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Toxicity of *Hottentotta saulcyi* venom against *Sesamia nonagrioides* on sugarcane plants

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Abstract

To evaluate the toxicity of *Hottentotta saulcyi* venom on *Sesamia nonagrioides*, we investigated the larval mortality, detoxification enzyme activity and histopathological alterations of the *S. nonagrioides* organs. Venoms were injected into the larvae at five doses (0.15µg, 0.35µg, 0.5µg, 0.75µg and 1µg; fifteen larvae per each dose) and the doses causing death were recorded during the experiment. This study demonstrated that *H. saulcyi* venom was able to cause external symptoms that eventually led to death of the intoxicated larvae. Moreover, this venom strongly inhibited glutathione S-transferases (GST) activity. Our data highlighted the relationship between the activity of GST enzyme and larval mortalities, so that the inactivation of this enzyme by scorpion venom may be responsible for the increasing the larval mortality. In the present study, the damage of midgut epithelium and malpighian tubules has been directly visualized and documented using histopathology methods. Clear histopathological alterations of intoxication were observed for all larvae injected with 1µg doses of venom, including a distinctive lysing and disorganizing of midgut epithelial cells that coupled with a progressive loss of the peritrophic membrane and the disappearance of microvilli.

Keywords: Glutathione S-transferases, *Hottentotta saulcyi*, malpighian tubules, midgut, *Sesamia nonagrioides*.

مسمومیت زهر عقرب *Hottentotta* **Saulcyi** در برابر *Sesamia nonagrioides* آفت نیشکر

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چکیده

به منظور ارزیابی سمیت زهر عقرب *Hottentotta saulcyi* علیه کرم ساقه‌خوار نیشکر (*Sesamia nonagrioides*)، درصد مرگ و میر لاروها، فعالیت آنزیم‌های سم‌زدایی و تغییرات هیستوپاتولوژی بافت‌های سزمیا مورد بررسی قرار گرفت. سم به لاروها در پنج دوز مختلف ۰/۱۵، ۰/۳۵، ۰/۵، ۰/۷۵ و ۱ میکروگرم (پانزده لارو به ازای هر دوز) تزریق شد و دوزهای که در طول آزمایش سبب مرگ و میر لاروها شده بودند ثبت شدند. این مطالعه نشان داد که زهر *H. saulcyi* قادر به ایجاد نشانه‌های ظاهری بوده که در نهایت منجر به مرگ لاروهای مسموم شده گردید. علاوه بر این، این سم به شدت فعالیت گلوتاتیون S-transferases (GST) را مهار کرد. داده‌های این آزمایش نشانگر وجود رابطه بین فعالیت آنزیم GST و مرگ و میر لارو می‌باشد، به‌طوری که غیر فعال شدن این آنزیم توسط زهر عقرب ممکن است مسئول افزایش مرگ و میر لارو باشد. در این مطالعه، آسیب‌های غشای روده و لوله‌های مالپیگی با استفاده از روش‌های هیستوپاتولوژیکی بررسی شد. در اثر مسمومیت، تغییرات هیستوپاتولوژیکی واضحی در تمام لاروهای تزریقی با دوز ۱ میکرولیتر سم مشاهده شد، از جمله تجزیه و بهم خوردن ساختار سلول‌های غشای روده میانی که با کاهش پیشرونده غشای پریتروفی و ناپدید شدن میکرو ویلی‌ها همراه بود.

واژه‌های کلیدی: روده میانی، لوله‌های مالپیگی، *Hottentotta saulcyi*, Glutathione S-transferases, *Sesamia nonagrioides*

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Introduction

Sugarcane plant is one of the most important sources of edible sugar, industrial used sugar, and sugar-rich products in the world. Two stem sugarcane borers species, *Sesamia cretica* and *Sesamia nonagrioides* (*Lepidoptera: Noctuidae*) are considered a major insect pests of sugarcane. Polyphagous *S. nonagrioides* is one of the most damaging pests of many host plants distributed in several agroecosystems specially sugarcane at Khuzestan sugar estate that affects the sugar production in economically significant level (Halabian *et al*. 2013). Currently used chemical pesticides had detrimental effects in non-pest and beneficial insects also they present hazardous effects on the health and environment (Bocquene and Galgani 1998; Zacharia 2011). Lack of success with these strategies has led to the increased interest in biological pest management. Recently, due to scorpion venom biological properties and proteinaceous nature (Boyer *et al*. 2009; Karatas 2003), it has been considered as a control agent in the biological pest management (Manzoli-Palma *et al*. 2003; Gurevitz *et al*. 1997). Meanwhile, scorpion venom mentioned as a potential source of bioactive substances and insectotoxins which majority of them is some polypeptides with neurotoxicity. Since the first insect-specific neurotoxin from scorpion venom was described (Gurevitz *et al*. 1997) this venom is featured as a potential candidate for bioinsecticide development (Manzoli-Palma *et al*. 2003). In light of these findings, the study of the scorpion venom toxicological and physiological activities on *S. nonagrioides* pest has not been reported so far. In the current project, *H. saulcyi* scorpion venom was tested for in vivo toxicity on *S. nonagrioides*. Moreover, the activity of the GSTs and AChE enzymes were examined in order to identify the toxic action of scorpion venom. Additionally, here, we described the effects of scorpion venom on internal tissues of *S. nonagrioides* using histopathological methods.

Material and methods

Venom preparations

The investigated *H. saulcyi* scorpions were obtained from Baghmalek and Ramhormoz cities in Khuzestan province, southwest of Iran and transported alive to Razi Laboratory of Scorpion Research. The crude venom was milked by applying mild electrical stimulation of the telson (20 V, 500 mA), lyophilized and preserved at -20°C until used. Whole venom preparations were done by dissolving 1 mg of the *H. saulcyi* lyophilized venom in 1 ml of the double-distilled water, and vortexing or shaking with glass beads at 30 Hz until homogeneously suspended. Protein concentrations of the venom preparation were measured by Lowry's method using BSA as standard (Lowry *et al*. 1951). To supply of four venom doses, different concentrations of whole venom preparation (0.15µg, 0.35µg, 0.5µg and 0.75µg) were diluted in phosphate buffered saline (PBS) up to 1µl.

Experimental insects

All experimental *S. nonagrioides* third instar larvae were collected from the stem of the sugarcane plant in Debel Khozai- Khuzestan sugar estate. Groups of larvae were placed into plastic boxes and the boxes were placed in a climatic chamber at 25 ± 1°C, 55–60 % humidity, under long day (LD) conditions (16:8, light: dark) photoperiod. Set of healthy homogeneous larva in the early 3rd instar stage and 70-80 mg body weight selected for bioassay.

Injections, paralysis assay and toxicity bioassay

To perform the paralysis assay, any changes in larval behavior patterns, such as falling outside the stems and immobilization (only moved their head when gently touched with a platinum loop), were scored as paralyzed. The toxicity bioassay was measured by injecting five different concentrations of venom preparation; 0.15µg, 0.35µg, 0.5µg, 0.75µg and 1µg and compared against a control that injected by PBS. Venom (1µl per insect) was injected between the second and third pair of legs using 10 µl Hamilton syringe. The larvae were kept under observation for 72h, post injection and the lethality effects of scorpion venom were recorded for each venom doses. Toxicity levels were calculated according to the method of Probit (Finney, 1971; Van der Valk and van der Meijden, 2014) using SPSS software and expressed as lethal doses (LD50). The collected larvae were flash frozen in liquid nitrogen for storage at −80 °C and subsequently used for extraction of enzyme extract. The injection and venom lethality bioassay experiments were replicated twice with at least fifteen larvae per treatment.

Assays of detoxification enzyme activities

Enzyme Extract Preparation: The midguts with contents of larval groups treated with 0.5µg, 0.75µg and 1µg

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doses of venom and the 1µl PBS as control (2 larvae per replication) were removed 24h after treatment. Each dissected midgut washed with 1× phosphate buffer and homogenized in 0.1 M potassium phosphate buffer, pH 6.5 on ice using a glass homogenizer. The homogenates were centrifuged at 10,000 g for 10 min at 4°C and the supernatants were pooled to use as an enzyme extract solution for enzymatic activity assays.

Glutathione S-Transferase (GST) activity determination assay: Activity of the GST was determined according to the earlier method of Habig *et al*. (1974) with little modifications. Enzyme activity was measured spectrophotometrically with reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB) as substrates. Briefly, samples were removed from -20°C freezer and allowed to thaw on ice. The reaction mixture (containing 20 ml of midgut extract, 1 mM CDNB added in 10 ml of ethylene glycol monomethyl ether, 5 mM reduced glutathione, and 0.1 M phosphate buffer, 0.15 M NaCl, 5 mM MgCl2, pH 7.0) was prepared and the activity of GST was determined in 1ml of reaction mixture for each assay. The reaction mixture was incubated at 25°C for 20 min then the increment in absorbance at 340 nm was recorded, every 1 minute, during 5 min. The enzyme activity (µmol substrate conjugated/min/mg protein) was determined using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB.

Acetyl cholinesterase (AChE) activity assay: AChE activity was determined by the procedure of Ellman *et al*. (1961) with some modifications. Briefly, 20 µl of thoroughly mixed midgut extract, 100 µl of 1 mM substrate (acetylthiocholine iodide (ACTC) for AChE assay) and assay mixture that contained 3 mL of 0.25 mM of 5', 5'-dithiobis-nitrobenzoic acid (DTNB) prepared in 0.05 M phosphate buffer were added into a cuvette and the mixture was stirred and allowed to stabilize for 60 sec. After mixing, change of absorbance per min was recorded at 410 nm at 25°C using spectrophotometer for 5 min. AChE activities were expressed in µmoles ACTC/min/mg protein.

Histopathological studies

Histopathological changes of the internal tissues of control and treated larvae (injected by 0.5µg and 1µg doses of venom) were studied. The experimental larvae that were alive after 24h of treatment were collected and fixed in 10% neutral buffered formalin for 72h and they processed and prepared as routine in pathology lab. After dehydration with ascending grades of alcohol and clarification with xylene, the specimens were embedded in paraffin wax, mounted, sectioned at 5 µm thickness and stained with hematoxylin and eosin (H&E) (Bancroft 1996). Finally, the stained sections were observed under light microscopy.

Statistical analysis

In this study, data were statistically analyzed using SPSS software (IBM SPSS Statistics, Version 22). One-way ANOVA was used to assess differences between the treated groups and control group in all measurements (Van der Valk and van der Meijden, 2014). In statistical analyses, P value of less than 0.05 was considered significant ($P < 0.05$).

Results and Discussion

Paralysis assay and bioassay of toxicity

In order to investigate the toxicity and anti-insect property of *H. Saulcyi* venom against *S. nonagrioides* larvae, protein content of the venom, 50% lethal doses (LD50) values and total mortality percentage of treated and control larval groups were measured. The protein content of the *H. saulcyi* venom was determined and its value was 8.9 mg/ml. The scorpion venom was injected into the *S. nonagrioides* larvae, in different doses, and the lethality test 12h, 24h, 48h and 72h post venom injection is presented in table 1. The LD50 in 12h after venom injection was found to be 1.26 µg/100 mg of larva while, 0.96 µg/100 mg of larva was found to be a lethal dose in 24h post treatment. Thus in 24h post venom treatment, the used toxin was found to be effective (Table 1). The maximum mortality of *S. nonagrioides* larvae was observed at 48 hours with LD50 of 0.39 µg/100 mg of larva.

According to the total mortality percentage observed in studied groups (Table 2), the statistically significant differences were found among high venom doses (1µg and 0.75µg) injected groups, and control group ($P<0.05$). In addition, the lethality of high venom doses injected groups is higher than other examined groups, for comparison, at 72h it

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was 1.14, 2.18 and 2.67 times higher than those observed in 0.5µg, 0.35µg and 0.15µg treated groups, respectively (Table 2).

Immediately after scorpion venom injection, the stem borers showed basic toxicity symptoms while, mortality was slightly delayed. The scorpion venom causes locomotive paralysis and then death in all larvae injected by high doses of scorpion venom. A half an hour after high doses of venom injection, all larvae being unable to hitch their legs and dropped outside the stem without any reflex until insect death. Before the death, the insects became motionless and them body begin to rigid, blackish, and died. Muscle paralysis occurred gradually for other groups and this status indicate the impending pest death. The larvae received 0.35 and 0.15µg doses of venom became paralyzed after venom injection and 35.41 and 29.16 per cent death was observed respectively, but no more significantly lethal effect was reported under studied conditions after 48 hours (Table 2). The surviving larvae recovered, returning into stem and started stem feeding after 48 hours. Of course, this fact should not be overlooked that this status in the larvae received the 0.5µg dose of venom is not imply and the mortality of those larvae continued to 72 h after injection.

Table 1. Toxicity values obtained in insect tests for *Hottentotta saulcyi* venoms.

Toxicity (µg/100 mg of larva) (95% Confidence Limits for dose)			
LD Values			
Time post treatment	**LD30**	**LD50**	**LD90**
12h	1.09 (0.97-1.38)	1.26 (1.1-1.74)	1.69 (1.39-2.62)
24h	0.68 (0.58-0.79)	0.96 (0.84-1.16)	1.66 (1.39-2.14)
48h	0.27 (0.22-0.32)	0.39 (0.35-0.44)	0.68 (0.61-0.78)
72h	0.21 (0.15-0.26)	0.35 (0.30-0.40)	0.69 (0.61-0.79)

LD=Lethal Dose

Table 2. Toxicity bioassay of *Sesamia nonagrioides* presented as larval mortalities percentages in different time post treatment.

		Treatment (Concentration)				
Time intervals (h)	**control**	**0.15µg**	**0.35µg**	**0.5µg**	**0.75µg**	**1µg**
12h	0^{c}	0^{c}	0^{c}	0^{c}	10.41 ± 2.09^{b}	18.75 ± 2.08^{a}
24h	0^{e}	10.41 ± 2.08^{d}	12.5 ± 2.41^{d}	22.92 ± 2.07^{c}	35.41 ± 2.09^{b}	50 ± 3.4^{a}
48h	2.08 ± 2.06^{d}	27.08 ± 2.07^{c}	33.33 ± 3.40^{c}	58.33 ± 3.42^{b}	100^{a}	100^{a}
72h	12.50 ± 2.41^{d}	29.16 ± 2.40^{c}	35.41 ± 2.08^{c}	64.58 ± 2.09^{b}	100^{a}	100^{a}

Significant difference between control and treated group using one-way ANOVA (P<0.05).
Values are presented as mean percentage ± S.E. of 15 insects per group. Statistical differences are indicated: a>b>c>d>e.

The distinguishing normal behavior pattern of *S. nonagrioides* larvae considers the control group as evermore takes place inside on stem from the initial stage of life. The effect caused by venom injections are clearly observed since toxicity symptoms started to emerge with an increase in motility and any abnormal behavior of larvae leads to drag them out of the stem. So, when the toxic effects of scorpion venom began to appear after injection, the larvae cannot feed and coming outside the plant stem. Thus, emerging the larvae out of the stem can be considered as symptom of the toxin effect in the first few minutes of injection. In the present investigation it has been shown that venom of *H. Saulcyi* has anti-insect properties similar to that found in *Scorpio maurus palmatus* (Lazarovici *et al*. 1982), *Buthus occitanus tunetanus* (Borchani *et al*. 1997), *Buthus tamulus* (Dhawan *et al*., 2002) and *Tityus serrulatus* scorpion (Manzoli-Palma *et al*. 2003). A preliminary study of the *Scorpio maurus palmatus* venom toxicity on blowfly larvae (Lazarovici *et al*. 1982) suggested that in contrast to the Buthidae venom that caused an immediate contraction paralysis, the *Scorpio maurus palmatus* venom leaded to slow progression in the severity of paralysis of larvae. Recently, the researchers

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indicated that the toxin from *Buthus tamulus* scorpion shows the toxicity towards the *Lepidopteran* species of insect *Helicoverpa armigera*, that causing severe and flaccid paralysis and the larva was dead within 24h (Dhawan *et al*. 2002).

Activity of GST and AChE enzymes

The *in vivo* effects of scorpion venom on larval detoxification enzymes activity was tested (Table 3). The midgut of larvae used for the examination of the GST and AChE enzymes activity were dissected from *S. nonagrioides* treated with three different doses of scorpion venom. Analysis of the enzymes activity data was determined that GST enzyme decreased significantly its levels of activity at the highest dose of scorpion venom treatment with regard to that of the control ($P<0.05$). In the presence of 1μg and 0.75μg doses of venom, the activity of GST diminished by 26 and 43 per cent ($P< 0.05$) respectively. Data showed that the GSTs activities in the larvae treated by 0.5μg dose of venom were not significantly affected. As previously reported by several researchers, GSTs is one of the important metabolic enzymes which involved in neutralizing the toxic effects, catalyze the xenobiotics and detoxifying reaction of a wide variety of toxic including insecticides, by conjugating them to glutathione, that lead to protecting nucleic acids and cells from oxidative damage (Soderlund *et al*. 1983; Motoyama, 1980). Consequently, GST maintained as the biomarker of oxidative stress and cellular damage. This enzyme is known to protect cells from oxidative damage by interfering with cellular detoxification of a fairly wide range of chemical substrates (Josephy 2010).

Table 3. Activity of the GSTs and AChE enzymes in *Sesamia nonagrioides* subjected to three doses of scorpion venom. The mean values of AChE and GSTs activity for larvae injected by 0.5, 0.75 and 1μg doses of scorpion venom and larvae injected by PBS as the control group are presented.

Treatment	GST activity (Iu/mg protein)*	% Inhibition	AChE activity (Iu/mg protein) **	% Addition
control	4.56± 0.25	-	1.01± 0.067	-
0.5μg	4.89± 0.32	-	1.97± 0.058	95
0.75μg	3.38± 0.37	26	1.81 ± 0.073	79
1μg	2.60± 0.028	43	1.61± 0.062	59

* One unit of GST activity is the amount of enzyme which produces 1 μmol of GS-DNB conjugate/min under the conditions of the assay.
** One unit of AChE activity is the amount of enzyme which produces 1 μmol of ACTC/min under the conditions of the assay.
Values are present as mean ± SE.

The current research revealed that the GST as a molecular target of toxin component can be effectively inhibited by scorpion venom. Despite the available evidence, it is likely that the detoxification mechanisms of scorpion venom in *S. nonagrioides* are related to the activity of this enzyme. Various studies showed different results, depending on the experimental animal, type and concentrations of toxin, exposure time and site of injection. However, scorpion venom toxicity and inhibitory effect on GSTs in *S. nonagrioides* have not yet been considered in detail. Similar inhibition effects have previously been reported for snake venom (Al-Quraishy *et al*. 2014). Likewise, Al Asmari *et al*. (2014) reported decreased activities of GST in case of treatment with venoms, which also agrees with our results. However, in contrast to our results, other studies showed increases in GST activity in venom-injected experimental larvae (Akef *et al*. 2017). Furthermore, induction effect of scorpion venom on GSTs was reported in human prostate cancer cells (Akef et al. 2017). The previously indicated studies showed that there is a clear correlation between level of GSTs and the insecticide resistance in pests (Mohan and Gujar 2003; Furlong and Wright 1994). Similarly, our investigation was showed that there is an obvious correlation between level of GSTs and the mortality rate (Figure 1). Based on the data recorded in Figure 1, high venom concentration resulted in an increase in mortality rate associated with inactivation of the GST enzyme. This fact has established that larval groups treated by lower doses of venom showed venom resistance while inactivation the GST in other groups leaded to decrease the resistance and increase the mortality rate. Relationship between activities of GSTs and larval mortality rate indicates the role of GST in detoxifying mechanism and host defense in *S. nonagrioides* to protect the cell against oxidative stress. However, this detoxification enzyme may also be susceptible to inactivation by scorpion venom.

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Additionally, the current study was to examine the effects of the scorpion venom on the AChE enzyme activity of *S. nonagrioides*. To check the changes in hydrolyzes the neurotransmitter acetylcholine in response to scorpion venom; we measured AChE activity levels in stem borers. The AChE activities of the *S. nonagrioides* midguts treated by scorpion venom summarized in Table 3. The scorpion venom doses treatment represented high AChE activity when compared to control larvae. As the data was showed the significantly highest AChE activity was observed in the larval group treated by 0.5μg scorpion venom regarding control larvae ($P<0.05$), whereas, this enzyme activity tended to decrease in other treated groups compared to 0.5μg treated group but not in comparison with the control. Overall, scorpion venom had an inducing effect on *S. nonagrioides* AChE activity. However, by increasing the doses of venom this progressive rate of enzyme activity has decreased, but still higher than that reported in the control group. This agrees with reports that showed an increase in AChE activity in rats when using *Androctonus crassicauda* venom as a treatment for 24 hours (Ozkan *et al*. 2007). Significant inhibition in AChE activity has been previously reported in humans (Ucar and Tas 2003) and insects (Babu *et al*. 1971) with different exposure time and concentration of scorpion venom. Ucar and Tas (2003) reported that the incubation of human plasma and erythrocyte lysates with the scorpion *Mesobuthus gibbosus* venom for 60 min leads to significant inhibitory in activities of AChE. Furthermore, the inhibitory effect of scorpion venom on acetylcholinesterase activity of muscular and nervous tissues of cockroach, 4h after venom application, were investigated previously (Babu *et al*. 1971). Also several studies reported that the insecticides such as organophosphates able to inhibit of ChEs activity and considered as the irreversible inhibitors of ChE activity (Bocquene and Galgani 1998; Ohbayashi *et al*. 1997). Acetyl cholinesterase (AChE,' EC 3.1.1.7) is one of the important regulatory enzyme of the cholinergic nervous system found in eukaryotes which assumes the role of hydrolyzes the neurotransmitter acetylcholine (ACh) and terminates synaptic signals. Reducing the concentration of Ach by its hydrolytic degradation into inactive products choline and acetic acid is an important reaction necessary to regulate the nervous transmission. Several researchers have emphasized that the cholinergic signs and clinical symptoms are closely related to venom concentration, because the low venom concentrations cannot stimulate the acetylcholine receptors. Therefore, the cholinergic signs and clinical symptoms only occur with high venom concentrations (Ozkan and Filazi 2004; Isbister *et al*. 2003). Hence, the discrepancy in the reported results may be related to the various experimental factors. It seems that several aspects have to be considered to perform enzyme activity assays of scorpion venoms using live larvae, such as: different experimental animal species, specific used venoms, exposure time and dosages of venom. The results of our study showed that venom dosages used for 24h not only have inducing effects on AChE activity but also have reversible effects. This may be due to this fact that the venom doses used are not sufficient to inactivate the acetylcholine receptors. The results of the enzyme activity could be verified by data obtained from the larval mortality bioassay (Figure 1).

Histopathologic results

The larval tissues of the *S. nonagrioides* were initially studied for damage caused by *H. saulcyi* venom exposure. However, prior to exposure time, malpighian tubules and midgut of larvae exhibited major alterations in histological degenerations induced by scorpion venom. To prove that the changes detected in tissues structure were not related to the injection procedure itself, the control larvae group was injected with PBS. No degeneration or damage to the malpighian tubules and midgut was observed for larvae injected with PBS. All treated larvae illustrated similar destruction in the midgut epithelial cells (Figure 2, 3 and 4) and malpighian tubules cells (Figure 5) compared to the control group. Therefore, changes in the larval midgut and malpighian tubules were considered as a consequence of scorpion venom intoxication. Figure 2 and 3 represents midgut sections in control animals and animals treated with *H. saulcyi* venom. Microscopic features of midgut epithelial cells in sections of the control larvae appeared normal with pale clear cytoplasm and regular brush border that closely attached to the basal lamina (Figure 2a and Figure 3a). In contrast, after injecting of the 0.5μg doses of venom, the

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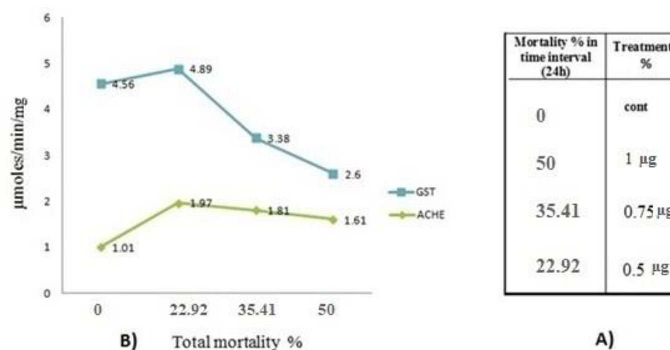
structure of the midgut epithelial cells began to disorganize, which were coupled with cell detachment from the basal lamina and deterioration of the gut epithelium in some sections (Figure 2b and 3b). The apical brush border is no longer continuous, and the remaining microvilli are disrupted and reduced. The observations in optical microscopy analysis, of the midgut of 0.5µg doses of venom treated larvae, showed that a part of the cells was disorganized and lysed (Figure 3b). In other hand, following the injection of the 1µg doses of venom, the epithelial cells of the midgut appear dramatically different from the PBS treated control (Figure 2c). The midgut epithelial cells became progressively more disorganized in appearance and clearly lysed; this was accompanied by the largescale extrusion and sloughing of cell contents and cell debris in to the gut lumen (Figure 2c). The midgut epithelium had completely broken down, prior to the eventual complete deterioration of the gute pithelium (Figure 2c), cell necrosis progressed (Figure 2c and Figure 4a-c), together with cell disintegration, chromatin and nucleoli degradation, their cytoplasmic and nuclear lysis, their local detachment from the basal lamina, and the disappearance of the microvilli (Figure 2c). Histopathologic examination revealed accumulation of pink fluid and inflammatory cells in the tracheal rings and between malpighian tubules of treated larvae (Figure 4). This is in line with the pathological effects of scorpion envenomation reported in humans, rabbits, rats and mice (Khosravi *et al*. 2017; Heidarpour *et al*. 2012; Das *et al*. 2013). According to the results of the present study, Khosravi *et al*. (2017) evaluated the clinical, histopathological and hematological effects of *Mesobuthus eupeus* venom on chicken organ. This finding of pathological examination is indicative of severe pulmonary and cerebral edema, tubular necrosis of the kidneys, hemorrhage in kidneys and heart. The previous studies in different experimental toxicants with various animals showed similar pathological changes (Abutaha *et al*. 2015; Al-Mehmadi and Al-Khalaf 2010). Yu *et al*. (2015) reported that the disruption of midgut function in treated larvae, leading to the death of larvae.



Mortality % in time interval (24h)	Treatment %
0	cont
50	1 µg
35.41	0.75 µg
22.92	0.5 µg

Fig. 1. Effects of *Hottentotta saulcyi* venom on GST and AChE enzymatic activities and larval mortality percentage. Relationship between activities of GST and AChE enzymes and larval mortality percentage. b) Larval mortality percentage in 24h after injection.

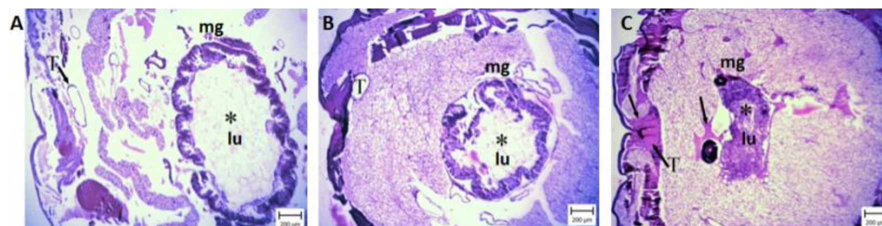


Fig. 2. Midgut cross sections of the *Sesamia nonagrioides* larvae: a) Control group, treated with phosphate buffered saline;

a) Larval group treated with 0.5µg doses of venom; c) Larval group treated with 1µg doses of venom.

b) Abbreviation: (mg) midgut; (Lu) Lumen; (T) tracheal rings; (stars) Effects caused by the treatments.

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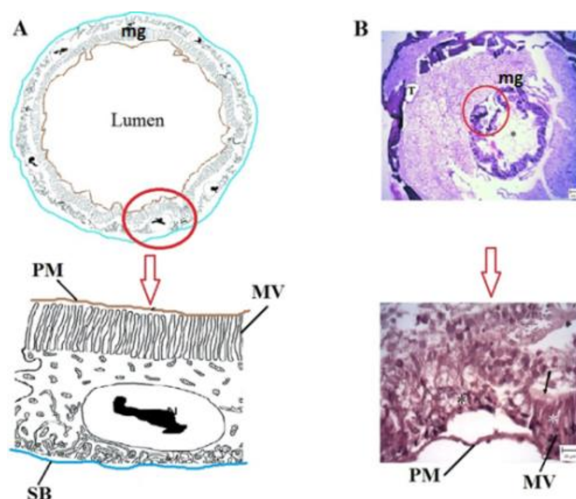

Fig. 3. a) Schematic representation of a cross-section of the region of the normal larval midgut showing the arrangement of peritrophic membrane (PM), microvilli (MV), epithelial cells (black), and serosal barrier (SB). b) Cross sections of the midgut of *Sesamia nonagrioides* larval group treated with 0.5µg doses of venom. Note to detachment of brush borders and necrosis of cells (black star) in comparison with adjacent normal area (white star) (H&E). The coding is the same as in a.

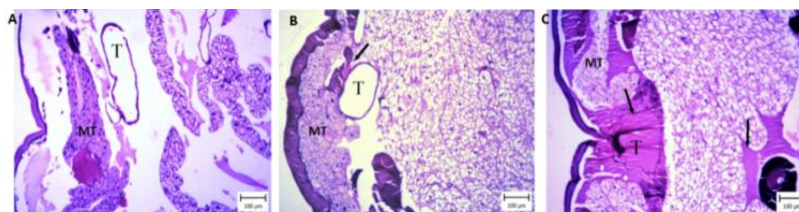

Fig. 4. Photomicrographs of effects of experimental *Hottentotta saulcyi* envenomation on study groups: a) Control, treated with phosphate buffered saline; b) larval group treated with 0.5µg doses of venom; c) larval group treated with 1µg doses of venom. Abbreviation: (MT) malpighian tubules; (T) tracheal rings. Accumulation of pink fluid and inflammatory cells in the tracheal rings and between malpighian tubules (arrows) in 1 and 0.5 µg treated group is noted.

To further demonstrate the pathological effects of scorpion venom in stem borer organs, we decided to monitor the malpighian tubules changes of control and treated larvae. The malpighian tubules epithelial cells of control larvae, which have distinct boundaries cytoplasm, are columnar in shape. Round or oval-shaped nuclei are situated roughly in the center of the cells. Light microscope examination of malpighian tubules from treated *S. nonagrioides* showed variable pathological changes, induced by scorpion venom (Figure 5a-b). Malpighian tubules of treated larvae exhibited necrosis in the proximal tubules, tubular cell edema, cellular infiltration, aggregation of inflammatory cell also cytoplasmic cells appear with numerous vesicles in various sizes. Furthermore, other severe pathological changes that were observed in the malpighian tubules of treated larvae are including; swelling of individual malpighian tubule cells, dilatation of the tubules and degenerations of tubular cells (Figure 5b). The tubular cells degeneration mainly projected into their nuclei, which the most of the cell nuclei of oedematus tubules were in the process of nuclear destruction and were not visible or appeared pyknotic (Figure 5b). Histopathological changes observed in the scorpion venom exposed of *S. nonagrioides* have not yet been reported but histopathological changes investigated in different experimental animals have been reported by the earlier workers (Heidarpour *et al*. 2012 in mice; Pipelzadeh *et al*. 2006 in rat). These findings correlate with the pathological findings of intestine and kidney organs in mice after envenomation by *Hemiscorpius lepturus* venom (Heidarpour *et al*. 2012). Several studies have been focused on the pesticide exposed insect malpighian tubules. Similar to present study narrowing of the lumen of the tubules, necrosis of Microvilli, degeneration of tubular cells in malpighian tubules of poisoned

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insects have been already noticed (Smitha and Vijaya Bhaskara Rao 2012). The findings of pioneer investigations reporting that excreting the toxic component in excretory organs (the malpighian tubules and hind gut considering as excretory organs of insect) often occur following the metabolized of them. Many different enzymes are known to be involved in metabolize of toxin in the insect which their activity occurs in the midgut, fat body and malpighian tubules (Smitha and Vijaya Bhaskara Rao 2012). Previously have been reported that the toxin able to inactivation of metabolizing enzymes (Al-Quraishy *et al*. 2014). Therefore, this lead to failure of detoxification mechanisms and increase the toxin accumulated in excretory organs that could be resulted to swellings, degeneration and necrosis of those tissues. On the other hand, the accumulation of toxic substances in excretory organs might have resulted in their extensive destruction, which can disrupt the detoxification mechanisms. All pathological changes of tissues associated with the progression of larval envenomation, such as swelling of the tubular cells, lead to reduce the size of the lumen, which results to the failure of detoxification mechanisms and cell death (Figure 5b). Smitha and Vijaya Bhaskara Rao (2012) suggested that one of the consequences of pathological degenerations is the intense stimulation of the nervous system which leads to progressive reduction of locomotive activity and ultimately death of the insects; therefore, there is a close relationship between the pathological changes and the progression of the clinical symptoms.

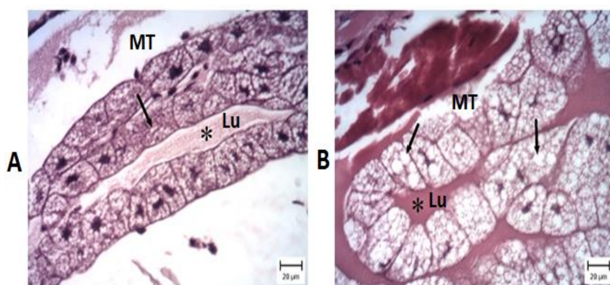

Fig. 5. Cross sections of the malpighian tubules of *S. nonagrioides* larvae: a) Control, treated with phosphate buffered saline; b) larval group treated with 0/5µg doses of venom. Abbreviation: (MT) malpighian tubules; (Lu) Lumen; (stars and arrows) Effects caused by the treatments. Note to the size of cells, size of vacuoles (arrows) and pink fluid in tubules (stars) in experimental groups (H&E).

Conclusion

The agricultural pests can be used as a model insect for scorpion toxicity study this will be future prospects of pest natural control by scorpion venom. Toxicity of scorpion venom for insects was studied by number of researchers but it is considered less concern about agricultural pests. In the present study, the anti-insect property of scorpion *H. saulcyi* venom was measured by toxicity bioassay, while elimination or accumulation of toxic compounds of venom in larvae was determined by enzymes activity assays and histopathologic evaluation. This study confirmed that the *S. nonagrioides* pest is very sensitive to scorpion venom. Mortality percentage recorded following the scorpion venom injection showed to be excellent indicators for stem borer envenomation. Our data emphasize that the used high venom concentration lead to increase the rate of clinical symptoms appearance. Paralysis occurred by venom injection as clinical symptoms was an intermediary state before insect death. The larvae receiving injections of scorpion venom due to suffering from paralysis and immobilization cannot be sticking in to the tunnel throughout the host plant stem and falling outside the stems. There was no change in movement patterns of control group injected by physiological solution were observed. We also showed that a 24h treatment of *S. nonagrioides* with the venoms caused a significant inhibition in the activity of the larval GST enzyme compared to the control group. Injection of *H. saulcyi* venom had considerable toxic effects on *S. nonagrioides* larvae and had specific impact on the larval detoxification enzyme may be due to the inefficiency of these enzymes to elimination of toxic compounds of venom in larvae. Therefore, GST activity in *S. nonagrioides* could be highlighted as a biomarker for larval envenomation. Considering that the whole larvae injected by high venom concentration were dead within 48 h, thus, these doses of scorpion venom is sufficient to pest mortality. The results obtained in this study can be verified by histopathological observations. The present histopathological observation of the larvae treated with venom of *H. saulcyi* revealed a similar manifestation trend of toxicity and paralysis. Therefore, this study showed a close relationship between the occurrence of external symptoms of

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envenomation and histopathological changes. Taken together the scorpion toxins can be considered as an effective component to develop the insecticides because this venom includes neurotoxins that delivered and rapidly spread to the circulatory system of the prey as part of the whole scorpion venom then reach their molecular targets.

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