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Assessing Toxicity and Some Biochemical Alterations for Nano-formulation of Emamectin Benzoate in *Spodoptera littoralis*

Gehan Y. Abdou[1], Abdel-Halim K.Y.[2] and Manal. A. Attia[3]

[1]Pests & Plant Protection Department, National Research Centre, Dokki, Giza, Egypt.
[2]Mammalian & Aquatic Toxicology Department, Central Agricultural Pesticides Laboratory (CAPL), Agricultural Research Center (ARC), 12618-Dokki, Giza, Egypt.
[3].Bioassay Research Department, Central Agricultural Pesticides Laboratory (CAPL), Agricultural Research Center (ARC), 12618-Dokki, Giza, Egypt.

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ABSTRACT

Emamectin benzoate (EMB) is a commonly used insecticide against *Spodoptera littoralis,* but its EC formulations are intolerance to light and temperature. This study aimed to develop and characterize a nano-emulsion of EMB (nano-EMB), assess the toxicity and biological effects of EMB and nano-EMB on *S. littoralis*, and investigate their interaction with certain enzymes. The results demonstrated the stability of nano-EMB with no phase separation during freeze-melt cycles, shaking and centrifugation. Characterization of nano- EMB using Transmission Electron Microscopy (TEM) revealed spherical droplets with size (39.0 -72.0 nm), and a zeta potential of 38.5 mV. The toxicity tests showed that, nano-EMB was 13 times more toxic to the 4th instar larvae of *S. littoralis* than EMB, with LC_{50} = 0.0014 and 0.0183 mg/l, respectively. The sub-lethal exposure to both EMB and nano-EMB affected the development of *S. littoralis* and significantly decreased adult emergence, fecundity, fertility, and longevity despite increased adult deformation; the highest effect was recorded in the larvae treated with LC_{50} of nano-EMB. Additionally, EMB caused a significant decline in acetylcholinesterase activity, which was low in larvae treated with LC_{25} or LC_{50} (0.029 and 0.032 µM/mg/min, respectively), compared with that treated with LC_{25} or LC_{50} of nano-EMB (0.038 and 0.037 µM/mg/min, respectively) While no significant changes were observed in α-esterase activity. Conversely, nano-EMB at LC_{50} significantly increased β-esterase activity (7.11 µM/mg/min) compared with the other treatments. The findings here suggest that nano-EMB could serve in integrated pest management programs, offering lower application rates and improved pest control efficacy.

Keywords: nano-emulsion, nano-emamectine benzozte, emamectine benzoate, insecticide toxicity, biochemical alternation, sub-lethal effects.

1. Introduction

Spodoptera littoralis (Boisd) (Lepidoptera: Noctuidae) is a polyphagous threat pest widespread in Africa, Europe and Asia. *S. littoralis* attacks both vegetable and field crops causing direct and extensive injury to crops (CABI, 2022) that required urgent control measures, including chemical control. Pesticides are essential for managing pests; the universal pesticide market was reported to reach $56 billion in 2012 (Atwood and Paisley-Jones, 2017). Yet, since the 1950s, the misuse of insecticides has led to the development of insecticide resistance (Moustafa *et al*., 2023) and environmental problems (Singh *et al.,* 2018). These conditions, along with the environmental concern, force researchers to discover alternatives or, at very least, specific chemicals to replace the traditional insecticides.

Emamectin benzoate (EMB), a macrocyclic lactone that derived from avermectin by the early 1980s belongs to the avermectin family which includes 16 members that originated from the natural fermentation of *Streptomyces avermitilis* (Pitterna *et al*., 2009). EMB was innovated after screening the biological activity of several avermectin derivatives; this particular compound was chosen for further

Corresponding Author: Gehan Y. Abdou, Pests & Plant Protection Dept., National Research Centre, Dokki, Cario, Egypt. E-mail: gehanabdou295@gmail.com

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enhancement in plant protection. EMB exhibited enhanced thermal tolerance and higher water solubility in comparison to the initial hydrochloride form (Jansson and Dybas, 1998), in addition to its wide range of toxicity (Ishaaya *et al*., 2002, Ahmed *et al*., 2020, and Chen *et al*., 2023). EMB is recommended and widely used in Egypt against several insects (APC, 2023). In particular, EMB showed potent insecticidal activity against the cotton leaf worm, *S. littoralis* (El-Sayed *et al*., 2020; and Salem *et al*., 2024). Unfortunately, avermectin derivatives are easy to degrade rapidly and are affected by environmental conditions (Song *et al*., 2019 and Zhou *et al*., 2019). Also, these insecticides bind the soil particles and expose them to soil microorganisms' decomposition (Zhang *et al*., 2022). Additionally, the performance of the current traditional pesticide formulations is not as perfect as required, as a major portion of the loss is drawn away from the target insect as a consequence of large particles and weak adherence (Fattahi *et al*., 2024; Xue *et al*., 2024), resulting in considerable environmental contamination and health problems (Zhao *et al*., 2023). Moreover, the existing commercial forms of EMB are light and temperature intolerant (Pitterna, 2007). Therefore, there is a growing demand to formulate products that achieve high efficiency and prolonged protection with a possible low environmental adverse impact

Nowadays, using nanotechnology tools in agriculture is a trending practice as they balance minimal concentration, maximize pest control, and reduce the cost (Amin *et al*., 2022). Nano-formulations present an aspirant key for sustainable insect management, whereas such formulations promote insecticide performance by enhancing the bioavailability of active ingredients, solubility, dispersion, controlled release, and decreasing environmental adverse effects (Sheeja *et al*., 2022; Sharma, 2023; Gupta *et al*., 2023; Liu *et al*., 2023 and Kumar *et al*., 2024). Although the current formulations of EMB contain a low relative concentration of active ingredients (a.i), they depend on a high content of surfactants that represent an ecological threat (Chen, 2011). Instead, nano-formulations could avoid organic solvents and reduce surfactants; few studies were achieved (Elabasy *et al*., 2019; Amin *et al*., 2022 and Kumar *et al*., 2024). Therefore, this study aimed to develop and characterize a nano-emulsion of EMB (nano-EMB) besides assess the toxicity and some biological effects of EMB and the developed nano-EMB against *S. littoralis* under laboratory conditions and the interaction of both formulations with certain enzymes.

2. Material and Methods

2.1. Chemicals

All chemicals used were purchased from Sigma-Aldrich Company unless otherwise declared.

2.2. Insects

A susceptible strain of *S. littoralis* obtained from Faculty of Agriculture, Cairo University, Egypt. This strain has been raised over several generations following the methodology described by Eldefrawi *et al*. (1964) in the laboratory. The insects were reared under controlled conditions (26±2 °C, relative humidity of 65% ± 5, and a 12:12 hr light-dark cycle), whereas larvae were fed fresh castor bean oil leaves, while the adult insects were fed a 10% sucrose solution.

2.3. Tested Insecticide

The tested pesticide, emamectin benzoate (Emar 5% EC), was obtained from The Central Agricultural Pesticides Laboratory (CAPL), ARC, Egypt and produced by SAUDI Veterinary Pharmacy Products Factory (SPPF), Saudi Arabia.

2.4. Preparation and characterization of nano-emulsion

A nano-emulsion of EMB (5%) was prepared as described by Abdel-Halim *et al*. (2021). The prepared nano-emulsion of EMB was subjected to different storage conditions of temperature and humidity to investigate its emulsifiable properties flowing the ICH guidelines Q1A (ICH, 2003). The nano-EMB emulsion was exposed for centrifugation at 3500 rpm for a period of 10 min to perceive phase separation. A dilution of 10 ml nano-EMB emulsion in 100 ml distilled water in a graduated cylinder was shaken in an up-and-down motion 30 times. Afterward, the solution was allowed to settle for 10 minutes to observe any oil separated layer or cream floating, or sediment parts. In addition, an aliquot of the nano-EMB emulsion was subjected to a freeze-thaw for six cycles between refrigerator (4^{o} C) and oven (48^{o} C) for 48 hrs in addition to three cycles between 21 and 25^{o} C. The prepared nano-EMB emulsion were characterized as follow: The morphology and structure of nano-EMB were analyzed using Transmission Electron Microscopy (TEM), Instrument model JOEL 1400 Plus, manufactured in Japan with a filament (80 KeV) to investigate the droplets shape and size. To prepare the sample a diluted solution of nano-EMB in deionized water ((1/100 v/v) was sonicated adequately, a drop of this solution was transferred to the film grid and dried, then observed (Bhatt and Madhav, 2011). For optimal visualization couple modes of a diffraction and bright-field imaging were employed. The dynamic light scattering mode (DLS) mean was used to estimate the particles size distribution of nano-EMB by zeta sizer instrument model: Malvern, ZS Nano, UK.

2.5. Bioassay procedure

Bioassay experiments were conducted to assess the efficacy of EMB and the prepared nano-EMB against the 4th instar larvae of *S. littorals* using the leaf dip method. Briefly, 6-8 dilutions of both EMB and its nano-emulsion were diluted in distilled water to give mortality ranging between 20 and 80%. Castor bean leaf desks were dipped in freshly prepared insecticide solutions or only water for the control group for 30 sec. and allowed to dry. The treated desks were introduced to the 4th instar larvae of *S.littoralis* (10 larvae/replicate and 4 replicates/each concentration). The treated larvae were set aside in an incubator (26± 2°C, 65 ±10% RH and a light–dark cycle of 12:12 hr). The larval mortality was recorded 48hr after treatment and the mortality percentages were calculated. The relative potency and toxicity index were calculated according to Sun (1950).

2.6. Effects of sub-lethal concentrations on biological parameters

Castor oil leaves were treated as mentioned above with the previously estimated LC_{25} or LC_{50} equivalent concentrations of both EMB and nano-EMB. Four hundred 4th instar larvae were fed on these treated leaves for each concentration (25 larvae per replicate). After 48hrs under the previously mentioned conditions, the survived larvae were transferred to clean jars with fresh untreated leaves and observed daily, kept at the same conditions. Data were recorded daily until pupae eclosion. Once pupae formation the pupae were weighted 24 hrs after eclosion. Larval and pupal durations were recorded, as well as the percentages of adult emergence. The emerged adults were fed sucrose solution and observed daily till death to determine their longevity. To investigate the other biological effects, two adult males were kept with one adult female to maximize the probability of successful mating (four replicates for each treatment aside from the control). The egg masses laid by each mated female were collected and counted daily to calculate the fecundity (number of eggs per female). The laid eggs were also observed daily to determine fertility (percentages of hatched eggs per female).

2.7. Biochemical quantification

2.7.1. Sample preparation

Groups of larvae were treated with the sub- lethal concentrations (LC_{25} and LC_{50}) of EMB or nano- EMB as described previously. After 48hrs of exposure, the alive larvae were collected and subjected to biochemical tests. The larvae (whole body) were homogenized in sodium phosphate buffer (20 mM & pH 7.4, 1:10 w/v) by a ceramic motor placed. The homogenate was centrifuged under cooling at 13000 rpm for 10 min, and the supernatant was separated and considered as enzymes source.

2.7.2. Acetylcholinesterase (AChE) activity

The AChE enzyme activity was determined using Ellman *et al.* (1961) method. The optical density of the developed yellow color was measured against the blank (containing all the reagents except the enzyme source) at 412 nm by a spectrophotometer, Spectronic 20 D, MILTON ROY Co. The activity of AChE was expressed as μM of the hydrolyzed acetylthiocholine iodide per mg protein per min.

2.7.3. Esterases activity

The activity of α and β-esterases was measured by the method of Van Asperen (1962). The samples were run on spectrophotometer and optical density was noted at 430 nm for α-esterase (CE), and 590 nm for β-esterase. The activity was expressed as μM /mg protein/min.

2.7.4. Glutathione-*S*-transferase activity

The activity of GST was determined following the spectrophotometric method described by Habig *et al.* (1974) the used substrate was 1-chloro, 2-4 dinitrobenzene (CDNB). The absorbance was recorded at 340 nm. the specific activity was expressed as nM/mg protein/min.

2.7.5. Total protein

Total protein in the whole larval body was determined by Lowry *et al.* (1951) method. Using Bovine serum albumin as a standard protein, the absorbance of the developed blue color was recorded by a spectrophotometry (750 nm).

2.8. Statistical analysis

The obtained mortality percentages were analyzed according to Finney, (1971) using Ldp line program software (http://embakr.tripod.com/ldpline/ldpline.htm) to estimate the LC_{25}, LC_{50} and LC_{90} values and their confidence limits in addition to slope. To compare the toxicity of EMB and nano-EMB the differences in toxicity were considered to be statistically significant whenever there was no overlap in confidence limits. All values obtained from biological and biochemical experiments are expressed as mean ± standard error and the variance was compared using one way ANOVA followed by a post hoc Student-Newman Keuls test (Costat Statistical Software, 1990), https://www.cohort.com.

3. Results and Discussion

3.1. Characterization of nano-EMB

The prepared nano-EMB showed stability during freez-melting cycl′s storage where cream or float phases were not showed up. Also, we did not observe any separated phase following the shaking and centrifugation processes. Images of TEM (Fig.1) show data about the nano droplet morphology, the droplet formed shape looks spherical that distinctive the appearance of oil in aqueous phase at a size in the range of 39.0-72.0 nm. The droplet size distribution curves of the nano-EMB by DLS had a droplet size average of 759.7 nm and a polydispersity index (PDI) value of 0.382 (Fig.2). Nano-EMB droplet indicated zeta potential value 38.5 mv (Fig. 3) that verify the emulsion excellent stability (Kovačević *et al*., 2014).

The range from 10 up to 500 nm was proven to represent a similar average size of o/w nano-emulsion droplet (Solans and Sole 2012; Gupta *et al*., 2016). The PDI value is in an inverse relationship with the homogenous distribution of particle size in the formula (Shakeel *et al*., 2007). The obtained DPI value in our study verified that nano-EMB was somehow homogeneous emulsion with particle size in the nano range. Our data are in harmony with previous research data whom stated that an appropriate nano-emulsion has the range of 20-200 nm (Ostertag *et al*., 2012; Massoud *et al*., 2018). Moreover, the outcome data of TEM for nano-EMB are similar with Singh and Vingkar (2008), Abdhesh and Gupta (2020), Mohammed and Naser, (2020), as they stated that, the nano-emulsion was spherical in shape.

Additionally, nano-EMB indicated zeta potential value 38.5 mv that verify the emulsion excellent stability (Kovačević *et al*., 2014).

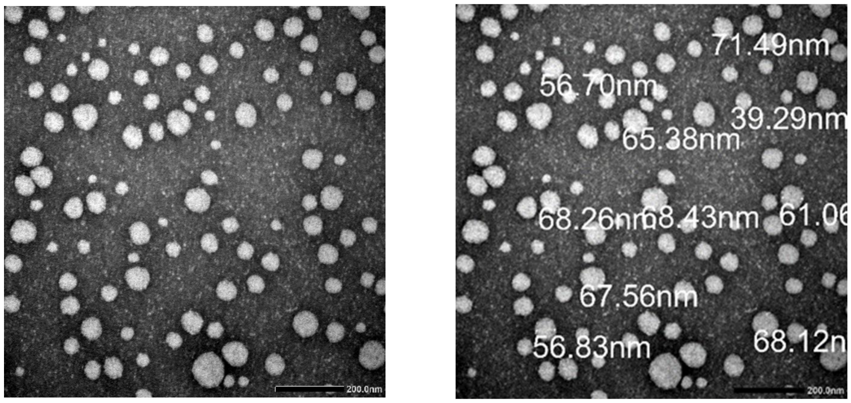

Fig. 1: Electron micrograph illustrates the shape and size of prepared nano-EMB at TEM visualized at 50000X.

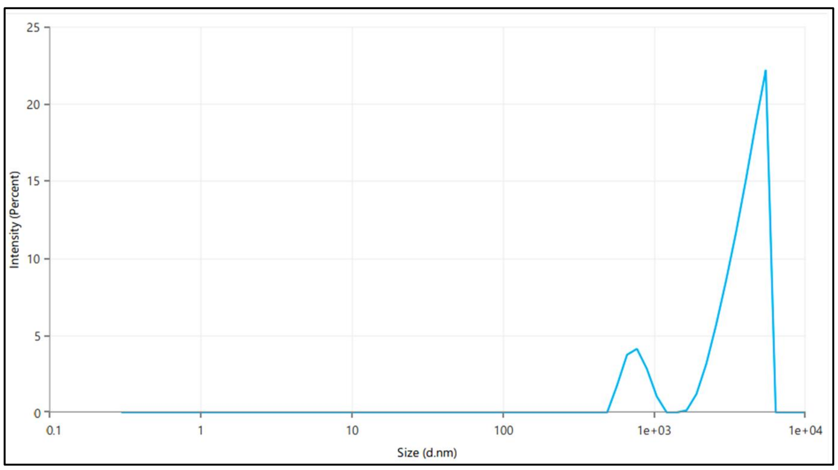

Fig. 2: Droplet size distribution measurement by dynamic light scattering (DLS) of Emamectin nano emulision.

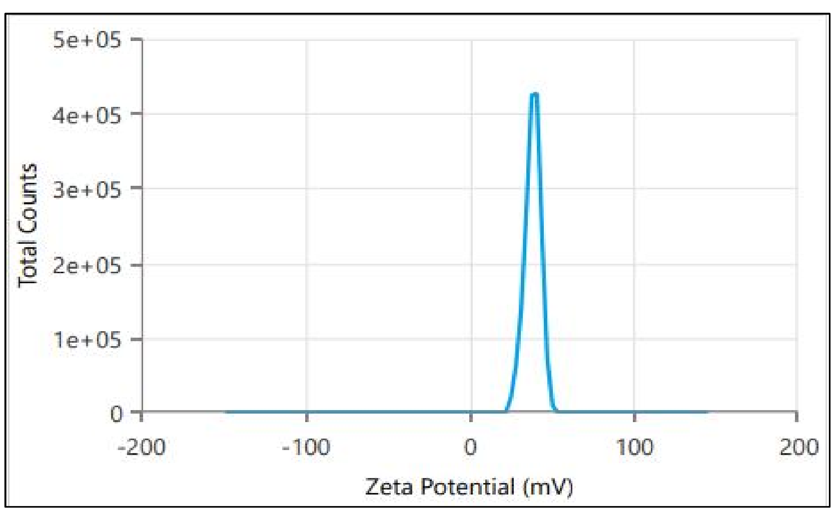

Fig. 3: Zeta potential measurement of nano- EMB using Malvern Zeta sizer Nano ZS.

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3.2. Assessing EMB and nano-EMB toxicity

The toxicity of EMB and its nano-emulsion against the 4th instar larvae of *S. littoralis* after 48 hrs of exposure (Table 1) revealed a significant elevation of nano-EMB toxicity compared with the toxicity of the original EMB; the LC_{50} values were 0.0014 and 0.0183 mg/l, respectively. Similarly, the LC_{25} value of nano-EMB was lower (0.0007 mg/L) than that of the EMB (0.0036 mg/L).

Table 1: Toxicity of emamectin benzoate (EMB) and its nano form against the 4th instar larvae of *S. littorals* 48 hr after exposure

	Insecticide	
	EMB	**Nano-EMB**
LC_{25} (mg/L) **(confidence limits)**	0.0036 (0.0013-0.0069)	0.0007 (0.0005-0.00009)
LC_{50} (mg/L) **(confidence limits)**	0.0183 (0.0102-0.0278)	0.0014 (0.0012-0.0017)
LC_{90} (mg/L) **(confidence limits)**	0.394 (0.248-0.761)	0.0049 (0.0038-0.0073)
Slope ± Variance	0.39 ± 0.114	2.23 ± 0.23
Relative potency	1	13
Toxicity Index	7.7	100

Toxicity index and Relative potency both calculated based on LC_{50} values

Moving to the higher concentrations, the LC_{90} values also showed an obvious increase in toxicity of nano-EMB (0.0049 mg/L) compared with the LC_{90} of EMB (0.394 mg/L), with no overlapping of its confidence limits. Moreover, the relative potency calculated according to the LC_{50} values showed that, nano-EMB was 13 times more potent than the original formulation of EMB, which was confirmed by the calculation of the toxicity index, which depends on the same values (100 and 7.7, respectively). In addition, the slope value of the concentration-mortality regression line of nano-EMB significantly increased to 2.23, associated with 0.39 of the original formulation of EMB; this reflecting differences in how the *S. littoralis* larvae responded to these different formulations. This increased slope value reflects not only the increased effectiveness of nano-EMB, but also highlights the potential for customized insecticidal approaches that utilize the unique properties of nanotechnology to improve pest control outcomes (Yousef *et al*., 2023).

3.3. Effects of sub-lethal concentrations on certain biological parameters

The effects of sub-lethal concentrations of EMB and nano-EMB on the 4th instar larvae of *S. littoralis* showed significant variations between treatments in all tested biological parameters ($P< 0.005$) (Table 2). Nano-EMB, particularly at the higher concentration (LC_{50}), showed slight prolongation in larvae and pupae duration (10.75 and 9.6 days) compared with the groups of the larvae treated with the LC_{50} of EMB. Also, the pupal weight significantly decreased in all treated larvae groups, whereas nano-EMB treatment at LC_{50} resulted in the lowest pupal weight (241mg), followed by the group treated with the LC_{25} (295 mg) of the same formulation. Moreover, pupa deformation and pupation percentages significantly affected in all treated larvae groups compared with the control group ($P< 0.005$), where nano-EMB treatment at LC_{50} showed a significant elevation in pupa deformation rate (20.9 %), followed by nano-EMB treatment at LC_{25} (8.05%), EMB at LC_{50} (5.25%), EMB at LC_{25} (4.18%) and the control group (1.04%).

Table 2: Influence of sub-lethal concentrations of emamectin benzoate (EMB) and its nano form (nano-EMB) with some biological parameters of *S. littoralis*

Insecticide	Conc.	Mean value ± SE				
		Larvae duration (days)	Pupae			
			Duration (days)	Weight (mg)	Deformation (%)	Pupation (%)
Control		9.25 ± 4.6 b	8.50 ± 0.2 b	384.25 ± 11.7 a	1.04 ± 0.0 d	95.90 ± 1.6 a
EMB	LC_{25}	9.63 ± 4.8 b	8.83 ± 0.2 ab	321.25 ± 9.7 b	4.18 ± 0.3 c	40.00 ± 0.2 b
	LC_{50}	10.13 ± 5.1 ab	9.43 ± 0.2 a	329.25 ± 20.1 b	5.25 ± 0.5 c	40.00 ± 3.7 b
Nano-EMB	LC_{25}	10.00 ± 5.0 ab	9.00 ± 0.2 ab	295.00 ± 5.1 b	8.05 ± 0.6 b	34.00 ± 3.3 bc
	LC_{50}	10.75 ± 5.4 a	9.6 ± 0.2 a	241.00 ± 7.3 c	20.9 ± 0.8 a	26.00 ± 1.2 c

Furthermore, nano-EMB showed a significant drop in pupation percent at LC_{25} and LC_{50} (34 and 26%) compared with EMB at the same sub-lethal concentrations (40 and 40%), respectively, or the control group (95.9%). In addition, EMB and nano-EMB significantly decreased adult emergence, fecundity, fertility, and longevity of *S. littoralis*, despite increased adult deformation ($P \leq 0.005$) (Table 3). The nano-EMB exhibited the lowest values of these parameters especially at the higher concentrations. Upon exposure of larvae to nano-EMB at LC_{25} or LC_{50}, the rate of adult emergence decreased to 61.25 and 54.15%, compared with larvae exposed to EMB at the same sub-lethal concentrations of 78.13 and 69.93%, respectively. As well, nano-EMB at LC_{25} or LC_{50} treatments showed low values for fecundity (792.75 and 684.5 eggs/female), fertility (73.2 and 64.1% of hatched eggs/female), and longevity (6.25 and 5.63 days) compared with EMB at the same sub-lethal concentration treatments for fecundity (860.25 and 808.25 eggs/female), fertility (82.48 and 71.55 % of hatched eggs/female) and longevity (6.5 and 6.13 days), respectively. In contrast, significant elevations in rates of adult deformation were observed in the groups treated with nano-EMB (11.88 and 29.15%) and EMB (9.48 and 21.08%) at LC_{25} and LC_{50}, respectively), while the minimal adult deformation percent was recorded in the untreated group (2.43%). The highest effect was recorded in the group treated with LC_{50} of nano-EMB.

Table 3: Influence of sub-lethal concentrations of emamectin benzoate (EMB) and its nano form (nano- EMB) with some adult biological parameters of *S. littoralis*

Insecticide	Conc.	Mean value ± SE				
		Adult emergence (%)	Adult Deformation (%)	Fecundity No. eggs/female	Fertility (Hatched eggs/female %)	Longevity (days)
Control		94.33±2.3a	2.43±0.3 d	984.25±33.3 a	92.70±1.1a	7.25±0.2 a
EMB	LC_{25}	78.13±3.7 b	9.48±0.6 c	860.25 ±24.8 b	82.48±1.2 b	6.50±0.2 b
	LC_{50}	69.93±3.4 bc	21.08±1.3 b	808.25±22.8 b	71.55±1.5 c	6.13±0.3 bc
Nano-EMB	LC_{25}	61.25±5.2 cd	11.88±0.8 c	792.75±12.2 b	73.2±2.2 c	6.25±0.2 bc
	LC_{50}	54.15±5.1 d	29.15±2.4 a	684.5±23.2 c	64.1±2.4 d	5.63±0.1 c

The toxicity elevation of insecticide nanoforms in comparison with their conventional formulations was reported in many literature (Abd Elnabi *et al*., 2021, Abdel-Halim *et al*., 2021, and Gao *et al*., 2022). Yang *et al*., (2017), prepared a nanodispersion formulation of EMB that showed enhanced toxicity against *Plutella xylostella* and *Myzus persicae*. Also, Ahmed *et al*., (2020) reported that nano-formulations of EMB prepared from a commercial formulation (Proclaim® 5%) caused a higher reduction in the *Tuta absoluta* population during two successful seasons. Moreover, Chen *et al*. (2022) stated increased toxicity of a prepared nano-formulation (which prepared by the co-solvent method) against *S. frugiperda* 3rd instar larvae compared with its EC formulation. The boosted potency of nano-EMB observed in our study could be attributed to their small size (Yang *et al*., 2017) and large surface area that may increase chemical reactivity and penetration into the cells (Gojova *et al*., 2007; Medina *et al*., 2007 and Pan *et al*., 2009, Anjali *et al*., 2010 Saini *et al*., 2014, Elabasy *et al*., 2019). As documented in the literature, nano-delivery enhances the affinity of active ingredients (a.i) resulting in high efficacy for nanoforms compared with the conventional ones. Such finding is in accordance with those obtained by Elmasry *et al*. (2020), where the addition of nano salt to the EMB formula increased the mortality percentage of *S. littoralis* and *T. absoluta* from 22.45 and 59.22 to 100% and decreased the lethal time from 10 to 3 days compared with the pesticide alone. As explained by Huang *et al.* (2018), the nanomaterials improve the dispersibility and stability of a.i and promote target ability. These are parallel with those obtained previously, where β-cypermethrin stabilized by a polymeric surfactant in a prepared nano-emulsion. The nano-emulsion exhibited good stability, even after 24 hrs of dilution in comparison to the commercial emulsions, due to the steric interaction between the polymeric inner surface and the pesticide (Wang *et al*., 2007). On the other hand, fabrication of EMB in nano-capsulation by using 1, 2-distearoyl-sn-glycero-3-phosphoethanol amine N-[amino (polyethylene glycol)-2000] increased dispersion, adhesion, and biocompatibility on maize foliage against *S. frugiperda;* the nano-capsules showed a strong toxicity with LC_{50} of 0.046 mg/L against the 3rd instar larvae after 48 hrs (Chen *et al*., 2022). Similarly, EMB-loaded liposome nano-vesicles (EMB-Lip-NV) improved pesticidal activity against *S. exigua* (Du *et al*., 2022).

Moreover, our results showed extended effects of EMB and nano-EMB on the development of *S. littotalis*, these effects were significantly higher in larvae treated with the nano-EMB in association with those treated with EMB, which indicates that, the nano-emulsion might enhance the bioavailability and potency of EMB; leading to more pronounced effects on pest development and reproduction (Moustafa *et al*., 2016; Kandil *et al*., 2020). The effects were more noticeable with the higher concentration (LC_{50}) of nano-EMB which is in accordance with results obtained by Zhang *et al*. (2023), they reported that nano-EMB showed pronounced effects against *S. frugiperda* with the LC_{25} compared with LC_{10}. Both EMB and nano-EMB prolonged larval and pupal durations. Nano-EMB at the highest concentration showed higher effects compared to other treatments which is parallel with the finding of enhanced toxicity of nano-EMB. These results are in accordance with the previous published data stating that EMB caused elongation of larval and pupation periods of *S. frugiperda* (Aly *et al*., 2024 and Fiaboe *et al*., 2023, Niu *et al*., 2024). These delays in development could be attributed to the disruption of ecdysteroid biosynthesis, as suggested by Niu *et al*. (2024), they found that EMB treatment reduced ecdysteroids levels in the 3rd instar larvae and pupae of *S. frugiperda*. Furthermore, our data revealed a significant reduction in pupal weight, pupation percentages and emergence percentage while increasing pupal deformation which was more obvious in nan-EMB. This finding is consistent with the increased rates of pupal malformation and decreased pupation rates observed in other studies (Moustafa *et al*., 2022 and Aly *et al*., 2024). The higher incidence of adult deformation and reduced adult emergence in nano-EMB-treated groups emphasizes the impact of this nano-emulsion on the physiological development of *S. littoralis*. Furthermore, our results revealed reductions in fecundity, fertility, and longevity in treated groups, particularly with nano-EMB which showed the greatest effects with the LC_{50}. These findings align with previous studies that reported similar reductions in fecundity and fertility in *Spodoptera* species following EMB treatment (Zhang *et al*., 2023; Liu *et al*., 2022). The significant decrease in the mean number of laid and hatched eggs and females produced from survival larvae treated with nano-EMB may be due to the possible accumulation of the tested formula in the larvae that survived treatment and has adversely affected the insect metamorphosis during its lifespan (Mao *et al*., 2018; Wang and Wang, 2014). Such reduction also may be linked to the ovicidal toxicity of EMB and its nano-emulsion, which has been documented in other insect species *T. absoluta* and *M. presica* (Moustafa *et al*., 2022; Yin *et al*., 2008) or it may be referring to their physiological effects (Yin *et al*., 2008 and Liu & Trumble 2005). Otherwise, it may be a result of the reduction in pupal weight that could lead to a decrease in adult vigor, which is crucial for survival and reproduction, as supported by findings from Fiaboe *et al*., (2023) and Zhang *et al*., (2023), who also observed decreased pupal w-eight and reduced adult emergence in *Spodoptera* species treated with sub-lethal concentrations of EMB. Moreover, the negative sub-lethal effects of EMB might disrupt the histological structure of the *S. frugiperda* parental generation, and then influence the reproductive parameters of the offspring generation (Mokbel and Huesien, 2020).

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3.4. Effects of sub-lethal concentrations on Biochemical responses

The activities of AChE, α-Esterase, β-Esterase and GST in *S.littoralis* 4th instar larvae exposed to various treatments of sub-lethal concentrations (LC_{25} and LC_{50}) of EMB and nano-EMB compared with the untreated larvae significantly affected by the treatments ($p < 0.005$), Figs (4-7). All treated larvae groups showed significantly decline in AChE activity ($p< 0.005$) compared to the control group, which had the highest value (0.044 µM/mg/min). The nano-EMB showed moderate activity with no significant difference between the LC_{25} and LC_{50} treatments (0.038 and 0.037 µM/mg/min, respectively), but they showed significantly higher than the activity showed by the EMB both LC_{25} and LC_{50} treatments. The EMB at LC_{25} treatment showed the greatest decline in AChE activity (0.029 µM/mg/min), followed by the EMB at LC_{50} (0.032 µM/mg/min). (Fig. 4). The α-esterase activity showed insignificant variation among the control, nano EMB at LC_{25} or LC_{50} treatments (5.21, 5.71. and 5.95 µM/mg/min respectively). While a significant decline in the α-esterase activity was observed in the larvae treated with EMB at LC_{25} and LC_{50} (4.37 and 4.30 µM/mg/min, respectively) compared with the larvae of the other treatments (Fig.5). Regarding to the β esterase activity, the data showed significant variations across the treatments ($p < 0.05$). Compared with activity in the control (6.93 µM/mg/min), the nano-EMB LC_{50} treatment showed the highest elevation in activity (7.11 µM/mg/min), even though, the EMB LC_{50} treatment showed the greatest decline in activity (5.25 µM/mg/min). The β esterase activity in larvae treated with Nano-EMB or EMB at LC_{25} indicated intermediate elevation levels in activity (6.41 and 5.55 µM/mg/min, respectively) (Fig.6). The GST activities varied significantly among the treatments ($p < 0.05$) showing another profile (Fig. 7). The highest GST activity was observed in the larvae treated with Nano-EMB at LC_{25} (0.090nM/mg/min).

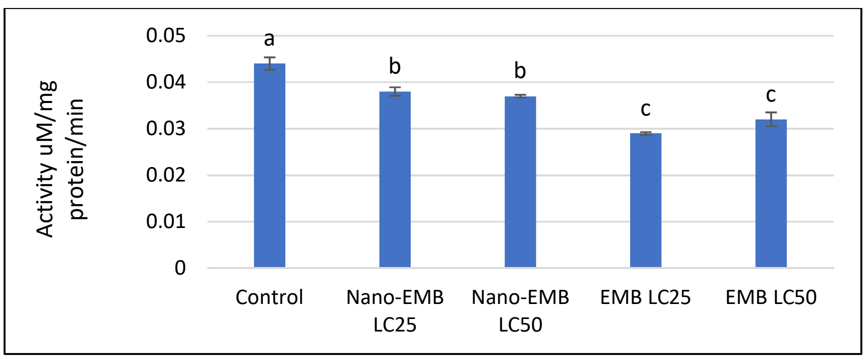

Fig. 4: Activity of acetyl cholinesterase (AChE) in the *S. littolaris* 4th instar larvae exposed to sub-lethal concentrations, LC_{25} and LC_{50}, of emamectin benzoate (EMB) and its nano emulsion (nano-EMB) for 48 hrs

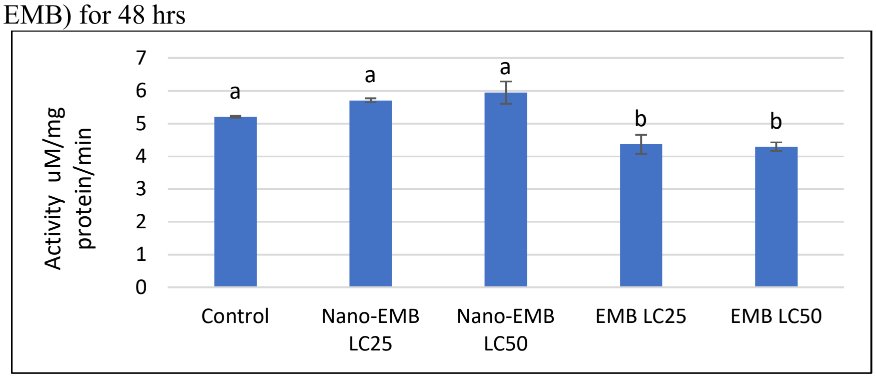

Fig. 5: α-Esterase activity in the *S. littolaris* 4th instar larvae exposed to sub-lethal concentrations, LC_{25} and LC_{50}, of emamectin benzoate (EMB) and its nano emulsion (nano-EMB) for 48 hrs

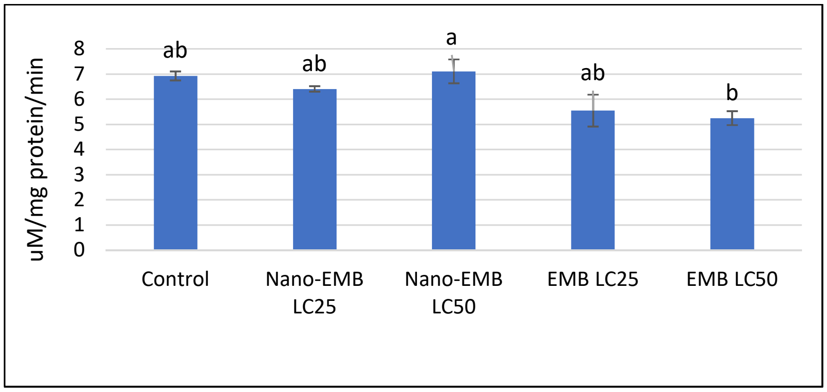

Fig. 6: *β*-Esterase activity in the *S. littolaris*4th instar larvae exposed to sub-lethal concentrations, LC_{25} and LC_{50}, of emamectin benzoate (EMB) and its nano emulsion (nano-EMB) for 48 hrs

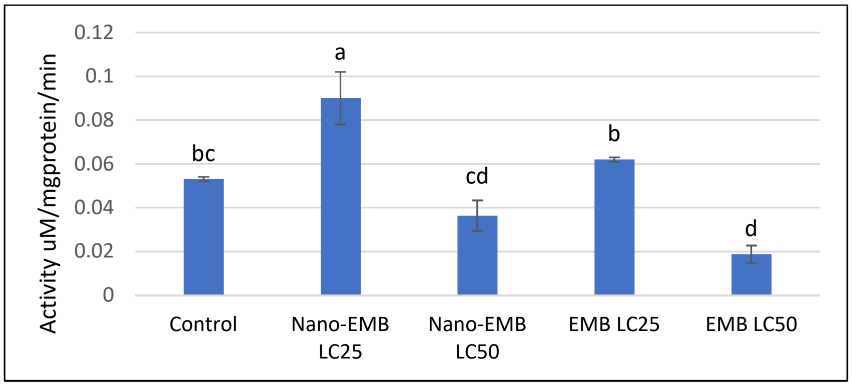

Fig. 7: Glutathion *S*-transferase (GST) activity in the *S. littolaris*4th instar larvae exposed to sub-lethal concentrations, LC_{25} and LC_{50}, of emamectin benzoate (EMB) and its nano emulsion (nano-EMB) for 48 hrs

In contrast the treatments of nano-EMB or EMB at LC_{50} showed significant declined in the enzyme activity (0.0188 and 0.0364 nM/mg/min, respectively) with respect to the untreated (0.053 nM/mg/min). The significant reduction in AChE activity observed in treated groups is aligns with findings by Khalifa *et al*. (2023), Chandi *et al*. (2023) and Salem *et al*. (2024) who reported a decrease in AChE activity in *S. littoralis* following exposure to EMB. In contrast Amin *et al*. (2023) noted a significant increase in AChE activity in the 4th instar larvae *of S. littolaris* treated with EMB and EMB+AgNPs.

The data suggests that the enzyme activities of *S.littorals* are differentially affected by the treatments. Nano-EMB at LC_{25} significantly increased GST and Beta esterase activities, while EMB at LC_{50} consistently showed the lowest enzyme activities across AChE, α-esterase, β-esterase, and GST. Our results indicated that no significant changes in α-esterase activities was observed with nano-EMB treatments this finding is in agreement with the enhanced toxicity obtained in larvae treated with nano-EMB as esterases play an important role in detoxifying the insecticide, as indicated by previous studies (Khalifa *et al*., 2023). The GST activity data present a more complex picture, with a significant increase observed in the nano-EMB LC_{25} treatment. This increase could be revealing of an adaptive response by the larvae, possibly linked to an enhanced detoxification processes or a stress response mechanism. Such an increase in GST activity has been associated with the detoxification of xenobiotics, as highlighted by Amin *et al*. (2023). However, the overall decline in GST activity with the higher concentrations (LC_{50}) of both EMB and nano-EMB is consistent with the findings of Khalifa *et al*. (2023) as they suggested that higher doses may overwhelm the detoxification system, leading to reduced enzyme activity.

4. Conclusion

This study shows enhanced toxicity of nano-EMB against *S. littoralis*. EMB, either in its original form or nanoform, significantly affected the biological and biochemical parameters. These findings suggest that nano-formulations could enhance insecticide efficacy and reduce application rates in integrated pest management programs. However, the long-term effects on pest population dynamics and resistance development may require further investigation. The employment of EMB, either in its nano or original forms, is considered a promising substitute for conventional insecticides. The nanoform of EMB that proved high efficiency against the larvae of cotton leafworm will allow the application of this nano-emulsion at low concentrations as an environmentally friendly formulation.

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