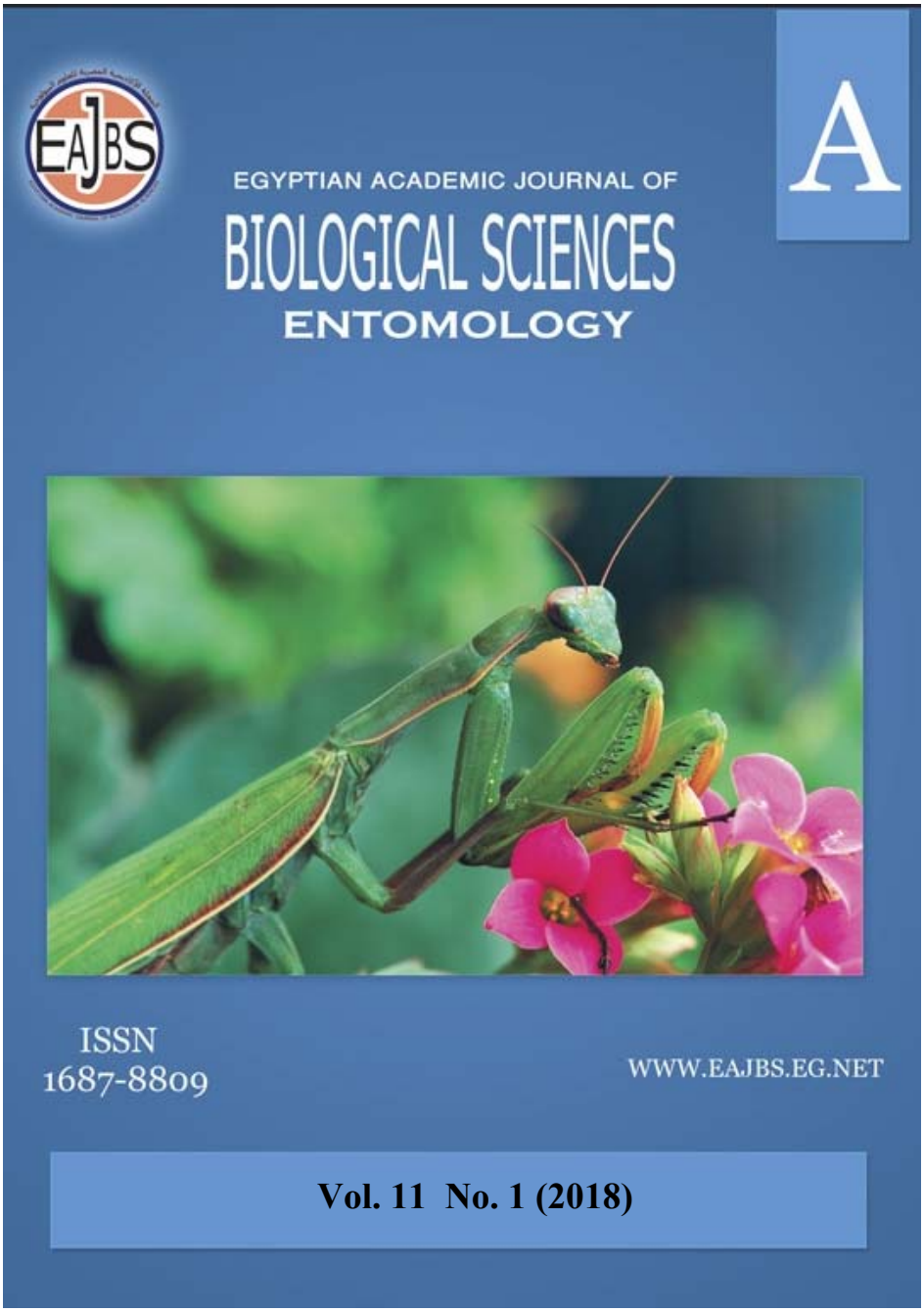

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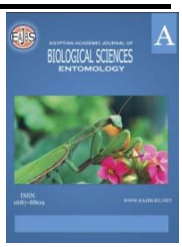

Development Rate and Ultrastructure Changes of Puparia of *Megaselia scalaris* (Loew) (Diptera: Phoridae) Induced by Azadirachtin.

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ABSTRACT

The effect of azadirachtin on the development and mortality of the scuttle fly, *Megaselia scalaris* (Loew) (Diptera: Phoridae) was investigated in the laboratory by using neem extract, Achook®, containing 0.15% azadirachtin. Different biological changes were recorded after treatment 1st larval instar with different concentrations of azadirachtin as larval mortality, pupation rate and adult emergence of. Azadirachtin-LC_{50} against the 1st instar larvae of *M. scalaris* was 13.79 mg/L. Exposing larvae to LC_{50} of azadirachtin caused many changes in the puparia such as having folded cuticle and swelling of the integument compared to control. Distortion of the anterior spiracle and papillae found on respiratory horns were also observed. The present study suggests that azadirachtin hampers the development rate of *M. scalaris* by reducing the insect population and causing several scanning electron microscopic changes in the puparia of the insect.

INTRODUCTION

Megaselia scalaris (Loew), scuttle fly or humpbacked fly, is a cosmopolitan insect with forensically importance (Disney 1994, Campobasso *et al*. 2004, Reibe and Madea 2010). Also, it has a medical importance as a cause humans myiasis (Hira *et al*. 2004, Mazayad and Rifaat 2005, Wakid 2008, Francesconi and Lupi 2012); vector of pathogens (Disney 2008) and contaminant of food products (Nickolls and Disney 2001, Brown and Oliver 2007). The insect is a polyphagous species and may be harmful to human health (Ghavami and Djalilvand 2014). These flies can explore a large variety of environmental and ecological niches (Disney 2008). Predation and parasitation of some arthropods by this species were also recorded (Zwart *et al*. 2005, Costa *et al*. 2007, Koch *et al*. 2013).

In recent years, due to toxicological concerns associated with the use of synthetic chemical insecticides in integrated pest management and increasing awareness about health protection, there has been an increased interest in the use of natural products (Stevensona *et al*. 2017, Kamaraj *et al*. 2018). Botanical insecticides may serve as alternatives to synthetic chemicals to develop safer control agents of *M.*

scalaris, as shown by some precedent studies (Mello *et al*. 2010).
Azadirachtin is a bio-insesticide, based on natural compound, excreted from *Azadirachta indica* and among the best-known substances tested for insect control (Duke *et al*. 2010, Abedi *et al*. 2014, Kamaraj *et al*. 2018, Devatha *et al*. 2018). It is used as contact insecticides, repellents, anti-fedant agent and responsible for the alteration of several biological parameters (growth rate, longevity, and reproduction) of different fly species harmful to humans and animals (Miller and Chamberlain 1989, Khan *et al*. 2007, Erler 2009, Boulahbel *et al*. 2015). Applying these biopesticides can be a viable alternative to the use of synthetic products that can be harmful toward the environment.

The objective of this study was to determine the efficacy, through laboratory bioassays of azadirachtin, against the first larval instar of *M. scalaris*. The effect of azadirachtin on the development rate and the pupal ultrastructure induced by treatment larvae with LC_{50} were recorded.

MATERIALS AND METHODS

Rearing Insects:

Larvae of *M. scalaris* were obtained from stock culture reared in Zoology laboratory, Department of Biological and Geological Sciences, Faculty of Education, Ain Shams University, Egypt. Larvae were reared on the modified artificial diet (Harrison and Cooper 2003) containing cornmeal (10 g), agar (1.5 g), dextrose (13.5 g), and yeast (0.7 g), dissolved in 74.3 ml of boiling distilled water. Adults of *M. scalaris* were fed with 10% sugar solution (10% sucrose) and cotton pads soaked in milk powder dissolved in water (10% w/v) provided to lay eggs.

Insecticides and Treatment:

The commercial product Achook® (0.15% EC Azadirachtin, Egyptian Agricultural development Co. Egypt) was used as the source of azadirachtin. Bioassay method was conducted using the dipping method. Six concentrations of azadirachtin were prepared as follows: 10, 50, 100, 1000, 5000 and 6666 µl of 0.15% azadirachtin was diluted in 100 ml distilled water to give final concentrations of azadirachtin 0.15, 0.75, 1.5, 15, 75 and 100 mg/L. Larval treatments were carried out by exposing the 1st instar larvae to various concentrations of azadirachtin. Four replicates of 20 larvae each per concentration and so for the control trials were set up. Larvae were gently dipped into treatment solutions for 30 sec with a dip net. Control group was obtained by dipping larvae in distilled water. Then the larvae were transferred to the rearing larval medium in glass beakers. Each beaker was covered with muslin cloth and Larvae were examined every 24 h for a total of 2 weeks. Bioassay was conducted at 27°C in 70±5% relative humidity, with a 12:12 h photoperiod. Larval mortality, larval duration, percent pupation, pupal weight and adult emergence were recorded hereafter. The percentage of larval mortality, pupation, pupal mortality and adult emergence were estimated using the equations:

Larval mortality % = number of dead larvae / number of tested larvae × 100 (1).
Pupation % = number of pupae formed / number of tested larvae × 100 (2).
pupal mortality % = [number of produced pupae – number of observed adults] / number of produced pupae × 100. (3).
Adult emergence % = number of emerged adults / number of tested larvae × 100 (4).

Pupal duration was calculated as the interval between the commencement of pupation and the commencement of adult emergence. All values calculated for each one and then the mean value was taken.

For the larvicidal activity of azadirachtin against *M. scalaris* larvae, Abbott's formula (Abbott 1925) was applied to correct the percentage of mortality. The log concentration–probability regression line, the lethal concentration values, and the corresponding 95% fiducial limits of the upper and lower confidence limits were determined using Probit regression (Finney 1971) by using SPSS 20.0. *M. scalaris* larvae were exposed to LC_{50} of azadirachtin to study the effect of this concentration on the pupal stage. Morphological malformation symptoms (any abnormal change in colour, shape, size or failure to develop to adult stage) were photographed and compared with those of the control assays.

Scanning Electron Microscopy (SEM) Study:

Three days old pupa resulted from normal and LC_{50}-treated 1st larvae were fixed in 2.5% glutaraldehyde for 24 h, then dehydrated in a graded ethanol series (70%-100%) and in 100% ethanol-acetone (1:1) solution, followed by four washes in 100 % acetone. After drying, the specimens were pasted to stubs and sputter coated with gold using Edwards sputter coater unit S 150 B (BOC Edwards, UK). Ultra-structural features of puparia surfaces were examined and photographed using different magnifications by JEOL- JSM T100 Model Scanning Electron Microscope (Tokyo, Japan), at the Research Park of Faculty of Agriculture, Cairo University, Giza, Egypt.

Data Analysis:

Results were expressed as mean ± standard deviations (SD). The statistical significance of differences between means was determined with SPSS 20.0 using Student's *t-test*. Data from four replicates of each tested concentration were analyzed, and $p<0.05$ was considered to be a statistically significant difference.

RESULTS

Data are given in (Table 1) indicated the biological activity of azadirachtin against the 1st instar larvae of *M. scalaris* the larval mortality % was concentration-dependent. The larval duration was significantly ($P<0.05$) affected by all concentrations used except the lowest concentration (0.15mg/L). The pupation % of the treated larvae was decreased as the concentration increased. The pupation % was 25 at the highest concentration 100mg/L and 81.25 at the lowest concentration 0.15mg/L. A toxic effect on the pupae resulted from treated larvae was observed. At 100 mg/L azadirachtin, pupae were deformed, and there was a total inhibition of adult ecdysis in those individuals that did pupate. The pupal duration was significantly prolonged at 1.5, 15 and 75 mg/L concentrations used compared with control. Also, the pupal weight was significantly ($P<0.05$) decreased at all the concentrations used when compared with the control. A remarkable reduction in the adult emergence % was also observed. The growth index was greatly affected by azadirachtin where it recorded 1.5, 3.9 and 5.1 at the concentrations 75, 15 and 1.5 mg/L respectively, compared to 10.9 for the control group.

The data obtained from the susceptibility test of larvae to azadirachtin are shown in Table 2. The estimated value of LC_{50} was 13.79 mg/l with 95% confidence limits (7.73 - 27.29 mg /L). Chi-square analyses indicated that there was a significant difference in survival of azadirachtin-treated insects ($\chi^2 = 5.45$; df= 4; P= 0.000) from that of the control.

The result presented in Figure 1. clearly indicated that LC_{50}-azadirachtin induced various degrees of developmental aberrations in pupae transformed from azadirachtin treated larvae. The most characteristic effect resulted from treatment

was the decrease in the size of the resulted pupa. Water treated group (control) showed normal pupal structure (Fig. 1 a) whereas, most of the pupae died as larval-pupal intermediates (Fig. 1 b), others are died as tapering anterior and constricted (Fig. 1 c), yellowish-albino coloured (Fig. 1 d), and broad posterior with curvature (Fig. 1 d). The pupa emerging from larvae treated with LC_{50} of azadirachtin failed to ecdysis as shown in Figure 2.

Table 1: Bioactivity of *M. scalaris* after dipping 1st larval instar with different concentrations of azadirachtin.

Azadirachtin (mg/L)	Larva mortality (%)	Larval duration (days) ± SD	Pupation (%)	Pupal mortality (%)	Pupal duration (days) ± SD	Pupal weight (mg)	Adult emergence % (a)	Developmental period (b)	Growth index (a/b)
100	75	4.70 ± 0.7**	25	100	0.00		0.00		
75	57.5	4.03 ± 0.6**	42.5	56.2	8.13 ± 0.9 **	8.62 ± 1.6**	18.75	12.16 ±1.5	1.5
15	45	3.91± 0.8**	55	29.06	7.77 ± 0.9 **	10.11 ±1.8**	45	11.68 ±1.7	3.9
1.5	33.75	4.11± 0.7**	66.25	22.8	5.93± 0.9 **	11.51±1.8**	51.25	10.04 ±2.5	5.1
0.75	27.5	3.50 ± 0.6 **	72.5	22.3	5.29 ± 0.8 ns	12.40 ± 1.4**	56.25	8.79 ±1.4	6.4
0.15	18.75	3.40 ± 0.6 ns	81.25	7.5	5.18 ± 0.7 ns	15.23 ± 2.0**	76.25	8.58 ±1.38	8.8
0.00	6.25	3.17± 0.5	93.75	4.02	5.08 ± 0.8	18.15 ± 1.1	90	8.25 ± 1.34	10.9

Significance level: n.s. (P>0.05), ** (P<0.01) as compared with control

Table 2: Probit analysis of azadirachtin treated 1st larval instar of *M. scalaris*.

Treatment	No. insects	LC_{50} (mg/L)	95% Fiducial limits (mg/L)	Regression equation	$\chi 2$	df
			Lower - Upper			
Azadirachtin	80	13.79	(7.73-27.29)	Y= 0.471*x + 0.059	5.45	4

Mortality was calculated from the difference between the numbers of tested larvae and those of resulted pupae.

SEM micrograph of the puparia of *M. scalaris* showed that the surface of the puparia, which had been molted from larvae dipped in distilled water, appeared normal, having a smooth integument covered by spinose setae, which had pointed ends, while the dorsal and lateral surfaces between each body segment bear short tubercles (Figs. 3 a and b), with the apex bearing minute spines (Fig. 3 c). Distinct rounded pair of anterior spiracles, located dorsolaterally on the prothorax, including two spiracular slits (Fig. 3 d). Just beneath the anterior spiracle, the intersegmental spines between the pro- and mesothorax were single pointed and arranged in five rows (Fig. 3 e). A pair of pupal respiratory horns, extruding through the posterolateral walls on 1st abdominal segment. These respiratory horns are long and slender, with their apex being slightly curved (Fig. 3 f). The surface of the horns bears numerous spirally arranged papillae from its base through the apex (Fig. 3 g). These papillae were shown at higher magnification with oval, domed-shaped, located on the convex base and has the single longitudinal straight opening (Fig.3 h).

In contrast, treatment with LC_{50} of azadirachtin during the 1st larval stage showed a lot of morphological changes on the puparial surface. These changes included, cuticle with loss of spines at the ventral surface of the thoracic region (Fig. 3 i), Also, the short tubercle on the cuticular surface showed swollen and loss of spines while many breaches occurred in some surface areas deformed and wrinkled cuticular surface and shrinkage cuticle (Fig. 3 j) The anterior spiracles were coated with thick residuum (Fig. 3 k), with distortion of the original features. Degeneration and corrugation of intersegmental spines rows between the pro- and mesothorax. (Fig. 4 l), the respiratory horns are covered with papillae (Fig. 3 m). These papillae are shown at higher magnification in Fig. 3n, a number of irregular swellings or blebs were present over the papillae.

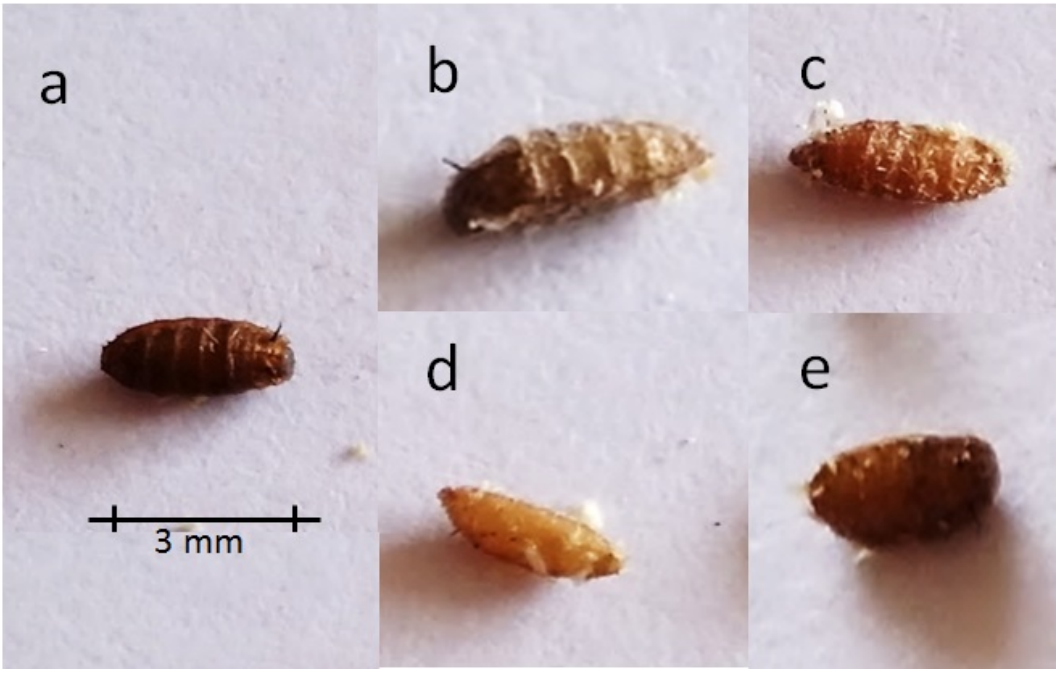

Fig. 1. Normal pupae of *M. scalaris* (a) compared to treated ones emerging from treated 1st larvae with LC_{50} of azadirachtin, (b-e); (b) larval- pupal intermediate, (c) constricted, (d) yellowish-albino coloured and (e) oval and broad posterior pupa.

Fig. 2. Photomicrographs of *M. scalaris* pupal stage, showing the pupal stage at higher magnification, Abnormal pupae of *M. scalaris* emerging from larvae treated with LC_{50} of azadirachtin (a) compared to untreated pupa (b), arrow shows the ecdysis position in treated pupa(c) and in control pupa (d), (scale bar is 3 mm).

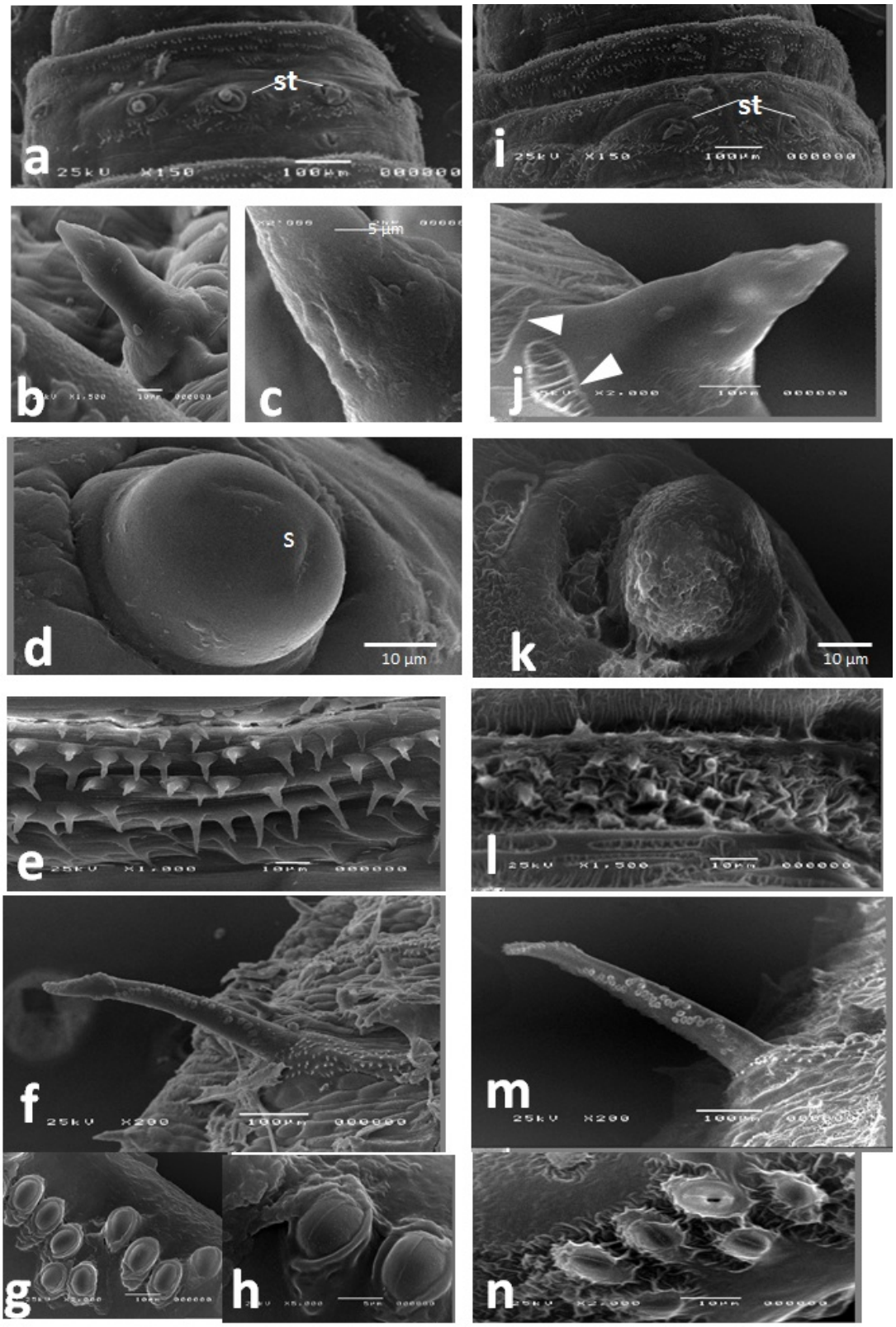

FIG. 3 (a-n) Scanning electron micrographs showing tegumental surface of pupa of *M. scalaris* (a-h), Untreated pupa and (i-n) pupa after treatment with azadirachtin (st, short tubercle; s, spiracle slits; arrow head, breaches in cuticle).

DISCUSSION

The successful control of an increasing number of insect species depends on substances inhibiting the developmental process of those insects (Kristensen and Jespersen 2003). From this point of view, the effects of six concentrations of azadirachtin on some biological aspects of the *M. scalaris* treated larvae (1st instar) and their subsequent developmental stages are observed. In the present investigation, the results showed that, azadirachtin have toxicity against the 1st instar larvae of *M. scalaris*. azadirachtin increased larval mortality and inhibited ecdysis. Azadirachtin has a larvicidal activity against other insects as *Haematobia irritans*, *Stomoxys calcitrans*, and *Musca domestica* (Miller and Chamberlain 1989); *Lutzomyia longipalpis* (Andrade Coelho *et al*. 2006); *Megaselia halterata* (Erler 2009); *Culex pipiens* and *Culiseta longiareolata* (Merabti *et al*. 2017). The insecticidal activity of azadirachtin was found to be related to its chemical structure similar to the insect ecdysone hormones (Zhong *et al*. 217)**.** In the present study, the larvicidal and pupicidal activity of the azadirachtin could be due to that azadirachtin acts as ecdysone blocker that breaks the insect life cycle by inhibiting the production of molting hormones. Furthermore, it blocks the cell cycle due to its inhibitory effect on microtubule polymerization (Salehzadeh *et al*. 2002) and the onset of apoptosis that explain the retarded development of the pests (Huang *et al*. 2011, 2014). Azadirachtin causes a direct damage to the DNA, apart from altering the activity of various genes and proteins (Lynn *et al*. 2012, Robertson *et al*. 2007). Moreover, Qiao *et al.* (2014) reported the neurotoxic effect of azadirachtin which might interfere with different endocrinological and physiological actions in insects.

In the present study, azadirachtin lengthened the larval duration and pupal duration in larvae treated with azadirachtin. Similar observations were also reported on *M. domestica* by Naqvi *et al.* (1995) and *Tirathaba rufivena* by Zhong *et al*. (2017) using azadirachtin. In addition, the percent pupation and the tendency of the treated pupae to develop to adults, obviously, decreased when they were treated as larvae with azadirachtin. The same findings were also reported for other insect species by using azadirachtin against *Aphis glycines* (Kraiss and Cullen 2008), *Plodia interpunctella* (Rharrabe *et al*. 2008, Lynn *et al*. 2012), *Aedes aegypti* (Koodalingam *et al*. 2014), *Drosophila melanogaster* (Boulahbel *et al*. 2015, Bezzar-Bendjazia *et al*. 2016, Oulhaci *et al*. 2018), *Lutzomyia longipalpis* (Andrade Coelho *et al*. 2014) and *Anopheles stephensi* (Siddiqui *et al*. 2003). The major retarding effect of azadirachtin on the development of *M. scalaris*, in the present study, can be explicated by the delaying effects of azadirachtin on the ecdysis and transformation of the insect. Azadirachtin is known to have a rapid effect by mimicking the activity of endocrine and neuroendocrine system (Morgan and Thornton 1973), thereby, interfering with the insect life cycle at different time points.

In the present study, Larvae of *M. scalaris* dipped in azadirachtin recorded a highly significantly lower pupal average weight and the average pupal weights dropped with increased concentration. The effect of the tested compound on reducing pupal weight of pupae treated as larvae agrees with the results obtained on *M. domestica* by Kilani *et al*. (1991) using *Azadirachta indica*, and *D. melanogaster* by Bezzar-Bendjazia *et al*. (2016) using azadirachtin. The decrease of pupal weight could be attributed to decrease in digestion and food assimilation in the treated larvae which reduce the total protein content needed for growth and development of tissues. These findings are in harmony with that of Schlüter (1985) who found that the

storage proteins in the fat body, which is necessary for pupation, did not occur after treatment of last larval instar of *Epilachna varivestis* with azadirachtin. It was suggested that azadirachtin might function by acting as a feeding deterrent (Kumar and Navaratnam 2013, Tiwari *et al*. 2014). This property adversely affects the ecdysteroid and juvenile hormones thus preventing pest proliferation. Moreover, Shu *et al.* (2018) reported that azadirachtin inhibited the growth of *Spodoptera litura* larvae by inducing apoptosis and destroying the structure of the midgut.

In the current experiments, azadirachtin induced morphological alterations of pupae of *M. scalaris*. Many abnormalities are observed like larva-pupa intermediate, heavy pigmentation of pupa and constricted pupa. These abnormalities also have been indicated in other insects as *D. melanogaster* (Bezzar-Bendjazia *et al*. 2016) and *Spodoptera littoralis* (Martinez and Emden 2001). *Diatraea saccharalis* (Schneider *et al*. 2017) after treated the last instar larvae or the prepupa with azadirachtin.

The deleterious effects of azadirachtin could be attributed to a disruption of ecdysteroid and juvenile hormone resulting of defective metamorphosis. Indeed, Insect ecdysone induces the larval-pupal metamorphosis in the absence of JH, but the presence of antagonist to JH during the JH sensitive periods (larval period) could lead to a new larval stage at ecdysis, or to the development of larval-pupal or larval-adult intermediates that are unable to give rise to normal adults (Kabir *et al*. 2013). Furthermore, Lai *et al*. (2014) showed that azadirachtin provoked potent growth inhibitory effects in Drosophila larvae by regulating the gene of cuticular protein which might be related to the deleterious metamorphic effects observed in the current study.

Morphological features of *M. scalaris* puparia play an important role in differentiating from other fly species, which is the initial requirement in forensic investigations if the species is found to associate with a corpse (Sukontason *et al*. 2006). Using scanning electron microscopic observations to determine the cuticular changes of *M. scalaris* puparia following exposure to azadirachtin has not been done before. In the present study, SEM showed morphological changes in the cuticular surface of pupa resulted from larvae dipped in LC_{50} of azadirachtin. These changes included, Bleb formation, breached integument, cuticular swelling, cuticle with loss of spines at the ventral surface, slight degeneration of anterior spiracles, deformed and wrinkled cuticular surface and shrinkage cuticle, degeneration of papillae on the respiratory horn. There were corrugation and thinning of cuticular surface and severely folded cuticle. None of the above phenomena were found in the controls. Similar results have been also reported by Siriwattanarungsee *et al.* (2008) exposed larvae and pupae of *Chrysomya megacephlala* and *M. domestica* to azadirachtin. Reed and Majumdar (1998) reported that insect cells treated with azadirachtin ultrastructurally displayed cell swelling and caused distortions of the cell surface, characterizing by blebbing and holes. Pérez-Serrano *et al*. (1994) attributed bleb formation and loss of integument organization resulting from any harmful condition due to stress responses.

CONCLUSIONS

This study revealed that azadirachtin interferes with the pupation rate and emergence of adults, corroborating to suppression in the development of *M. scalaris*. The results showed that, azadirachtin have toxicity against the 1st instar larvae of *M. scalaris* and causes the alteration in ultrastructural morphological features of *M. scalaris* puparia. It reveals that, the bioinsecticide, azadirachtin is considered as an effective insecticide, eco-friendly for controlling scuttle fly, *M. scalaris*.

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