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Research Article

The adverse impact of nano formulation of neem oil, *Azadirachta indica* against the desert locust, *Schistocerca gregaria*

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Abstract. Botanical biopesticides are recognized for their efficacy in combating a variety of crop pests. They are characterized by their biodegradability and affordability. The application of nanotechnology is viewed as a promising avenue for advancing pest control methods. This study revealed a detrimental impact associated with the conversion of neem oil into its nano form using high-energy sonication. Transmission Electron Microscopy (TEM) analysis indicated that prepared nano particles were spherical and smooth with mean particle size ranged from 90 to 120 nm. The effects of both bulk neem oil and its nano-emulsion were evaluated against the fifth instar nymphs of *Schistocerca gregaria* under semi-field conditions. Topical applications of both formulations, at doses of 5 and 2.5 ppm, resulted in a significant increase in mortality rates, as well as nymphal malformations and failed molting, in comparison to the control group. However, the crude form of neem oil demonstrated greater effectiveness than the nano formulation. The chitinase activity and total protein were influenced by both formulations. Notably, the nano emulsion resulted in a significant extension of duration period in the survived nymphs. Gas chromatography/mass spectrometry (GC/MS) analysis indicated the absence of linoleic acid in the nano sample, which may account for the superior efficacy of the bulk sample over the nano formulation. The prolonged ultrasonic treatment during preparation of neem oil nano-emulsion adversely affected the active biocompounds presented in neem oil, leading to a reduction in its bioactivity against the fifth instar nymphs of *S. gregaria* compared to its crude form.

Keywords: Desert locusts, Total protein, GC/MS, Nano emulsion, enzyme activity, green pesticides

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Introduction

The desert locust crisis impacts numerous countries and regions. Typically residing in 30 countries, desert locusts have the potential to expand their range to as many as 60 during periods of population surges. These insects are among the oldest migratory pests globally, inflicting extensive damage to crops. Outbreaks of the desert locust species *Schistocerca gregaria* are not a recent occurrence. Among various locust species, *S. gregaria* is particularly harmful. Although locusts belong to the grasshopper family, they exhibit behavioral changes that enable them to form dense, mobile swarms and migrate over considerable distances. An invasion of desert locusts represents a serious threat to food security, as these insects can consume an amount of food equivalent to their body weight each day. For example, a swarm occupying a single square kilometer can contain a range of 40 - 80 million adult locusts, and a small swarm of 1 km^2 can consume as much food in a single day as would be required to feed 35,000 individuals (FAO, 2020). Desert locusts undergo an incomplete metamorphosis characterized by three developmental stages: egg, hopper (nymph), and adult. Locusts are classified as solitarious when they exist in low population densities and as gregarious when found in high densities. The desert locust, *S. gregaria*, exhibits significant plasticity in its behavioral, coloration, morphological, and physiological characteristics in response to factors such as crowding, temperature, and other external conditions. As the population of solitarious hoppers increases, their behavior shifts, leading to aggregation and the formation of groups, particularly when vegetation begins to desiccate. Additionally, their coloration alters, resulting in the appearance of dark spots (FAO, 2001).

In recent times, insecticides have faced considerable criticism due to their environmental hazards, the development of resistance among insect populations, and their impact on non-target organisms. The understanding of the necessity to minimize the use of chemical pesticides and safeguard the ecological environment is steadily advancing. In the management strategies for desert locusts, we are also diligently seeking new pesticides that pose minimal risks to non-target organisms, operators, residents, livestock, and the surrounding environment (Bayoumi *et al.*, 2022; Van Huis *et al.*, 2007; Lomer *et al.*, 2001). As a result, the pursuit of new environmentally friendly solutions has become a significant undertaking (Nassar *et al.*, 2018). Researchers prioritize strategies aimed at preventing desert locust populations from escalating to harmful levels. The application of oils for locust control offers the benefits of being environmentally friendly and ecologically safe, thereby ensuring sustainable management. Plants are regarded as one of the most abundant sources of natural bioinsecticides, which can be utilized as pest control agents. This includes the use of botanical toxicants, repellents, synergists, growth regulators, and antifeedants to combat various insect pests (Disha *et al.*, 2020; El-Shazly *et al.*, 2019; El-Sonbati *et al.*, 2014).

Azadirachtin, recognized as the primary active ingredient in neem oil, is predominantly found in the seeds and has been shown to significantly enhance mortality rates, interfere with metamorphosis, and reduce overall activity levels in locusts and grasshoppers. Azadirachtin, for instance, has the capability to diminish food intake, disrupt the success of moulting from the fifth instar to the adult stage, induce the occurrence of over-aged nymphs, inhibit the growth of desert locusts, and influence their reproductive and developmental processes. Neem oil has the potential to reduce the reproductive capacity and egg-hatching success of desert locusts, while also influencing their growth and development (Bashir & El Shafie, 2014). It acts swiftly, decomposes rapidly, and exhibits low toxicity to mammals (Lomer *et al.*, 2001). So, using neem oil has the advantages of environmental friendliness, ecological security, hence controlling locusts continuously. The present study is an attempt through semi-field conditions to evaluate the efficacy of azadirachtin in both forms bulk and nano against *S. gregaria*.

Materials and methods

Mass rearing of insects

The insects were obtained from the stock culture maintained for several generations at Locust and Grasshopper Research Department, Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza, Egypt. Insects were reared under crowded conditions in the laboratory according to (Ouedraogo *et al.* 2002) in specific closed cages, fed on brunches of Egyptian clover, *Trifolium alexandrinum*, leaves in winter and Sesban, *Sesbania sesban*, in summer. The locust's cages were kept at 30±2°C and 30-50% relative humidity.

Preparation of nano capsules

Polyethylene glycol (PEG) loaded nanoemulsion was formulated utilizing an oil-in-water (o/w) emulsification technique, based on a modified approach outlined in references (Zhang *et al.*, 2008; Mohammad *et al.* 2022). The PEG-neem nanocapsules were synthesized through the miniemulsion polymerization method. Neem oil (obtained from Luna Company, Egypt) was emulsified with distilled water in a 1:1 (v/v) ratio using Tween 80, with stirring conducted for 10 minutes. The resulting emulsion was then introduced into a 3% PEG solution at a 1:1 (v/v) ratio while maintaining drop wised in continuous mechanical stirring at ambient temperature. The emulsion underwent sonication for 60 minutes using an ultrasonic cleaner, model WUC-DO3H, operating at 290W and 60 Hz, followed by an additional 3 minutes of sonication with a high-energy ultrasonic probe (model VCX750, 750W, 20 kHz). The suspension of the loaded nanocapsules was allowed to equilibrate overnight, resulting in the formation of nanocapsules dispersed in an aqueous solution.

Transmission Electron Microscopy (TEM)

The morphological shape of prepared nano-formulation was carried out using TEM (Jeol, JEM-2100). The nano-capsule suspension was diluted with distilled water and deposited onto a carbon coated copper grid and particle sizes were determined by TEM; the diameter of the particles is shown using the scale bar of the photo (Fig.1).

Gas chromatography/mass spectrometry (GC/MS)

The analysis of crude and PEG nano-capsules via GC-MS was conducted utilizing a gas chromatography-mass spectrometry instrument located at the Department of Medicinal and Aromatic Plants Research, National Research Center. The equipment employed was a TRACE GC Ultra Gas Chromatograph (THERMO Scientific Corp., USA), which was coupled with a thermo mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system featured a TG-5MS column (30 m x 0.32 mm i.d., 0.25 µm film thickness). The analyses were performed with helium as the carrier gas at a flow rate of 1.0 mL/min and a split ratio of 1:10, following a temperature program that commenced at 60°C for 1 minute, then increased at a rate of 3.0°C/min to 240°C, where it was maintained for an additional minute. Both the injector and detector were set to 240°C. Samples were diluted (1:10 hexane, v/v) and 1 µL of the mixtures was injected consistently. Mass spectra were acquired through electron ionization (EI) at 70 eV, covering a spectral range of m/z 40-450.

UV spectrophotometric examinations

The active ingredient contents of loaded nano-capsules were assessed through a refluxing process. A total of 1g of the loaded nano-emulsions were refluxed with 10 ml of methanol at 65°C for one hour to ensure complete extraction of the encapsulated oil. Following this, the samples were centrifuged at 10,000 x g for 10 minutes. The absorbance of the methanol solution containing the extracted neem oil was measured at 214 nm according to Jerbin *et al.* (2012), using a CHEM-7 UV spectrophotometer, with absolute methanol serving as the blank. The oil concentration was determined using a calibration curve derived from pure oil samples within a specific concentration range for neem oil. The encapsulation parameters were established following the methodology outlined by Kumar *et al.* (2014) as detailed below:

$$\%EncapsulationEfficiency(EE) = \frac{Theamountofoilmeasured \in thesupernatant}{Totalamountofoil} x100$$

$$\%Loadingcapacity(LC) = \frac{Theamountofoilmeasured \in thesupernatant}{Totalweightofnano - capsules} x100$$

Bioassay tests

Five topical applications were conducted to investigate the effects of neem oil from *A. indica*, in both its nano and bulk forms, on *S. gregaria*. The treatments were administered to one-day-old fifth instar nymphs following their most recent fourth molt. The treatments included two doses of neem oil at concentrations of 5 ppm and 2.5 ppm. So, the insects were inoculated (using the micro pipette) with 10 and 5 µl per nymph for the two doses, respectively. These concentrations were based on the assumption that a drop of 5µl of the solution will reach the insect by using Ultra Low Volume (ULV) sprayer. So, we converted this volume to ppm and utilized it and its double as two concentrations. Additionally, a control group of untreated nymphs was included. The control and experimental nymphs were maintained individually in plastic cylinder containers (10 cm X 25 cm), with six replicates for each group, comprising five nymphs per replicate. As the capsule ingredients are not separately used to control the insect, they were used as built-in parts of the treatments, not in the untreated check. During the experiment, the nymphs were provided daily with branches of Egyptian clover, *T. alexandrinum*, leaves and were monitored until they either molted into adults or died. The duration of each nymphal instar and appearance of any malformations (insects shown damage in their legs, wings, and antennae after molting into adults) were meticulously recorded, and the entire procedure was conducted under semi-field conditions (not controlled but ambient temperature and humidity).

Biochemical activity

Preparation of enzyme extracts

The biochemical impacts of neem oil, whether in bulk or nano-formulations, were investigated, focusing on total protein and chitinase activity. The treatment involved the topical application of neem emulsions (both nano and bulk) to fifth instar nymphs of *S. gregaria* at concentrations of 2.5 and 5 ppm. Enzyme activity and total protein levels were assessed after 24, 48, and 72 hours. The nymphs were homogenized in sodium phosphate buffer (0.1M, pH 7.0) at a ratio of 0.1 g of body weight to 1.5 ml of buffer. The homogenates were then centrifuged at 10,000 xg for 15 minutes at 4°C, and the supernatant was utilized as the enzyme source. This supernatant was subsequently stored at -20˚C until further analysis.

Evaluation of total protein

Total protein concentration was determined using a colorimetric method with the Protein - Biuret kit from Biogiagnostic Company, following the procedure outlined by Gornall *et al.* (1949). The sample mixture consisted of 25 µl of the sample and 1 ml of the protein reagent (alkaline cupric sulfate). Similarly, the standard mixture included 25 µl of the standard and 1 ml of the protein reagent. All mixtures were thoroughly combined and incubated for 10 minutes at 37°C. The absorbance of the sample (ASample) and the standard (AStandard) was measured against a reagent blank at a wavelength of 550 nm. The color remained stable for one hour. The protein concentration was calculated using the following equation:

$$\text{Protein Concentration (mg/g insect body weight)} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \text{ X Conentration of Standard X } \frac{1}{\text{Insect body weight (g)}}$$

Determination of Chitinase activity

Chitinase activity was evaluated utilizing 3,5-dinitrosalicylic acid reagent to quantify the free aldehydic groups of hexosaminase released during chitin digestion, following the methodology outlined by Ishaaya & Casida (1974). The specific activity of chitinase is reported as µg of N-acetyl glucosamine released per minute per milligram of sample.

Statistical analysis

One-way analysis of variance (ANOVA), followed by Tukey's HSD test (SPSS software) was used to analyze data. Statistically significant means were separated at $P < 0.05$.

The mortality was corrected according to Abbott's formula (Abbott, 1925)

$$\text{Corrected mortality\%} = \left(1 - \frac{\text{n in T after treatment}}{\text{n in Co after treatment}}\right) * 100$$

Where: n = Number of insect population, T = treated, Co = control

Results

Transmission Electron Microscopy

The morphological characteristics and particle sizes of the synthesized neem nano-emulsion were analyzed using TEM, as depicted in Fig. 1. The particles of the nano-formulation exhibited an almost spherical morphology with a smooth surface texture. The preparation of PEG nano-capsules containing neem oil was conducted through a multi-step process utilizing a high-energy ultrasonic technique. The resulting emulsion of neem oil and PEG manifested in the form of core-shell capsules, where the inner core comprised the oil and the shell was constituted of PEG serving as the loading material. The average sizes of the cores ranged from 30 to 100 nm, while the dimensions of the core-shell capsules varied between 90 and 120 nm.

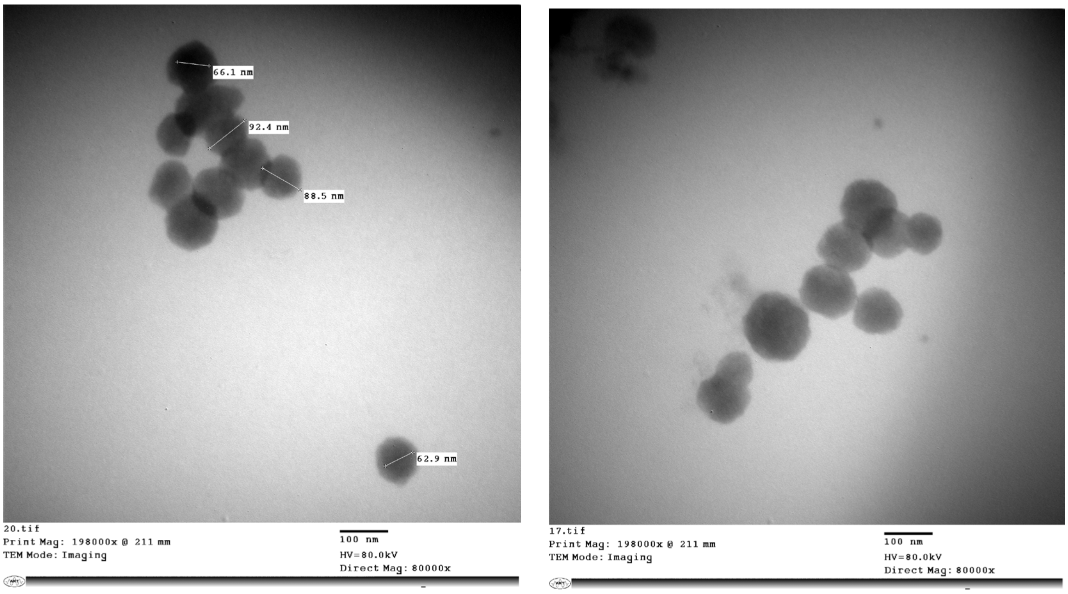

Fig. (1): Transmission electron microscopy of the prepared neem nanoemulsion loaded on PEG

GC/MS analysis of crude and Poly Ethylene Glycol (PEG) loaded emulsion

The chemical composition of neem oil, specifically in its crude and PEG-loaded emulsion forms, was analyzed using GC/MS, as depicted in fig. 2 and 3, and detailed in table 1. The spectral analysis revealed the presence of eight distinct peaks. The compounds within each emulsion were identified, showing percentages of 98.75% for the crude emulsion and 97.63% for the nano emulsion. Oleic acid, the predominant compound in both emulsions, accounted for 55.64% in the crude form and 63.37% in the PEG-loaded nanoemulsion. This was followed by palmitic acid, which constituted 19.02% and 20.56% in the respective emulsions. Notably, linoleic acid exhibited a significant reduction during the nano preparation process, with values of 14.90% and 0.99% for the crude and nano emulsions, respectively. Stearic acid was present at 5.98% in the crude emulsion and 8.14% in the nano-loaded emulsion. Additionally, hexadecadienoic acid was found at 2.10% and 1.84%, respectively, while the final peak, representing eicosanoic acid, showed values of 1.11% and 1.59%.

Determination of Encapsulation Efficiency (EE) and Loading Capacity (LC) of prepared loaded nano-formulations

The incorporation of neem oil into PEG nano-capsules has been studied. The results in table (2) showed that Encapsulation Efficiency (EE%) was 72.58±0.99%. Loading Capacity (LC%) was 40.328±0.55.

Insecticidal activity of neem oil nano and bulk formulations on the 5th nymphal instar of *S. gregaria*

The findings are displayed in Table (3), Fig. (3), they illustrate the impact of neem *A. indica* nanoemulsion and crude oil on the fifth nymphal instar of *S. gregaria*. After the topical application treatment, the mortality rates for the nano-emulsions at concentrations of 5 and 2.5 ppm were recorded at 73.33% and 56.11%, respectively. Contrary to expectations, the mortality percentages for the crude oil treatments at both 5 and 2.5 ppm were significantly higher, achieving 100% mortality, in stark contrast to the 0.0% observed in the control group.

Table 1: Gas chromatogram analysis of neem crude oil and PEG loaded nano emulsion

NO.	R.T.	Compounds	Area %	
			Crude oil	Nano emulsion
1	31.85	Palmitic acid, methyl ester	19.02	20.56
2	33.18	Hexadecanoic acid, 14-methyl-, methyl ester	--	0.48
3	35.70	Linoleic acid, methyl ester	14.90	0.99
4	36.03	Oleic acid, methyl ester	55.64	63.37
5	36.14	Elaidic acid, methyl ester	--	0.66
6	36.57	Stearic acid, methyl ester	5.98	8.14
7	37.67	7,10-Hexadecadienoic acid, methyl ester	2.10	1.84
8	40.90	Eicosanoic acid, methyl ester	1.11	1.59

Table 2: Values of Encapsulation Efficiency (EE%) and loaded capacity (LC%) of the prepared neem oil nano-emulsion

Parameter	Mean ± SE	Lower Bound	Upper Bound
EE%	72.58 ± 0.99	68.31	76.85
LC%	40.328 ± 0.55	37.95	42.69

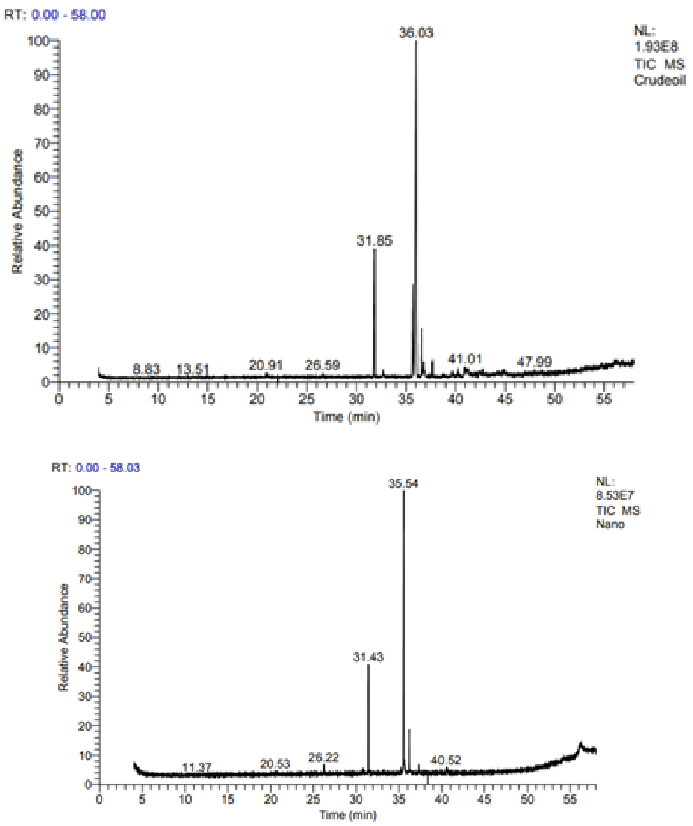

Fig. (2): Upper graph: Gas chromatogram/mass spectrometry analysis of neem crude emulsion. Lower graph: Gas chromatogram/mass spectrometry analysis of neem PEG-loaded nano emulsion.

Effect of Nano and bulk formulations of Neem Oil on Different Biological Aspects of S. gregaria-the 5th nymphal instar

A notable increase in the number of over-aged nymphs was recorded during the treatment period for nymphs exposed to 2.5 ppm nanoemulsion, amounting to 21.25 days. While no significant differences were observed between the 5 ppm nanoemulsion and the control group, the durations were 18.8% and 18.39 %, respectively. Furthermore, the data presented in Table (3) and Fig. (3 and 4) indicated a highly significant decrease in the rate of adult emergence, with 100% of the control nymphs successfully developing into adults, in contrast to 0% emergence in both the 5 ppm and 2.5 ppm crude oil treatments. In the case of nanoemulsions at 5 ppm and 2.5 ppm, the adult emergence rates were recorded at 26.67% and 43.89%, respectively. It is important to note that both doses of nanoemulsion (5 ppm and 2.5 ppm) resulted in 100% malformation, as illustrated in Fig (4), where the insects exhibited damage to their legs, wings, and antennae after molting into adults. Conversely, no such deformities were observed in the control group.

The research indicated that nano-emulsion of neem oil produced morphogenesis effects at all administered concentrations of 5 and 2.5 ppm, disrupting the morphogenesis of newly molted nymphs of *S. gregaria* when applied to the fifth-instar nymphs (Fig. 4). This treatment led to varying degrees of malformations, including significantly or slightly curled bodies, as well as deformities in the antennae and legs. These abnormalities arose from the nymphs' inability to molt into the subsequent nymphal instar or their transition into malformed adult forms. The malformations observed included nymphs that could not completely shed their last nymphal exuviae, which adhered to their bodies, resulting in curled legs and coiled, underdeveloped short wings, along with individuals displaying characteristics of an intermediate nymphal-adult stage. Daily observations revealed a marked antifeedant effect of neem in both crude oil and nano-emulsion treatments on *S. gregaria* nymphs, irrespective of the doses used. The growth inhibition attributed to neem was evident in both treatment types. The application of both crude and nano treatments on the fifth-instar nymphs of *S. gregaria* resulted in a reduction in body mass compared to the control group. Additionally, the amount of fecal waste produced by the desert locusts serves as a reliable measure of their food intake. Consequently, the daily production of fecal pellets was diminished and appeared dry and small in *S. gregaria* fifth-instar nymphs treated with crude oil (5 and 2.5 ppm) and subsequently with nano-emulsion (5 and 2.5 ppm). In contrast, the control insects produced a typical quantity of fecal matter with a standard shape and size. In this study, it was noted that the topical application of *A. indica* crude oil at doses of 5 and 2.5 ppm to the nymphs of *S. gregaria* influenced their body coloration prior to death, with the cuticle color changing from yellow with black patches to a uniformly black appearance in all deceased specimens (Fig. 5).

Table 3: Percentage mortality, duration, adult emerge and adult malformation of *S. gregaria* against two doses of neem oil and its nano and bulk forms

Oil type	Dose (ppm)	Corrected mortality% Mean ± SE (Confidence limit)	Nymphal duration days Mean ± SE (Confidence limit)	Adult emergence% Mean ± SE (Confidence limit)	Adult malformation% Mean ± SE (Confidence limit)
Nano	5	73.33 ± 4.22^{b} (62.5-84.17)	18.8 ± 0.39^{b} (17.92-19.68)	26.67 ± 4.22^{c} (15.83-37.51)	100
	2.5	56.11 ± 2.91^{c} (48.64-63.59)	21.25 ± 0.90^{a} (19.12-23.38)	43.88 ± 2.91^{b} (36.41-51.37)	100
Bulk	5	100^{a}	-	0^{d}	-
	2.5	100^{a}	-	0^{d}	-
Control	0	0^{d}	18.39±0.30^{b} (17.75-19.03)	100^{a}	0
F Value		324.9**	8.86**	324.9**	-

Mean ±SE within the same column followed by the same letter are not significantly different (ANOVA and Tukey's HSD test at $P < 0.05$). Note: All bulk (crude oil)-treated individuals were shortly dead in the tested concentrations which negated comparison with nano formulations.

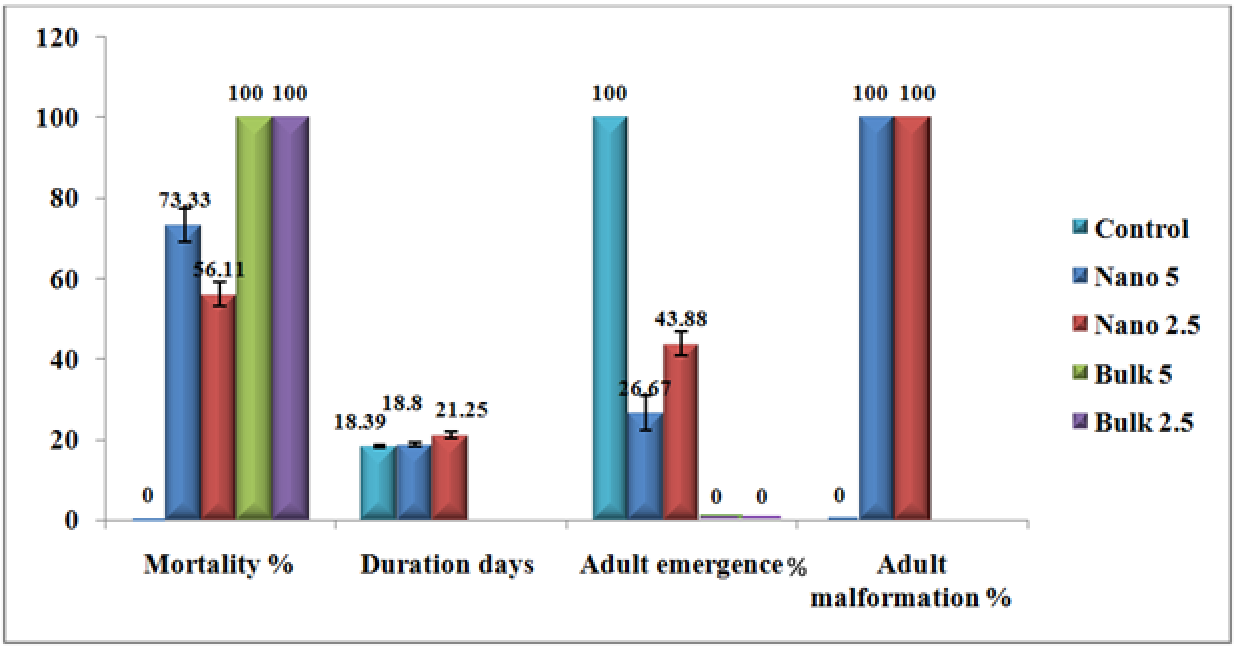

Fig. (3): Mortality%, Nymphal duration days, Adult emergence% and Adult malformation% of *S. gregaria* against two doses nano and bulk forms of neem oil.

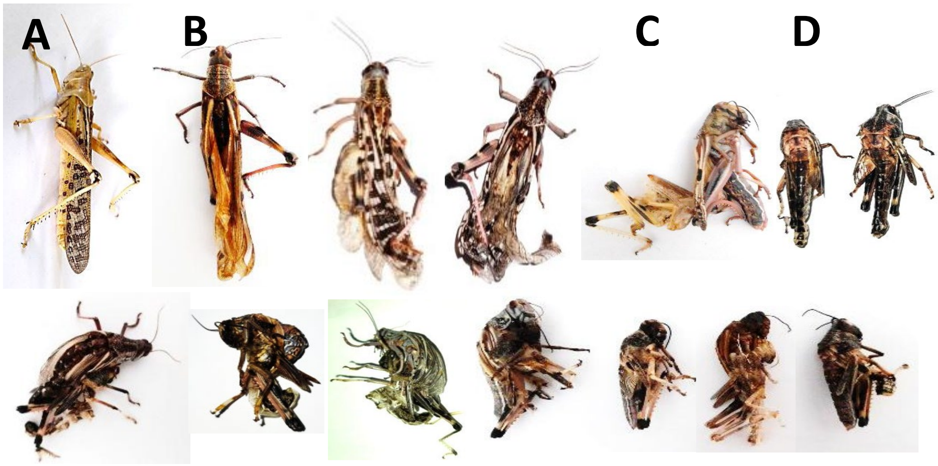

Fig. (4): Molting failure of treated *S. gregaria* 5th nymphal instars with nano-emulsion of neem *at* 5 and 2.5 ppm. A: Normal adult. B: Curled and twisted wings. C: The old cuticle is linked to the developed adults. D: Nymphs were unable to transition into adult and died without completing the molting process.

Physiological effects (biochemical analysis)

The information presented in Tables 3 and 4 illustrates the impact of neem nanoemulsion and crude oil on the total protein and chitinase activity of *S. gregaria* nymphs after 24, 48, and 72 hours. The parameters examined were influenced by various treatments and dosages of neem nanoemulsions in comparison to the control group. Table (4) indicates that a 5 ppm dose (contact treatment) significantly affected both total protein and chitinase activity. A notable reduction in total protein was observed 24 hours post-treatment with the nanoemulsion when compared to the control. Conversely, the crude oil treatment did not alter total protein levels during the same timeframe; however, after 72 hours, a significant decrease was noted in total protein levels when compared to both the control and the nano treatment (F value 7.44). Additionally, chitinase enzyme activity exhibited a significant decline after 24 hours in the nano treatment relative to the control, while a significant increase was recorded in chitinase activity following the crude oil treatment during the same period (F value 3.40).

Table (5) presents the effects of a 2.5 ppm dose (contact treatment) on total protein and chitinase activity. A significant decrease in total protein was observed 24 hours post-contact treatment with crude oil, whereas the nano treatment did not affect total protein levels in comparison to the control (F value 3.72). Furthermore, chitinase activity significantly decreased after 24 hours of nano treatment, while crude oil treatment led to a significant increase in chitinase activity during the same period when compared to the control nymphs (F value 21.80).

Fig. (5): Body color of treated *S. gregaria* 5th nymphal instars with crude oil of neem with 5 and 2.5 ppm. A: Normal nymphs. B: treated nymphs.

Table 4: Effects of 5 ppm *A. indica* nano and bulk emulsions on certain biochemical aspects of the 5th instar nymphs of *S. gregaria*

Chemical parameter / Treatment		Total protein Mean ± SE mg/g insect body weight	Lower Bound	Upper Bound	Chitinase Enzyme Mean ± SE µg NAGA/min/mg sample	Lower Bound	UpperBound
Nano Formulation	24 h	33.25 ± 4.13bc	15.46	51.04	14.87 ± 0.14bcd	14.27	15.47
	48 h	32.68 ± 1.35bc	26.86	38.51	21.25 ± 3.43ab	6.50	36.00
	72 h	36.20 ± 1.75ab	28.66	43.74	22.65 ± 2.51a	11.87	33.43
Bulk Formulation	24 h	26.29 ± 1.86c	18.30	34.27	18.07 ± 3.33abc	3.73	32.40
	48 h	43.24 ± 1.67a	36.05	50.44	12.89 ± 2.11cd	3.79	21.98
	72 h	41.44±1.44bc	35.26	47.62	10.57 ± 1.34d	4.80	16.33
Control	24 h	35.46 ± 1.36abc	29.59	41.33	17.61 ± 1.4abc	11.59	23.63
	48 h	35.87 ± 0.28ab	34.65	37.09	15.74 ± 1.42bcd	9.63	21.85
	72 h	40.70±.78ab	37.34	44.07	14.32 ± 0.69bcd	11.37	17.27
F value		7.44**			3.40*		

**Highly significant. Each value represents the mean of 3 replicates±SE Values with different letters within the same row are significantly different (ANOVA and Tukey's HSD test at $P < 0.05$).

Discussion

TEM analysis

This study demonstrated that the inner cores of active ingratiate (neem oil) were developed as a single core of smaller particles within each capsule. To achieve the reduced size of the capsules, both a cleaner sonicator and a high-energy ultrasonic probe were employed. It was indicated that the utilization of the high-energy ultrasonic probe is a crucial step in producing small nano-capsules. Our findings were consistent with those reported by Bayoumi *et al.*, (2022). However, the authors noted that relying solely on mechanical stirring is inadequate for producing uniformly sized nano-capsules, and extending the sonication time, either prior to or following the addition of oil to the loading material, resulted in smaller capsules. In this study, the capsule sizes were larger than those reported by Youssef *et al.* (2018), who prepared peppermint/alginate nano-capsules and found that the diameter of peppermint oil nano-capsules ranged from 70 to 100 nm. The application of sonication after cross-linking with calcium chloride contributed to a reduction in the diameter of the nano-capsules without the use of a solvent. A similar observation was made by Lertsutthiwong *et al.* (2008), who reported that the size of the prepared nano-capsules was influenced by the use of high-energy sonication, sonication duration, and increased dilution with distilled water.

Mortal potency of the neem formulations against nymphs of *S. gregaria*

The research conducted by Linton *et al.* (1997) demonstrated that the application of azadirachtin to *S. gregaria* during the 5th instar stage significantly increased mortality rates among these insects, with the death rates in the treated group being markedly higher than those in the control group. Specifically, mortality rates for the 5th instar *S. gregaria* were recorded at 56% and 64% for dosages of 7 µg and 5 µg of azadirachtin, respectively, in contrast to 5.3% mortality in the control group. Notably, 40% of the insects treated with 7 µg and 5 µg of azadirachtin perished during the ecdysis process, while only 5.3% of the control group experienced similar outcomes. Furthermore, Hamadah *et al.* (2013) observed that all insects in the 4th and 5th nymphal instars of *S. gregaria* succumbed to treatment with high doses of Neemazal from *A. indica*, with mortality rates reaching 60% and 70% at lower concentration levels, respectively. Azadirachtin has also been shown to exert toxic effects on the 4th nymphal instars of *S. gregaria*, with potential mortality rates of 100% after 72 hours of treatment with clover leaves emerged in a concentration of 25%, while a lower concentration of 5% resulted in 65% mortality (Al-Maroug *et al.* 2022). The oils derived from *A. indica* seeds exhibited mortality rates ranging from 65% to 100% in *S. gregaria* (Nicol *et al.*, 1991; Schmutterer & Frekes, 1990). The observed partial or complete mortality in *S. gregaria* can be linked to feeding inhibition, which leads to prolonged starvation and eventual death (Hamadah *et al.*, 2013). Additionally, the mortality of desert locusts due to plant extracts may disrupt metamorphosis in the final instar nymphs of *S. gregaria*, potentially as a result of hormonal regulation disturbances. It has been suggested that the prevention of metamorphic ecdysis and subsequent death may be achieved by modulating the ecdysteroid peak or influencing the release of eclosion hormone (Al-Sharook *et al.*, 1990).

Table 5: Effects of 2.5 ppm *A. indica* nano and bulk emulsions on certain biochemical aspects of the 5th instar nymphs of *S. gregaria*

Chemical parameter / Treatment		Total protein Mean ± SE mg/g insect body weight	Total protein Lower Bound	Total protein Upper Bound	Chitinase Enzyme Mean±SE µg NAGA/min/mg sample	Chitinase Enzyme Lower Bound	Chitinase Enzyme Upper Bound
Nano Formulation	24 h	36.20±2.77^{ab}	24.30	48.10	14.15±1.78^{cd}	6.51	21.79
	48 h	33.09±0.72^{ab}	30.02	36.16	13.38±1.034^{d}	8.93	17.83
	72 h	36.04±1.73^{ab}	28.58	43.50	19.05±0.40^{b}	17.33	20.78
Bulk Formulation	24 h	30.06±2.57^{b}	18.98	41.13	29.81±0.93^{a}	25.81	33.81
	48 h	32.51±1.59^{ab}	25.65	39.38	17.07±0.26^{bc}	15.94	18.20
	72 h	30.71±1.81^{b}	22.92	38.51	15.20±0.77^{cd}	11.90	18.51
Control	24 h	40.70±0.78^{a}	37.34	44.070	17.61±1.4^{bc}	11.59	23.63
	48 h	35.87±0.28^{ab}	34.65	37.09	15.74±1.42^{bcd}	9.64	21.85
	72 h	35.46±1.36^{ab}	29.60	41.33	14.32±0.69^{cd}	11.37	17.27
F value		3.72*			21.80**		

**Highly significant. Each value represents the mean of 3 replicates ± SE Values with different letters within the same row are significantly different (ANOVA and Tukey's HSD test at $P < 0.05$).

Neem oil has been shown to disrupt the growth and developmental processes in desert locusts. Specifically, instars treated with azadirachtin, referred to as over-aged nymphs, exhibit an extended instar duration, allowing them to survive for several weeks longer than the typical duration. Furthermore, over-aged nymphs of *L. migratoria* and *S. gregaria* that were treated with azadirachtin demonstrate activity in both the corpus allatum and juvenile hormone.

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A study by Linton *et al.* (1997) indicated that 20 days post-treatment, all insects in the control groups had reached adulthood, while only 12% of those treated with 5µg of azadirachtin achieved adulthood. In contrast, a higher dosage of 7mg resulted in the production of over-aged nymphs in *S. gregaria*. Neemazal has been observed to inhibit the development of both penultimate and final instar nymphs, significantly prolonging the nymphal duration in penultimate instars, particularly at concentration levels of 3.7, 1.8, 0.9, and 0.4%, with durations of 19.0±2.8, 17.7±3.2, 13.2±2.8, and 15.5±3.9 days, compared to 9.6±2.6 days for control nymphs. A similar inhibitory effect was noted for the last instar nymphs, especially at concentrations of 1.8% and 0.9%, which resulted in durations of 15.8±4.0 and 15.9±3.9 days (Hamadah *et al.*, 2013), respectively, versus 12.3±1.2 days for control nymphs. The application of Neemazal on penultimate instar nymphs of *S. gregaria* resulted in delayed development, characterized by an extended developmental duration and a regression in the developmental rate relative to the concentration level (Nicol *et al.*, 1991). This prolonged developmental duration serves as a significant indicator of inhibited growth in desert locusts following treatment with *A. indica* seed oil. Additionally, azadirachtin notably extended the duration of fourth instar nymphs of *S. gregaria*, with the most significant prolongation observed at concentrations of 10% and 15%, yielding durations of 16.7 and 17.6 days, respectively vs. 10.2 days of control (Al-Maroug *et al.*, 2022).

In alignment with the findings of the current study on *S. gregaria*, the application of neem in its nano form on the 5th instar nymphs resulted in a delay in development when compared to the control group. There exists a quantitative relationship between juvenile hormone and ecdysteroids (Schmutterer & Frekes, 1990). The temporal alteration of this balance is crucial for the successful development of the insect; thus, a postponement of the ecdysteroid peak prior to molting, as noted by Bashir and El Shafie (2014) following azadirachtin injection, may induce 'solitarisation effects.' This disruption caused by azadirachtin within the hormonal system is also linked to malformations, molting disturbances, and developmental delays. Azadirachtin has been shown to affect the success of molting between the 5th instar and adult stages, inhibiting the growth of desert locusts. Neem oil similarly regulates growth and development in desert locusts. The treatment or ingestion of azadirachtin can result in diminished feeding levels in insects, which is essential for achieving the necessary body mass prior to ecdysis. Commonly observed abnormalities in insects treated with azadirachtin include mortality during the molting process, where the insect fails to shed the old cuticle. More than 45% of molts in Azadirachtin-treated insects resulted in death during ecdysis, whereas no mortality was recorded in the control group. Research indicates that Azadirachtin treatment significantly affects the loss of molts in insects, likely due to their inability to ingest sufficient air volumes to rupture the old cuticle and facilitate the expansion of the new one during ecdysis (Linton *et al.* 1997; Hamadah *et al.*, 2013). The same phenomenon was observed in the treated 5th instar nymphs of *S. gregaria* in the present study. Neemazal, an extract derived from neem, disrupts the metamorphosis process in *S. gregaria* by generating nymphal-adult intermediates, which may interfere with the normal ecdysone or ecdysteroid levels required for proper metamorphosis. Azadirachtin has been documented to inhibit ecdysteroid levels. Furthermore, azadirachtin treatment impedes neurosecretion of prothoracicotropic hormone, leading to physiological disruptions (Hamadah *et al.*, 2013).

Description of Nymphal Performance Affected by neem

Neem formulations effected malformation

The utilization of nano formulations in varying doses has prompted morphogenesis changes in *S. gregaria*, leading to different levels of malformations characterized by nymphal-adult intermediate stages. This investigation indicates that azadirachtin induces morphogenesis effects in *S. gregaria* following treatment of fourth instar nymphs, resulting in the above-mentioned malformations. These abnormalities arise when nymphs fail to molt into the subsequent nymphal instar or when the molting process culminates in malformed adult stages. This study documents that the nymphal morphogenesis of *S. gregaria* is disrupted by azadirachtin treatment administered to penultimate instar nymphs. Observed deformities include the inability of nymphs to shed the last nymphal exuviae, which remain attached to their bodies, resulting in an adult-like form with curled legs and incompletely developed short wings, alongside the emergence of nymphal-adult intermediate stages (Al-Maroug *et al.*, 2022). Previous research done by Nassar *et al.* (2018) demonstrated various impacts on the transformation of immature adult forms due to botanical extracts, with some exhibiting no effects or contradictory outcomes, contingent upon the activity of the plant species and the susceptibility of the insect species. Consequently, azadirachtin has been shown to influence both the hormonal system of *S. gregaria* and juvenile hormone levels (Al-Maroug *et al.*, 2022). Our findings corroborate Josephrajkumar *et al.* (1999) who reported morphogenesis defects in adult *S. gregaria* following neem oil treatment of final instar nymphs. The performance of *S. gregaria* may be compromised by changes in ecdysteroid titers, which can lead to alterations in lysosomal enzyme activity and subsequent morphological abnormalities.

Neem formulations effected feeding

The antifeedant characteristics of azadirachtin are well-documented. This compound has been shown to decrease food intake and hinder the growth of desert locusts (Linton *et al.* 1997). The effects of *A. indica* are linked to its saponin constituents, which may influence cell membranes, thereby impairing digestion and nutrient absorption (Bogumil & Wieslaw, 2006, De Geyter *et al.*, 2012). Azadirachtin interferes with gut functionality, resulting in a flaccid, partially filled gut, diminished protein digestion efficiency, and lowered feeding rates. Injections of azadirachtin produce a significant antifeedant response in 5th instar nymphs of *S. gregaria*, as indicated by the average daily production of fecal pellets (Linton *et al.*, 1997). Consistent with these findings, insects treated with both doses of bulk and nano formulations exhibited a pronounced antifeedant effect on *S. gregaria*, evidenced by a decrease in daily fecal pellet output, which was notably smaller and drier in comparison to the control group.

Neem formulations effected nymphs color

Indent imbalances in the levels of ecdysteroids and juvenile hormones are evident in the effects associated with elevated juvenile hormone titers. These effects include the presence of green hemolymph, brown and green cuticles, a lack of black pigmentation, and supernumerary molts in locusts, as well as cuticular melanization that results in black spots linked to low ecdysteroid levels and the absence of juvenile hormone (Schmutterer & Frekes , 1990, Nicol & Schmutterer, 1991, Gelbic & Ne´mec, 2001). The role of the bright yellow and black coloration observed in gregarious late-instar nymphs of *S. gregaria* remains ambiguous. However, it has been proposed that this coloration serves as warning signals, as these gregarious nymphs, who inhabit desert environments, consume plants that contain alkaloids and other toxic compounds (Cullen *et al.*, 2017). During the nymphal stage of *S.*

gregaria, the shift in coloration, which is influenced by population density and the consumption of native toxic host plants, leads to the development of warning coloration, known as aposematism (Sword *et al.* 2000). Numerous studies have indicated that juvenile hormone promotes green body coloration in the nymphal stages of various acridid species (Pener & Simpson, 2009). In regions with ample vegetation, *S. gregaria* exhibits green phenotypes and solitary behavior. Yet, as vegetation diminishes due to arid conditions, these locusts undergo a phase change to a gregarious form, displaying yellow with black patterns (FAO, 2001). Despland & Simpson (2000) indicated that food quality significantly impacts mortality and development in desert locusts. High-quality food sources may enhance population growth by hastening the emergence of later instar hoppers, reducing mortality rates, and enabling the production of two generations within a single season. Based on these findings and our data, we deduce that the color change to black in fifth-instar nymphs occurred following treatment with neem in bulk form, likely due to the cessation of feeding and an imbalance in ecdysteroid and juvenile hormone levels.

The active components responsible for the insecticidal properties of botanical insecticides are typically lipophilic, allowing them to permeate the cell membranes of insects and disrupt their digestive and neurological systems (Da Cruz *et al.* 2020). Liu *et al.*, (2013) reported that compounds such as terpenoids, alkaloids, and steroids, which are synthesized by plants, contribute to this insecticidal activity. Research has identified key chemical compounds, including α-terpinyl acetate, α-terpineol, 4-terpineol acetate, and linalool, as significant agents that can effectively kill and repel insects. Analysis using gas chromatography-mass spectrometry (GC/MS) revealed a substantial reduction in linoleic acid due to the nano formulation process, suggesting that an extended ultrasonic treatment of three minutes negatively affected the stabilization of linoleic acid. Furthermore, Youssef & Abdelmegeed (2021) noted that the preparation of a *Mentha piperita* alginate nanoemulsion did not significantly alter the oil compounds, with the exception of the absence of eucalyptol, as only two minutes of ultrasonic treatment was employed in their methodology. This may account for the enhanced efficacy of bulk oil in nano formulations as an insecticidal product.

Botanical formulations effected biochemical parameters

Proteins are essential components within cells, serving various functions including hormonal regulation (Sugumaran, 2010). They also contribute structurally, similar to carbohydrates and lipids. Furthermore, the synthesis of proteins is crucial for the maintenance of growth and reproductive processes in the body (Dimetry *et al.*, 2019). Azadirachtin has been shown to influence pest development by interfering with the hormones that regulate moulting and metamorphosis (Amin *et al.*, 2019). Mahmoud *et al.* (2014) indicated that the expression of proteins in adult *S. gregaria* treated with azadirachtin was reduced. Specifically, when adult locusts were administered a dose of 7.5 µg/g body weight, the total number of protein bands increased slightly from 10 in the control group to 11 in the treated group. However, when the dosage was raised to 15 µg/g body weight, the number of protein bands decreased from 10 to 8, and this number remained unchanged even with a further increase to 30 µg/g body weight. Additionally, protein expression can decline when insects are fed a diet containing azadirachtin or when larvae are injected with it. Reda *et al.* (2009) proposed that the reduction in hemolymph protein levels in *S. gregaria* may result from the detrimental effects of neem on the neurosecretory cells in the brain, which are responsible for protein secretion. This reduction may also stem from inadequate stimulation of neurosecretory activity, leading to a decrease in the protein levels in hemolymph necessary for oocyte development. Consistent with these findings, the current study demonstrated a decrease in total protein levels following neem treatments (both nano and bulk oil), particularly after 24 and 48 hours compared to the control group. It was noted that the reduction in hemolymph proteins in adult *S. gregaria* treated with Hexaflumuron, a chitin-synthesis inhibitor, may indicate a diminished capacity for protein synthesis. This decrease in hemolymph proteins can inhibit oogenesis and ovarian development, ultimately resulting in the sterility of the adult insect. Azadirachtin shows promise as a biopesticide for managing insect pests (Amin *et al.*, 2019). The process known as apolysis involves the separation of epidermal cells from the old cuticle through the secretion of molting fluid and the formation of the ecdysal membrane, marking the initiation of ecdysis. This molting fluid is rich in proteases and chitinases, which are enzymes responsible for breaking down the primary components of the old endocuticle. In this study, a notable increase in chitinase activity was observed in nano and bulk formulations after 24, 48, and 72 hours following treatment. Additionally, Abdel-Aal (2006) reported that chlorfluazuron significantly enhanced chitinase activity in *Spodoptera littoralis*. These findings are consistent with previous research reported by Al-shannaf *et al.* (2012), they indicated that insect growth regulators, such as chlorfluazuron and pyriproxyfen, led to substantial increases in chitinase enzyme activity, with a reported increase of 130% in the larvae of the American bollworm, *Helicoverpa armigera*. The observed rise in chitinase activity may serve as a detoxification mechanism, which is considered as a main reason for the black color appeared on the treated nymphs and the individuals resulted from them.

Conclusion

The current research revealed the detrimental effects of topically administered neem *A. indica* on fifth-instar *S. gregaria* in semi-field settings. Prolonged sonication adversely impacted the key compound (Linoleic acid) within the nano formulation, leading to diminished pesticidal efficacy compared to the neem extract. Both formulations negatively influenced biological parameters, including mortality rates, failed molting, and deformities, in addition to affecting biochemical activities such as chitinase activity and total protein levels. Consequently, the nano formulation developed through this method does not seem to be an effective control agent for *S. gregaria*. Interestingly, bulk (crude oil)-treated individuals were shortly dead in the tested concentrations so that their durability was not compared with nano formulations.

Author's Contributions

Nadia Zekry Dimetry: investigation, methodology, supervision, formal analysis, visualization, draft preparation; **Mahfouz Mohamed Mostafa Abd-Elgawad**: conceptualization, formal analysis, visualization, final review and edit; **Dalia Abdallah Youssef**: investigation, methodology, formal analysis, visualization, draft preparation, final review and edit; **Samira Mohamed Nabeel Abd-El Wahed** investigation, methodology, formal analysis, visualization, draft preparation, final review and edit.

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Data Availability Statement

All data supporting the findings of this study are available within the paper.

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Ethics Approval

Insects were used in this study. All applicable international, national, and institutional guide lines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Conflict of Interest

The authors declare no conflict of interest.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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Research Article

تاثیر مخرب فرمولاسیون نانو روغن چریش، *Azadirachta indica*، روی ملخ صحرایی، *Schistocerca gregaria*

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چکیده: آفت‌کش‌های زیستی گیاهی به دلیل اثربخشی‌شان در مبارزه با انواع آفات زراعی شناخته شده‌اند. اهمیت این آفت‌کش‌ها، قابلیت تجزیه زیستی و مقرون‌به‌صرفه بودنشان می‌باشد. کاربرد فناوری نانو به عنوان راهی امیدوارکننده برای پیشرفت روش‌های کنترل آفات در نظر گرفته می‌شود. این مطالعه، تأثیر مخربی را در ارتباط با تبدیل روغن چریش به شکل نانو با استفاده از امواج فراصوت پرانرژی نشان داد. تجزیه و تحلیل میکروسکوپ الکترونی عبوری (TEM) نشان داد که نانوذرات تهیه شده، کروی و صاف بودند و میانگین اندازه ذرات آنها از 90 تا 120 نانومتر متغیر بود. اثرات روغن چریش فله‌ای و نانوامولسیون آن بر روی پوره‌های سن پنجم ملخ صحرایی، *Schistocerca gregaria* در شرایط نیمه‌مزرعه ارزیابی شد. کاربرد موضعی هر دو فرمولاسیون، در دوزهای 2.5 و 5 ppm، منجر به افزایش قابل توجه میزان مرگ و میرو همچنین ناهنجاری‌های پورگی و پوست‌اندازی ناموفق، در مقایسه با گروه کنترل شد. با این حال، فرم خام روغن چریش، اثربخشی بیشتری نسبت به فرمولاسیون نانو نشان داد. فعالیت کیتیناز و پروتئین کل تحت تأثیر هر دو فرمولاسیون قرار گرفتند. نکته قابل توجه این است که نانو امولسیون منجر به افزایش قابل توجه مدت زمان زنده ماندن پوره‌ها شد. تجزیه و تحلیل کروماتوگرافی گازی/طیف‌سنجی جرمی (GC/MS)، عدم وجود اسید لینولئیک در نمونه نانو را نشان داد، که ممکن است دلیل اثربخشی برتر نمونه خام نسبت به فرمولاسیون نانو باشد. تیمار اولتراسونیک طولانی مدت در طول تهیه نانو امولسیون روغن چریش، بر ترکیبات زیستی فعال موجود در روغن چریش تأثیر منفی گذاشت و منجر به کاهش فعالیت زیستی آن درروی پوره‌های سن پنجم *S. gregaria* در مقایسه با فرم خام آن شد.

کلمات کلیدی: ملخ صحرایی، پروتئین کل، جی سی مس، امولسیون نانو، فعالیت آنزیم، آفت‌کش‌های سبز

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