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Toxicity, and biological and biochemical effects of hexaflumuron on the elm leaf beetle, *Xanthogaleruca luteola* (Col.: Chrysomelidae)

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Abstract

The elm leaf beetle, *Xanthogaleruca luteola* Müll., is the most important pest of elm trees in Iranian cities. Insect growth regulators (IGRs) such as hexaflumuron are recommended to use in urban areas for their low toxicity to human and environment. In this study, the impact of the chitin synthesis inhibitor, hexaflumuron, was evaluated on some biochemical and biological characteristics of the elm leaf beetle under laboratory conditions. The toxicity of this insecticide investigated on the last instar larvae of *X. luteola* using leaf dip method (25 ± 2 ºC, 75 ± 10% relative humidity, 16 h (light): 8 h (dark)). LC_{30} and LC_{50} values were calculated at 53.45 and 122.02 ppm, 72 h post treatment, respectively. Biological and biochemical characteristics were evaluated for 3rd instar larvae, following 72 h feeding on the elm leaves treated with LC_{50} and LC_{30} concentrations of hexaflumuron. The LC_{30} and LC_{50} concentrations of hexaflumuron increased the duration of larval stage, 10.04 ± 0.24 and 9.27 ± 0.43 days, respectively, compared with the control, 7.07 ± 0.413 days. There were no significant differences in the duration of pupal stage for LC_{30} and LC_{50} concentrations and control group. Hexaflumuron significantly decreased adult longevity compared with the control. Several morphological abnormalities were also observed in treated larvae and pupae. The results showed significant reductions of carbohydrate, protein, glycogen and lipid contents at the LC_{30} and LC_{50} concentrations of hexaflumuron. The activities of the detoxifying enzyme glutathione S-transferase, general esterases (α-esterases and β-esterases) and the immunological enzyme phenoloxidase were significantly affected by hexaflumuron. Although the results are clearly indicative of the adverse effects of sublethal concentrations of hexaflumuron on the beetle pest, further investigations are required to improve the efficiency of the chemical for being viably used in integrated pest management programs against the elm leaf beetle.

Key words: hexaflumuron, general esterase, glutathione S-transferase, morphological abnormality, phenoloxidase

چکیده

سمیت و اثرات بیولوژیکی و بیوشیمیایی هگزافلومورون روی سوسک برگ‌خوار نارون *Xanthogaleroca luteola* (Col.: Chrysomelidae)

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سوسک برگ‌خوار نارون، *Xanthogaleruca luteola* Müll. یکی از آفات مهم درختان نارون در شهرهای ایران است. تنظیم‌کننده‌های رشد حشرات مانند هگزافلومورون به دلیل سمیت کم برای انسان و محیط زیست، برای استفاده در محیط‌های شهری توصیه می‌شوند. در این مطالعه اثر هگزافلومورون (بازدارنده سنتز کیتین) روی بعضی از ویژگی‌های بیوشیمیایی و زیستی سوسک برگ‌خوار نارون در شرایط آزمایشگاهی مورد ارزیابی قرار گرفت. سمیت این حشره‌کش روی لارو سن آخر *X. luteola* با استفاده از روش غوطه‌ورسازی برگ نارون در دمای ۲ ± ۲۵ درجه سلسیوس، رطوبت نسبی ۱۰ ± ۷۵ درصد و دوره نوری ۱۶ ساعت روشنایی و ۸ ساعت تاریکی انجام گرفت. مقدار LC_{30} و LC_{50}، ۷۲ ساعت بعد از تیمار به‌ترتیب ۵۳/۴۵ و ۱۲۲/۰۲ پی‌پی‌ام تخمین زده شد. ویژگی‌های زیستی و بیوشیمیایی لارو سن سوم ۷۲ ساعت بعد از تغذیه از برگ‌های نارون تیمارشده با غلظت‌های LC_{30} و LC_{50} هگزافلومورون ارزیابی شد. غلظت‌های LC_{30} و LC_{50} هگزافلومورون، طول دوره لاروی را به‌ترتیب ۰/۲۴ ± ۱۰/۰۴ و ۰/۴۳ ± ۹/۲۷ روز در مقایسه با شاهد (۰/۴۱۳ ± ۷/۰۷ روز) افزایش داد. تفاوت معنی‌داری در طول دوره شفیرگی بین غلظت‌های LC_{30} و LC_{50} و شاهد مشاهده نشد. هگزافلومورون طول عمر حشره بالغ را در مقایسه با شاهد به‌طور معنی‌داری کاهش داد. همچنین ناهنجاری‌های ریختی در لاروها و شفیره‌های تیمارشده مشاهده شد. نتایج نشان داد که غلظت‌های LC_{30} و LC_{50} هگزافلومورون میزان کربوهیدرات، پروتئین، گلیکوژن و چربی را در لاروهای تیمارشده به‌طور معنی‌داری کاهش می‌دهند. سطح فعالیت آنزیم سم‌زدای گلوتاتیون اس- ترانسفراز، استرازهای عمومی (آلفا و بتا استراز) و آنزیم درگیر در ایمنی، یعنی فنل‌اکسیداز، به‌وسیله هگزافلومورون به‌طور معنی‌داری تحت تأثیر قرار گرفت. هرچند این نتایج به‌روشنی بیانگر اثرات مضر غلظت‌های زیرکشنده هگزافلومورون روی سوسک برگ‌خوار نارون است، برای بهبود کارایی این ترکیب با کاربرد مؤثر در مدیریت تلفیقی این آفت، تحقیقات بیشتری لازم است صورت گیرد.

واژگان کلیدی: هگزافلومورون، استرازهای عمومی، گلوتاتیون اس- ترانسفراز، ناهنجاری‌های ریختی، فنول‌اکسیداز

Introduction

Most of the conventional insecticides for the control of insect pest are neurotoxic compounds which also possess several adverse effects on human health, environment and non-target organisms (Bai & Koshy, 2004). In the last two decades, researches have focused on the development of compounds that are more selective, have short pre-harvest intervals and are

environment friendly (Paoletti & Pimentel, 2000). As a result, a number of insecticides with novel mode of actions have been developed including new class of chitin synthesis inhibitors, juvenile hormone (JH) analogous, ecdysone agonists, and botanical insecticides (Kellouch & Soltani, 2006; Zhu *et al*., 2012).

The insect growth regulators (IGRs) have specific target site actions which adversely interfere with the growth and development of insects (Zhu *et al*., 2012) such as reproduction and metamorphosis (Yan-Yan *et al*., 2010). The acute toxicity of IGRs to insect has been reported as LC_{50} or LD_{50}. However, in addition to mortality induced by IGRs, their sublethal effects on biochemical and biological parameters must be considered. Sublethal concentrations of IGRs may be having adverse effect to different stages of pests through interfering with metabolisms. Cutler *et al*. (2005) reported reduced egg viability in adults of *Leptinotarsa decemlineata* (Say) (Col.: Chrysomelidae) on novaluron-treated potato foliage. Also, the ovicidal effects of hexaflumuron and lufenuron reported on *Plutella xalostella* (L.) (Lep.: Yponomeutidae) (Mahmoudvand *et al*., 2010). El-Barkey *et al*. (2009) observed that hexaflumuron increased in larval and pupal duration and decreased percentage of pupation and adult emergence of *Pectinophora gossypiella* (Saunders) (Lep.: Gelechiidae). Also, activities of glutathione S-transferases (GST), carboxylesterase, and other metabolic enzymes can be affected by these compounds. General esterases and glutathione S-transferases (GSTs) are important detoxifying enzymes in metabolism of synthetic and non-synthetic insecticides (Mouches *et al*., 1986; Vanhaelen *et al*., 2001). General esterases are a large and diverse group of hydrolyzers, which break down numerous insecticides with esteric bounds (Mouches *et al*., 1986). GSTs are a large family of multi-functional enzymes that catalyze the conjugation of glutathione with different xenobiotic such as insecticides (Clark, 1989). Some studies have indicated the effects of IGRs on general esterases and GSTs (Ali, 2008; Baker *et al*., 2010). Phenoloxidases (POs) are the enzymes that have crucial role in the immunity systems and molting processes in insects. Some studies have demonstrated that POs can be either inhibited or activated by some IGRs (Soltani *et al*., 1984). Nasr (2011) showed that oxymatrine, chlorfluazuron and chlorpyrifos significantly decreased the activity of POs in *Bombyx mori* L. larvae. These alterations in biochemical parameters may be manifested as reductions in fecundity and fertility (Liu & Trumble, 2005).

The elm tree is susceptible to more than 80 insect pest species. The most important insect pest of this tree is *Xanthogaleruca luteola* Müll. which feeds on its leaves during the larval and adult stages. Severe infestation can lead to defoliation, physiological disorders and increase the susceptibility of the trees to other pests, pathological agents and environmental stresses (Arbab *et al*., 2001). Because any kinds of Chemical control of *X. luteola* in urban areas will pose risks to residents, the application of IGRs such as hexaflumuron with low toxicity to humans and environment is highly encouraged.

Hexaflumuron [1-[3, 5-dichloro-4-(1, 1, 2, 2-tetrafluoroethoxy) phenyl]-3-(2, 6-difluorobenzoyl) urea] is a benzyl phenyl urea (BPU) insecticide with insecticidal lethal effect on the larvae of Lepidoptera, Coleoptera, Homoptera, and Diptera. This compound inhibits chitin synthesis and interrupts the molting process in insects. It is ovicidal and also effective through ingestion and contact (El-Barkey *et al*., 2009; Mahmoudvand *et al*., 2011). This insecticide has low toxicity on human, vertebrates and environment (Mahmoudvand *et al*., 2011). The impacts of hexaflumuron's lethal and sub-lethal doses on insects have been extensively studied (Coppen & Jepson, 1996; Marco & Castanera, 1996; Kellouch & Soltani, 2006; Karimzadeh *et al*., 2007; Zhu *et al*., 2012). Elm leaf beetle is considered as an economically important pest on elm trees in urban areas, where the use of common insecticides is heavily discouraged for the safety of residents. We decided to study the effect of hexaflumuron on this urban pest for adverse effects of

IGRs on insects. We studied the biochemical effect of hexaflumuron on the energy reserves (carbohydrate, glycogen, protein, and lipid), detoxification enzymes, phenoloxidase as well as its biological properties.

Materials and methods

Insect rearing

Eggs and larvae of *X. luteola* were collected from the elm tree *Zelkova carpinifolia* (Pallas) Koch (Rosales: Ulmacese) in a pesticide-free park in the Caspian coast city of Rasht. Insect colonies were maintained in the laboratory conditions at 25 ± 2 ºC; 16: 8 h (L: D); 75 ± 10 % RH. Larvae were reared in transparent plastic jars 10 × 20 cm in which the lids contained holes covered with muslin cloth. In order to maintain humidity, the bottom of the jars were covered with compressed wet sponges and the leaves containing larvae were vertically placed over the sponges. Fresh leaves were provided on daily basis.

Bioassay

Hexaflumuron (10 EC; Hexaflumuron Kavosh Kimia) was supplied by Kavosh Kimia Kerman Company, Iran. Leaf dip method was used for bioassays. Elm leaf discs (2.5 cm diameter) were dipped in five concentrations (50, 69.5, 98.62, 139.95, 200 ppm; prepared in water) of hexaflumuron for 30 s. The controls received the leaf discs immersed in distilled water. The treated leaf discs were allowed to dry at room temperature for 45 minutes and then the third instar larvae (10-12 h old) were placed on it. This experiment was repeated 5 times for each treatment and 10 larvae of the same age were used in each replicate. Mortality was recorded after 72 h and the LC_{50} and LC_{30} were estimated using POLO-PC software (Leora Software, 1987). A larva was considered dead when it did not move after prodding with a camel's hair brush.

Effect of hexflumuron on the developmental stage of *X. luteola*

Larval duration after treatment with two concentrations (i.e. LC_{50} and LC_{30}) of hexflumuron was evaluated. The leaf discs were dipped in different concentrations for 30 s. The third instar larvae (10-12 h) were transferred to treated leaves. Living larvae were transferred to fresh leaves after 72 h and left to continue their development until the pupal stage. Pupae and adults were placed individually in plastic jars (10 × 20 cm) after emergence and the period of each stage was recorded.

Determination of total carbohydrates level

The carbohydrate level was measured according to Yuval *et al.* (1998). The third instar larvae were homogenized in 62.5 µL of 2% Na_2SO_4 72 h after treatment with LC_{50} and LC_{30} concentrations. Then 469 µL of chloroform: methanol (1:2) was added to the homogenate and samples were centrifuged for 10 min at 8000 × g. To determine the amount of total carbohydrates, 150 µL of supernatant was taken and mixed with 100 µL of distilled water. The sample was mixed with 500 µL of anthrone reagent (500 mg anthrone dissolved in 500 ml concentrated H_2SO_4) for 10 min at 90 ˚C. Then, the rate of absorbance was measured at 630 nm. The amount of total carbohydrate was determined by standard curve using maltose (Sigma) as standard. This experiment was repeated 6 times for each treatment for each individual larva.

Determination of Glycogen level

Glycogen content was determined in the pellet achieved after centrifugation. The pellet was washed in 400 µL of 80% methanol three times. Then, 125 µL distilled water was added to the pellet. The mixture was heated for 5 min at 70 ºC. Subsequently, 100 µL of the solution was mixed with 500 µL anthrone reagent and heated for 10 min at 90 ºC (Yuval *et al.*, 1998). The absorbance rate was measured at 630 nm. The amount of glycogen in sample was quantified by a standard curve using glycogen (Sigma). This experiment was repeated 6 times with individual larvae.

Determination of total protein value

Total protein was measured based on the Bradford method with some modification (Bradford, 1976) using bovine serum albumin as standard. Initially, two insects were homogenized with 100 µL phosphate buffer (pH 7.0) and centrifuged for 10 min at 10000 × g. Later, 10 µL of supernatant was mixed in 500 µL Bradford's reagent and the absorbance rate was recorded at 630 nm. This experiment was repeated six times for each individual larva.

Determination of total lipid level

Lipid content was determined according to Yuval *et al*. (1998). Two larvae were homogenized in 62.5 µL of 2% Na_2So_4. 469 µL of chloroform: methanol (1: 2) was added to the homogenate and the samples from each treatment were centrifuged for 10 min at 8000 × g. 125 µL of supernatant was transferred to a micro tube and placed in an oven at 40 ºC until completely dried. Then, 125 µL H_2SO_4 (98% Merck) was added to each tube and heated for 10 min at 90 ºC. A mixture of 30 µL of supernatant and 270 µL of vanillin reagent was left at room temperature for 30 min. The absorbance rate was recorded at 545 nm. The amount of lipid was determined using cholesterol as standard. This experiment was repeated 6 times with individual larva.

Assay of general esterase

The living third instar larvae, after treatment with hexaflumuron concentrations, were killed by deep freezing 72 h post treatment. The samples were homogenized in phosphate buffer containing 0.2% triton X-100 (pH 7.0) and centrifuged for 10 min at 10,000 × g. The supernatant was used as enzyme source for esterase activity. In the case of GST activity, the sample was homogenized in phosphate buffer without triton X-100.

The activity of general esterase was determined according to Van Asperen (1962) method. α-naphtyl acetate (α-NA) and β- naphtyl acetate (β-NA) (10 mM) were used as substrates. 13 µL of supernatant (enzyme) was transferred to micro plate and mixed with 112 µL phosphate buffer (pH 7.0), 25µL substrate and 50 µL fast blue RR salt (1 mM). The absorbance rate was recorded kinetically at 450 and 540 nm due to formation of α-naphtol and β-naphtol, respectively. Enzyme activity was expressed as µmol/min/mg protein.

Assay of GSTs activity

GST activity was determined using the method of Habing *et al*. (1974). 1-chloro 2, 4-dinitrobenzene (CDNB) was used as the substrate. An amount of 10 µL of supernatant (enzyme) was mixed with 110 µL phosphate buffer (pH 7.0), 100 µL of GSH and 80 µL of CDNB. The absorbance rate was recorded at 340 nm in kinetic mode. Enzyme activity was expressed as µmol/min/mg protein.

Determination of POs activity

Phenoloxidase activity was determined in larval homogenate according to the method of Robb (1984). Ten µL enzymes was mixed with 90 µL phosphate buffer (pH = 7.0) and 100 µL substrate (containing 3 methyl -2- benzo thiazolinon-hyorazon hydrochlorid (MBTH), D-methyl Formid (DMF), H_3PO_4, and L-3, 4-dihydroxyphenyl alanine (L-DOPA)). Finally, absorbance rate was recorded at 492 nm every 30 seconds and enzyme activity expressed as µmol/min/mg protein.

Statistical analysis

For determination of median lethal and LC_{30} concentrations, POLO-PC software (Leora Software, 1987) was used. The data from the experiments was subjected to analysis of variance (ANOVA) using SAS software. The least significance among treatments were compared using Tukey's multiple range test (SAS Institute, 1997).

Results and discussion

LC_{30} and LC_{50} values were calculated at 53.45 and 122.02 ppm, respectively (table 1). The results

(table 3) showed that hexaflumuron significantly decreased total carbohydrate content in LC_{50} (0.167 ± 0.043) as compared with control (0.402 ± 0.077) (F = 5.6; df = 2, 6; P = 0.0263). But, no significant differences were observed between LC_{50} and LC_{30} concentrations. Amount of total lipid significantly reduced after larval treatment with LC_{50} and LC_{30} concentrations (0.136 ± 0.074 and 0.195 ± 0.064, respectively) compared with the control (0.529 ± 0.056) (table 3). However, no significant differences were observed between the LC_{50} and LC_{30} concentrations (F = 14.3; df = 2, 6; P = 0.0052). The glycogen level significantly reduced in LC_{50} and LC_{30} concentrations (0.116 ± 0.034 and 0.22 ± 0.021, respectively) (F = 141.4; df = 2, 9; P < 0.0001) (table 3). The concentration of LC_{50} and LC_{30} had a significant reduction in total protein compared with the control (0.048 ± 0.00081, 0.0527 ± 0.00095, 0.0821 ± 0.0064, respectively) (F = 107.32; df = 2, 6; P < 0.001) (table 3). By measuring the detoxifying enzymes including general esterases and GST, it was found that GST is probably the main detoxifying enzyme for hexaflumuron detoxification in *X. luteola*. LC_{30} and LC_{50} concentrations significantly increased GST activity as compared with the control, especially LC_{50} concentration (0.271 ± 0.1, 2.759 ± 0.695 and 0.229 ± 0.089, respectively) (F = 12.53; df = 2, 6; P = 0.0072) (table 4). The activity of α-esterase significantly increased in LC_{50} concentrations as compared with the control (81.33 ± 2.8 and 54.11 ± 3.7, respectively) (table 4). The activity of β-esterase in LC_{50} concentrations increased (19.07 ± 2.63), although no

Table 1. The LC_{50} and LC_{30} values, confidence limit (95%) and regression slope after 72 h exposure to hexaflumuron in larvae of *Xanthogaleruca luteola.*

Insecticide	Slope ± Se	X^2 (df)	LC_{50} (ppm) (95% confidence limits)	LC_{30} (ppm) (95% confidence limits)
Hexaflumuron	2.89 ± 0.471	1.042 (3)	122.02 (91.38-193.87)	53.45 (20.76-74.03)

Table 2. Life stages duration (mean ± SE) of *Xanthogaleruca luteola* after treatment with hexaflumuron**.**

Treatments	3rd Instar larval duration (days)	Pupal duration (days)	Adult longevity (days)	No. of deformed insects
LC_{50}	9.27 ± 0.433a	5.68 ± 0.491a	12.18 ± 1.36b	0.3 ± 0.065a
LC_{30}	10.04 ± 0.242a	5.8 ± 0.423a	12.66 ± 1.38b	0.14 ± 0.049b
Control	7.07 ± 0.413b	6.14 ± 0.331a	20.18 ± 1.5a	0.0c

Means with different letters in each column are significantly different based on Tukey's test ($p \leq 0.05$).

Table 3. The effects of hexaflumuron on energy reserves (mean ± SE) of *Xanthogaleruca luteola* larvae.

Energy reserves (µg/larvae)	Control	LC_{50}	LC_{30}
Carbohydrate	0.402 ± 0.077a	0.167 ± 0.043b	0.211 ± 0.023ab
Lipid	0.529 ± 0.056a	0.136 ± 0.074b	0.195 ± 0.064b
Protein	0.0821 ± 0.0064a	0.048 ± 0.000811c	0.0527 ± 0.00095b
Glycogen	0.649 ± 0.0087a	0.116 ± 0.034c	0.22 ± 0.021b

Means with different letters in each column are significantly different based on Tukey's test ($p \leq 0.05$).

Table 4. The effects of hexaflumuron on some enzyme activities (mean ± SE) of *Xanthogaleruca luteola* larvae.

Specific activity (µmol/min/mg protein)	Control	LC_{50}	LC_{30}
Glutation S-transferase	0.229 ± 0.089b	2.759 ± 0.695a	0.271 ± 0.1005b
Eterase,α-naphtyl	54.11 ± 3.7b	81.33 ± 2.8a	59.92 ± 3.4ab
Eterase, β-naphtyl	10.34 ± 2.02b	19.07 ± 2.63a	6.31 ± 2.03b
Phenoloxidase	3.808 ± 0.58c	6.078 ± 0.43b	16.12 ± 0.21a

Means with different letters in each column are significantly different based on Tukey's test ($p \leq 0.05$).

Fig. 1. *Xanthogaleruca luteola*: (a) normal larva, (b) normal larva and pupa, (c-b) larval-pupal intermediates (15 mm).

significant differences were observed between LC_{30} and control (table 4). The activity of the PO in larvae treated with LC_{50} and LC_{30} concentrations significantly increased (6.078 ± 0.43 and 16.12 ± 0.21, respectively) ($F = 206.28$; df= 2, 6; $P < 0.0001$) (table 4).

In this study, hexaflumuron showed a high toxicity on 3rd instar larvae of *X. luteola*. The application of two different concentrations of hexaflumuron on the larvae increased their larval duration (table 2). This result is in consistent with the results of Khosravi & Jalali Sendi (2013) who showed that *Thymus vulgaris* essential oil significantly increased larval duration in *X. luteola*. Khajepour *et al*. (2011) stated that hexaflumuron increased larval duration in *Ephestia figulilla* Gregson (Lep.: Pyralidae). Valizadeh *et al*. (2013) reported a similar result using Neem product on *X. luteola*. Kandil *et al*. (2010) wrote that the benzylphenyl urea compounds, lufenuron and chlorfluazuron, had significantly increased the larval duration in *P. gossypiella*

Higher level of energy consumption occurs during detoxification of insecticides (i.e. increased GST activity in this research). This phenomenon may be leads to lower or higher larval duration or a reduction in reproductive performance (Bovin *et al*., 2001) which is evident also in present results.

We observed morphological abnormities in larval-pupal stage (fig 1.) similar to the results of

Gelbic *et al*. (2001), Baker *et al.* (2010) and Khajepour *et al*. (2011).

No significant differences in pupal duration were observed in our results (table 2) that are similar to the results by Josan & Sing (2000), Willrich & Boethel (2001) and Ashouri *et al.* (2014). The treatment of 3rd instar larvae of *X. luteola* with hexaflumuron significantly decreased longevity of resultant adults compared to the control (table 2). We believe that the longevity is related to what has been gained during larval stage. However, the presence of toxic material and reduction in energy reserves, which has been involved in detoxification process in the larval stage, has caused the short longevity (Bovin *et al*., 2001).

The results clearly showed that hexaflumuron significantly reduced total carbohydrate content (table 3). Kandil *et al*. (2010) had observed the same results by using lufenuron and chlorfluazuron against *P. Gossypiella*. El-Gammal *et al*. (1989) experimental application of fenoxycarb against *Schistocerca gregaria* (Forskål) (Orth.: Acrididae) was identical to our finding. Depending on physiological conditions, lipid and carbohydrate levels in the insect's bodies are being used to deal with chemical stress (Miranda *et al*., 2003). Therefore, it is likely that reduction of carbohydrate content may be due to the effect of anti-feedant and an increase in metabolism under toxicant stress (Remia *et al*., 2008).

The reduction in total lipid in the present study (table 3) is similar to those reported by Abdel-Aal (2006) using chlorfluazuron against *Spodoptera littoralis* (Boised.) (Lep.: Noctuidae). Lohar & Wright (1993) stated that reduction in lipid content in treated insects might have been due to the effect of insecticide on the adipokinetic hormone that modulates the lipid metabolism (Sak *et al*., 2006).

The protein plays a fundamental role in biochemical reactions and hormonal regulation in all known species and is integrated in the cell as a structural element (Sugumaran, 2010). The amount of total protein decreased as the concentration of hexaflumuron increased (table 3). The reason behind this phenomenon could be the break-down of protein in to amino acids and their entry to TCA cycle as keto acids (Shekari *et al*., 2008). Baker *et al*. (2009) reported that reduction of protein level might be due to the destructive effect of IGRs on some of the cerebral neurosecrotory cells of the brain. Our result is in agreement with Baker *et al*. (2009) who reported reduction in total protein in *S. gregaria* after treatment with hexaflumuron. The protein content decreased in house fly, *Musca domestica* Vicina (Dip.: Muscidae) after treatment with lufeuuron and hexaflumuron (Assar *et al*., 2010). Bouaziz *et al*. (2011) reported a total protein content reduction after application of novaluron in *Culiseta longiareolata* (Macquart) (Dip.: Culicidae). Valizadeh *et al*. (2013) observed a significant decrease in the amount of total protein in the treated larvae of *X. luteola* with neem.

Glycogen is a multibranched polysaccharide of several glucose residues which exists as storage form of carbohydrate in animals (Klowden, 2007). We observed hexaflumuron decreased the amount of glycogen of *X. luteola* (table 3). Similar results were also reported by Behroozi *et al.* (2011) when they treated larvae of *Onceria terebinthina* Strg. (Lep.: Lymantriidae) with chlorfluazuron. Valizadeh *et al*. (2013) also reported a reduction in the amount of glycogen in *X. luteolla* treated with neem.

One of the most important constituents of insect cuticle is chitin. The starting point for making of this polysaccharide is glucose, which may come from storage of trehalose or glycogen (Gordon & Burford, 1984). Therefore, changes in amount of glycogen could upset the homeostatic mechanism in insects (Nath, 2003; Oguri & Steele, 2007; Valizadeh *et al*., 2013).

GSTs are a large family of multi-functional enzymes that catalyze the conjugation of glutathione with various xenobiotic compounds such as insecticides (Mannervik & Danielson, 1988). They are involved in the detoxification of xenobiotics and protection of organisms from oxidative damage (Hayes & Pulford, 1995; Yu, 1996). In the current study, the activities of GST increased in treated larvae (table 4).

Valizadeh *et al*. (2013) and Mohammadzadeh Tamam *et al*. (2014) reported higher GST activities in the larvae of *X. luteola* treated with neem and spinosad, respectively. Sharifi *et al*. (2013) also reported enhanced activities of GST in *Ephestia kuehniella* (Zeller) (Lep.: Pyralidae) after pyriproxyfen treatment. An increased activity of GST occurred in the larvae of *Galleria mellonella* (L.) (Lep.: Pyralidae) treated with teflubenzuron (Chiang & Sun, 1993).

In this study, significant differences were observed on α-esterase and β-esterase activity (table 4). Similar results were also reported by Valizadeh *et al*. (2013) who reported increase of general sterases in the larvae of *X. luteola* treated with neem. Saleem *et al*. (1995) and Mead (2006) showed reduction in the activity of α- and β-esterases in the larvae of *S. littoralis* treated with buprofezin, diafenthiuron and triflumuron. Liu *et al*. (2008) reported that GST and general esterase activities did not change in the larvae of *Osterinia furnacalis* Guenee (Lep.: Pyralidae) after being fed on fraxinello-treated food. Flufenoxuron significantly decreased the activity of α- and β-esterases in the larvae of *S. littoralis* (Baker *et al*., 2010).

Insect immunity consists of both humeral and cellular defensive reactions (Lavine & Strand, 2002) and PO has major roles in humeral defense. PO correlates with resistance to some parasites and pathogens species as well as being involved in the processes of coagulation, melanization and wound healing (Nigm *et al*., 1997). The results indicated that hexaflumuron significantly increased PO activity (table 4). The identical results were obtained when hexaflumuron was used against *S. littoralis* (Yan-Yan *et al*., 2010) and diflubenzuron and pyriproxyfen against *S. littoralis* (Farag, 2001). Also, Piri *et al*. (2014) showed a greater PO activity level in *Glyphodes pyloalis* Walker (Lep.: Pyralidae) larvae treated with LC_{30} and LC_{40} spinosad concentrations compared with LC_{10}, LC_{20} and the control.

In conclusion, the results indicated that hexaflumuron can affect larval, pupal and adult development by disrupting the essential energy sources in *X. luteola*. This compound alters the activities of detoxification enzymes and phenoloxidases in the immunity system. Our findings indicate that hexaflumuron has great capabilities as an environmentally-friendly alternative to synthetic chemical insecticides against *X. luteola*.

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