Effects of Azadirachtin on Embryological Development of the Desert Locust Schistocerca gregaria Forskål (Orthoptera: Acrididae)

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Effects of azadirachtin on embryological development of the desert locust Schistocerca gregaria Forskål (Orthoptera: Acrididae)

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Abstract

Azadirachtin prolonged the incubation period and reduced hatchability of desert locust eggs. Many embryos suffered morphological deformation of the compound eyes and legs and incomplete development of abdominal segments. Histologically, the brain was poorly developed, this being reflected in the compound eyes, neurosecretory cells and entire ventral nerve cord. The embryonic cuticle was insufficiently deposited. The electrophoretic mobilities of the egg proteins after the addition of 200 ppm of azadirachtin were characterized by SDS-PAGE analysis and revealed the appearance of new protein bands and the disappearance of others. We conclude that after suitable field trials, the use of azadirachtin may be a viable alternative to chemical insecticides for control of grasshoppers in Egypt.

Key words

grasshoppers, azadirachtin, deformation, histogenesis

Introduction

The search for naturally occurring compounds as alternative means of pest control was renewed (Perkins 1985). More recently, the use of plant-derived chemicals such as azadirachtin, which is widely used in experimental insect control and insect endocrinology during postembryonic and adult stages, has been emphasized. However, findings about its effect on embryonic development are to date limited (Mordue & Blackwell 1993, Simmonds *et al.* 2004). Azadirachtin affects egg growth (vitellogenesis) in some insect species (Ghazawi *et al.* 2007). Rate of vitellogenesis depends primarily on concentrations of circulating free juvenile hormone controlled essentially by the brain. A malformed brain should signficantly affect the development of an insect.

The aim of this research was to clarify the action of azadirachtin, as a natural alternative to chemical insecticides, on the development of *S. gregaria* embryos manifest in external morphogenesis and histological deformities occurring inside the eggs. This study also extended to investigating the possible changes that may occur in egg proteins, the principal components of different organs.

Materials and methods

Schistocerca gregaria were raised, derived from a colony maintained in Cairo University for several years. Insects were fed on clover (*Trifolium alexandrinum*) from October till June, then on maize (*Zea maize*). They were reared in electrically heated cages (60 × 60 × 40 cm) at a constant temperature of 30±1°C, uncontrolled relative humidity 60-80% and 14 h light. For oviposition, insects were provided with pots filled with sieved, washed and sterilized sand that was kept constantly wet.

For experimental purposes, egg-pods were removed daily from these pots; the eggs were separated from the sand with a fine brush, put on a wet cotton piece in Petri dishes, and incubated at 30±1°C.

To study the effect of azadirachtin on *S. gregaria* eggs, serial concentrations (100, 200, 300, 400 and 500 *ppm*) were each applied directly onto 50 eggs for each concentration. Five eggs from each of 10 egg pods were taken to comprise each treatment group of 50 eggs. These pods were taken from 10 different females (Abdel Rahman 2004). Eggs were immersed in the assigned concentration in a Petri dish for 1 to 2 s and then removed to another Petri dish containing a piece of wet cotton and incubated at 30±1°C. This procedure was replicated three times.

The chosen embryonic stages were: eggs at 0 d old; 1 d old (*i.e.*, about the time the blastoderm is formed); 8 d old [*i.e,* blastokinesis is established (Jones 1964)]. We recorded total incubation period, percentage of hatchability, and percentage of deformed embryos. Control eggs were treated with acetone solution for 1 to 2 s. To recognize the effect of azadirachtin on embryonic development, control and treated eggs were dechorionated in 5% hypochlorite solution for few seconds and then washed with distilled water and examined (Slifer 1945). Per cent development (% D) is determined as 100 (mean incubation period of egg stage/total mean egg incubation period).

Histological preparation took place by fixing eggs in Bouin's fluid; eggs were washed in 80% alcohol and then dehydrated in increasing alcoholic concentrations to 100%, cleared in xylene and infiltrated in paraffin wax of melting point of 58°C. Serial sections were cut at 6μ thick and stained with haematoxylin and eosin (Awad 2008). The stained samples were examined for histopathological changes using a light microscope.

Statistical analysis.— Data for the incubation period were analysed by *t*-test, using the free software "Grapfpad" at http://www.graphpad.com/quickcalcs/ttest1.cfm. Mortality was corrected using Abbott's formula (1925) and curves made using Probit analysis by Finny (1971). To facilitate such calculations, data were run on a simple program developed by Abdel Rahman (2001) using Microsoft Excel©. Malformations, morphologically and histopathologically, were analysed using a correlation test by Microsoft Excel©.

SDS/PAGE of egg proteins under reducing conditions.—Electrophoresis on SDS/PAGE slab gels was carried out according to Laemmli (1970). Proteins were denatured with 2% SDS and 5% 2-mercaptoethanol by heating at 75°C for 15 min and loaded on SDS slab gels containing 10% acrylamide. Three replicates of each treated sample were centrifuged at 10,000 r.p.m. for 5 min, before being loaded on the gels. Electrophoresis was carried out at a constant voltage of 2 mA/

Table 1. Effects of different concentrations (ppm) of azadirachtin on the mean incubation period ($\bar{x}$ ±SD) of *S. gregaria* eggs incubated at 30°C.

	Dose (ppm)																	
day	Control	100			200			300			400			500		
	$\bar{x}$ ±SD	$\bar{x}$ ±SD	*t*-value	*df*	$\bar{x}$ ±SD	*t*-value	*df*	$\bar{x}$ ±SD	*t*-value	*df*	$\bar{x}$ ±SD	*t*-value	*df*	$\bar{x}$ ±SD	*t*-value	*df*
0	16.2±1.28[a]	16.38±1.56[a]	0.4011	49	17.23±0.83[a]	2.6831	49	17.14±1.35[a]	1.7619	43	18.25±1.26[b]	3.042	40	18.33±1.53[b]	2.742	39
1		17.63±1.41[a]	3.6064	52	16.93±1.73[a]	1.6302	50	17.57±1.13[a]	2.6305	43	18.2±0.84[b]	3.371	41	-*		
8		16.41±1.74[a]	0.542	68	16.55±1.64[a]	0.9335	56	16.84±1.54[a]	1.7412	56	16.55±1.63[a]	0.719	47	19±1.41[b]	5.229	43
	% hatch															
0	76	30			24			14			8			6		
1		32			26			14			10			0		
8		64			40			38			22			14		
	% deformed															
0	0	10			16			20			30			46		
1		12			18			22			30			48		
8		0			0			0			0			0		

Number used: 50 eggs, * no. hatching took place, values followed by different letter are significantly different ($p \leq 0.05$)

sample for about 90 min (the voltage should start at about 70 to 80 V, but increased during the run). After electrophoresis, gels were stained with Coomassie brilliant blue R-250. For determination of molecular weights, the gel was calibrated with the following marker proteins: 116, 97.4, 66.2, 37.6, 28.5, and 18.4 kDa (Sigma Chemical Company, St. Louis, USA) and the quantification analyses were made using an Image densitometer G 700 (Bio-Rad, USA).

Results

I. Biological parameters of azadirachtin against S. gregaria *embryos.*—Azadirachtin (Table 1) had an insecticidal effect on *S. gregaria* embryos, with a statistically significant increase in the incubation period apparent in embryos treated with 400 and 500 ppm azadirachtin ($p \leq 0.05$). The values recorded were 18.25±1.26 d and 18.33±1.35 d at 0 and 1 d respectively, when compared to the control value 16.2±1.28. Mortality of the embryos showed a dose-dependent manner. Eggs treated either at 0 or 1 d (Table 2, Fig. 1) showed interference within 95% confidence limits, while eggs treated at 8 d did not; *i.e.*, the value of the LC_{50} for the latter group is significantly different ($p \leq 0.05$). The percentage of hatching decreased gradually, reaching 8 and 10% after 400 ppm azadirachtin at 0 and 1 d, respectively, with 30% malformed embryos. When the embryos were treated with 500 ppm azadirachtin at 0 and 8 d, the incubation periods were 18.33±1.53 and 191.41 days compared to the control value 16.2±1.28. No hatching took place for eggs treated at 1 d.

II. Morphological deformation of embryos.—The effects of azadirachtin were manifest as morphological deformations. Embryos showed dose-dependent deformities when eggs were treated in earlier stages. When treated just after blastokinesis however, there was no malformation in embryos and hatchability was significantly higher than in treated earlier stages ($p \leq 0.05$) (Table 1). Deformities recorded were asymmetric development of the whole embryo (Fig. 2), asymmetric development of the compound eyes and appendages, as well as incomplete formation of abdominal segments.

III. Histopathological Studies.—Azadirachtin affected the brain drastically. The optic lobe of the brain was imperfectly developed and hence the compound eye was poorly developed (Fig. 3). Moreover, the eye was asymmetrically developed (Fig. 2). The ommatidia were entirely damaged.

The neurons of the brain as well as the neurosecretory cells were widely spaced and impaired in structure and contained no neurosecretory materials, as compared with normal brain (Fig. 4).

The corpora allata (CA) arise in early embryonic development (40%D) from intersegmental invaginations anterior to the maxillary segment (Gassier 1998). Azadirachtin caused poorly developed CA as seen in Figure 5. The embryonic cuticle of the treated embryos was

Table 2. Mortality rates caused by azadirachtin on embryos of *S. gregaria* treated with azadirachtin at different intervals.

days	LC_{50} (ppm)	X^2	Slop ± SE	95% confidence limits
0	79.02	3.33	1.64 ± 0.106	60.40 - 103.38
1	95.33	4.35	1.98 ± 0.119	78.47 - 115.80
8	243.96*	5.27	2.54 ± 0.061	225.76 - 263.62

* significant $p \leq 0.05$

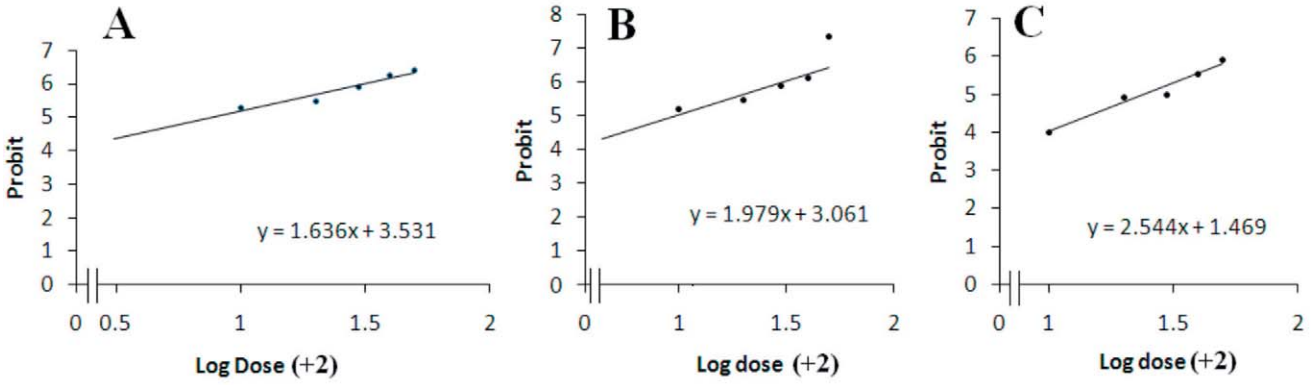

Fig. 1. Regression lines representing the mortality rate of *S. gregaria* embryos treated at 0, 1 and 8 d (A, B, and C respectively) with serial concentrations of azadirachtin.

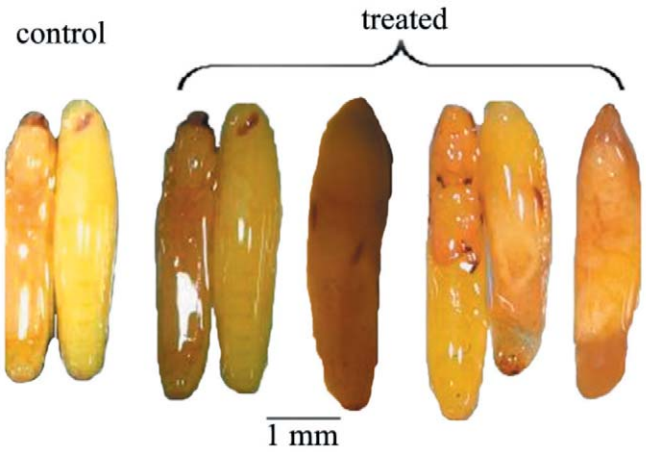

Fig. 2. Effect of azadirachtin on morphology changes of *S. gregaria* embryos (90%D). For color version, see Plate VIII.

insufficiently deposited (Fig. 6). Figure 7 summarizes percentages of the embryos suffering such malformations, showing how these increased depending on the dose ($p \leq 0.05$, n=5). The malformations are correlated with each other, ($R^2 = 0.932$, df = 4).

IV. Electrophoretic pattern of egg proteins.—The electrophoretic mobility of egg proteins from *S. gregaria* treated with 200, 300, and 400 ppm azadirachtin, revealed the appearance of new protein bands and the disappearance of others in comparison to the control group. With SDS polyacrylamide gel electrophoresis (PAGE), the proteins of control eggs were shown in 10 bands ranging from 126.69 to 17.435 KDa by using Coomassie brilliant blue (COBB) stain (Table 2, Fig. 6). Total bands in the eggs treated with 200, 300, and 400 ppm azadirachtin were 7, 7, and 8 protein bands, respectively. The protein bands of 126.69, 108.45, 71.45, and 23.76 KDa disappeared in egg proteins treated with 200, 300, and 400 ppm azadirachtin. New proteins of 45.51, 37.60, 32.86, and 29.45 KDa appeared in embryos treated with 200 ppm azadirachtin, new proteins 69.76, 46.49, 32.74, 29.45, 25.76, and 19.75 KDa in embryos treated with 300 ppm azadirachtin, and new proteins 74.22, 33.46, and 19.95 KDa in embryos treated with 400 ppm azadirachtin.

Discussion

The present study revealed that azadirachtin had drastic effects on the development of *S. gregaria* embryos. Our results suggest that the use of azadirachtin disrupted the hormonal balance in the eggs treated with different concentrations of azadirachtin, resulting gradually in a decreased percentage of hatching after 400 ppm azadirachtin at 0 and 1 d, respectively. The destruction of the brain led to the malformation of the neuroendocrine cells secreting prothoracicotropic hormone. This hormone activates the corpora allata and the prothoracic glands responsible for regulating growth (Wigglesworth 1972). This led to the malformations that took place in the present study.

Such results were also obtained by Ghazawy *et al.* (2008). Gelbic *et al.* (2006) mention that the use of metyrapone, which has an action similar to that of juvenoids on *S. littoralis*, also inhibited the synthesis of vitellogenins (*i.e.*, proteins which are precursors of vitellin) or their transport with a consequent decrease in hatchability. This fact is in congruence with the suggestion made above that azadirachtin inhibits the action of juvenile hormone. Juvenile hormone is necessary for the synthesis of vitellogenins. Also, the disrupted hormonal balance in embryos treated with different concentrations of azadirachtin, resulted in the production of malformed embryos which mostly failed to give rise to normal embryos. Vennard *et al.* (1998) studied the effect of juvabione on the embryonic course of *S. gregaria*, and reported that the development was blocked during blastokinesis. This was associated with several external and internal morphogenesis aberrations leading to prevention of hatching. Histopathological changes showed a parallel sequence of morphogenic aberrations and developmental disruptions. This impact varied according to the time of treatment.

The data of the present study also show the possible changes that may occur in the chemical composition of the egg proteins of *S. gregaria* treated with, 200, 300, and 400 ppm azadirachtin. There seemed to be a complex interaction between the malformed embryos and protein banding patterns within the eggs, leading to the appearance of new proteins or disappearance of normal proteins. The disappearance of the protein bands may be attributed to the presence of toxins, which could increase protease activity leading to protein hydrolysis. It may be suggested from the present study, that the decrease of protein contents of the eggs treated with azadirachtin caused growth inhibition of the embryos, also leading to prolongation of the incubation period of eggs treated with different

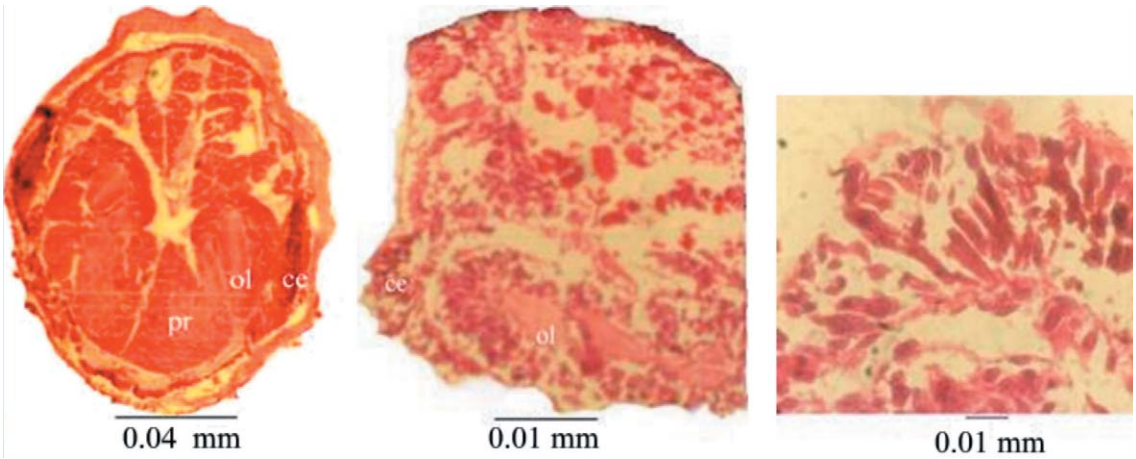

Fig. 3. Transverse sections in the head of a 90% D embryo showing the effect of azadirachtin on brain and compound eye formation. Left control, middle treated, right a magnified portion of the compound eye. ce, compound eye; pr, protocerebrum; ol, optic lobe. For color version, see Plate VIII.

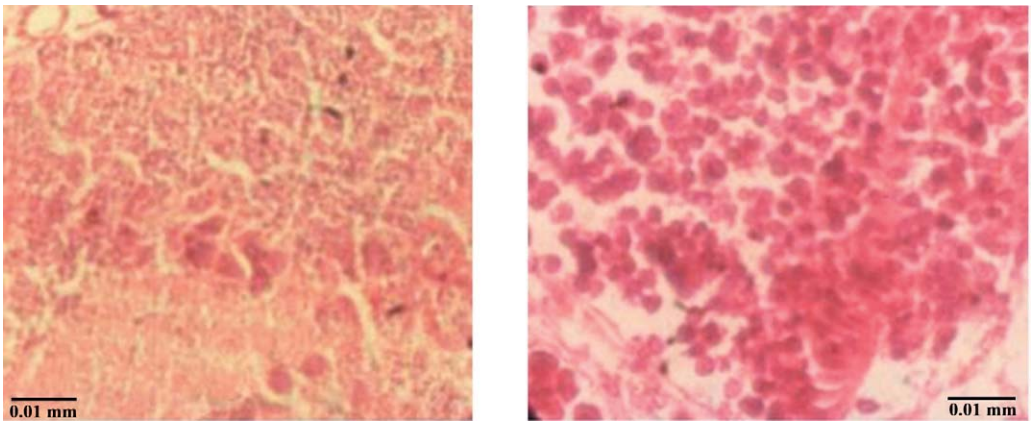

Fig. 4. A magnified portion of the brain at 90%D showing developed neurosecretory cells (left) and loose cells in the treated brain (right). For color version, see Plate VIII.

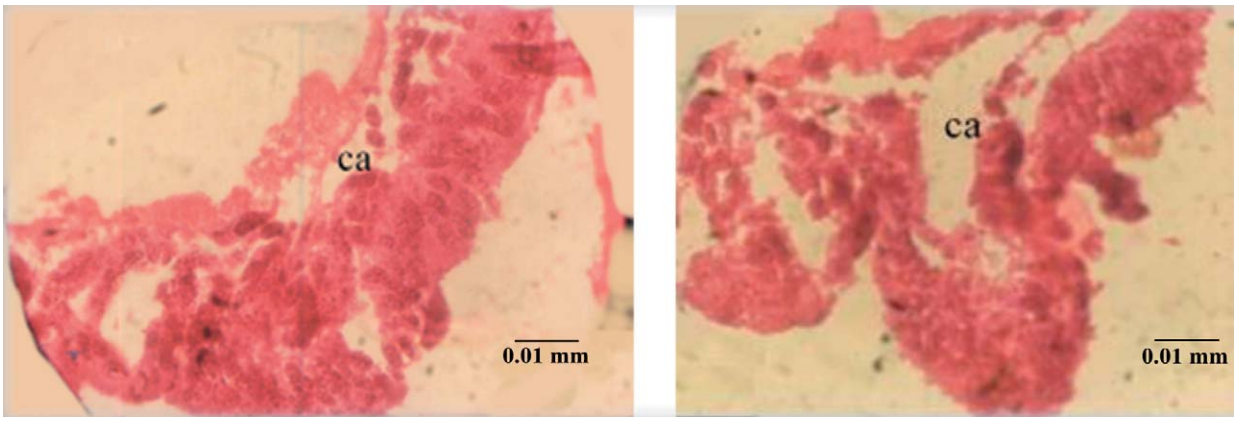

Fig. 5. Development of the corpora allata at 40%D: control (left) treated (right). For color version, see Plate VIII.

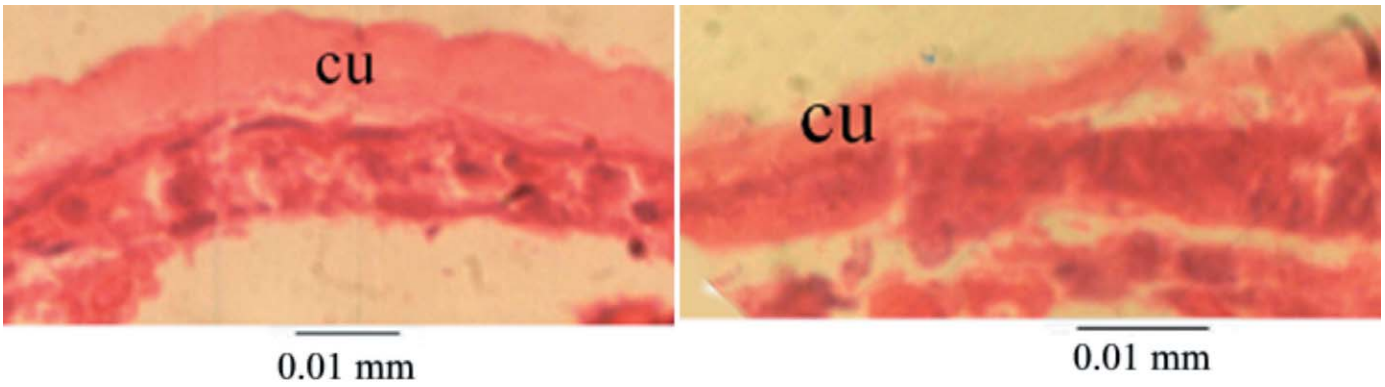

Fig. 6. Deposition of the cuticle in control (left) and treated embryo at 90%D. For color version, see Plate VIII.

concentrations of azadirachtin.

We recommend that in future this study be extended in an attempt to explain the external morphogenic aberrations, histological deformities and possible changes that may be occurring in egg proteins. Using newly available molecular biological techniques could help give insight to the role of egg hormones in modifying normal gene activity, hence revealing the mechanisms of action of normal hormones during insect embryogenesis.

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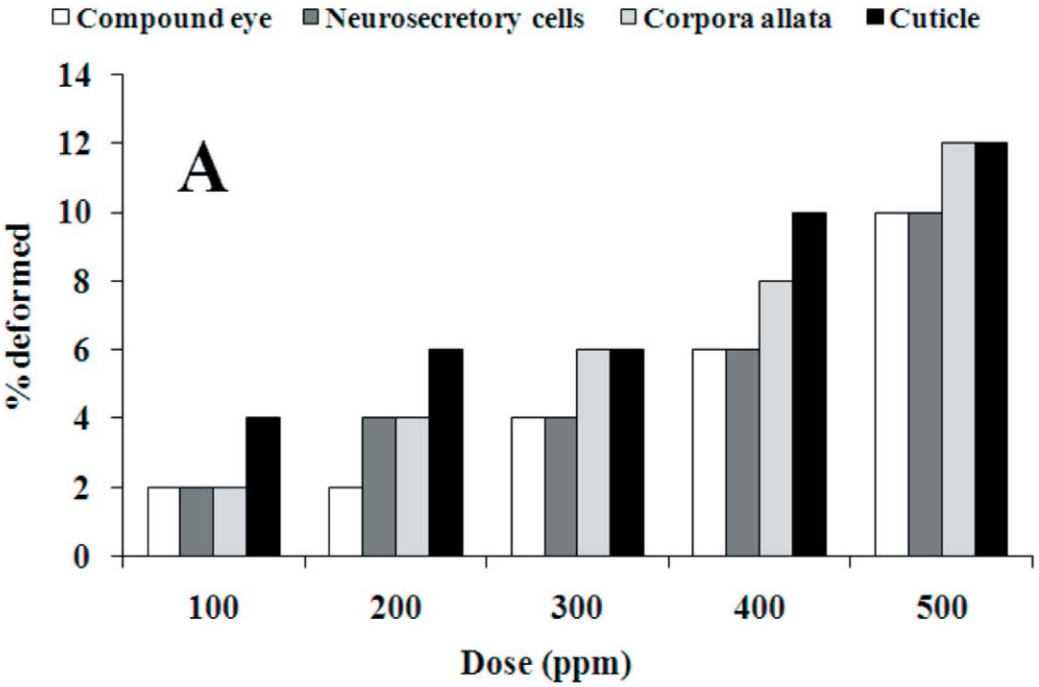

Fig. 7a. Per cent deformed of selected organs of *S. gregaria* embryos O-days old.

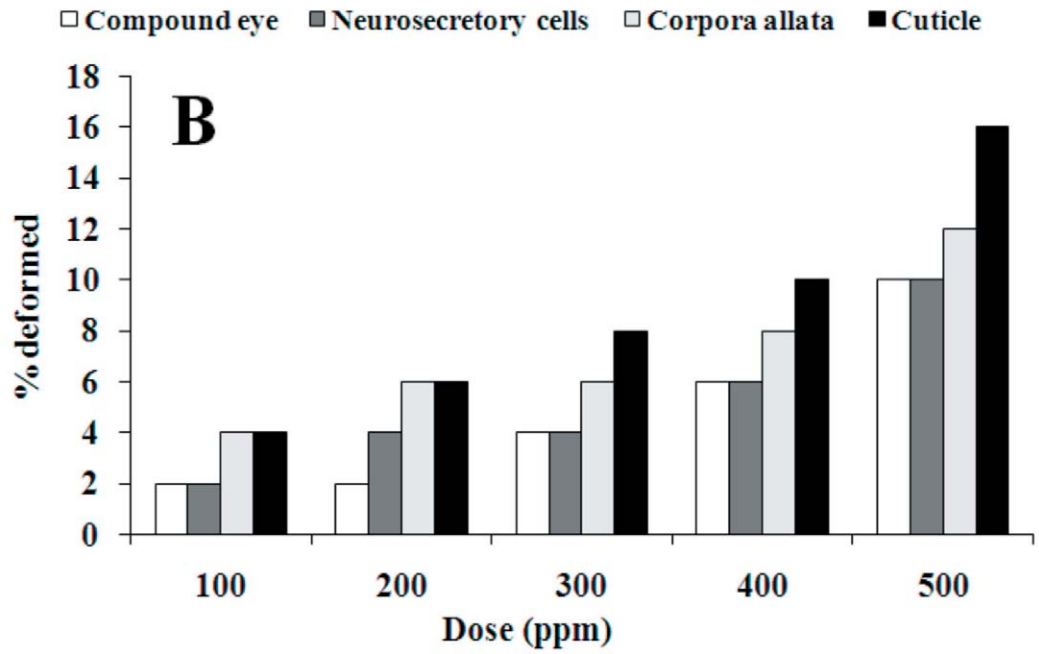

Fig. 7b. Percent deformed of selected organs of *S. gregaria* embryos 1-day old.

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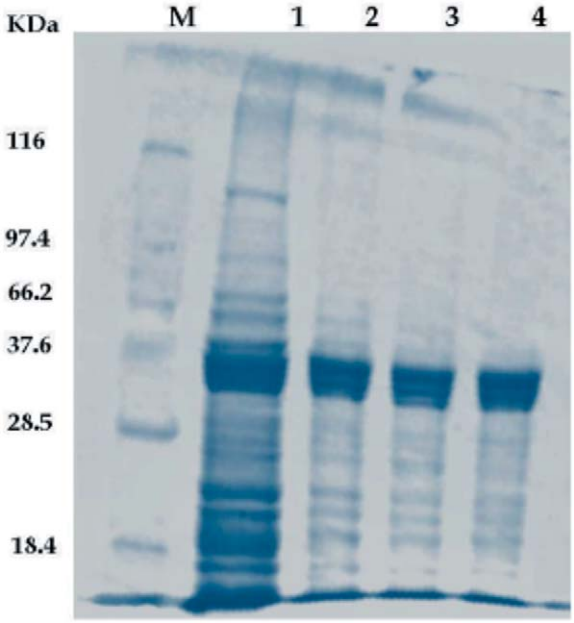

Fig. 8. SDS-PAGE under reducing conditions of *S. gregaria* eggs treated with azadirachtin. Lane M, Standard molecular weight marker (Sigma); Lane 1, control eggs homogenate (1 mg); Lane 2, Lane 3, Lane 4, treated with 200, 300, 400 ppm (1 mg) respectively.

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