The effect of Neem oil in controlling Khapra beetle *Trogoderma granarium* (Dermestidae : Coleoptera) and reducing its associated fungal isolates in wheat grains

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**Abstract.** The khapra beetle, *Trogoderma granarium* is an important pest of stored wheat worldwide. The current study was carried out to evaluate the lethal and the sublethal three concentrations of neem oil on *T. granarium*. We also assessed the effect of neem oil on the growth of fungal species associated with *T. granarium* infected wheat grains. The results demonstrate that treating of wheat grains with 5% neem caused the highest mortality of *T. granarium* (82.3%). The results were also shown that application of neem oil decreased the percentage of weight loss of wheat grains and the percentage of egg hatching. In addition, the total number of fungal isolates associated with *T. granarium* infecting neem-treated wheat grains were 4 fungal isolates, compared to 13 fungal isolates from untreated wheat grains. The results demonstrate that neem oil has potential use as a biological control agents of *T. granarium*; however, further studies under commercial storage conditions are required.

1. **Introduction**

*Trogoderma granarium* Everts (Khapra beetle) is one of the most serious pest of stored products throughout the world, including Iraq due to its high infestation potential [1]. One of the most important damage caused by storage insect pests including *T. granarium* is their ability to transfer fungal spores. Al–Saedy [2] reported the ability of *Sitotroga cerealella* to transfer and spread the conidia of *Aspergillus flavus*. Welson [3] was also found that insects help to transfer the fungal spores in storage through the adhesion of fungal conidia on their bodies. The chances of contact between stored insects and fungal conidia in the presence of mucous substances on the insect's body which help attaching the fungal conidia to their bodies [4]. Some of these fungi, especially *A. flavus* and *A. niger* have been found to be able to produce dangerous compounds known as Mycotoxins, which are secondary metabolites lead to acute and chronic toxicity, mutagenicity and teratogenicity in human and animal health [5].

Synthetic insecticides are still the main way to control *T. granarium*. However, the extensive usage of synthetic insecticides has been associated with many problems [6]. These problems include mainly; insect resistance development coupled with the resurgence of treated primary insects, toxicity to man, animals and other non-target organisms, and environmental contamination [7]. Moreover, these problems have encouraged the development of alternative methods of managing this insect pest in storage.

Several plant extracts and plant oils were used as alternatives to minimize or replace the use of synthetic pesticides against different insect pests [8]. Among the studied plant extracts and oils, several studies have been reported the efficacy of neem oil as a potential insecticidal [9], antifeedant, growth retardant [10], and repellent against stored insect pests including *T. granarium* [11]. However, There is no information about the
effect of neem oil on decreasing the ability of *T. granarium* to spread of fungal species. Thus, the current study was aimed to evaluate different concentrations of neem oil against third instar larvae of *T. granarium*, and to investigate sublethal effects of neem oil on the reducing fungal isolates that transfer by *T. granarium* and their capacity to produce mycotoxins.

2. Materials and Methods

2.1. *Trogoderma granarium* rearing

The stock culture of *T. granarium* originated from wheat grain stores in Najaf, Iraq, in 2017, and was maintained in the Entomology Laboratory, Faculty of Agriculture, University of Kufa, Najaf, Iraq. Insects (50 male and female pairs) were reared on whole sterilized wheat grains (200 g per jar) placed in 300-ml plastic jars secured with a muslin cloth and rubber bands, and maintained at 30 ± 2°C and 55 ± 3% RH in continuous darkness.

2.2. Wheat grains

Wheat grains were obtained from the local markets in Najaf, Iraq. The samples were taken to the laboratory in sterilized polyethylene bags. They were sterilized at -20°C for 48 hours and then with 70% ethanol to eliminate any insect or fungal infections.

2.3. Potato Dextrose agar (*Potato Dextrose*)

200 g of potato tubers, were cut into small pieces and boiled with 500ml distilled water for 20-30 minutes in a glass beaker. After the boiling period, then filtered content by a piece of gauze. 10 g of sucrose and 17 g of agar were dissolved in 500 ml of distilled water and then the potato leach and full size to the litre. The media were distributed in flasks according to its use, and their vents were sealed with cotton bolts and sterilized with the sprinkler device at 121°C and 15 lb / kg for 20 minutes. After that the flasks were left to cool and then placed in the refrigerator until use. This medium was used to isolate and grow the fungi.

2.4. Preparation of different concentrations of neem oil

Neem oil was obtained from the local markets in Najaf, Iraq. Three concentrations of neem oil (1, 3, 5%) were used. To obtain these concentrations, 1, 3, and 5 ml of neem oil was diluted in 99, 97 and 95 ml of acetone. The suspensions were then agitating for 20 minutes on a magnetic stirrer, and were shaken again before they were used in experiments.

2.5. The effect of Neem oil on the mortality of *T. granarium*

To determine the efficacy of neem oil against third instar larvae of *T. granarium*, the above mentioned concentrations were used which sprayed with two application methods. In the first application method, five plate replicates, each with 10 third larval instars were tested for each concentration and control (acetone only). To prevent insect larvae from crawling, individual insects were cooled at 4°C for 10 min and larvae were then introduced. For each concentration, 1 ml was applied once on each plate containing *T. granarium* using a 250 ml trigger water sprayer. Then, 2 g of sterilized wheat grains were placed on each plate. Plates were incubated at 30 ± 1 and 45 ± 3 % RH and mortality was recorded after 1, 5, 10, 15 and 20 days post-treatment. The percentage of weight loss of wheat grains was calculated after 20 days of treatment using the formula described by Mousa *et al.* [12].

\[
\text{Weight loss} \% = \left( \frac{\text{grain weight before insect infection} - \text{grain weight after insect infection}}{\text{grain weight before insect infection}} \right) \times 100
\]

In the second application method, five plate replicates, each with 10 third larval instars were tested for each concentration and control (acetone only). For each concentration, 1 ml was applied once on each plate containing 2 g of sterilized wheat grains and then each plate was infested with 10 third instar larvae of *T. granarium*. Plates were incubated at 30 ± 1 °C and 45 ± 3 % RH and mortality was recorded after 1, 5, 10, 15 and 20 days post-treatment and the percentage of weight loss of wheat grains was calculated using the formula described above.
2.6. The effect of neem oil on the percentage of egg hatching
Five male-female (1–2 d old) pairs were introduced in 8-cm plastic cub covered from the top and bottom with muslin cloth secured using a rubber band which placed into 9-cm sterile Petri dish containing black papers to facilitate recognizing the eggs. After 24 h, male-female pairs were removed and the number of eggs produced were recorded. 1 ml of neem oil at a concentration of 5% was once on each Petri dish and were kept at 30 ± 2°C, 45 ± 5% RH for 7 days and then the number of hatching eggs was recorded.

2.7. Treatment of wheat grains with neem oil
In this experiment, 10 ml of neem oil were mixed with 1kg of sterilized wheat grains for 30 min using plastic bags. Then, treated wheat grains were transferred to glass jars for 3 days in order to saturate of grains with neem oil. Afterwards, three replicates each with 100 g of treated wheat grains were transferred to sterilized glass jars, covered with a muslin cloth, and kept at 30 ± 2°C, 45 ± 5% RH for 3 months. In addition, three replicates each with 100 g of untreated wheat grains were transferred to sterilized glass jars as described in above (control). After this storage period, 1 g of wheat grains of each jar in each treatment was taken and placed on the center of PDA in Petri dishes and incubated at 25°C for 7 days. After 7 days, growing fungi were identified using the taxonomic keys mentioned by Hoching and Pitt [13]. The number of isolates or colonies for each growing fungus was calculated from samples taken from wheat grains.

2.8. Treatment of larvae of T. granarium with neem oil
Third larval instars of T. granarium were treated with neem oil at a concentration of 5%. Three replicates each with 100 g of sterilized wheat grains placed in sterilized glass jars, covered with a muslin cloth were each infested with 20 treated third larval instars of T. granarium. In addition, three replicates each with 100 g of sterilized wheat grains were transferred to sterilized glass jars and each infested with 20 untreated third larval instars of T. granarium (control). After this storage period, 1 g of wheat grains of each jar in each treatment was taken and placed on the center of PDA in Petri dishes and incubated at 25°C for 7 days. After 7 days, growing fungi were identified using the method described above.

2.9. Statistical analysis
The analysis was performed using GenStat software (VSN International 2016). Cumulative mortality was corrected for natural death in the control using Abbott’s formula [14]. Normality of data distribution was assessed using the Shapiro-Wilk test. Corrected mortalities were logit transformed when necessary to meet the assumption of normality. One-factor repeated measurement ANOVA was used to determine the effect of the different connotations of neem oil on the corrected mortality of T. granarium. Mean comparisons were performed using LSD test at the 5 % level of significance ($P \leq 0.05$).

3. Results and Discussion

3.1. The effect of Neem oil on the mortality of T. granarium
Corrected mortality varied significantly among concentrations of neem oil ($P < 0.001$), with a high mortality rate (61.59 %) recorded 61.59 caused by a neem concentration of 5%. The effect of days after application on the percentage of corrected mortality was also significant ($P < 0.001$), with a high mortality rate recorded for all concentrations 20 days after treatment (Table 1). Results were also shown that the percentage of weight loss of wheat grains was significantly affected by concentrations of neem oil ($P < 0.001$), with low percentage of weight loss (8.25%) recorded for grains treated with neem oil at a concentration of 5%, compared to 33% in the control treatment.
Table 1. Corrected mortality (±SE) of third instar larvae of T. granarium infected wheat grains treated with three different concentrations of neem oil after 1, 5, 10, 15 and 20 days of application.

| Concentration of neem oil | Corrected mortality (±SE) |
|---------------------------|---------------------------|
|                           | 1 day     | 5 days    | 10 days   | 15 days   | 20 days   |
| 1%                        | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 3.3 ± 0.2 | 13.86 ± 0.7 |
| 3%                        | 0.0 ± 0.0 | 3.3 ± 0.1 | 10.34 ± 1.2 | 20.68 ± 0.8 | 24.19 ± 1.1 |
| 5%                        | 13.3 ± 0.4 | 26.99 ± 2.1 | 42.33 ± 1.6 | 53.86 ± 3.2 | 61.59 ± 2.9 |

L.S.D(0.05) for Concentration = 0.61; L.S.D(0.05) for days = 0.68; L.S.D(0.05) for interaction = 1.36

Corrected mortality varied significantly among concentrations of neem oil ($P < 0.001$), with a high mortality rate (40%) recorded 61.59 caused by a neem concentration of 5%. The effect of days after application on the percentage of corrected mortality was also significant ($P < 0.001$), with a high mortality rate recorded for all concentrations 20 days after treatment (Table 2). Results were also shown that the percentage of weight loss of wheat grains was significantly affected by concentrations of neem oil ($P < 0.001$), with a low percentage of weight loss (18.7%) recorded for grains treated with neem oil at a concentration of 5%, compared to 37% in the control treatment.

Table 2. Corrected mortality (±SE) of third instar larvae of T. granarium treated with three different concentrations of neem oil after 1, 5, 10, 15 and 20 days of application.

| Concentration of neem oil | Corrected mortality (±SE) |
|---------------------------|---------------------------|
|                           | 1 day     | 5 days    | 10 days   | 15 days   | 20 days   |
| 1%                        | 0 ± 0.0   | 6.7 ± 0.3 | 10 ± 0.1  | 10 ± 0.5  | 20 ± 0.4  |
| 3%                        | 0 ± 0.0   | 6.7 ± 0.4 | 10 ± 0.6  | 13.3 ± 0.2 | 23.3 ± 1.2 |
| 5%                        | 0 ± 0.0   | 13.3 ± 0.3 | 20 ± 0.9  | 33.3 ± 2.1 | 40 ± 1.7  |

L.S.D(0.05) for Concentration = 0.58; L.S.D(0.05) for days = 0.65; L.S.D(0.05) for interaction = 1.31

3.2. The effect of neem oil on the percentage of egg hatching

Neem oil had a significant effect on the percentage of egg hatching ($P < 0.001$), with the lowest rate of egg hatching (20%) recorded in neem treated eggs, compared to 80% in the control treatment. This is due to the entry of oils into the egg through the opening of the pecker through the eggshell and then lead to the death of the fetus or incomplete development or that the oils may prevent the entry of oxygen to the fetus inside the egg, and these results agree with the findings of former study [18] that Palm oil extract reduced the rate of hatching eggs of South cowpea beetle.
3.3. The effect of neem oil on the level of wheat grain contamination with fungal species

The results showed that the number of fungal species isolated from wheat grains, which infected with \( T. \) \textit{granarium} was significantly affected by application of neem oil \((P \leq 0.01)\). Where, the total number of fungal isolates isolated from wheat grains treated with neem oil was 4 fungal isolates, compared with 13 fungal isolates from untreated wheat grains (Table 3).

Table 3. Number of fungal species and their isolates which isolated from wheat grains treated with neem oil and then infected with \( T. \) \textit{granarium}, compared to untreated wheat grains.

| Treatment          | \( A. \) flavus | \( A. \) niger | Fusarium sp. | Alternaria sp. | Penicillium sp. | Total |
|--------------------|-----------------|----------------|---------------|-----------------|-----------------|-------|
| Acetone only       | 4               | 1              | 0             | 5               | 3               | 13    |
| Neem oil           | 0               | 0              | 1             | 0               | 1               | 2     |
| L.S.D\((0.05)\)    | 0.40            | 0.18           | 0.26          | 0.56            | 0.33            |

3.4. Treatment of larvae of \( T. \) \textit{granarium} with neem oil

The results showed that there was a significant difference in the number of fungal species isolated from wheat grains infected with neem-treated \( T. \) \textit{granarium} or untreated \( T. \) \textit{granarium} \((P \leq 0.01)\). Where, the total number of fungal isolates isolated from wheat grains infected with neem-treated \( T. \) \textit{granarium} was 7 fungal isolates, compared with 20 fungal isolates isolated from wheat grains infected with untreated \( T. \) \textit{granarium} (Table 4). Our results are in agreement with previous study [19] who reported that water extracts of Sidr and Eucalyptus leaves significantly decreased the number of fungal isolates growth associated with stored insect pests on wheat grains.

Table 4. Number of fungal species and their isolates which isolated from wheat grains treated with neem oil and then infected with \( T. \) \textit{granarium}, compared to untreated wheat grains.

| Treatment          | \( A. \) flavus | \( A. \) niger | Fusarium sp. | Alternaria sp. | Penicillium sp. | Total |
|--------------------|-----------------|----------------|---------------|-----------------|-----------------|-------|
| Acetone only       | 6               | 3              | 2             | 6               | 3               | 20    |
| Neem oil           | 0               | 2              | 0             | 4               | 1               | 7     |
| L.S.D\((0.05)\)    | 0.52            | 0.64           | 0.31          | 0.80            | 0.38            |

4. Conclusion

The study documents that that lethal and sublethal effects of neem oil against \( T. \) \textit{granarium}. The results indicated that neem oil might be useful as an alternative to synthetic insecticides for the control of \( T. \) \textit{granarium}. However, further experiments are required to be carried out in commercial storage conditions.
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