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A preliminary laboratory study of thymol nanoemulsion effect on naturally nosema-infected honey bee workers

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Abstract: Honey bees health is a common current concern due to the colony collapse disorders worldwide as a result of diseases, pests and pollution. The implications of excessive and unwise use of pesticides have been motivated researchers to seek safe control strategies as essential oils of medicinal plants. Thymol is a natural component of thyme essential oil known for varroacidal and antimicrobial properties but there are no reports about thymol nanoemulsion effect on honey bees. There was an epidemic of nosema during the experiment and *Nosema* spp. spore count indicates to potency of the treatments but honey bee worker mortality percentages indicate to their safety limits. This experiment was carried out under lab condition for 16 consecutive days. The highest mortality percentages were recorded with thymol nanoemulsion (NTh) 0.01 ppm, thymol (Th) 100 ppm, NTh 10 ppm and NTh 50 ppm. The highest spore count was recorded in NTh 0.1, 50 and 100 ppm with significant differences. The highest sugar syrup consumption rate was recorded in NTh 0.1 and 50 ppm. This may be due to high *Nosema* spore count that consumes more carbohydrates for germination and propagation in the worker midgut. Pollen consumption insignificantly increased until the 7th day and decreased due to the worker hypopharyngeal glands could not secret royal jelly and reserved it because no brood to feed in such experiments of caged bees. Thymol nanoemulsion of 25, 10, and 1 ppm seem to be promising as a novel health-supporting and nosema-control approach. Further investigation is needed to confirm these findings for more reliability, especially with honey bees and their vital role in cross-pollination and environmental sustainability.

keywords: honey bees, diseases, control, thymol, nanothymol

1.Introduction

In the last decades, it was recorded a severe global decline in honeybee colony numbers, causing problems for pollination and beekeeping. So, honey bees health is a major current concern due to the Colony Collapse Disorder (CCD) **[1, 2]**. This is due to pathogens, pesticide residues, and climatic changes. Though, the precise weight of each factor is still unknown **[3, 4]**. The *Varroa* mite is the most common and damaging of *Apis mellifera*, causing important stress worldwide and even colony losses **[5]**. It absorbs hemolymph and fat body of brood and adult honeybees **[6]**. The second common agent is the microsporidian fungi *Nosema* spp. **[7, 8]**.

The antibiotic fumagillin is the only known effective treatment for nosemosis **[9]**. Despite quick degradation, its residues may be still in the hive products for up to 6 months **[10]**. Also, the unwise use of acaricides resulted in residues and mite resistance **[11]**.

Motivated by these problems, scientists have sought nonchemical control strategies **[12]**. Plants are the greatest mine of tremendous bioactive organic compounds **[13]**. Essential oils are a different and valuable approach because of safety to bees and mammals and low impacts on the environment **[14]**. Thymol is a natural major constituent of thyme essential oil

and has antimicrobial properties **[15]**. Also, it has antifungal activity against *Nosema* **[16]** and can control *Varroa* **[17]**. Nonetheless, recent researches showed residues of thymol in honey **[18]** and beeswax **[19]**.

Nanotechnology is one of the most important technologies. The amounts required of nano-pesticides are very small for effective pest control, reducing their load on the environment **[20]**. This technique in insect pest management field can be an alternative to overcome pesticide implications **[21]**. The effect of nanothymol on honey bees still unknown yet. It is necessary to test its safety before application on honey bee diseases. This is a pilot trial to study nanothymol effect on honey bee workers to determine the appropriate concentrations against nosemosis and varroosis within the safety limits.

2. Materials and methods

2.1. Apiary and bees

The study was carried out in Entomology laboratory at faculty of science Mansoura University. Honey bee workers were brought from a private outdoor apiary at Meet Fares village, Bani Ebeid district, Dakahlia Governorate (31°04'50.4"N & 31°35'51.8"E. The honeybee race was the local Carniolan hybrid. The study was conducted during spring of 2021 that was recognised with an epidemic of nosemosis. One naturally *Nosema*-infected colony was chosen as the source of bees during the time course of the experiment. It did not receive the usual treatments for honey bee diseases during the time course of study.

2.2. Tools and feeding:

Clear rounded plastic containers, measuring 12 cm in diameter × 10 cm in depth, were used after slight handling. Every container has 60 pores ≤ 2 mm for ventilation. Also, a bottom hole is appropriate for insertion of 5 ml plastic syringe, whose needle and tip were totally eliminated. The syringe was vertically hanged for the workers inverted feeding with sugar syrup. Eppendorf tube was tip cut, filled with 1.5 g clover pollen, and laterally inserted. The container inner wall was provided with a small piece of wax foundation of 5 × 5 cm, simulating the familiar hive **(Fig. 1)**.

Fig. 1. The handled plastic containers.

2.3. Chemicals:

2.3.1. Thymol

The tested thymol is a standard substance was THYMOL POWDER A. R., which procured from Alpha Chemika Co., Molecular formula of thymol is $C_{10}H_{14}O$ and its molecular weight is 150.22. It is a white powder product with a net content weight of 500 g when packed with purity of 99.0%, melting point of 48-50°C and boiling point of 232°C. This product is packed not for culinary or medicinal uses but for servicing industries, quality control laboratories, and research purposes.

2.3.2. Thymol nanoemulsion

2.3.2.1. Thymol nanoparticle preparation

Thymol nanoparticles (ThNP) were obtained from 5 samples of the white thymol powder (5 g for each) that were dried in a dryer (Binder, Germany) and adjusted at 37 ± 1°C for 12 h. The minimum particle size of thymol was obtained by the ball milling and its high-energy collision. This technique has been used as a solvent-free method for the reduction of the particle size without possible change in its structure. A Photon ball mill machine (Photon Scientific Co.), utilizing zirconium oxide grinding spheres of variable size, was used to reduce the particle size. Each reaction vessel was charged with one sample of thymol and stirred in the grinding bowl with a rotation speed of 250-500 rpm for about 30 min that was gradually increased to the maximum speed of 5000 rpm for 4 h **[22, 23]**. These procedures were carried out in the Advanced Materials Research Laboratory, Spectroscopy Department, National Research Centre, Dokki, Cairo, Egypt.

2.3.2.2. Thymol nanoemulsion concentrations

The thymol emulsion stock solution was freshly prepared by dissolving 1 g thymol powder in 1 ml tween 80 as a surfactant and then completed to 100 ml warm distilled water,

resulting in a final concentration of 10 mg/ml (10,000 ppm). In the same way, the thymol nanoemulsion stock solution was freshly prepared.

The concentrations of thymol emulsion and thymol nanoemulsion were prepared from the 2 stock solutions. Thymol and thymol nanoemulsion (100 ppm) concentration was obtained by adding 1 ml of the stock solution (10 mg/ml) to 99 ml of 50% sugar syrup (1:1, w/v). The subsequent concentrations of thymol nanoemulsion were prepared by serial dilutions from the 100 ppm solution with different volumes of 50% sugar syrup. It was prepared 50, 25, 10, 1, 0.1 and 0.01 ppm for the other different experimental groups.

2.4. Experiment groups

Group (1): control (fed only syrup 50%)

Group (2): thymol (100 ppm)

Group (3): nanothymol (100 ppm)

Group (4): nanothymol (50 ppm)

Group (5): nanothymol (25 ppm)

Group (6): nanothymol (10 ppm)

Group (7): nanothymol (1 ppm)

Group (8): nanothymol (0.1 ppm)

Group (9): nanothymol (0.01 ppm)

According to the experiment groups, it comprised 9 groups with 4 replicates for each. Each replicate was one of the aforementioned handled clear plastic containers having 25 newly emerged workers (n = 100).

2.5. Experiment procedure:

There was an epidemic of nosemosis during this experiment. One sealed brood frame was selected from the infected chosen hive, incubated in the lab at 34 ± 1°C and 60 ± 2% relative humidity. The newly emerged worker bees were considered zero day old and randomly placed into the plastic containers according to the groups. Each container was provided with 5 ml of 50% sugar syrup (1:1, w/v) and about 1.5 g clover pollen. All were incubated at 33 ± 1°C and 60% ± 2% relative humidity for 24 h and the dead worker bees were replaced by new ones before the beginning of the experiment **[24]**.

The next day to workers emergence was considered the 1st day of the experiment. All feeding materials were replaced day after day with freshly prepared equal amounts. All containers were incubated at 60 ± 2% relative humidity and 33 ± 1°C, which was decreased to 31 and 30 ± 1°C on the 2nd and 3rd day, respectively and continued until the 16th day of the trial. The application of thymol and nanothymol concentrations was on the 10th day, coinciding with the apex of *Nosema* life cycle of 6-10 days. This period is required for *Nosema* spp. spores germination and propagation in the midgut tissue to produce other mature spores that transport to body tissues via hemolymph and some will be defecated **[25]**.

Numbers of dead worker bees and food consumption amount were recorded day after day. Furthermore, it could not be neglected the impact of natural nosema infection on these findings. So, the spore count was recorded in each group on the 16th day at the end of the experiment. The effects of thymol and nanothymol on honey bee workers were evaluated in comparison to the control group. Moreover, the appropriate and safe concentrations could be determined.

Nosema spp. infection was identified by collecting 10 honey bee workers from the chosen hive entrance. The alimentary canals were removed under stereomicroscope. The infection was classically identified with the white colour instead of the normal amber colour. Then, the infected workers were placed with distilled water (one worker/ml) in a mortar and manually smashed. One drop of the suspension was mounted on a glass slide and examined by a light microscope with 400× for *Nosema* spores **[26]**.

The spore suspension was strained by a glass funnel containing a small clean piece of gauze. The spores were recorded by a haemocytometer slide (Neubauer grid) and the pore count was calculated with the following equation:

$$\text{Spore count} = \frac{\text{Spore count of 5 blocks} \times 4 \times 10^6}{80} \text{ spores/ml}$$

The spore count was used to calculate average infection level, which if was less than 10^4 spores/ml, it was considered not detected (ND) **[27]**. Quantification of *Nosema* infection level ensures that all experimental groups had a comparable initial infection level. Presence of

Nosema spores and infection severity based on spore count per bee.

2.5. Statistical analysis:

The data checked for normality and homogeneity of variance with Kolmogorov-Smirnov and Levene tests, respectively and expressed as mean percentages with standard deviations. Parametric data were analysed with One-Way ANOVA followed by LSD test for post-comparison as well as Pearson correlation coefficient. If the data were non-parametric, Kruskal-Wallis test was used followed by Mann-Whitney (U) test as well as Kendall's Tau correlation coefficient. The statistical significance difference was accepted at $p \leq 0.05$ with a double-sided (two-tailed) distribution. All data were statistically analysed using SPSS 20.0© software.

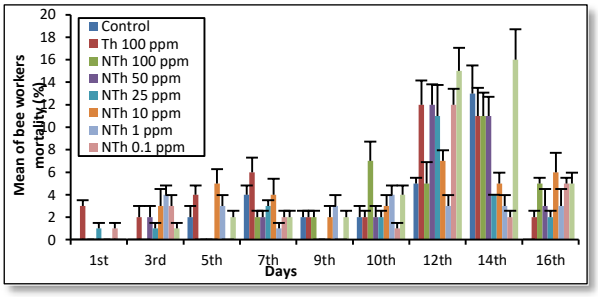

Fig. 2. Mortality percentages of honey bee workers.

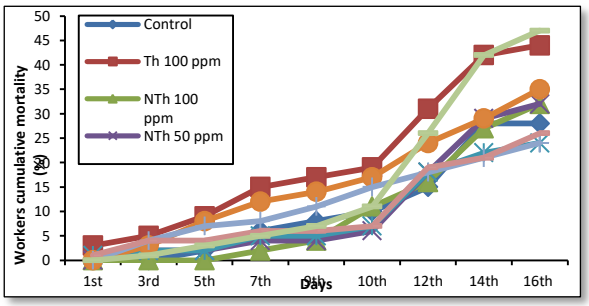

Fig. 3. Cumulative mortality percentages of honey bee workers.

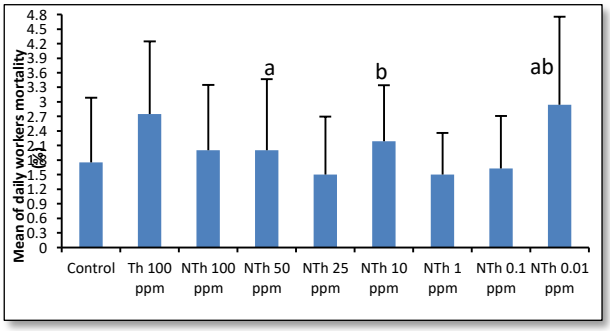

Fig. 4. Daily mortality percentages of honey bee workers.

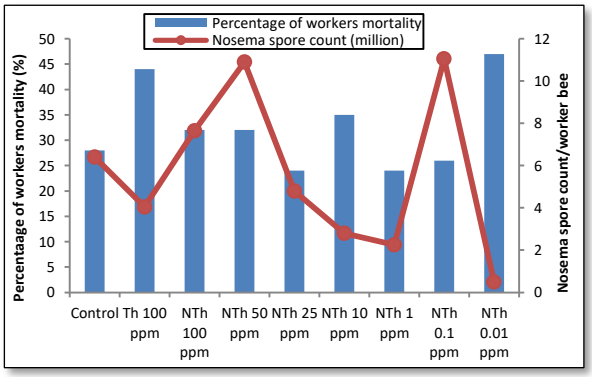

Fig. 5. Total mortality percentages of honey bee workers with *Nosema* spore count.

3. Results

3.1. Mortality of honey bee workers

The mean percentages of dead honey bee workers until the 10th day of experiment were low. Then, they significantly and greatly increased from the 10th to 14th days and again decreased on the 16th day **(Fig. 2)**. Also, the cumulative mortality percentages assured these findings. The highest mortality percentage means were recorded in thymol (Th) 100 ppm group followed by nanothymol (NTh) 10 ppm and NTh 1 ppm **(Fig. 3)**.

The mean daily workers mortality significantly increased in case of NT 0.01 ppm (2.94%) in comparison with NT 10 ppm (2.19%) and NTh 50 ppm (2%) groups **(Fig. 4)**. The highest counted numbers of *Nosema* spp. spores were recorded in NTh 0.1 ppm (11.05 million) and in NTh 50 ppm (10.9 million) followed by NTh 100 ppm (7.65 million) and the control groups (6.4 million spores). Groups treated with 1, 10, 25 NTh ppm and Th 100 ppm showed the lowest spore count and moderate mortality percentages of honey bee workers. NTh 0.01 ppm group exhibited the lowest record (0.5 million spores) but showed the highest workers mortality (47%) **(Fig. 5)**.

3.2. Sugar syrup consumption

In general, it was observed gradual increase in sugar syrup consumption until the 10th day. Then, it slightly decreased on the 12th day. Then, it is significantly and greatly decreased on the 14th and 16th days **(Fig. 6)**. The highest total means of sugar syrup consumption rates were recorded in NTh 50, 0.01, 0.1 ppm and Th 100 ppm with values of 29.9, 29.8, 29.5 and 28 µl/worker/day **(Fig. 7)**.

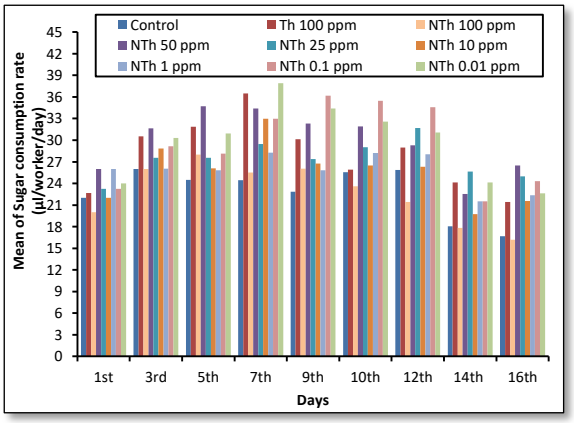

Fig. 6. Sugar syrup consumption rate by honey bee workers.

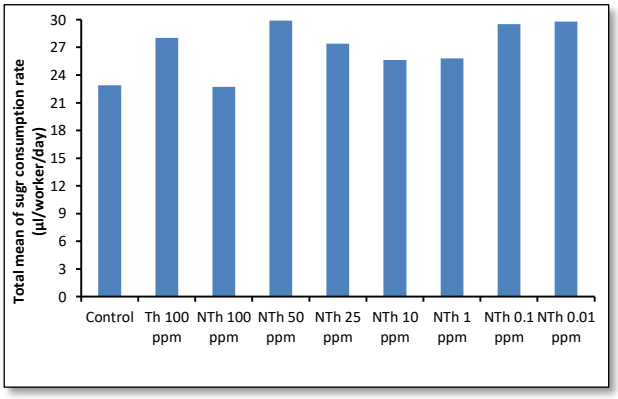

Fig. 7. Total sugar consumption rate of honey bee workers.

3.3. Pollen consumption

Generally, the findings showed that there are significant high increases in pollen consumption during the first days of the experiment. It was recorded great significant decreases on the 7th day that nearly continued until the end of the experiment **(Fig. 8)**. Th 100 ppm group exhibited the highest total mean pollen consumption rate of 1.3 mg/worker/day. There were insignificant differences in pollen consumption rate between the experimental groups **(Fig. 9)**.

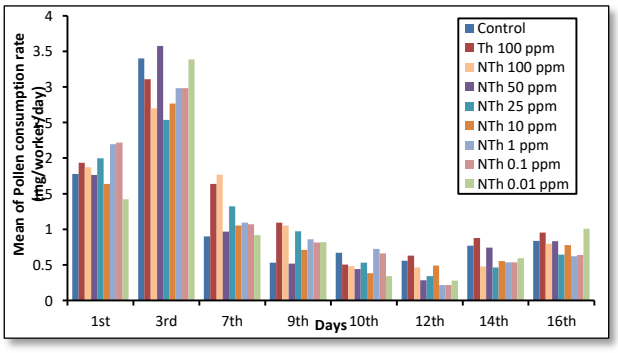

Fig. 8. Mean of pollen consumption by honey bee workers.

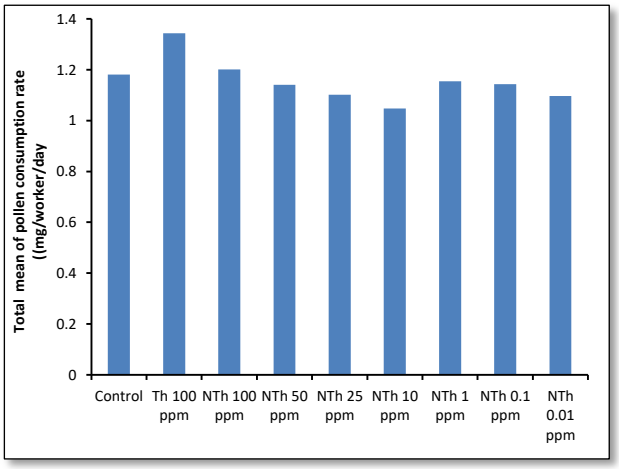

Fig. 9. Total pollen consumption rate by honey bee workers.

4. Discussion

Thymol is known for *Varroa* control and the choice of 100 ppm concentration as the upper limit in this study is owed to its moderate safety degree for honey bees in sugar syrup or candy. It significantly increases their lifespan than those fed sugar syrup only under laboratory conditions according to **[28]**. Also, **[29]** found Apiguard (powdered thymol-based formulation) has no harmful effects on honey bees mortality; while, **[30]** found undesired effects. Moreover, it was proved that higher concentrations of powdered thymol as 0.5% has fewer adverse impacts on honey bees comparable to 3% and 6% that cause increases in mortality percentages. It was proved that feeding type, either as liquid (sugar syrup) or solid (honey candy, sugar candy) has no undesirable impacts on the survival or measured parameters of honey bees **[31]**. Similarly, and even with smaller concentrations, it was postulated that 0.05% and 1% thymol still have adverse impacts on honey bees **[32]**.

It was recorded the increase in thymol evaporation along with the increase of temperature **[33]**. Thymol is most efficacious at a temperature range between 15 and 35°C and its evaporation is not influenced by air humidity **[34]**. The present experiment was carried out under controlled conditions of temperature in this range.

The mortality percentages recorded in honey bee workers might be owed to the adverse effects of thymol 100 ppm as repellency and interference with the colony pheromones but

decreases *Nosema* spp. spore load. This agrees with **[35]** who reported decline in spore loads with thymol. Otherwise disagrees with their findings of positive effects of thymol on honey bees health. Moreover, supplementation with thymol did not induce negative effects in *Nosema*-infected bees. However, their results indicate that in *Nosema*-free bees, thymol itself could cause certain disorders (affecting bee survival, decreasing oxidative capacity, and downregulation of some immune-related gene expressions), showing that one should be careful with preventive, uncontrolled, and excessive use of thymol. Moreover, thymol is known for its antibacterial and antioxidant properties **[15, 16]**.

This is the first study that deals with the effect of nanothymol on honey bees and there are no researches on this topic. In general, it was noticed that the concentrations of thymol nanoemulsion as 25, 10 and 1 ppm exhibited ameliorative effect on nosemosis, decreasing the spore count. The lowest and highest concentrations showed higher *Nosema* spore count and higher records of honey bees worker mortalities. However, some studies revealed the antimicrobial activities of nanothymol **[36, 37]**.

The sugar syrup consumption greatly increased until the 10^{th} to 12^{th} day of the experiment. This coincides with the apex of the life cycle of *Nosema* spp. The worker bees are infected because the sealed brood frame was picked up from a naturally-infected colony. The newly emerged adult workers are infected due to feeding on infected brood food. Also, infection was pooled within each container of workers through trophalactic behaviour. *Nosema* spores invade the worker midgut cells, producing vegetative cells that divide to form mature spores, which reinfect the epithelial cells and propagate to form mature spores again until the 10^{th} day. So, the syrup consumption increased due to need of the contagion for energy. After that, they decreased due to less propagation and defecation. Then the consumption decreased again because *Nosema* spp. needs lower carbohydrates. This is in consistence with the findings reported by **[25, 38]**.

The amount of pollen consumed by honey bee workers was the highest in case of thymol 100 ppm. This may be due to the high repellency effect of this high concentration when compared with the others. So, honey bee workers exhibited decreased consumption in the sugar syrup containing this concentration. This explains their tendency to feed on pollen more than syrup. This could be supported by findings of **[39]**. They stated that the disease impairs the digestion of pollen, thereby shortening the life of the bee by reducing protein intake and compromising nutritional status and metabolic integrity. *Nosema*-induced mortality is most noticeable in spring, as bees are restricted from cleansing flights by cold weather. The necessary winter confinement of honey bees in cold climates puts beekeeping operations especially at risk of *Nosema* infection in Northern countries. Moreover it coincides with **[40]** who cited that the hypopharyngeal glands secretion peak is during 6-10 days old workers. Then, it decreases as there is no brood to feed and transformed to a reservoir due to stop secretion.

5. Conclusion

This is the first pilot study carried out to evaluate the effect of nanothymol on honey bee workers. Controlled laboratory conditions concise the results and can give rise to more reliable findings. Thymol 100 ppm and lowest concentrations of nanothymol as 0.01 and 0.1 are not suitable for bees or control nosemosis because of high workers mortalities or high *Nosmea* spore count. Nanothymol of 25, 10 and 1 ppm would be more promising. These needs further studies for more reliability of this novel control approach.

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