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Battol Qasem Kteo
Faculty of Agriculture, University
of Kufa, Najaf, Najaf Governorate,
Iraq

Efficacy of Neem seed extract (*Azadirachta indica*) against *in vitro* preserved larvae of the hairy grain beetle (*Trogoderma granarium* Everts)

Battol Qasem Kteo

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Abstract

This study was conducted in the laboratory to evaluate the efficacy of cold aqueous extract of neem seeds, *Azadirachta indica*, against larvae of the hairy grain beetle, *Trogoderma granarium* Everts, and to determine its effect on wheat seed viability. The cold aqueous extract of neem seeds was prepared and used at three concentrations, 2, 4 and 6%, with distilled water as the control treatment. Its efficacy was tested on the second, third and fourth larval instars, with 10 larvae per replicate and three replicates for each treatment. Mortality was recorded after 24, 48, 72 and 96 hours of treatment. The viability of wheat seeds treated with 6% extract was also tested. The results showed a significant effect of extract concentration, exposure period and their interaction on larval mortality, with the 6% concentration clearly superior to the 2 and 4% concentrations and the control treatment. The second larval instar showed the highest response to the extract, with the highest mortality rate of 63.3% at 6% concentration after 24 hours of treatment, while the highest mortality rates in the third and fourth instars were 43.3 and 26.7%, respectively, at the same concentration and exposure period. The results showed differences in the sensitivity of the larval instars to the extract, with the second larval instar being the most affected, followed by the third and then the fourth. The efficacy of the extract also appeared early, especially at the 6% concentration, with differences in larval response according to exposure period and larval instar. On the other hand, the use of neem seed extract at 6% did not cause a significant decrease in wheat seed viability, as the germination rate was 100%, which was the same rate recorded in the control treatment. These results indicate the possibility of using the aqueous neem seed extract as one of the promising plant alternatives in integrated management programs for the hairy grain beetle, while maintaining the germination ability of wheat seeds.

Keywords: Azadirachtin, larval mortality, khapra beetle, neem oil, stored grain pests

Introduction

Global agriculture highly depends on grains including stored grains because of their nearness to the food security (Ziegler *et al.*, 2021). They are major providers of energy, carbohydrates, and also contribute primarily for the provision of plant proteins and essential nutrients to humans, being among the most critical strategic food crops that humans rely on for their survival in everyday life (Poutanen *et al.*, 2022). Grain products, especially wheat grains, are exposed to different types of insect pests. Insects belonging to the order Coleoptera are among the most important pests that attack stored grains, accounting for 75% of the other pests. One of the most famous of them is the hairy grain beetle (khapra), *Trogoderma granarium* Everts (Abbasi *et al.*, 2021), which is one of the most important and widely distributed stored-product insects and one of the most resistant to pesticides (Ramadan *et al.*, 2023). It can tolerate difficult environmental conditions, as its larvae can survive for 23 months without food in a long state of dormancy until food becomes available. It also causes crop damage and reduces its marketing value because heavy infestation leaves marks and an odor on the grains or infested food material, making it unacceptable and useless to humans. Larval feeding also contaminates grains with its wastes and greatly reduces the germination rate of infested grains. It also increases the possibility of infestation by secondary insect pests and fungi, and consequently increases the damage (Kteo, 2019; Eesa & Mohammadali, 2024) [2, 10, 11, 13, 16, 17].

Corresponding Author:
Battol Qasem Kteo
Faculty of Agriculture, University
of Kufa, Najaf, Najaf Governorate,
Iraq

methods, but the random use of chemical pesticides led to the appearance of resistant insect strains, in addition to the fact that pesticides are harmful to other living organisms and the environment. Therefore, many studies have recently been conducted on the use of plant parts or their extracts as materials to protect grains from infestation by stored-product insects (Asiry and Zaitoun, 2020), which are among the important environmentally friendly pesticides used in integrated pest management programs (Tavares *et al.*, 2021). Many researchers have extracted chemical compounds from the leaves, seeds and fruits of many plants that act as feeding deterrents, repellents, growth regulators and others (Domingue *et al.*, 2022). Neem extracts are environmentally friendly botanical pesticides because they biodegrade rapidly and have low toxicity to non-target organisms when used according to recommendations. Their content of several active compounds also reduces the possibility of resistance development in pests compared with many traditional chemical pesticides (Chatterjee *et al.*, 2023). Therefore, this study aimed to evaluate the use of environmentally friendly methods, represented by the aqueous extract of neem seeds, *Azadirachta indica*, to protect wheat seeds from infestation by the hairy grain beetle, *Trogoderma granarium*. This approach aims to provide a natural alternative to chemical pesticides that contributes to reducing dependence on importing chemical pesticides from abroad and reducing the environmental and health effects resulting from their use. The study also seeks to promote the use of safe and effective plant extracts within integrated pest management programs to protect stored crops while maintaining the environment and reducing its pollution. Therefore, the study aimed to conduct a laboratory evaluation of the efficacy of the cold aqueous extract of neem seeds, *Azadirachta indica*, against larvae of the hairy grain beetle, *Trogoderma granarium* Everts, and to evaluate the effect of the extract on wheat seed viability ^[1, 3, 18, 19].

Material and Methods

Collection and rearing of the hairy grain beetle (khapra) *T. granarium*

A pure culture of the insect was obtained from the Entomology Laboratory at the College of Agriculture, University of Kufa. This insect had been previously identified as the hairy grain beetle by Professor Dr. Akram Ali Mohammed of the Plant Protection Department at the College of Agriculture, University of Kufa. The insect was reared in 15 x 9 cm glass bottles, each containing 200 g of sterilized wheat seeds. The bottle openings were covered with cotton woolen cloth and secured with rubber bands. The bottles were then incubated at a temperature of 30 ± 1°C and a relative humidity of 55-65% to ensure colony maintenance (Hassan, 2015). Colonies were maintained by periodically replenishing the insect's food supply by introducing additional bottles containing 200 g of sterilized wheat and infesting them with sufficient numbers of adult beetles and larvae ^[15, 21].

Wheat grain samples

The wheat grains were purchased from the local markets in Najaf Governorate and the samples were brought to the laboratory in sterilized polyethylene bags. Sterilized by freezing for 48 hours at -20°C to kill any infestation of fungi or insects before use for insect rearing or carry out the laboratory experiments as required for the study.

Preparation of neem seeds cold aqueous extract

Neem seeds were obtained from the agricultural sector in Iraq

and ground in an electric mill. The powder was then stored in sealed nylon bags in the refrigerator until use. Fifty grams of the ground neem seed powder were weighed and placed in a clean 1000 ml glass container. A quantity of 500ml of cold water was added and the contents were subject to magnetic stirring for an hour. This technique is used to obtain the complete and uniform mixing of solutions and liquids while producing better mixing of particles or substances, and preventing clump or agglomeration formation (Mohammed and Hawar, 2022). This mixture was left to infuse for 24 hrs at room temperature. Then, the extracted was filtered using Whatman No.1 filter paper to obtain the impurity free filtered extract. The extract was left in the glass for days in order to dry it completely. Drying was followed by careful and gentle skimming of the extract with a teaspoon. The dried extract powder was transferred to a tightly capped glass container and stored in a cold and dry atmosphere, away from exposure to direct sunlight, until its preparation as dried concentrated extract (Aritonang *et al.*, 2019). The required concentrations were made by adding distilled water to 6 g of the dry matter to give 6%, 2% and 4% concentrations of these were prepared from this. Only distilled water was used in the control treatment ^[21, 22].

Effect of Neem Seed Extract on the Mortality of Khapra Larvae:

In this experiment, the effect of 2, 6, and 4% concentrations of neem seed extract on the mortality rates of second, third, and fourth larval instars was tested. Larval stages were collected by placing pairs of adults in a plastic dish and observing egg hatching. After successive molts and larval size was observed and the different larval stages were isolated. Thirty replicates, consisting of 10 larvae in each Petri dish in 5 g of sterile uninfested wheat grain, were set up in Petri dishes for each of the concentrations. The dishes were then sprayed vertically from a distance of 20 cm using a hand sprayer at a rate of 1 ml per dish. These plates were then dried in the lab for 30 minutes. After that, the plates were placed in the incubator, and after 24 hours, the initial killing results were read and recorded. The dead stages were extracted and discarded, and then the plates were returned to the incubator. After 48 hours, the same steps were repeated. After 72-96 hours, the final killing results and the extraction of dead stages were recorded each time. For the control, the plates were sprayed with distilled and sterile water only.

Effect of neem seed extract on wheat grains viability

Five grams of wheat grains were mixed with neem seed extract at a concentration of 6% then, ten seeds were taken and placed in Petri dishes containing cotton moistened with water. The process was repeated with three replicates, while nothing was added to the wheat in the control treatment. The germination rate was calculated after 7 days from the beginning of the treatment.

Experimental Design and Statistical Analysis

The laboratory experiments were conducted according to a complete randomized design with one factor and three replications. The data were analyzed and ANOVA tables were used using the computerized GenStat 12th software (Payne *et al.*, 2009). Means between treatments and experimental factors were compared using the Least Significant Difference (LSD) criterion at a probability level of 0.05 (Hoshmand, 2018).

Results and Discussion

Efficacy of neem seed extract in mortality of *T. granarium* 2nd larval instar

The results (Table 1) showed significant differences in the mortality rate of second-instar larvae according to the concentration of neem seed extract, time period post exposure and the interaction between the two factors. The 6% concentration showed the highest mortality effect, with a mortality rate of 63.3% after 24 hours of treatment, which was the highest value recorded among all treatments and times for this larval instar. The rate dropped to 3.3% at the concentration of 4%; the concentration of 2% did not show mortality in the initial 24 hours. The response to the 6% concentration continued

with mortality of 27.3% after 48 hours and 25.0% after 72 hours before decreasing to 16.7% after 96 hours (values from the figure). The effects of the two concentrations of 2 and 4% were noticed to be limited and variable during the exposure periods and the mortality rates recorded at these concentrations were much lesser when compared with 6%. Second-instar larvae appears to have been highly sensitive to the extract as early as the 1st 24 hours of treatment, the 6% concentration was the most effective than the lower concentrations.

Table 1: Mortality (%) of the *Trogoderma granarium* second larval instar as effected by neem seed extract at different concentrations at different time periods post exposure

Average (Concentration effect)	Mortality% after treatment (hours)96	Mortality% after treatment (hours)72	Mortality% after treatment (hours)48	Mortality% after treatment (hours)24	Neem seeds extract (mg ml ⁻¹)
1.1	0	3.3	0		2
2.3	0	3.5	3.4		4
2.3	16.7	25	27.3		6
	5.57	10.6	10.2	24	Average (Time effect)
Interaction = 0.71	Time = 0.36	Concentration = 0.36			L.S.D _{0.05}

Third larval instar

The weedicide effect of neem seed extract is apparent, as the third instar larvae show differences as compared to second instar and the differences between the concentrations and the exposure duration and the interaction between concentration and duration was also significant (Table (2)). There was a high mortality in the 6% concentration (mortality 43.3% after 24 hours of treatment), 4% concentration (mortality 3.3% after 24 hours of

treatment), and no mortality after 24 hours in 2% concentration. As the duration of exposure increased, only 5.9% of the 6% sample died after 48 hours; there was no death in either the 2% or 4% samples during the remainder of the period of exposure. These results indicate that the response of third-instar larvae to the extract was lower than that of second-instar larvae, although the 6% concentration maintained its clear superiority over the other concentrations.

Table 2: Mortality (%) of the *T. granarium* 3rd larval instar as effected by neem seed extract at different concentrations at different time periods post exposure

Average (Concentration effect)	Mortality% after treatment (hours)96	Mortality% after treatment (hours)72	Mortality% after treatment (hours)48	Mortality% after treatment (hours)24	Neem seeds extract (mg ml ⁻¹)
0	0	0	0	0	2
0.825	0	0	0	3.3	4
12.3	0	0	5.9	43.3	6
	0	0	1.97	15.5	Average (Time effect)
Interaction = 1.52	Time = 0.76	Concentration = 0.76			L.S.D _{0.05}

Fourth larval instar

The results of (3) showed that fourth-instar larvae were the least sensitive to neem seed extract compared with the second and third instars. The 6% concentration recorded the highest mortality rate of 26.7% after 24 hours of treatment, compared with 3.3% at the 4% concentration, while the 2% concentration did not record any mortality. After 48 hours, the 6% concentration did not record mortality, while the rate was 3.4%

at the 4% concentration. After 72 hours, a mortality rate of 13.6% appeared at the 6% concentration, while the treatments of the other two concentrations did not record mortality. No mortality rates were recorded in the different treatments after 96 hours. In comparison among the larval instars, it is clear that the decrease in sensitivity with advancing age was more evident in the fourth instar, which recorded the lowest maximum mortality rate compared with the second and third instars..

Table 3: Mortality (%) of the *T. granarium* 4th larval instar as effected by neem seed extract at different concentrations at different time periods post exposure

Average (Concentration effect)	Mortality% after treatment (hours)96	Mortality% after treatment (hours)72	Mortality% after treatment (hours)48	Mortality% after treatment (hours)24	Neem seeds extract (mg ml ⁻¹)
0	0	0	0	0	2
1.675	0	0	3.4	3.3	4
10.075	0	13.6	0	26.7	6
	0	4.5	1.13	10	Average (Time effect)
Interaction = 1.13	Time = 0.56	Concentration = 0.56			L.S.D _{0.05}

General comparison among larval instars

Tables (1, 2, 3) show that the 6% concentration was superior to the other concentrations, giving the highest mortality rate in all stages after 24 hours of treatment. The mortality rate increased with increasing concentration. The highest recorded mortality rate for the extract was in the second larval instar, surpassing the other stages in mortality. This is consistent with the findings of Mekhlif (2007) in his study on the effect of concentrated saffron fruit extract on the immature stages of the insect *Spodoptera ciliata* (Lepidoptera). The extract showed high toxicity one day after treatment, and the insect's life cycle was affected by the extract, lengthening the larval and pupal stages and shortening the pre-pupal stage. After the 8th day of treatment extract was found non-toxic. Neem extracts have been proven to have insecticidal activity as reported in Michel *et al.*, (2023). High mortality was found in insects, *Tenebrio molitor* when the neem LE is used. The study demonstrated that the insecticidal action of its extract was both concentration-dependent and time dependent, which is in agreement with the present study which revealed that the mortality percentage of the hairy grain beetle larvae increased as the concentration of the aqueous extract of neem seeds increased [4, 20].

Singh *et al.* (2023) [5] also indicated that plant extracts have insecticidal effects on stored grain pests. The plant extracts depressed insect populations and influenced different life stages, suggesting the potential of using plant extracts as a part of an IPM strategy for stored grain. This study's results regarding differences in sensitivity of larval stages to the plant extract agree with those of Kavallieratos *et al.*, 2023 [6], who reported in their study that plant extracts can be effective green pesticides against a number of stored-product pests, as they indicated toxicities that varied with the nature of the extract used as well as its concentration and the tested insect. Similarly, Henagamage (2023) [7] reported toxic activity of some plants extracts namely: *Lantana camara* and *Tithonia diversifolia* extracts against the hairy grain beetle *Tribolium castaneum*, and the mortality rate was positively related to concentration of the extracts used which is in line with the positive relationship between extract concentration and larval mortality presented in the current work. The results of this study were also similar to those obtained by Draz *et al.* (2024) [14], who studied the effect of some plant

essential oils and nano-emulsions on different storage insects' pests including *Sitophilus oryzae* L. and *Tribolium castaneum* rusty flour beetle. They found that the insect mortality rate increased with increasing concentration. The effect of the plant extract used in the study may be due to its content of active chemical compounds and volatile oils that can spread and penetrate insect tissues in a way similar to pesticides, or they may act by contact with the insect body surface, where the chemical compounds penetrate the insect cuticle through thin areas, causing paralysis and then death (Afifi, 2002) [8]. The effect of the extract may also be due to its content of alkaloid compounds, toxic substances or other active compounds that enter through the respiratory openings and affect the nervous system.

Effect of neem seed extract on wheat seed germination rate

Results indicated that the seed germination percentage of wheat seeds were not significantly different between the treatment of wheat seeds with 6% Neem seed extract regime and the control (Figure 1). There was 100% germination in both treatments which means extract used in the test concentration did not adversely affect wheat seed germination in the experiment. This finding, based on a stable germination rate for treated and untreated seeds suggests that the insecticidal activity of this extract was not associated with an inhibitory effect on germination and hence is likely to be safe in treating grain without negatively impacting seed viability. This finding corroborates that of Ekwueme *et al.* (2025) [9] who reported the absence of any adverse impact of nano-forms of plant extracts on grain viability and germination. Similarly, the values obtained in the present study compare positively with that obtained by Mahmoud (2017) [12] who stated that cowpea grains treated with the plant extract did not suffer any significant decrease in germination rate when compared with the control treatment. This outcome has practical relevance as the finding that there was no effect on seed viability in combination with the mortality effect neem seed extract had on the larvae of the hairy grain beetle, could indicate a potential use for the plant to serve as an alternative in integrated management programs for stored-grain pests.

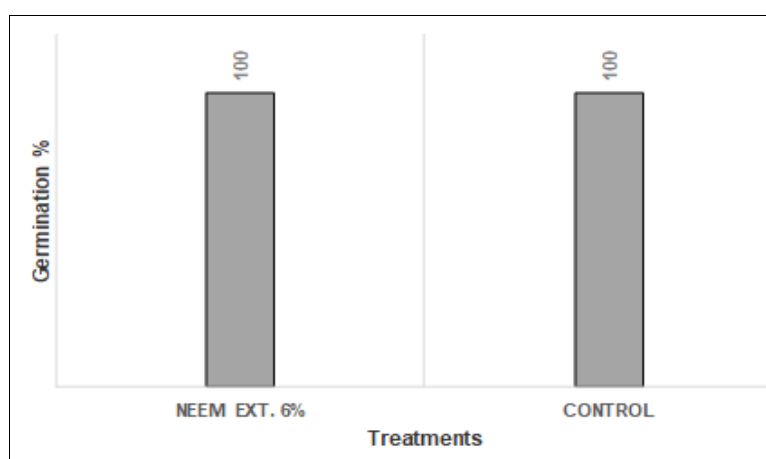


Fig 1: Germination percentages of wheat seeds affected by neem seed extract

Conclusion

The results of the study showed that the aqueous extract of neem seeds has high biological efficacy in controlling hairy grain beetle larvae, as it caused a significant increase in mortality rates compared with the control treatment. The results indicated that

the efficiency of extract was directly proportional to the concentration, as the concentration was increased, the efficiency was also increased. For all of the larval stages observed, the 6% concentration was the most effective in causing adult death compared to the 2 and 4% concentrations and had a significant

difference. But larval sensitivity was different, 2nd instar larva being sensitive than 3rd and 4th instar larvae. However, seed viability was not significantly affected by the aqueous extract; so it may be used as one of the potential plant alternatives for the hairy grain beetle in the integrated management program without jeopardizing seed quality or germination potentials.

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