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Biochemical and Toxicological Effects of Emamectin Bonzoate against *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae)

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ABSTRACT

The cotton leaf worm, *Spodoptera littoralis* (Boisd.) is a key pest of cotton and a wide range of economically important crops, vegetables, and fruits in Egypt and worldwide. Emamectin benzoate is a broad-spectrum bioinsecticide that has long been used to control lepidopteran pests such as S. littoralis. The toxicity of emamectin benzoate against *S. littoralis* fourth-instar larvae and its biochemical effects on their acetylcholinesterase (AChE), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined. The toxicity of emamectin benzoate against larvae of *S. littoralis* was increased by the increase of exposure time. The LC_{50} values (lethal concentrations causing 50% larval mortality) caused by emamectin benzoate against *S. littoralis* larvae were 8.8×10^{-2}, 1.1×10^{-2} and 4.8×10^{-3} ppm a.i. at 2, 3 and 4 DAT, respectively. Overall, the application of emamectin benzoate on larvae at concentrations of 4.8×10^{-4}, 9.6×10^{-4} and 2.4×10^{-3} ppm a.i. (concentrations equivalent to 10, 20 and 50% of the estimated LC_{50} value at 4 DAT, respectively) increased the activity of AChE, AST, ALT of the treated larvae. The increase rate in the activity of those enzymes was dependent on both the concentration and exposure time for emamectin benzoate.

INTRODUCTION

Egyptian cotton is widely regarded as the best in the world (FAO 2022). It is vital to the country's economy because it meets domestic and export demands while also significantly contributing to agriculture, industry, employment, and export income (FAO 2022). *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) is a highly damaging pest of economically important crops, vegetables, and fruits (EL-SHEIKH *et al.,* 2013). It is the major insect pest registered on cotton in Egypt and elsewhere (Sabry and Khedr 2014; EL-SHEIKH *et al.,* 2013), not only for cotton but also for a wide range of economically important crops, vegetables, and fruits (Elbarky *et al.,* 2008). It is a damaging pest of

Egyptian crops because of its wide host range, which includes 40 plant families (Zaka *et al.*, 2014; Holloway 1989), avidity, and reproductive potential (K. Ahmed *et al.*, 2019). Larvae attack plants and commonly consume fruits, vegetables, flowers, and other plant parts. Infestation frequently results in complete leaf defoliation and plant development disruption (K. S. Ahmed *et al.*, 2019). The main method of controlling this pest is chemical control, and several insecticidal applications are thus used to protect the infested crops (Zaka *et al.*, 2014). As a result, the subsequent use of chemical insecticides for control has resulted in the development of resistance to several insecticides, including avermectins, carbamates, organophosphates, and pyrethroids (Ahmad 2009; Frank *et al.*, 1990; EL-SHEIKH *et al.*, 2013). Emamectin benzoate is a semisynthetic derivative of the Avermactin family bioinsecticide abamectin (Aziz and Mohamed 2019). It is a broad-spectrum insecticide for lepidopteran pests, including *Spodoptera littoralis* (Putter *et al.*, 1981; Jansson *et al.*, 1997; Ishaaya *et al.*, 2002). Emamectin benzoate performs its biological action on the insects by interfering with gamma-aminobutyric acid action (GABA) (Fritz *et al.*, 1979). It is a chloride channel modulator that prevents muscle contraction, stops insect feeding, and eventually kills the insect (Ishaaya *et al.*, 2002; Aziz and Mohamed 2019). It is toxic to arthropods via contact and ingestion, and death occurs within four days (Jansson *et al.*, 1997). The absence of glutamate-gated chloride channels in mammals compared to insects is a critical reason for avermectins, including emamectin, being safe against mammals (Arena *et al.*, 1995; Jansson *et al.*, 1997).

Acetylcholinesterase (AChE) is a key enzyme in the nervous system of insects, where it terminates neurotransmission by hydrolyzing the neurotransmitter acetylcholine (Papachristos *et al.*, 2004; McHardy *et al.*, 2017). It breaks down ACh into acetic acid and choline (McHardy *et al.*, 2017). This inhibits ACh activity, particularly on ACh release and accumulation, at the junctions of various cholinergic nerve endings with their effector organs or post-synaptic sites (McHardy *et al.*, 2017). According to Assar *et al.*, (2016), it has been found that emamectin benzoate stimulates the activity of AChE. Aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) are essential aminotransferase enzymes in humans and insects which catalyse the interconversion of amino acids and oxoacids via amino group transfer (Trang and Khandhar 2021). As a result, AST and ALT enzymes are known to play critical roles in the mobilisation of L-amino acids for gluconeogenesis and to act as linkages among carbohydrate and protein metabolism under different physiological, pathological, and induced environmental stress conditions (Conway 2011; Ramaswamy *et al.*, 1999). In the current work, we assessed the toxicity of emamectin benzoate against *Spodoptera littoralis* fourth-instar larvae. Furthermore, the biochemical effects of emamectin benzoate on the activity of AChE, AST, and ALT were investigated.

MATERIALS AND METHODS

Chemicals:

Emamectin benzoate 5% SG (Proclaim)® was provided by Syngenta Egypt. Potassium phosphate, l-glutamic acid (L-Glu), pyridoxal 5'-phosphate (PLP), water and methanol (HPLC grade) were supplied by Sigma Aldrich, Italy. Sodium acetate, sodium hydroxide, 3-mercaptopropionic acid (3-MPA), o-phthaldialdehyde (OPA), boric acid and glacial acetic acid were provided in analytical grades by Sigma Chemical Corp (St. Saint Louis, MO, 63103 USA). Alanine transaminase and aspartate transaminase enzymes were assayed with Diamond Diagnostic kits (Diamond Co. Egypt).

Insect Colony:

Larvae of *Spodoptera littoralis* used in the current study were collected from a culture previously reared at the laboratory of the Plant Protection Department, Faculty of

Agriculture (Saba Basha), Alexandria University, Egypt. The culture was reared on fresh leaves of Castor (*Ricinus communis*) in one-liter Mason jars covered with a piece of gauze following the method previously reported by Abdel-Salam and Hassan (1962). A number of 15 fourth instar larvae were added into each jar containing fresh castor leaves and transferred daily into clean jars. Once larvae pupated, they were sexed and 12 pupae were put into a new jar. A piece of cotton moistened with 10% sugar solution and 2 leaves of *Nerium oleander* were added to each jar to allow the emerged adults to deposit their eggs. The hatched larvae were then transferred on fresh Castor leaves in new jars. The insect culture was kept under controlled environmental conditions (25±2 ºC, 65±5% RH*) (Eldefrawi et al., 1964; Metayi et al., 2015).* The homogenized fourth-instar larvae with the same age and weight were usually used in all following bioassay and biochemical studies.

Leaf-dip Bioassay:

The lethal toxicity of the emamectin benzoate 5% SG (Proclaim)® (Syngenta) on the 4th instar larvae of *S. littoralis* was determined by the leaf-dip bioassay (Abdel-Halim *et al.,* 2019). The insecticidal solution was prepared in distilled water. Homogenous pieces of the Castor leaves were dipped in the prepared insecticidal solutions for 10 sec., held vertically to allow the excess solution to drip off and let to well-dry at room temperature. The treated piece of Castor leaves was transferred to plastic cups, and 20 starved 4th-instar larvae (same age and weight) were placed into each cup. Controls were just dipped in the distilled water. Each treatment was replicated fifth. All treatments were kept under controlled environmental conditions (25±2 ºC, 65±5% RH). Mortality percentages of larvae were recorded at 1, 2, 3 and 4 days after treatment (Saroukolai *et al.,* 2010; Eldefrawi *et al.,* 1964; Shawer 2017; Shawer e*t al.,* 2018a; Shawer e*t al.*, 2018b). Larvae were considered dead if they did not move when lightly touched with a camelhair needle (Shawer *et al.,* 2018a). Mortality was then corrected according to Abbott's formula (Abbott 1925; Miller *et al.,* 2010) as follows:

$$\text{Corrected mortality\%} = \frac{(\text{Mortality of treated insects} - \text{Mortality of control})}{(100 - \text{Mortality of control})} \times 100$$

LC50 values (lethal concentrations causing 50% larvae mortality) were calculated using the Probit analyses according to Finney (1971).

Biochemical Studies:

Activity of AChE

Samples were first prepared by feeding starved fourth-instar larvae of *S. littoralis* on the castor leaves previously immerged in the insecticidal solution of emamectin benzoate at concentrations equivalent to 10, 20 and 50% of the estimated LC_{50} value at 4 DAT. Larvae were fed on the castor leaves dipped in the distilled water considered as a control treatment. Head capsules of the treated larvae were collected at 1, 2, 3 and 4 DAT and washed with an ice-cold saline solution (0.9% NaCl) repeatedly. Six g of the head capsules were homogenized (Kinematica Polytron™ PT 2500E, Kinematica 11090108) in 15 mL of 50 mM phosphate buffer (pH 7.4). The homogenate was filtered through two layers of cheesecloth. The filtrate was centrifuged (IEC-CRU 5000 cooling) at 5000 rpm for 30 min at 4 ºC. The supernatant was used for the determination of AchE activity following the colorimetric method previously described (Ellman *et al.,* 1961). A 100 µl of the prepared supernatant was added to 3 mL of a mixture of 2 mM acetylthiocholine iodide (ASChI) and 2 mM dithiodinitrobenzoic (DTNB) (ratio, 1:1). The changes in the absorbance at a wavelength of 412 nm were recorded using a spectrophotometer (Sequoia-Turner Model 340) (Krzyżowski *et al.,* 2020). An assay mixture without an enzyme was used as the blank.

The Activity of AST and ALT:

The activity of Aspartate transaminase/glutamic oxalo acetic transaminase (AST/GOT) and glutamic pyruvate transaminase/alanine transaminase (GPT/ALT) were determined in the 4th instar larvae of *S. littoralis* following the method described by Reitman and Frankel (1957) using the Diamond Diagnostic kit (Diamond Co., Egypt). Starved fourth-instar larvae of *S. littoralis* were fed on the Castor leaves previously dipped in the insecticidal solution of emamectin-benzoate at concentrations of 10, 20 and 50% of the estimated LC_{50} at 4 DAT. Larvae were fed on the Castor leaves were dipped in the distilled water only acted as controls. Ten-treated larvae were collected and homogenized in the distilled water (pH: 7) (1: 10 w/v) using a Pyrex® Ten Broeck Homogenizer (Thomas Scientific) at 1, 2, 3 and 4 DAT. The homogenate was centrifuged (5000 rpm, 30 min.) at 4ºC using IEC-CRU 5000 cooling centrifuge. A hundred µL of the supernatant was added to 500 µL of 100 mM phosphate buffer (pH 7.2), containing 80 mM L-aspartate and 4 mM ά-ketoglutarate. The mixture was incubated for 30 min. at 37 ˚C. Then, 500 µl of 4 mM 2, 4-dinitrophenylhydrazine (a developing color reagent) was added and the solution was incubated for 20 min at room temperature. After that, 5 mL of 0.4 N NaOH was added, mixed and left at room temperature for 5 min. A mixture without the enzyme source was used as a blank. The absorption was measured by the spectrophotometer (Milton Roy Spectronic 601) at 546 nm. Activities of AST/GOT and ALT/GPT were determined at 1, 2, 3 and 4 DAT as IU/mg protein/hr and calculated as percentages of control.

Statistical Analysis:

Adults' mortality data were subjected to analysis of variance (ANOVA) (CoStat Statistical Software, 1990). The standard deviation of five replications was calculated. Duncan's least significant difference (LSD) test (Duncan 1955). Differences were considered significant at $\alpha = 0.05$. The LdP line computerised software programme was used to calculate the probit analyses of LC50 values and their fiducial limits (confidence intervals) according to Finney (1971).

RESULTS

Leaf-dip Bioassay:

Mortality results of the leaf-dip bioassay study indicated that one day of exposing larvae to the treated leaves was not enough to show the toxic effect of emamectin-bonzoate since all the tested concentrations were not able to cause any adult mortality at 1 DAT. While, the toxic effect of emamectin benzoate against the 4th instar larvae of *S. littoralis* was clear at 2, 3 and 4 DAT (Table 1 and Fig. 1). The LC_{50} values were 8.8×10^{-2}, 1.1×10^{-2} and 4.8×10^{-3} ppm a.i. at 2, 3 and 4 DAT, respectively. The highest lethal activity of emamectin benzoate was caused at 4 DAT, and the lowest was at 2 DAT. It is generally observed that the toxicity of emamectin benzoate against the 4th instar larvae of *S. littoralis* was increased by the increase of exposure time.

Table 1. LC_{50} values of emamectin benzoate against the 4th instar larvae of *S. littoralis* at different exposure times following the leaf-dip technique**.**

DAT[1]	LC_{50} (ppm a.i.)	95% Conf. limits (ppm a.i.)		Slope
		Lower limit	Upper limit	
2	8.8×10^{-2}	0.035	1.342	0.923
3	1.1×10^{-2}	0.0077	0.016	1.394
4	4.8×10^{-3}	0.0033	0.0069	1.321

[1] DAT=days after treatment.

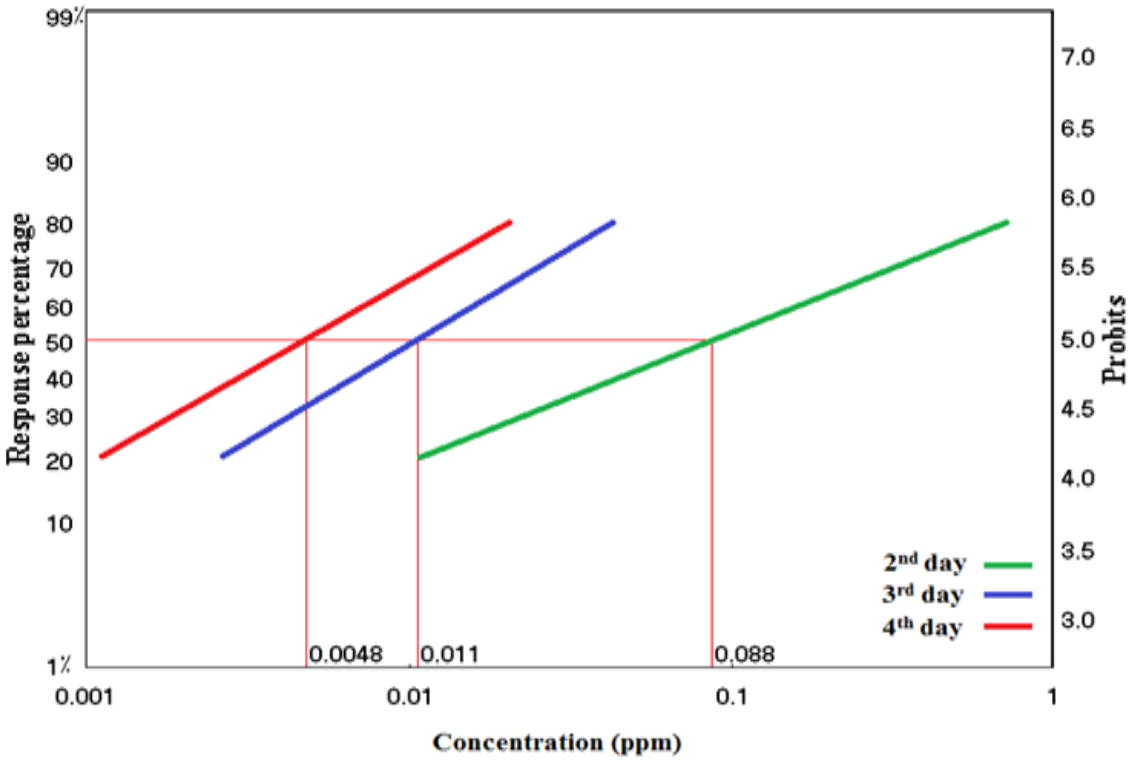

Fig. 1. LdP lines of emamectin benzoate against *S. littoralis* 4th instar larvae at different exposure times.

Biochemical Studies:

Activity of Acetylcholinesterase:

The activity of AChE of *S. littoralis* 4th instar larvae exposed to Castor leaves dipped in the insecticidal solution of emamectin benzoate at lethal concentrations (10, 20 and 50% of the estimated LC_{50} at 4 DAT) for different exposure times (1, 2, 3 and 4 DAT) is presented in Table 2. All the applied concentrations of emamectin benzoate significantly increased the activity of AChE of the treated *S. littoralis* larvae compared to controls at 1, 2, 3 and 4 DAT. The activities of AChE were 133.9, 149.9 and 160.11% in comparison with the activity of control, when *S. littoralis* larvae were exposed to emamectin benzoate at concentrations of 50, 20 and 10% of LC_{50} at 1 DAT, respectively. The AChE activities increased further by increasing the exposure time. The highest increase of AChE activity (186.31%) over the control was recorded by using emamectin benzoate at a concentration of 2.4×10^{-3} **(**ppm a.i.) at 2 DAT. The activity of AChE in *S. littoralis* larvae caused by emamectin benzoate was clearly dependent on its concentration and time of exposure.

Activity of ALT:

The activity of ALT of *S. littoralis* 4th instar larvae exposed to Castor leaves dipped in the solution of emamectin benzoate at different concentrations (10, 20 and 50% of the estimated LC50 at 4 DAT) for different exposure times (1, 2, 3 and 4 DAT) is shown in Table 3. Exposing *S. littoralis* larvae to different concentrations of emamectin benzoate resulted in an increase of ALT activity. However, this activity increase was almost not very clear at 1 and 2 DAT, as all concentrations of emamectin benzoate showed very similar ALT activity results to control. While the evaluated concentrations increased the ALT activity by about 46-80% over control at 3 DAT. The activity continued to increase while the exposure time to emamectin benzoate was increasing. The highest ALT activity (175%) was achieved by the higher concentration of emamectin benzoate (2.4×10^{-3} ppm) at 4 DAT compared to

control. This is followed by the concentrations of 9.6×10^{-4} and 4.8×10^{-4} (ppm), giving 168.2 and 154.4% ALT activity, respectively.

Table 2. Effect of emamectin benzoate on the activity of acetylcholinesterase of *S. littoralis* larvae at different exposure times.

DAT[1]	Concentration (ppm a.i.)	Specific Activity (S.A.) (ΔO.D/mg protein/hr) ± SD	Activity (%)
1	4.8×10^{-4}	0.092±0.0052 [c]	133.91
	9.6×10^{-4}	0.103±0.0049 [b]	149.92
	2.4×10^{-3}	0.110±0.0061 [b]	160.11
	Control	0.069±0.0033 [d]	100
2	4.8×10^{-4}	0.106±0.0037 [b]	154.2
	9.6×10^{-4}	0.127±0.0053 [a]	184.86
	2.4×10^{-3}	0.128±0.0041 [a]	186.31
	Control	0.069±0.0033 [d]	100
3	4.8×10^{-4}	0.104±0.0026 [b]	151.38
	9.6×10^{-4}	0.119±0.0055 [a]	173.21
	2.4×10^{-3}	0.120±0.0039 [a]	174.67
	Control	0.069±0.0033 [d]	100
4	4.8×10^{-4}	0.099±0.0028 [b]	144.10
	9.6×10^{-4}	0.111±0.0034 [a]	161.57
	2.4×10^{-3}	0.117±0.0038 [a]	170.30
	Control	0.069±0.0033 [d]	100

[1] DAT=days after treatment.

Table 3. Effect of emamectin benzoate on the activity of ALT of *S. littoralis* larvae at different exposure times.

DAT[1]	Concentration (ppm a.i.)	Specific Activity (S.A.) (IU/mg protein/hr) ± SD	Activity (%)
1	4.8×10^{-4}	373.8 ± 4.13 [d]	97.7
	9.6×10^{-4}	382.0 ± 8.41 [d]	99.8
	2.4×10^{-3}	398.9± 6.78 [c]	104.31
	Control	382.4 ± 8.19 [d]	100.0
2	4.8×10^{-4}	336.1 ± 5.94 [d]	87.8
	9.6×10^{-4}	348.2 ± 4.97 [d]	91.05
	2.4×10^{-3}	460.2 ± 6.74 [c]	120.3
	Control	382.4 ± 8.19 [d]	100.0
3	4.8×10^{-4}	557.1 ± 8.12 [b]	145.6
	9.6×10^{-4}	608.2 ± 9.15 [b]	159.04
	2.4×10^{-3}	689.2 ± 4.98 [a]	180.2
	Control	382.4 ± 8.19 [d]	100.0
4	4.8×10^{-4}	590.5 ± 8.35 [b]	154.4
	9.6×10^{-4}	643.2 ± 7.89 [a]	168.2
	2.4×10^{-3}	670.7± 6.77 [a]	175.20
	Control	382.4± 8.19 [d]	100.0

[1] DAT=days after treatment.

Activity of AST:

It is obvious that exposing *S. littoralis* 4th instar larvae to varying concentrations of emamectin benzoate for various exposure times resulted in an increase in AST activity in particular at 3 and 4 DAT (Table 4). At 1 and 2 DAT, there were significant differences in the estimated AST activities in all concentrations of emamectin benzoate (with the exception of 2.4×10^{-3} ppm at 2 DAT) and the controls. All treatments significantly increased the activity of AST over controls at 3 and 4 DAT. The application of emamectin benzoate at a concentration of 2.410^{-3} ppm at 3 DAT, resulted in the highest AST activity percentage over controls (141.24%).

Table 4. *In vivo* effect of emamectin benzoate on the activity of AST of *S. littoralis* larvae at different exposure times

DAT[1]	Concentration[2] (ppm a.i)	Specific Activity (S.A.) (IU/mg protein/hr) ± SD	Activity %
1	4.8×10^{-4}	956.8 ± 8.51 [d]	99.2
	9.6×10^{-4}	988.2 ± 8.67 [d]	102.4
	2.4×10^{-3}	1007.1 ± 9.16 [d]	104.46
	Control	964.1 ± 6.97 [d]	100.0
2	4.8×10^{-4}	955.7 ± 7.98 [d]	99.12
	9.6×10^{-4}	972.6 ± 6.45 [d]	100.88
	2.4×10^{-3}	1090.8 ± 9.34 [c]	113.14
	Control	964.1 ± 6.97 [d]	100.0
3	4.8×10^{-4}	1210.4 ± 6.21 [b]	125.54
	9.6×10^{-4}	1254.4 ± 7.36 [b]	130.11
	2.4×10^{-3}	1361.7 ± 6.49 [a]	141.24
	Control	964.1 ± 6.97 [d]	100.0
4	4.8×10^{-4}	1211.8 ± 6.97 [b]	125.6
	9.6×10^{-4}	1279.2 ± 5.99 [a]	132.68
	2.4×10^{-3}	1320.4 ± 9.11 [a]	136.91
	Control	964.1 ± 6.97 [d]	100.0

[1] DAT=days after treatment.

DISCUSSION

In the present study, the toxicological and biochemical effects of the insecticide emamectin benzoate on the 4th instar larvae of *S. littoralis* were studied. The LC_{50} values were used to measure the toxic effects of the insecticide against the treated larvae at 1, 2, 3 and 4 DAT. One day after treatment was not enough time to show the toxic effect of emamectin benzoate against larvae. The greatest activity ($LC_{50}=4.8\times10^{-3}$ ppm a.i.) of emamectin benzoate against the 4th instar larvae of *S. littoralis* was recorded on the 4th day of the exposure time. It is generally observed that the toxicity of emamectin benzoate against larvae of *S. littoralis* is positively correlated with the increase of exposure time to it. These findings are consistent with those obtained by Rashwan (2013), who noticed that increasing the exposure time increased the activity of emamectin benzoate.

AChE is the target site of several neurotoxic insecticides and plays an important role in neurotransmission by hydrolyzing the neurotransmitter acetylcholine in cholinergic synapses of the nervous system (Papachristos *et al.*, 2004). Changes in AChE activity disrupt cholinergic transmission in the head ganglia and ventral nerve cord, resulting in uncoordinated leg movement (Dohi *et al.*, 2009; Lang et al. 2012). Several articles have described the influence of emamectin benzoate on AChE activity (Dohi *et al.*, 2009; Krzyżowski *et al.*, 2020; Lang *et al.*, 2012). The biochemical results of the current study indicated that exposing the 4th instar larvae of *S. littoralis* to emamectin benzoate increased the activity of AChE. The increase in AChE activity could be due to one or more of the following factors: increasing AChE expression, decreasing AChE metabolism, or increasing AChE activity itself (Choi *et al.*, 2003). Total ATPases may be inhibited by emamectin benzoate (Choi *et al.*, 2003). According to its function, this will result in the accumulation of ATP, which may stimulate AChE expression (Choi *et al.*, 2003). On the other hand, acetylcholinesterase activities in the head of *S. littoralis* 4th instar larvae treated with emamectin benzoate at 1, 2, and 3 DAT were clearly reduced compared to the control, according to Megahed *et al.* (2013). It was found that the change percentage of AChE activity reached its maximum level at 2 DAT and its minimum level at 3 DAT.

Additionally, the results of this study showed that emamectin benzoate significantly increased the activity of AST and ALT in the treated *S. littoralis* 4th instar larvae. These findings are consistent with those previously reported (Megahed *et al.* 2013; Abou-Taleb *et al.*, 2009; Ramaswamy *et al.* ,*1*999; İçen *et al.*, 2005; El-Shershaby *et al.*, 2008; Ghoneim *et al.*, 2016; Assar *et al.*, 2016). The researchers have found that exposing *S. littoralis* 4th instar larvae to different concentrations of emamectin benzoate at different times increased the activity levels of these enzymes. When *Sarotherodon mossambicus* (Peters) was subjected to sub-lethal and lethal concentrations of the carbamate pesticide carbaryl, Ramaswamy *et al.* (1999) found that the activity levels of the AST and ALT enzymes were raised. Some insect growth regulators (IGRs) including pyriproxyfen, flufenoxuron, or chlorfluazuron (Abdel-Aal 2003; Ghoneim *et al.*, 2016); flufenoxuron (Ghoneim et al. 2016), and novaluron, cyromazine, or diofenolan (Tanani *et al.*, 2016; Ghoneim et al. 2016), have been shown to increase GOT activity in *Spodoptera littoralis* larvae. According to Radwan *et al.*, (1992), one possible mechanism for increased AST and ALT levels is tissue damage caused by increased synthesis and/or decreased metabolism of both enzymes. And this may be due to the presence of reversible binding between bioinsecticides and the enzymatic site of action on the enzyme surface.

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