 450000 spores after the second month, which is consistent with the results reached by (20), where he indicated that nettle extract reduced the number of spores in workers infected with the pathogen *Nosema ceranae* after three weeks of using nettle extract, as well as decreased the number of spores in workers infected after treatment with thyme extract study by (7) to examine the impact of thymol supplementation(thymol is chemically known as 2-isopropyl-5-methylphenol is a dietary monoterpene phenol and is abundantly found in certain plants such as *Thymus vulgaris* (1,2) on the expression of immune-related genes, oxidative stress parameters, bee survival, spore loads, and bees infected with the microsporidian parasite *N. ceranae*. , the results show that thymol has primarily beneficial impacts on health when administered to *Nosema*-infected bees, including raising immune-related gene levels and oxidative stress parameter values as well as lowering *Nosema* spore loads, thymol supplementation had no adverse effects on bees that were *Nosema*-infected, as well. The mechanism of thymol's anti-*Nosema* activity, according to (13) is thought to be based on its contact with the *Nosema* spore, which interferes with the plasma membrane and prevents spore germination. When using nettle extract in the treatment of the pathogen *Nosema ceranae*, the number of spores was reduced, and its effectiveness was better than thyme extract in reducing the number of spores (20).These results are consistent with the results of this study.

Conclusion

The plant extracts proved effective in combating the pathogen *Nosema ceranae*, as well as the microbial preparation of *Lactobacillus* bacteria. Nettle extract proved good efficacy in reducing the number of spores of the pathogen.

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