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Hematological and histopathological impacts of nano-emamectin benzoate against the larvae of the cotton leafworm, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) under laboratory conditions

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Abstract

Background: Insects withstand foreign substances and infection by expressing robust defense responses, which are mediated by hemocytes, fat body, midgut, and many other tissues. The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval), is a polyphagous pest with considerable economic importance in Egypt and globally. Many control strategies were employed to control this pest. Nowadays, there is a trend to use nanotechnology tools in agricultural practices as they balance minimal concentration and maximum pest control, safe concentration, and reduce the cost of pest control. The present study aimed to evaluate the hematological and histopathological response of *S. littoralis* larvae post-treatment with sublethal concentrations of emamectin benzoate and its nanoform, besides the silver nanoparticles.

Results: The results revealed the high toxicity of emamectin benzoate and its nanoform (LC_{50} values were 0.0524 and 0.023 ppm, respectively). The results also showed that all tested compounds significantly influenced the mean number of laid eggs/female. The emamectin benzoate nanoform (837.3 ± 52.09) was the most efficient compound compared to the control (1999.3 ± 46.5). The hematological responses against the EMB + AgNP were lowered total hemocyte counting (22.41 ± 1.3) compared to the untreated larvae (38.08 ± 0.83). In addition, there were some histopathological changes in the midgut tissues. They were represented as destroying the integrity of the epithelial cells and the ciliated border. The columnar cells began to disintegrate, and the peritrophic membrane became vacuolized. In contrast, the cuticle layers were not affected by various treatments.

Conclusion: We can conclude that the employment of emamectin benzoate, either in its original form or as its nanoform, is considered a promising substitute for conventional insecticides. The nanoform of emamectin benzoate proved its high efficiency against the larvae of the cotton leafworm, which may allow the application of this formulation at low concentrations.

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Keywords: *Spodoptera littoralis*, Nanopesticides, Hematological studies, Histopathological changes, Total hemocytes counting

1 Background

The Egyptian cotton leafworm, *S. littoralis* (Boisduval), is considered one of the economically important insect pests in Egypt and worldwide. Larvae of *S. littoralis* cause considerable destruction as they feed on leaves, fruiting points, flower buds, and occasionally on bolls [5, 29, 32]. Larvae generally prefer young leaves, consuming other plant parts, causing plant defoliation, interference with plant development, destruction of growth points and flowers, and sometimes, hollowing out the seed bolls, which often causes them to wilt and drop [12]. As a result, many control strategies were applied to manage the damage of this pest. Using conventional synthetic insecticides have been a traditional strategy for controlling the Egyptian cotton leafworm. However, the extensive use of these chemical compounds gave rise to many disadvantages, resistance, resurgence, and residues [15]. In addition, these compounds adversely affect the natural enemies of insect pests [17]. Accordingly, the need to find alternatives to conventional chemical insecticides represents a challenge for researchers. Nanotechnology is a resurgent implementation in pest management programs [8]. Nanotechnology deals with particles on the nanoscale; between 1 and 100 nm. This scale initiates several unique characteristics of materials, making them suitable for many applications in different fields, including agriculture, chemistry, physics, medicine, and biology [27]. Nanotechnology provides a new path to establishing insecticide formulations with high activity and low environmental risk [35]. Nanocarriers can increase the solubility of active compounds while protecting them from volatilization and degradation. The efficiency improvements can generate better results using lower doses and application numbers, reducing environmental contamination and risks to human health [10]. Different nanoparticle formulations are used in agriculture as herbicides, insecticides, fungicides, acaricides, fertilizers, and growth regulators, among others [36]. Several studies have demonstrated the potential of formulations of biopesticides associated with polymeric nanoparticles [13]. Silver nanoparticles (AgNPs) represent a powerful eco-friendly pest management alternative and an improved crop production material. Moreover, adding AgNPs to pesticides can increase toxicity to exposed pests and reduce unexpected side effects on plants [37]. Insects withstand chemical substances and pathogen invasion through potent immune responses that are mediated by hemocytes, the fat body, the midgut, and many other tissues [25, 41]. Foreign substances and pathogens are recognized by the immune system which then activate the immune signaling pathways that amplify the immune response, induce the production of factors with antimicrobial activity, and activate effector pathways [25, 41]. The impact of emamectin benzoate and silver nanoparticles was well documented. However, the present study could be considered a first record study for the immunotoxicological effect of emamectin benzoate and its nanoform as well as the AgNPs against the larval instar of *S. littoralis*. In this context, the present study aims to avoid the environmental hazards posed by extensive amounts of synthetic pesticides. Moreover, the present work is studying the possibility of developing a much more effective control method of insecticide in nanoform, which may also lead to decreasing the cost price of insect pest management programs. The target insect model is the cotton leafworm, *S. littorals*.

2 Methods

2.1 Tested insects

A laboratory strain of *S. littoralis* was obtained as egg masses from the Research Division of the cotton leaf worm, Plant Protection Research Institute, Agricultural Research Center, Dokki, Giza, Egypt. These eggs were kept in plastic cups covered with gauze under laboratory conditions of 27 ± 2 °C and 65 ± 5% R.H. until hatching. The newly hatched larvae were offered fresh and clean castor bean leaves, *Ricinus communis*, and were checked daily for adding more leaves if needed [16, 19]. The fourth instar larvae were employed for further investigations.

2.2 Tested compounds

Jasper® 3.4% ME (emamectin benzoate) (18% solvent, 78.6% water, 3.4% emamectin benzoate) (EMB) was supplied from the Egyptian group for agricultural development (EGAD) at the rate of application of 120 cm^3/feddan. Silver nanoparticles (AgNPs) (15 ml, PS: 10–20 nm) were synthesized and supplied from Nano Gate company, Nasr City, Cairo governorate, Egypt. It was supplied at a concentration of 200 ppm. Emamectin benzoate nanoform loaded on AgNPs (EMB + AgNPs) (15 ml, 0.45% of Jasper) was synthesized and supplied at a concentration of 200 ppm from Nano Gate company, Nasr City, Cairo governorate, Egypt. The EMB + AgNPs were prepared by adding 2 ml of EMB to 14 ml of AgNPs. The mixture was then stirred for 15 min. on a sonicator to confirm the homogenous attachment of nanoparticles.

2.3 Toxicological studies

A toxicity test was carried out using the leaf-dipping technique to determine the LC_{50} and LC_{90} values of the tested compounds for the fourth instar larvae [6]. Dry and clean castor bean leaves were dipped for 10 s in six different concentrations of the tested compounds, then left to air dry at room temperature, and then offered to the fourth instar larvae in clean jars, each jar containing 20 larvae. Four replicates were used for each concentration of each treatment. Leaves dipped in water served as an untreated group. The LC values of tested compounds were statistically analyzed for significance using "LdPLine®" software (Swaroop and Organization). Regression lines obtained the LC_{50} and LC_{90} values according to (Finney 1971) using "LdPLine®" software.

2.4 Treatment and further analysis

The tested compounds were evaluated for their impacts on the late sixth instar larvae, treated as fourth instar larvae with the obtained LC_{50} of the tested compounds. The survived larvae were categorized into two groups. The first group was kept until adult emergence, mating, egg laying, and egg hatching to evaluate the impact of sublethal concentrations on mean larval duration, mean pupal duration, mean pupal weight, rate of pupation, rate of adult emergence, fecundity, and fertility. The second group was kept till the late sixth instar for further hematological and histopathological studies.

2.5 Hematological studies

2.5.1 Total hemocyte counts and viability percentage

Insect physiological saline solution consisted of NaCl (8.8 gm), KCl (0.2 gm), and CaCl (0.3 gm) per liter. The pH was adjusted to 6.7–6.8. Diluting solution consisting of trypan blue (0.4%) in insect physiological saline solution. Hemolymph was collected directly from pooled samples (8–10 larvae) using Thoma white blood cell diluting pipette to the 0.1 mark [44]. Diluting solution was taken up to 11 marks on the pipette. The mixture was hand shaken for three minutes and then dispensed to both chambers of the hemocytometer. After about one minute, the total number of cells, recognized as viable and dead cells, in the 64 squares of the four corners were counted. Dead hemocytes were stained with trypan blue, whereas living cells were not [26]. Cells within the lines and left and bottom boundary lines of the four corners squares were counted. The total number of cells was multiplied by a factor of 250 to give a number of cells/mm^3 of hemolymph. If one count differed from the other by more than 5000, the preparation was discarded, and another hemolymph sample was subjected to counting. This procedure was replicated at least 10 times for each determination. Viability % was calculated using the formula given by Horohov and Dunn [26] as follows:

$$\text{Viability\%} = \frac{\text{No. of viable cells}}{\text{Total no. of cells}} \times 100 \quad (1)$$

2.5.2 Differential hemocyte counts

Fresh hemolymph from the late sixth larval instar was smeared on a clean glass slide, air dried and then fixed for 2 min with ethanol. Blood films were stained with Giemsa stain freshly prepared by mixing stock Giemsa: distilled water (1:10 V/V) for 15 min. After a brief wash in distilled water, slides were dipped for about 30 s in tap water. Smears were air dried for 24 h, mounted in Canada balsam, and examined. Differential hemocyte counts were accomplished by observing and differentiating at least 100 cells from random fields on each slide. This procedure was repeated 10 times for each treatment. The identification of morphological cell types was based on differences in size, morphology, and staining affinity.

2.6 Histopathological studies

2.6.1 Specimen preparation

The late sixth instar larvae, which survived treatment with the sublethal concentrations of the tested compounds, were dissected for their midguts and cuticles. The dissected tissues were immediately fixed in Bouin's fluid for 6 h. separately. The tissues were rinsed in serial 70% ethyl alcohol to remove excess Bouin's fluid. Tissues were then preserved in 70% ethyl alcohol for further analysis. The tissues were then left in paraffin wax serials to prepare paraffin blocks for sectioning with rotary microtome at a diameter of 5 µm and received on glass slides. The sections were deparaffinized, rehydrated, and stained in hematoxylin for 12 min and in eosin Y for 5 min. The sections were dehydrated, cleared in xylene, and then mounted in neutral balsam. Control sections of non-treated larvae were also carried out as previously. The mounted sections were examined and photographed using light microscopy with a digital high-definition camera. The sections were photographed at 40X magnification.

2.7 Statistical analysis

Means were tested for significance by the one-way analysis of variance (ANOVA) using SPSS statistics 17.0 release 17.0.0 software (Statistical Package for Social Sciences, USA) at $P \leq 0.05$ [40]. Differences between the treatments were determined by Duncan's multiple range test [14] ($P \leq 0.05$) using SPSS statistics 17.0 release 17.0.0 software (Statistical Package for Social Sciences, USA) [40]. All tested data were parametrically distributed.

3 Results

3.1 Toxicity of the tested compounds

The results presented in Table 1 showed the rate of larval mortality of the fourth instar larvae of the cotton leafworm, *S. littoralis* (Boisd.), treated with different concentrations of the tested compounds; Jasper® (EMB), silver nanoparticles (AgNP), and the nanoform of the tested compounds (EMB + AgNP). The LC_{50} and LC_{90} values were determined for the fourth instar larvae. The results showed that the nanoform of EMB + AgNP was the most toxic compound against the fourth instar larvae as detected from the low value of LC_{50}. In addition, the EMB and AgNP exhibited low toxicity against the fourth instar larvae according to the LC_{50} values (Table 1). All tested compounds show instant larval mortality.

3.2 Biological studies

Table 2 shows the effect of the LC_{50} of EMB, AgNPs, and EMB + AgNPs on mean larval duration, mean pupal weight and mean pupal duration of the treated fourth instar larvae of *S. littoralis*. The results showed a highly significant shortening of the mean larval duration when treating the fourth instar larvae with the LC_{50} of EMB + AgNPs, followed by EMB and AgNPs ($P = 0.0044^{**}$). Furthermore, there was a reduction in the mean pupal weight of pupae treated as fourth instar larvae with the LC_{50} of the tested compounds ($P = 0.0015^{**}$). The reduction in mean weight pupal was not significant in the case of EMB and AgNPs treatment. However, the lessening of pupal weight caused by EMB + AgNPs was significant compared to the control. Furthermore, the results showed that treatment of the fourth instar larvae with LC_{50} of the tested compounds has a varied influence on the mean pupal duration of the survived pupae.

Table 1 Susceptibility of tested compounds against the fourth instar larvae of the cotton leafworm, *S. littoralis*

Tested compounds	Lethal concentration (LC) (ppm)		Fiducial limits (C. I. 95%) (ppm)		Slope
			Lower	Upper	
Jasper® (EMB)	LC_{50}	0.0524^{**2}	0.0615	0.0449	1.69 ± 0.12
	LC_{90}	0.3005	0.4296	0.2278	
AgNP	LC_{50}	0.079^{**3}	0.109	0.0506	1.01 ± 0.11
	LC_{90}	2.008	4.3108	1.2251	
EMB + AgNP	LC_{50}	0.023^{*1}	0.0298	0.0165	1.05 ± 0.12
	LC_{90}	0.3767	0.6991	0.2472	

Numbers 1, 2, and 3 refer to sorting high toxic compounds according to the LC_{50} value

* Corresponding to high toxicity according to LC_{50} value

** Corresponding to moderate toxicity according to LC_{50} value

Table 2 Impact of the LC_{50} of the tested compounds on mean larval duration, pupation rate, mean pupal weight, and mean pupal duration of the fourth instars larvae of *S. littoralis*

Tested compounds	Mean larval duration (days) ± S. E	Mean pupal weight (gm) ± S. E	Mean pupal duration (days) ± S. E
Jasper® (EMB)	13.3 ± 0.34^{b}	0.28 ± 0.03^{b}	14 ± 0.59^{a}
AgNP	14.6 ± 0.33^{a}	0.29 ± 0.2^{a}	14.3 ± 0.58^{a}
EMB + AgNP	11.6 ± 0.59^{b}	0.24 ± 0.1^{c}	12.3 ± 0.33^{b}
Control	15.3 ± 0.34^{a}	0.30 ± 0.03^{a}	14.6 ± 0.34^{a}
Df	3	3	3
F value	10	14	6
P value	0.0044^{**}	0.0015^{**}	0.0191^{*}

Means followed by the same letter in a column are not significantly different at the 5% level of probability (Duncan's Multiple Range Test) [14]

EMB + AgNPs treatment significantly decreased the mean pupal duration compared to control, followed by EMB alone. The AgNPs alone have no significant impact on the mean pupal duration ($P = 0.0191^{*}$).

Data presented in Table 3 showed the effect of sublethal concentration of tested compounds on the pupation rate and adult emergence rate. The results revealed a significant reduction in the pupation rate, approximately 50%, compared to the control group. The most effective compound on the pupation rate was EMB + AgNPs, followed by EMB and AgNPs. In addition, the adult emergence rate has been influenced by treatment as a latent effect of the tested compounds. EMB + AgNPs was the most effective formula as the lowest adult emergence percentage (70.3%) was obtained, followed by EMB and AgNPs.

The results in Table 4 showed the impact of sublethal concentrations of the tested compounds on fecundity and fertility as a latent effect. The results showed that all tested compounds significantly influenced the mean number of laid eggs/female, and EMB + AgNPs was the most efficient compound compared to control.

Table 3 Effect of the LC_{50} of the tested compounds on the rate of pupation and adult emergence of survived individuals treated as fourth instar larvae of *Spodoptera littoralis*

Tested compounds	% Pupation	% Adult emergence
Jasper® (EMB)	46.6^{c}	89.3^{b}
AgNP	50.6^{b}	91.6^{b}
EMB + AgNP	43.6^{d}	70.3^{c}
Control	100^{a}	100^{a}
Df	3	3
F value	2833	641
P value	0.0000***	0.0000***

Means followed by the same letter in a column are not significantly different at the 5% level of probability (Duncan's Multiple Range Test) [14]

Table 4 Effect of EMB, AgNP, and EMB + AgNP on fecundity and fertility of the fourth instars larvae of *S. littoralis*

Tested compounds	Mean no. of laid eggs/female	Mean no. of hatched eggs/female	%Egg hatchability	% Sterility
Jasper® (EMB)	911.6 ± 13.5^{c}	857.6 ± 51.3^{c}	94.07^{b}	5.93^{b}
AgNP	942 ± 59.9^{b}	897.6 ± 57.04^{b}	95.28^{ab}	4.72^{bc}
EMB + AgNP	837.3 ± 52.09^{d}	730.3 ± 56.40^{d}	87.22^{c}	12.78^{a}
Control	1999.3 ± 46.5^{a}	1924.3 ± 53.09^{a}	96.24^{a}	3.76^{c}
Df	3	3	3	3
F value	917,174.75	915,708.75	50	50
P value	0.0000***	0.0000***	0.0000***	0.0000***

Means followed by the same letter in a column are not significantly different at the 5% level of probability (Duncan's Multiple Range Test) [14]

Table 5 Effect of tested compounds on the hemocyte viability percentage and the total hemocytes count in the late sixth instar larvae of *S. littoralis* after treatment as the fourth instar larvae

Tested compounds	% Hemocytes viability	Total hemocyte counts (cells/mm^3) $\times 10^3$
Jasper® (EMB)	81.40 ± 0.7^{c}	26.40 ± 1.0^{c}
AgNP	88.35 ± 1.7^{b}	35.80 ± 1.2^{b}
EMB + AgNP	77.72 ± 0.8^{d}	22.41 ± 1.3^{d}
Control	95.63 ± 0.6^{a}	38.08 ± 0.83^{a}
Df	3	3
F value	188.75	168.75
P value	0.0000***	0.0000***

Means followed by the same letter in a column are not significantly different at the 5% level of probability (Duncan's Multiple Range Test) [14]

In addition, treatment with tested compounds reduced the mean number of hatched eggs compared to control. Moreover, the tested compounds have impacted the hatchability percentage, and the sterility percentage and EMB + AgNPs were the most efficacious of all tested compounds (Table 4).

3.3 Effect of tested compounds on total hemocyte counts

The effect of treatment of the fourth instar larvae with the LC_{50} of the tested formulae; EMB, AgNPs, and EMB + AgNPs, on the viability and total count of hemocytes in the late sixth instar larvae was listed in Table 5. Relative to control, the percentage of hemocyte viability was significantly decreased in treated larvae due to the toxic effect of tested compounds. After treatment, the viability percentage decreased to 77.72, 81.4, and 88.35% due to treatment with EMB + AgNPs, EMB, and AgNPs, respectively. Furthermore, the total counts of hemocytes in sixth instar larvae after treatment as fourth instar larvae with the LC_{50} of tested compounds were significantly decreased compared to control. However, treatment with AgNPs showed no significant effect on the total hemocyte count.

3.4 Effect of tested compounds on differential hemocyte counts

Five hemocyte cells were recognized in the hemolymph based on the size and shape of the nucleus and soma, presence/absence of cytoplasm, type, size, and the number of inclusions (Fig. 1). These cells were prohemocytes, plasmohemocytes, granulocytes, spherocytes, and oenocytoids. Prohemocytes were round/elliptical, small-sized cells with compact, relatively large nuclei (Fig. 1a). Whereas, plasmohemocytes were small to large polymorphic cells with round/elongated, centrally located nuclei (Fig. 1b). On the other hand, granulocytes were small to large, oval, or spherical cells and exhibited granular cytoplasm (Fig. 1c). Spherocytes were spherical and were larger than granulocytes. Their nuclei were small, centrally, or eccentrically located (Fig. 1d). Oenocytoids exhibited variable sizes and shapes (Fig. 1e). The differential hemocyte counts in the normal late sixth instar larvae of *S. littoralis* showed a maximum of 39.0% of plasmohemocytes, followed by 30.6% granulocyte, 12.6% prohemocytes, 10.3% spherocytes, and 7.3% oenocytoids (Table 6). Treatment with the LC_{50} of EMB + AgNPs significantly decreased the percentage of prohemocytes, granulocytes, spherocytes, and oenocytoids. In contrast, the plasmohemocytes percentage was increased due to treatment. Furthermore, treatment with LC_{50} of AgNPs caused no significant effect on plasmohemocytes, granulocytes, spherocytes, and oenocytoids percentages. Moreover, treatment with LC_{50} of EMB caused a significant increase in prohemocytes, granulocytes, and oenocytoids percentages. At the same time, no significant influence was observed in the percentage counts of plasmohemocytes and spherocytes (Table 6).

3.5 Histopathological effects on cuticle

The standard structure of the cuticle of the late sixth instar larvae of *S. littoralis* is shown in Fig. 2a. As

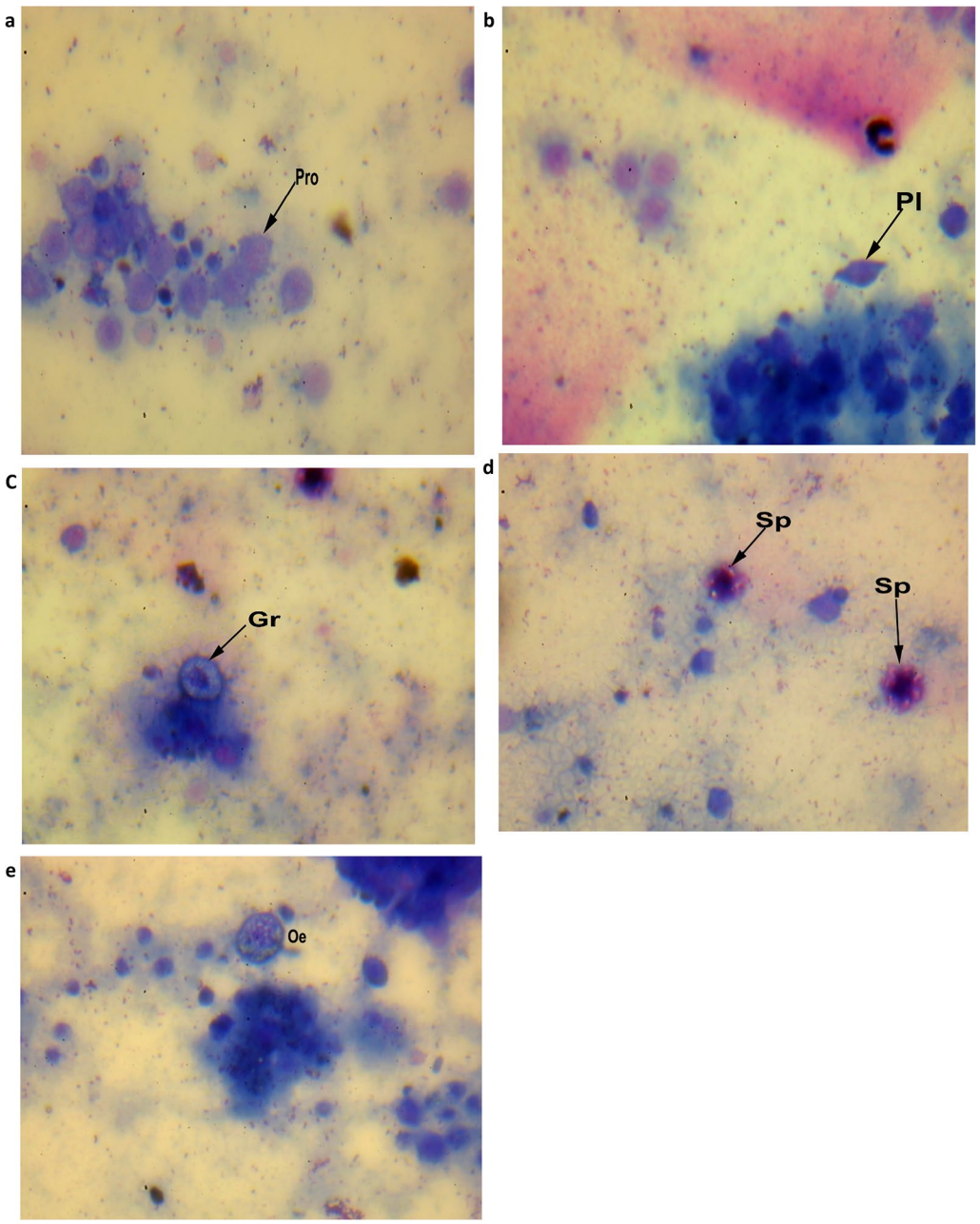

Fig. 1 Light microscopic graph of hemocytes obtained from the whole hemolymph of untreated sixth instar larvae of *Spodoptera littoralis* stained with Giemsa staining: **a**: Prohemocytes (Pro), **b** Plasmohemocytes (Pl), **c** Granulocytes (Gr), **d** Spherocytes (Sp), and **e** Oenocytoid (Oe) (×40)

shown in Fig. 2), the normal integument is mainly composed of three layers, two non-cellular layers, epicuticle and endocuticle, and one cellular layer, the hypodermis which is rested on an inner basement membrane. The histopathological influences of tested compounds are presented in Fig. 2b-d. As shown in the photomicrographs, treatment with tested compounds did not influence the larval integument compared to control. The cuticle layers remained unchanged, and the muscle layer was not affected.

3.6 Histopathological effects on midgut

The histological and histopathological features of the midgut of the late sixth instar larvae treated as fourth instar larvae with the LC_{50} of the tested compounds are shown in Fig. 3a–d. The midgut of the cotton leafworm

Table 6 Effect of tested compounds on the differential hemocytes counts (%) in the late sixth instar larvae of *S. littoralis* treated as fourth instar larvae

Tested compounds	% Differential hemocytes counts				
	Prohemocytes	Plasmohemocytes	Granulocytes	Spherocytes	Oenocytoids
Jasper® (EMB)	10.3 ± 1.8^{b}	38.0 ± 1.7^{c}	36.6 ± 4.5^{a}	10.3 ± 0.3^{a}	4.6 ± 1.8^{b}
AgNP	9.3 ± 0.9^{bc}	41.3 ± 4.7^{b}	32.6 ± 5.5^{b}	10.6 ± 3.5^{a}	6.0 ± 2.6^{a}
EMB + AgNP	8.6 ± 0.3^{c}	51.3 ± 6.0^{a}	27.3 ± 4.1^{d}	8.6 ± 1.2^{b}	3.6 ± 1.2^{b}
Control	12.6 ± 0.9^{a}	39.0 ± 3.3^{c}	30.6 ± 1.2^{c}	10.3 ± 1.9^{a}	7.3 ± 1.4^{a}
Df	3	3	3	3	3
F value	8.75	106.75	42.75	4.75	10
P value	0.0066**	0.0000***	0.0000***	0.0347*	0.0044**

Means followed by the same letter in a column are not significantly different at the 5% level of probability (Duncan's Multiple Range Test) [14]

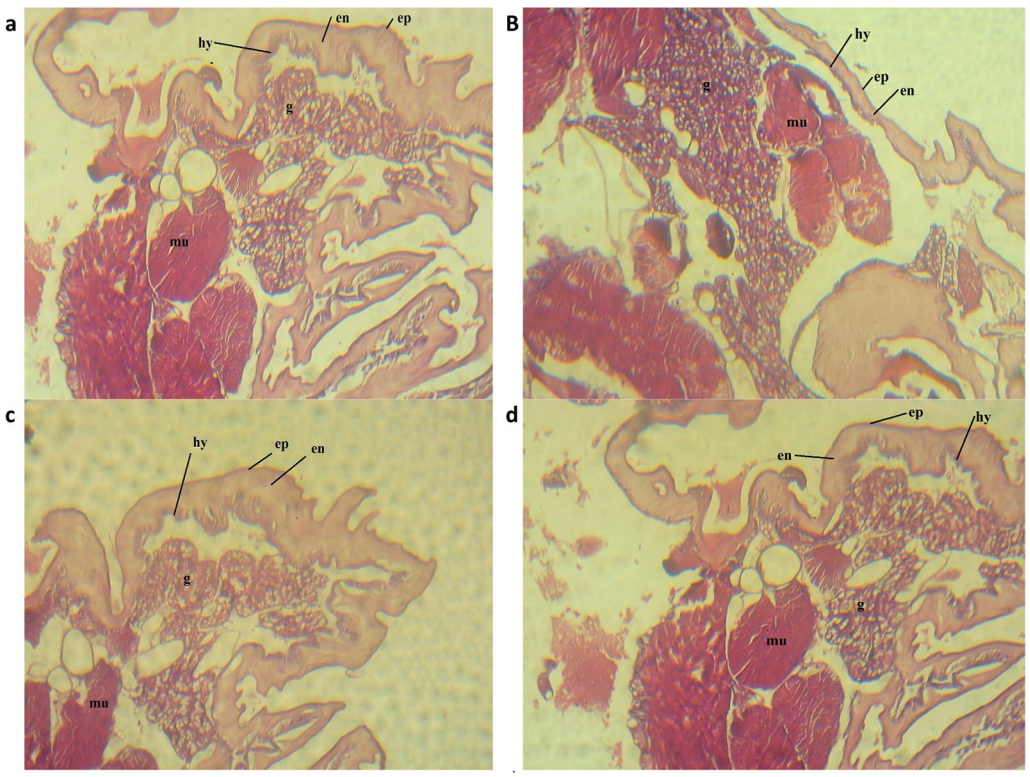

Fig. 2 Photomicrograph of T.S. in the cuticle of the late sixth instar larvae of *Spodoptera littoralis* showing the structure of larval cuticle: **a** the normal, **b** EMB treatment, **c** AgNPs treatment, and **d** EMB + AgNPs treatment (ep: epicuticle, en: endocuticle, hy: hypodermis, mu: muscle, g: cytoplasmic granules) (×40)

is commonly a simple tube. The midgut epithelium lining consists of columnar cells resting on a basement membrane with a more or less oval centrally located nucleus (Fig. 3a). The columnar cells are interspersed apically with goblet cells and basally with regenerative cells. They exhibited a delicate brush border confronting the midgut lumen. The peritrophic membrane is observed. Figure 3b presents the histopathological changes due to treatment with EMB LC_{50}. The regenerative cells lost their integrity between the columnar cells separated from the basement membrane but kept their defined integration. The brush border appeared slightly swallowed, and the peritrophic membrane was unattached and disintegrated. The LC_{50} treatment of AgNPs showed thickness and deformities in epithelial cells. The basement membrane was separated from the base of the columnar cells. The ciliated border

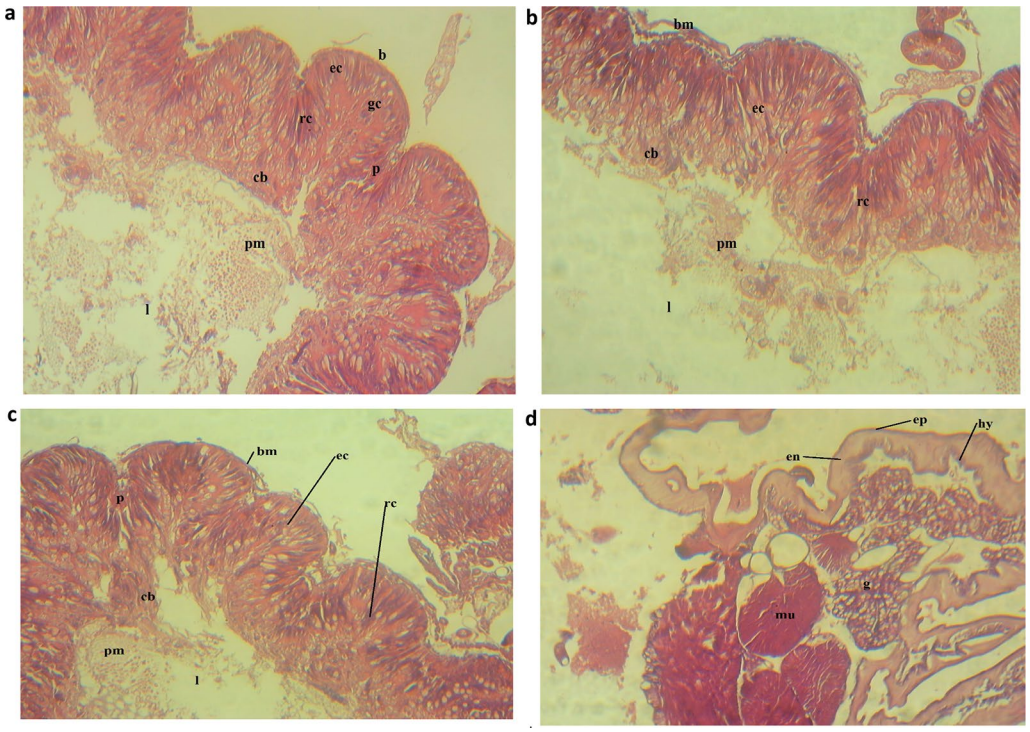

Fig. 3 Photomicrograph of T.S. in the midgut of the late sixth instar larvae of *Spodoptera littoralis* showing the structure of larval midgut: **a** the normal, **b** EMB treatment, **c** AgNPs treatment, and **d** EMB + AgNPs treatment (b: basement membrane, ec: epithelium cells, rc: regenerative cell, gc: goblet cell, p: papillary crypt, cb: ciliated border, pm: peritrophic membrane, l: lumen) (×40)

lost its order, elongated, disintegrated, and separated from the cell border into the gut lumen. The peritrophic membrane was disintegrated and partially disappeared (Fig. 3c). Treatment with LC_{50} of EMB + AgNPs destroyed the integrity of the epithelial cells and the ciliated border. The boundaries between columnar cells completely disappeared, and the columnar cells began to disintegrate. The peritrophic membrane became vacuolized and inconsistent (Fig. 3d).

4 Discussion

The present study aimed to avoid the environmental hazards of extensive amounts of synthetic pesticides. Moreover, the present work is studying the possibility of developing a much more effective control method of insecticide in a nanoform, which may also decrease the price of insect pest management programs. The target insect model is the cotton leafworm, *S. littorals*. The present study showed that the toxicity of the nanoform of EMB was higher than EMB in its original form at lower concentrations, suggesting the safety of nanopesticides as their environmental hazards can be neglected. The results showed that all tested formulae were toxic to the fourth instar larvae and EMB + AgNPs exhibited the highest toxicity according to the low LC_{50} and LC_{90} values compared to the rest formulae. The high toxicity of nanoform of EMB could be due to the condensation of the active ingredients in the nanopesticides compared to the original formula, which may lead to an increase in the toxic effect of the nanoformulation than the original one [46]. The nanoformulation of pesticides possesses a high penetration ability through insect tissues, increasing their toxicity over their original formulae [18]. The toxic effects of nanoparticles (NPs) can be attributed to their small size and large surface area, thereby increasing chemical reactivity and penetration in the living cells [23, 31, 34]. Obtained results agreed with [2, 7, 30], who tested the larvicidal activity of different nanopesticides against *S. littoralis* larvae. Furthermore, the results showed the extended effect of the tested formulations against larval, pupal, and adult stages. The results showed reduced mean larval and pupal duration. In addition, the mean weight of obtained pupae that survived treatment was reduced compared to the control. The results showed a significant reduction in the mean number of laid and hatched eggs/females produced from survived larvae. This reduction may be due to the possible accumulation of the tested formulae in the larvae that survived treatment that has adversely affected the insect metamorphosis during its life span [30, 42]. Our results were agreed

with [3, 4, 9, 20, 33], who tested EMB and nanopesticides against different insects.

Furthermore, compared to control, the total hemocyte counts have reduced in larvae that survived treatment with LC_{50} of EMB, AgNPs, and EMB + AgNPs. In addition, the viability percentage of hemocytes was also decreased in all treatments. The differential hemocyte counts showed five types of hemocytes: prohemocytes, plasmohemocytes, granulocytes, spherocytes, and oenocytoids. Treatment with the LC_{50} of EMB + AgNPs significantly decreased the percentage of prohemocytes, granulocytes, spherocytes, and oenocytoids, while the plasmohemocytes percentage was increased. Furthermore, treatment with LC_{50} of AgNPs caused no significant effect on plasmohemocytes, granulocytes, spherocytes, and oenocytoids percentages. Moreover, treatment with LC_{50} of EMB caused a significant increase in prohemocytes, granulocytes, and oenocytoids percentages, while no significant influence was observed in the percentage counts of plasmohemocytes and spherocytes. Hemocytes are the cells residing in the hemolymph. They mediate the cellular arm of the insect immune response, especially phagocytosis and, in many insects, encapsulation [11]. The decline in total hemocyte counts might depend on the test insect, different insects, insecticide, and concentration tested [39]. Moreover, it was reported that treating insects affected normal functioning, such as phagocytosis, encapsulation, nodule formation, and coagulation of the hemocytes. These hematological studies help to understand the insect body's physiological mechanisms that would help develop a successful pest management program [38]. Furthermore, the reduction in total hemocyte counts may be due to nodulation, encapsulation, degranulation of some cell types, or the inhibition of the brain hormone secretion [1]. The increment in some hemocyte types can be due to the recovery of the immune system to hazards [21, 28]. It is noteworthy that granulocytes and plasmohemocytes have variable responses than other types present under exposure to different insecticides [21, 22, 24, 28]. In insects, the midgut plays a central role in the insect's immunity response [11]. The results showed that the tested formulae exhibited histopathological impacts against the midgut of the late sixth instar larvae that survived treatment as fourth instar larvae. However, the cuticle of treated larvae showed no alteration. In the present study, an inhibition in the rate of development of *S. littoralis* larvae that survived treatment could be related to the changes observed in the midgut tissue after treatment with the tested formulations. These changes are observed as midgut damage, digestive dysfunction, and nutritional metabolism disorders; however, the exact mechanism is unknown. The tested formulations could induce oxidative stress and apoptosis in various non-nerve tissues [43, 45].

5 Conclusion

The present study is considered the first to show the latent effect of EMB, as an alternative insecticide for conventional ones, AgNPs, as a carrier, and EMB + AgNPs, the nanoform of EMB, on the immune response of the cotton leafworm, *S. littoralis*. The obtained results showed the effectiveness of these formulations against all stages of S. littoralis. The immune response of the tested insect was obvious in influenced life span, impacted rate of pupation and adult emergence, and reduced fecundity and fertility of resulting females. More studies are needed to understand the influence of the EMB and its nanoform on the vitellogenin expression.

Abbreviations

AgNPs: Silver nanoparticles; CaCl: Calcium chloride; EGAD: The Egyptian group for agricultural development; EMB: Emamectin benzoate; EMB + AgNPs: Emamectin benzoate nanoform loaded on AgNPs; KCl: Potassium chloride; L:D: Light: Dark; ME: Microemulsion; NaCl: Sodium chloride; NPs: Nanoparticles; ppm: Part per million; R.H.: Relative humidity.

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Author contributions

This work was carried out in collaboration among all authors. Authors HSHA, MSSA, TAAE, and EEAE designed the study. HSHA and TAAE performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MSSA, TAAE, and EEAE managed the analyses of the study. Authors HSHA managed the literature searches. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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